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EXPRESSION OF THE REPRESSOR ELEMENT 1 SILENCING TRANSCRIPTION FACTOR (REST) IS REGULATED BY IGF-I AND PKC IN HUMAN NEUROBLASTOMA CELLS

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ABSTRACT

The repressor element 1-silencing transcription factor (REST) was first identified as a protein that binds to a 21-bp DNA sequence element (known as repressor element 1 (RE1)) resulting in transcriptional repression of the neural-specific genes [Chong et al., 1995; Schoenherr and Anderson, 1995]. The original proposed role for REST was that of a factor responsible for restricting neuronal gene expression to the nervous system by silencing expression of these genes in non-neuronal cells. Although it was initially thought to repress neuronal genes in non-neuronal cells, the role of REST is complex and tissue dependent.

In this study I investigated any role played by REST in the induction and patterning of differentiation of SH-SY5Y human neuroblastoma cells exposed to IGF-I. and phorbol 12-myristate 13-acetate (PMA) To down-regulate REST expression we developed an antisense (AS) strategy based on the use of phosphorothioate oligonucleotides (ODNs). In order to evaluate REST mRNA levels, we developed a real-time PCR technique and REST protein levels were evaluated by western blotting.

Results showed that nuclear REST is increased in SH-SY5Y neuroblastoma cells cultured in SFM and exposed to IGF-I for 2-days and it then declines in 5-day-treated cells concomitant with a progressive neurite extension. Also the phorbol ester PMA was able to increase nuclear REST levels after 3-days treatment concomitant to neuronal differentiation of neuroblastoma cells, whereas, at later stages, it is down-regulated. Supporting these data, the exposure to PKC inhibitors (GF10923X and Gö6976) and PMA (16nM) reverted the effects observed with PMA alone. REST levels were related to morphological differentiation, expression of growth cone-associated protein 43 (GAP-43; a gene not regulated by REST) and of synapsin I and □ III tubulin (genes regulated by REST), proteins involved in the early stage of neuronal development. We observed that differentiation of SH-SY5Y cells by IGF-I and PMA was accompanied by a significant increase of these neuronal markers, an effect that was concomitant with REST decrease.

In order to relate the decreased REST expression with a progressive neurite extension, I investigated any possible involvement of the ubiquitin–proteasome system (UPS), a multi-enzymatic pathway which degrades polyubiquinated soluble cytoplasmic proteins [Pickart and Cohen, 2004]. For this purpose, SH-SY5Y cells are concomitantly exposed to PMA and the proteasome inhibitor MG132. In SH-SY5Y exposed to PMA and MG 132, we observed an inverse pattern of expression of synapsin I and β- tubulin III, two neuronal differentiation markers regulated by REST. Their cytoplasmic levels are reduced when compared to cells

exposed to PMA alone, as a consequence of the increase of REST expression by proteasome inhibitor. The majority of proteasome substrates identified to date are marked for degradation by polyubiquitinylation; however, exceptions to this principle, are well documented [Hoyt and Coffino, 2004]. Interestingly, REST degradation seems to be completely ubiquitin-independent.

The expression pattern of REST could be consistent with the theory that, during early neuronal differentiation induced by IGF-I and PKC, it may help to repress the expression of several genes not yet required by the differentiation program and then it declines later. Interestingly, the observation that REST expression is progressively reduced in parallel with cell proliferation seems to indicate that the role of this transcription factor could also be related to cell survival or to counteract apoptosis events [Lawinger et al., 2000] although, as shown by AS-ODN experiments, it does not seem to be directly involved in cell proliferation. Therefore, the decline of REST expression is a comparatively later event during maturation of neuroroblasts *in vitro*. Thus, we propose that REST is regulated by growth factors, like IGF-I, and PKC activators in a time-dependent manner: it is elevated during early steps of neural induction and could contribute to down-regulate genes not yet required by the differentiation program while it declines later for the acquisition of neural phenotypes, concomitantly with a progressive neurite extension. This later decline is regulated by the proteasome system activation in an ubiquitin-indipendent way and adds more evidences to the hypothesis that REST down-regulation contributes to differentiation and arrest of proliferation of neuroblastoma cells.

Finally, the glycosylation pattern of the REST protein was analysed, moving from the observation that the molecular weight calculated on REST sequence is about 116 kDa but using western blotting this transcription factor appears to have distinct apparent molecular weight (see Table 1.1): this difference could be explained by post-translational modifications of the proteins, like glycosylation. In fact recently, several studies underlined the importance of O-glycosylation in modulating transcriptional silencing, protein phosphorylation, protein degradation by proteasome and protein–protein interactions [Julenius et al., 2005; Zachara and Hart, 2006].

Deglycosylating analysis showed that REST protein in SH-SY5Y and HEK293 cells is O-glycosylated and not N-glycosylated. Moreover, using several combination of deglycosylating enzymes it is possible to hypothesize the presence of Gal-β(1-3)-GalNAc residues on the endogenous REST, while β(1-4)-linked galactose residues may be present on recombinant REST protein expressed in HEK293 cells. However, the O-glycosylation process produces an immense multiplicity of chemical structures and monosaccharides must be sequentially hydrolyzed by a series of exoglycosidase. Further experiments are needed to characterize all the post-translational modification of the transcription factor REST.

INTRODUCTION

1. THE TRANSCRIPTION FACTOR REST

Generation of cellular diversity during development requires coordination of gene expression mediated by both positive and negative transcriptional regulators. Regulation of gene expression is critical for nervous system development and function. The nervous system relies on a complex network of signaling molecules and regulators to orchestrate a robust gene expression program that leads to the orderly acquisition and maintenance of neuronal identity. Identifying these regulators and their target genes is essential for understanding the regulation of neuronal genes and elucidating the role of these regulators in neural development and function.

The repressor element 1-silencing transcription factor (REST), also known as the neuron restrictive silencer factor (NRSF), was first identified as a protein that binds to a 21-bp DNA sequence element (known as repressor element 1 (RE1), also known as neuron restrictive silencing element (NRSE)) resulting in transcriptional repression of the neural-specific *voltage-gated type II sodium channel (nav1.2)* and *superior cervical ganglion 10* genes [Chong et al., 1995; Schoenherr and Anderson, 1995].

The original proposed role for REST was that of a factor responsible for restricting neuronal gene expression to the nervous system by silencing expression of these genes in non-neuronal cells. REST is usually expressed in nonneuronal cells and is rarely expressed in neurons *in vivo* [Ballas et al., 2005]. However, REST was also found to be expressed in certain mature neurons in adults [Shimojo et al., 2004; Griffith et al., 2001], suggesting that it is either reexpressed or that its expression is maintained in a specific type of neurons. Moreover, subsequent studies suggest other additional and more complex roles for REST.

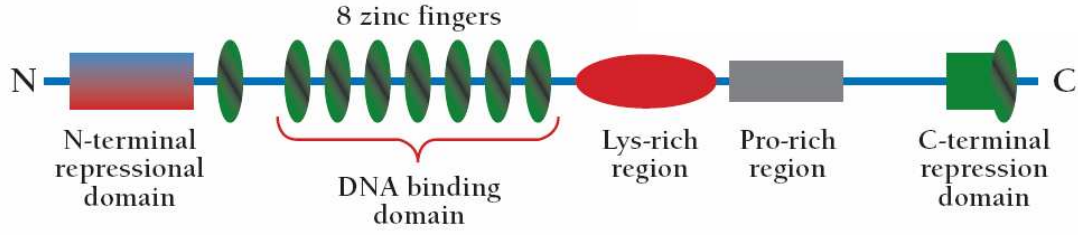


Figure 1.1 *Domains of the REST protein.*

For example, REST expression has been detected within hippocampal neurones and expression levels have been shown to rise in response to epileptic and ischaemic insults [Palm et al., 1998; Calderone et al., 2003], supporting an important repressor role for REST within neurones. Additionally, REST also plays a pivotal role during neuronal degeneration in Huntington's disease [Zuccato et al., 2003]. Whilst outside of the nervous system, REST is critically important for directing the appropriate expression of the foetal cardiovascular gene programme in heart and vascular smooth muscle [Kuwahara et al., 2003; Cheong et al., 2005] and acting in a classical tumour suppressor gene [Westbrook et al., 2005]. Currently, a complete understanding of the true roles played by REST is lacking.

REFERENCE	**CELL LINE**	**MOLECULAR WEIGHT (KDA)**
Chong et al. 1995	PC12	116
Wood et al. 2003	PC12 and JTC-19	120
Cheong et al. 2005	Murine endothelial cells	
Qiang et al. 2005	Primary cortical neurons (mouse)	150
Thiel et al.1998	NS20Y, NG108-15, CHO-K1	190-210
Zuccato et al.2003	Neuronal murine cells	200 and 160-180
Pance et al. 2006	PC12(A35C), K562	
Grimes et al. 2000	Transfected HEK293	200
Jia et al. 2006	Rat cortical neurons	
Westbrook et al. 2005	Colorectal cancer cell lines	
Di Toro et al. 2005	SH-SY5Y	
Kim et al.2006	HEK293, SH-SY5Y, HB1, F3, PC12, C6, C17.2	
Kuratomi et al. 2007	Rat cardiomyocytes	

Table 1.1 *Molecular weight of REST protein in different models and cell lines.*

By fluorescence in situ hybridization REST gene was assigned to the chromosomic region 4q12 [Cowan et al., 1996] and is 3365 bp long [GeneBank NM_005612].

REST is a 116-kDa zinc finger protein that contains a DNA-binding domain and two repressor domains: one at the N-terminal and the other at the C-terminal of the protein (Fig. 1.1) [Ballas et

al., 2005b]. The DNA-binding domain of REST binds to a 21-bp consensus DNA sequence, the RE-1 binding site/neuron-restrictive silencer element (RE-1/NRSE), present in the target gene's regulatory regions.

Although the molecular weight calculated on REST sequence is about 116 kDa, using western blot this transcription factor appears to have distinct apparent molecular weight (Table 1.1): this difference could be explained by post-translational modifications of the proteins.

In addition, several splice variants of REST were discovered [Palm et al., 1998] encoding proteins with five or four zinc finger motifs. Two variants of REST termed REST4 and REST5 were only detected in neuronal tissues. These transcripts are generated by alternative splicing of a neuron-specific exon (exon N) located between exons V and VI. REST4 retains the N-terminal repression domain and five of the eight zinc fingers that function as a DNA-tethering domain. The neuron-specific isoforms have an insertion of 16 nucleotides (REST4), followed by an in-frame stop codon [Palm et al., 1998]. The neuron-specific splicing of REST is conserved in human, mouse and rat [Palm et al., 1999]. The biological function of REST4 is controversial. Although REST4 itself does not bind to the NRSE, it acts as a dominant negative by inhibiting the binding of REST to the NRSE sequence. REST4 itself does not act as a transcriptional repressor but rather regulates the repressor activity of NRSF/REST [Shimojo et al., 1999].

Depending on the cellular context, the binding of REST to a variety of cellular factors can produce an array of mechanisms that can be used to differentially influence transcription of each of its many target genes in a cell context-dependent manner. The specific effect generated by the REST complex can be further affected by the neighbouring transcriptional activators or repressors bound to the particular target gene chromatin, and the resultant effect then will determine the gene expression [Ballas et al., 2001].

1.1 REST gene network

Although it was initially thought to repress neuronal genes in non-neuronal cells, the role of REST is complex and tissue dependent. In order to provide some insight into REST function *in vivo*, bioinformatic analyses have identified REST targets on the basis of DNA sequence alone. The relatively long binding site for REST (21 bp), large number of conserved nucleotides (which results in a high information content) and large number of known functional binding sites have made the RE1 site amenable to such bioinformatic analysis (Figure 1.2). Four independent groups [Bruce et al., 2004; Jones and Pevzner, 2006; Mortazavi et al., 2006; Wu and Xie, 2006]

have carried out genome-wide analyses to predict the complete set of RE1 sites and their associated genes.

These bioinformatic data have been used to predict the set of genes that could be regulated by REST, in order to develop hypotheses about its physiological function. Evidently, bioinformatic searches can be powerful predictors of transcription-factor function. Nevertheless, it is important to remember that the effectiveness of this technique relies heavily on the accurate identification of bona fide target genes. This is more difficult if the binding site is relatively short and, consequently, occurs frequently in the genome by random chance. The length of the binding site and the number of invariant bases within that site are therefore crucial factors in identifying a genuine target-gene subset using this method.

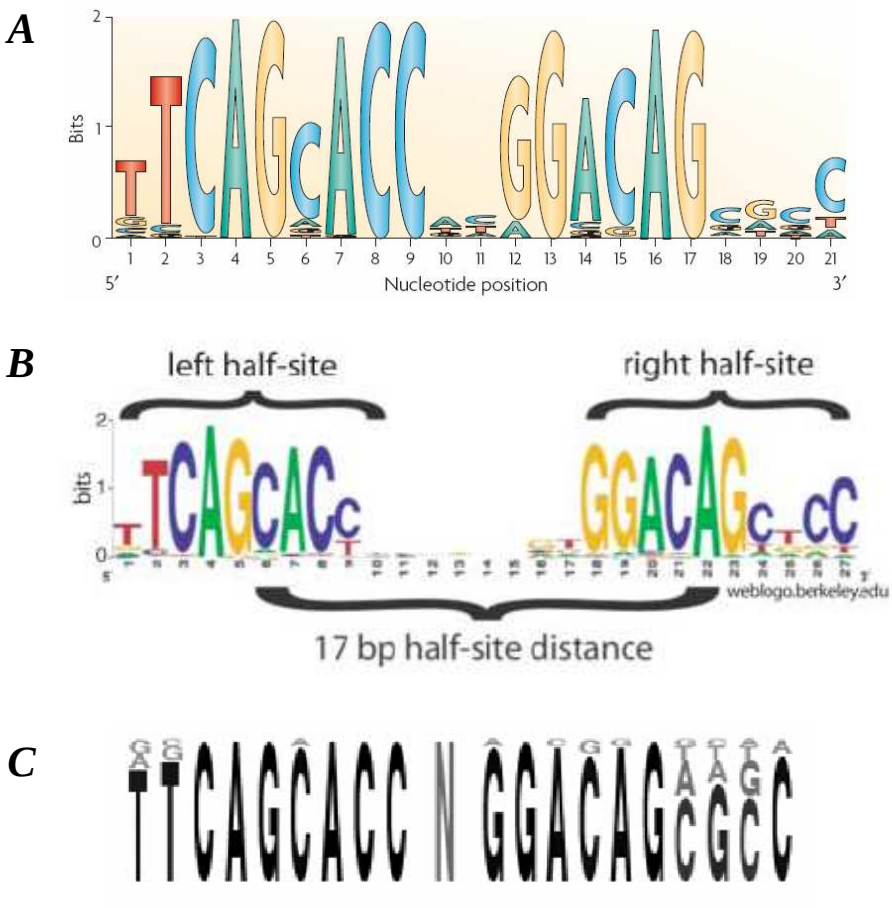


Figure 1.2 *The sequence logos used to identify RE1 sites in the genome: A. Classical RE1 sequence; B. expanded RE1 motif; C. compressed RE1 motif.*

Although the majority of work on the REST-RE1 system has concentrated on the repression of gene expression, it has become increasingly clear over the years that the RE1 possesses enhancer

activity as well as silencer activity. In a transgenic analysis of the gene that encodes the β2-nicotinic acetylcholine receptor (β2-nAChR), a point mutation introduced into the gene's RE1 resulted in loss of reporter gene expression in dorsal root ganglia and sympathetic ganglia [Bessis et al., 1997]. A transgenic study of the neuronally expressed gene that encodes the L1 cell adhesion molecule [Kallunki et al., 1997] similarly showed that disruption of the RE1 caused loss of expression in certain populations of neuronal cells, particularly in the hippocampus. Recently, RE1s have also been implicated to enhance expression of the genes encoding dynamin [Yoo et al., 2001], atrial naturietic peptide [Kuwahara et al., 2001], and corticotropin-releasing hormone [Seth et al., 2001]. Although the mechanisms that determine whether a RE1 will act to silence or enhance gene expression have remained elusive, some inroads have been made. Foremost, the position of the RE1 within the gene appears to influence strongly how this element affects gene expression: in fact REST-binding site are not restricted to the promoter region of target genes.
Moreover recently novel REST-binding motifs were discovered. Using SACO, it was seen unexpectedly that REST-binding site is both compressed and expanded compared with the original motif (Figure 1.2). Interestingly, close inspection of the original 21 bp RE1 motif indicated that nucleotides 10 and 11 are two of the least-highly conserved nucleotides across mammalian species. This is precisely where the additional nucleotides were either inserted (for the expanded sites) or removed (for the compressed sites). These results strongly suggest that in these instances, REST interaction is complex and may involve association with another DNA binding factor or binding through a DNA element unrelated to the known RE1 [Otto et al., 2007].

1.2 REST co-repressors

Eukaryotic DNA is wrapped around histones to form a complex known as chromatin. The base unit of chromatin, a nucleosome, is composed of eight histones that are encircled one-and-three-quarter times by 147 bp of DNA. Nucleosomes are arranged to form a structure that facilitates the packaging of an entire genome into a nucleus. In addition to this structural role for histones, post-translational modifications of their N-terminal tails give rise to changes in nucleosome–DNA interactions that are important for regulating transcription. Acetylation and methylation modifications of residues within histones were first described more than 40 years ago [Allfrey et al., 1964], closely followed by histone phosphorylation and, more recently, ubiquitination. These modifications have a well established role in regulating transcription.

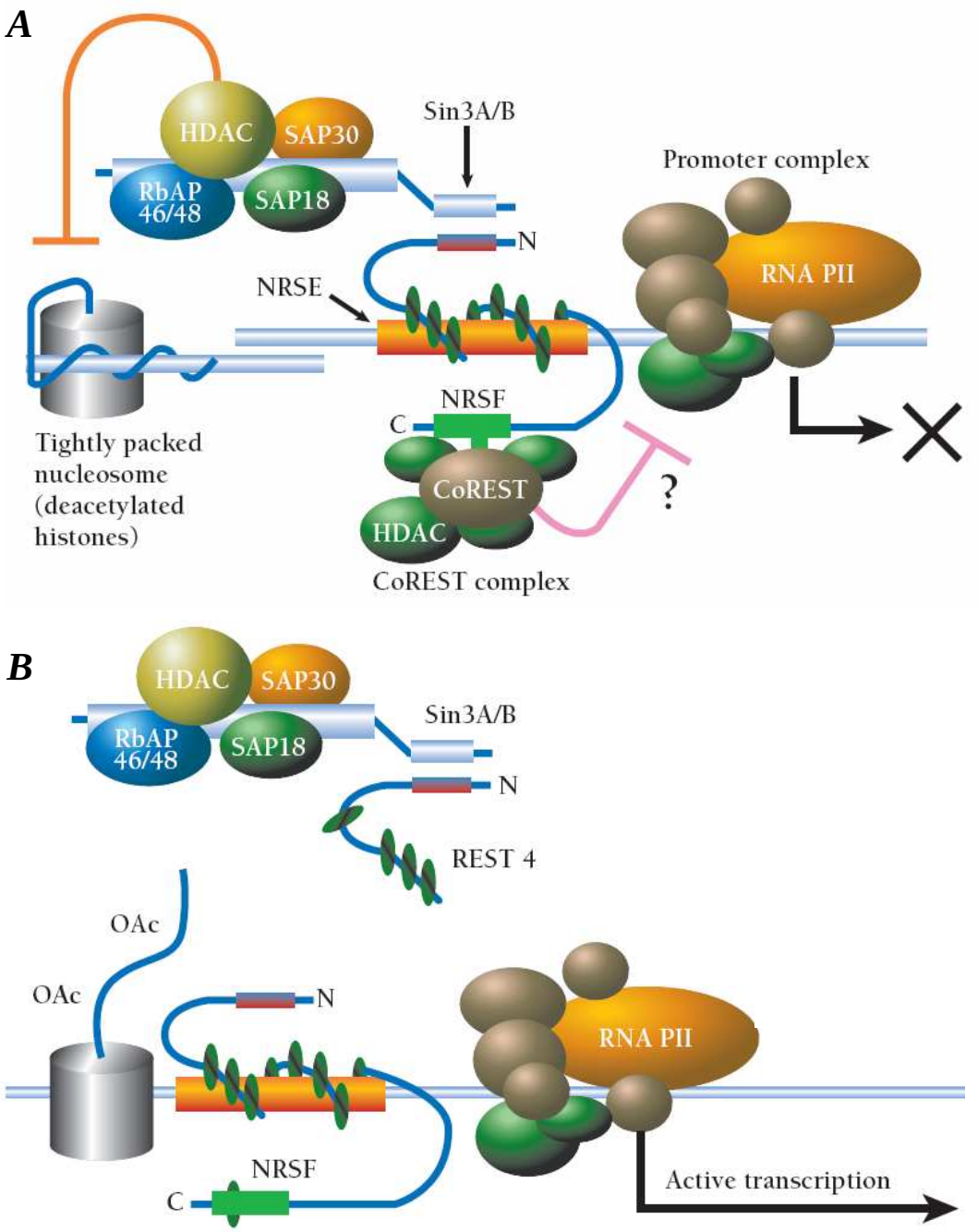


Figure 1.3 ***A.*** *Dual mechanism of repression via the REST-RE1 interaction. The N-terminal domain of RE-1-bound REST interacts with Sin3, which in turn recruits histone deacetylase (HDAC) and associated proteins to the promoter region. HDAC deacetylates lysine residues of nucleosomal core histones, which consequently limits the accessibility of DNA to transcription factors; the resulting inhibition of RNA polymerase activity (RNA PII) is indicated schematically by an X. In addition, a zinc-finger domain proximal to the C terminus of REST interacts with CoREST. CoREST recruits an HDAC complex that includes HDAC1 and HDAC2.* ***B.*** *Proposed mechanism for activation of gene expression by derepression. Although REST is schematized to bind to the RE1, the REST4 protein competes with REST for association with Sin3 corepressor complex. Thus sequestered away from interacting with REST, the corepressor–HDAC complex cannot remove acetyl groups (AcO) from nucleosomal core histones, and the RNA polymerase machinery (RNA PII) is thus able to form a transcriptionally active complex with the promoter.*

REST binds to the 21 bp RE1 site through eight zinc finger domains [Chong et al., 1995; Schoenherr and Anderson 1995]. REST-mediated gene repression is achieved by the recruitment of two separate corepressor complexes, mSin3 and CoREST [Andres et al., 1999; Roopra et al., 2000] (Figure 1.3), in addition to other corepressors such as the histone H3 lysine 9 (H3K9) methylase, G9a (also known as EHMT2) [Tachibana et al., 2001], and the NADH-sensitive corepressor, CtBP26. G9a and CtBP can be recruited directly by REST but have also been found associated with the CoREST complex. The mSin3 complex contains two class I histone deacetylases (HDACs), HDAC1 and HDAC2, the retinoblastoma-associated proteins RbAp48 (also known as RBBP4) and RbAp46 (also known as RBBP7) that are thought to interact with histones, and several other proteins with unclear function. The class II HDACs HDAC4 and HDAC5 are also associated with the mSin3 complex, although this association is lost upon phosphorylation of the HDACs by calcium/calmodulin-dependent protein kinase (CAMK). The CoREST complex contains HDAC1 and HDAC, a histone H3K4 demethylase, LSD1, the chromatin-remodelling enzyme, BRG1 (also known as SMARCA4), as well as CtBP, G9a, and a methyl-CpG-binding protein, MeCP2 (Figure 1.4). Thus, REST interacts with several complementary chromatin-modifying activities, with the recruitment of some of these enzymes being regulated by intracellular signals such as CAMK-mediated phosphorylation and NADH levels [Ooi et al., 2007].

To repress gene transcription, REST coordinates chromatin changes that are brought about by combinations of distinct chromatin-modifying enzymes. First, BRG1 is recruited by the C-terminal repression domain of REST, as part of the CoREST complex, and stabilizes REST binding to RE1 sites *in vivo* [Ooi et al., 2006] (Fig. 1.4). The BRG1-mediated increase in REST occupancy at RE1 sites requires both the bromodomain (which is known to bind acetylated histone H4K8) and the ATP-dependent remodelling activity of BRG1.

So, by remodelling chromatin, BRG1 seems to allow REST to gain better access to its chromatin targets by remodelling the local chromatin environment.

Once associated with the chromatin, REST mediates repression through histone-deacetylase, histone-demethylase and histone-methylase activities [Ooi et al., 2007]. Studies of co-repressor complexes that are recruited by REST have shown that these activities are interdependent, and are likely to occur in an ordered, stepwise fashion to mediate efficient gene repression (Figure 1.4). *In vitro*, the N- and C-termini of REST seem to form distinct repression domains that interact with different co-repressor complexes, and expression of REST proteins that contain either repression domain show partial repressor function in cell lines.

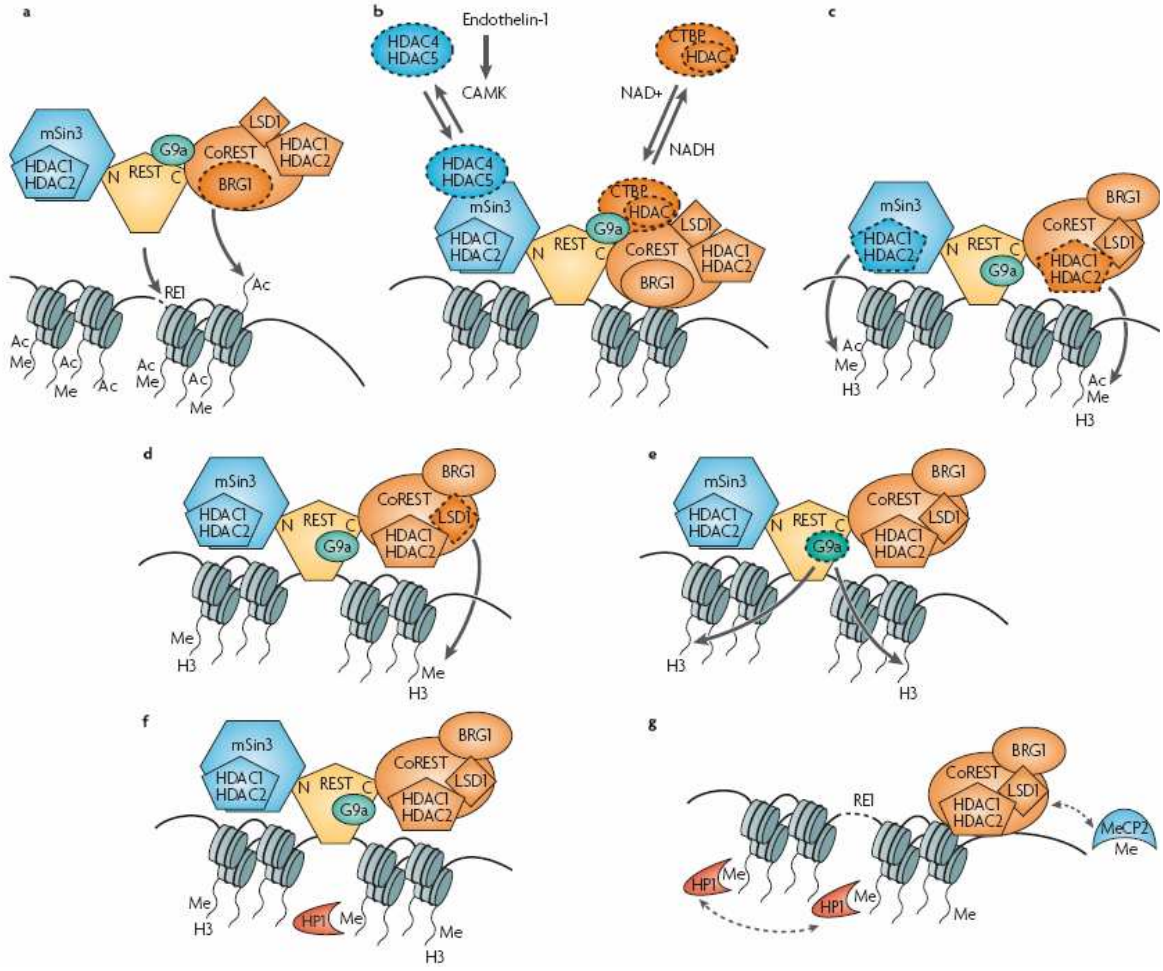


Figure 1.4 *REST corepressors.* ***a*** *The initial step in REST repression is the recruitment of this trranscription factor to repressor element 1 (RE1) sites. The RE1 sites are recognized by the zinc-finger domain of REST and interaction with DNA is stabilized by the ATP-dependent chromatin-remodelling enzyme, BRG1. The bromodomain of BRG1 recognizes acetylated histone H4 lysine 8 (H4K8), and increased H4K8 acetylation results in increased REST recruitment. When bound to chromatin, the BRG1 complex repositions nucleosomes with respect to DNA, presumably allowing REST to form a more stable interaction with the DNA51.* ***b*** *The N-terminus of REST interacts with the mSin3 complex, which contains histone deacetylases HDAC1 and HDAC2 The C-terminus of REST interacts with the CoREST complex, which contains HDAC1, HDAC2, BRG1, the H3K4 demethylase LSD1 and the H3K9 methylase G9a.* ***c*** *Once REST is associated with DNA, HDACs that are recruited by mSin3 or CoREST remove acetyl groups from H3 and H4 lysine residues.* ***d*** *The removal of acetylation from H3K9 stimulates LSD1 activity, which removes di- and monomethylation marks from H3K431.* ***e*** *Removal of H3K9 acetylation by HDACs also provides a substrate for G9a-mediated methylation, although it is unclear whether G9a is recruited directly by REST or as part of the CoREST or CtBP complexes.* ***f*** *The result of REST recruitment and its associated corepressors is that several modifications that are associated with active gene transcription are removed, and at least one mark that is associated with gene repression is added, forming an altered chromatin landscape.* ***g*** *During neuronal differentiation, REST is lost, although CoREST and the methyl-CpG-binding protein MeCP2 can remain at RE1-containing genes when these are methylated. Long-term silencing of RE1-containing genes might involve a compact chromatin state that is mediated by interactions of HP1 proteins that are recruited to adjacent nucleosomes. (Ac, acetylated; Me, methylated).*

The REST N-terminus interacts with the mSin3 complex [Grimes et al., 2000; Huang et al., 1999], which is important for the function of several repressors, including Mad/Max, an inhibitor of cell proliferation, and p53, a tumour-suppressor protein.

Meanwhile, the C-terminal repression domain of REST interacts with the CoREST complex. CoREST is widely expressed in neuronal and non-neuronal cells [Andres et al., 1999].

Although the N- and C-terminal domains of REST can function in isolation, it is debatable whether they should be considered as distinct and independent of one another. Together, they provide multiple layers of control that give rise to robust repression of target genes.

Furthermore, overexpression of REST mutants that lack either repression domain results in only partial repression of RE1-containing promoters in multiple cell lines [Andres et al., 1999; Grimes et al., 2000; Huang et al., 1999], suggesting that, at least for some REST-regulated genes, the combinatorial action of the enzymes that are recruited to both repression domains is important.

The two co-repressors Sin3A and CoREST, have different spatio-temporal expression profiles through development [Grimes et al., 2000]. CoREST is found primarily in the head mesenchyme at around embryonic day 8.5 and becomes more widespread later in development, whereas Sin3A has a more ubiquitous expression pattern. This difference in availability of co-repressors will likely determine REST's effect on RE1-containing genes within a cell. It is likely that specific promoter architecture will also influence the activity of co-repressor complexes. The ability of promoter architecture to influence repressor function has been documented for the Rb protein, which represses transcription by recruiting HDAC to E2F sites [Brehm et al., 1999].

The mSin3 and CoREST complexes contain HDAC1 and HDAC2, and it is likely that either co-repressor complex is sufficient to deacetylate H3K9 and H3K14 [Bingham et al., 2007], a process that is required for subsequent demethylation of H3K4 by the CoREST-associated demethylase, LSD1. Deacetylation of H3K9 and K14 can also inhibit the association of the bromodomain-containing MLL complex, which contains a H3K4-specific methylase that antagonises REST function during neuronal differentiation [Wynder et al., 2005]. Presumably, reducing MLL recruitment would reduce the drive for H3K4 methylation, thereby abrogating its antagonistic effects with LSD1.

Once H3K9 is deacetylated, it becomes dimethylated by the histone methylase, G9a. Recruitment of G9a can be achieved independently of the CoREST complex, possibly as a part of a CtBP-containing complex. G9a has also been found to be associated with the CoREST complex, although it is unknown whether this is a functionally important mechanism for G9a recruitment by REST. The addition of methyl groups to H3K9 might occur in order to prevent its re-acetylation and the resultant recruitment of MLL. Demethylation of H3K9 might then be

possible only under the appropriate conditions, for example, by histone replacement or through a H3K9 demethylase.

This stepwise activity of REST's co-repressors leads to the removal of several marks that are associated with transcriptional activation, followed by their replacement with marks that are associated with transcriptional repression (Figure 1.4). This mode of action is relevant in the repression of many genes: histone hyperacetylation and H3K4 methylation are widely associated with transcriptional activation, whereas histone hypoacetylation and H3K9 methylation are widely associated with gene repression. In most cases, however, gene repression will involve multiple DNA-binding proteins, each recruiting individual, complementary corepressor complexes through a solitary corepressor interaction domain.

Through the action of its corepressors, REST mediates both transient repression and seeds long-term silencing at specific genes in different tissues. Repression of some genes, such as *Nppa* in cardiac tissue and *Kcnn4* (a calcium-activated potassium channel gene) in vascular smooth muscle, requires the continued presence of REST at their binding sites [Ballas et al., 2005; Kuwahara et al., 2003]. However, at other loci, such as *Bdnf* (brain-derived neurotrophic factor) and *Calb1* (calbindin), repression is maintained following the loss of REST in development. During neural stem-cell differentiation, although REST binding is lost from these RE1s, the CoREST complex remains bound and continues to mediate the repression of the associated genes. This continued repression seems to be facilitated by DNA methylation and the recruitment of MeCP2 [Ballas et al., 2005] (Figure 1.4). A recent structural study has shown that CoREST can bind DNA directly through one of its two SANT (SWI3/ADA2/N-CoR/TFIIIB) domains [Yang et al., 2006]. Moreover, CoREST and MECP2 can be co-immunoprecipitated in Rat-1 fibroblast cells [Lunyak et al., 2002]. Together, these data suggest that CoREST and MeCP2 form a stable repressive complex in the absence of REST at RE1-regulated genes that contain methylated DNA.

The mSin3 complex can also form stable interactions with chromatin, binding to histones, to maintain repression subsequent to H3 deacetylation but independent of H4-acetylation levels. It remains to be seen whether such stable mSin3 recruitment occurs at RE1 sites in the absence of REST.

Collectively, these data highlight potential mechanisms by which REST and other transcription factors that recruit mSin3 and/or CoREST could mediate long-term gene repression after only transient repressor occupancy by initiating stable corepressor-chromatin interactions. It is unclear how long such repression would last; in neurons, synaptic activity results in the loss of the corepressors from chromatin and increased expression of targeted genes [Ballas et al., 2005]. This process might be the result of MeCP2 phosphorylation, or DNA demethylation leading to loss of

MeCP2 recruitment. In other cell types, the complexes would probably be removed during cell division and would need to be re-established following mitosis

Although REST can recruit multiple corepressors, not all complexes are recruited at all genes. For example, in Rat-1 fibroblasts and mouse neural stem cells, REST recruits mSin3 and CoREST to the *Scn2a2* promoter, but only mSin3 is recruited at the *Stmn2* promoter [Lunyak et al., 2002]. The consequences of recruiting one complex as opposed to another will be a potential difference in the chromatin marks that are produced. Such differences in chromatin modifications might underlie the observation that REST binding can mediate both short-term repression and long-term silencing. The difference between stable and transient repression of REST targets is important when REST levels are reduced, for example, in neuronal differentiation, cardiac hypertrophy, vascular smooth muscle proliferation and colon cancer.

In addition to regulating chromatin structure, the REST complex can modulate transcription rates. Its binding to the TATA-box-binding protein (TBP) inhibits the formation of the pre-initiation complex [Murai et al., 2004], whereas its interactions with RNA polymerase II small CTD phosphatases (SCPs) inhibit the activity of the polymerase [Yeo et al., 2005]. Interactions that target the REST complex to promoters in this way could be particularly important for those RE1s that are located far from their corresponding transcription start sites.

1.3 Role of REST in neurogenesis and neural plasticity

Earlier studies revealed that REST is not expressed at high levels in differentiated neurons during embryogenesis. Using a mouse REST probe, the presence of REST in most nonneuronal cells, but not in neurons, was found in mouse embryos aged 11.5 to 13.5 days, suggesting that REST functions as a cellular brake for neurogenesis [Chong et al., 1995]. Gene-deletion experiments with REST-/- mice, also indicated that this transcription factor is required for repression of multiple neuronal genes during embryogenesis. In support of this view, it was subsequently observed that REST is transcribed in NSCs but that its transcription is blocked as the cells undergo neuronal differentiation [Ballas et al., 2005; Paquette et al., 2000]. Although REST repressor function is required to repress the transcription of multiple neuronal differentiation genes, its absence alone is not sufficient to activate these multiple target genes. In fact, gene deletion studies with REST-/- mice, described above indicated that the absence of REST in vivo resulted in the expression of very few of the REST target genes, such as the neuron-specific tubulin gene, in a subset of nonneuronal tissue, followed by embryonic lethality [Chen et al.,

1998]; this lack of REST did not activate other REST target genes. From this observation, the expression of multiple neuronal differentiation genes required both the absence of REST function and the presence of other promoter/enhancer-specific positive activators.

To further study the impact of REST-mediated repression on neurogenesis, a recombinant transcription factor, REST-VP16 was constructed [Immanemi et al.,2000], in order to examine what happened when the REST target genes were activated. The regulated expression of REST-VP16 in an NSC line generated from newborn mouse cerebellum rapidly converted the cells into a mature neuronal phenotype, enabling them to survive in the presence of mitotic inhibitors, which is a characteristic of mature neurons, and showed physiological activities such as synaptic vesicle recycling and glutamate-induced calcium influx [Su et al., 2006]. These results showed that direct activation of REST target genes in NSCs is sufficient to cause neuronal differentiation. These results also suggested that such simultaneous activation of REST target genes were not prohibitory for neuronal differentiation.

Although many studies have focused on determining the mechanism of REST-mediated regulation of neurogenesis by using neural stem cells, a novel and fascinating aspect of this regulation was discovered by Gail Mandel's group, who used the staged conversion of ES cells into NSCs and further differentiated the NSCs into mature neurons in the presence of retinoic acid [Ballas et al., 2005]. These researchers then validated their findings in cortical progenitors and cortical neurons isolated from mouse embryos. They found that REST and its corepressor complex use two distinct mechanisms to regulate the target neuronal genes. In the first mechanism, the RE-1 site of the promoter is occupied by the REST-corepressor complex, which represses chromatin such that the removal of REST from the chromatin derepresses these genes, resulting in expression (class I neuronal genes). In the second mechanism, the RE-1 site of the promoter is occupied by the REST-corepressor complex; in addition, distinct but adjacent methylated CpG sites are occupied by CoREST/HDAC and methyl-CpG-binding protein-2/mSin3/HDAC complexes. In this scenario, removal of REST from the chromatin alone does not activate these genes (class II neuronal genes) because of the additional repressor complexes located on the methylated CpG sites. The class II genes, however, can then be activated upon application of a specific stimulus, such as membrane depolarization, which relieves the additional chromatin repression and provides neuronal plasticity. Mandel's group also found that the REST gene is transcribed in both ES cells and NSCs. In ES cells, the REST protein is expressed at high levels, and the REST-corepressor complex occupies and strongly represses its target genes. As the ES cells transition into NSCs, most of the REST protein is degraded by the proteasomal pathway. In NSCs, as the level of the REST protein decreases, the REST-corepressor complex

still occupies and represses its target genes but less strongly than in ES cells, causing the target genes to be poised for transcription. Then, as the NSCs exit the cell cycle and differentiate into mature neurons, REST gene transcription is blocked by the binding of the unliganded retinoic acid receptor-repressor complex to the retinoic acid receptor element found on the REST gene promoter. In the absence of active REST transcription, the REST protein level diminishes, resulting in the expression of class I genes. Proper physiological stimulus will then activate the class II genes and contribute to neuronal plasticity.

REST has an essential role in development: mice that lack REST die by embryonic day 11.5 of embryogenesis. Although these mice appear normal until embryonic day 9.5, after this, widespread apoptotic death results in malformations in the developing nervous system and restricted growth [Chen et al., 1998]. Despite the fact that most studies have identified REST as a transcriptional repressor in non-neuronal cells, several lines of evidence suggest that it might have a role in transcriptional activation of RE1-containing genes during neurogenesis, and in adult neural stem cells. Unexpectedly, mutation of the *Chrnb2* (nicotinic acetylcholine receptor-β2) and *L1cam* (L1 cell-adhesion molecule) RE1s in the mouse result in loss of expression of these genes in some neuronal populations. Furthermore, inhibition of REST function in developing *X. laevis* embryos results in reduced expression of the RE1-containing genes *Scn2a2*, *Stmn2* and *Tubb3* (N-tubulin) in cranial and ganglia neurons.

REST-mediated repression of RE1-containing genes seems to be cell-type specific. For example, in cells that express the RE1-containing *Nppa*, such as mouse and rat cardiac myocytes, REST is recruited to the *Nppa* RE1 site and mediates *Nppa* gene repression [Kuwahara et al., 2003]. However, *Nppa* is silent in rat PC12 cells even though REST is not expressed there. Interestingly, ectopic expression of REST in these cells does not result in REST binding to the *Nppa* RE1 site, even though it is recruited to the *Chrm4* (muscarinic acetylcholine receptor M4) and *Chat* (choline acetyltransferase) RE1 sites. Furthermore, the *Chrm4* gene is not reactivated by REST inhibition in the rat fibroblast JTC-19 cell line [Wood et al., 2003]. These data show that REST is not recruited to all RE1 sites in all REST-expressing cells.

These observations suggest that a compact chromatin conformation, which is refractory to transcription-factor recruitment, is important for the silencing of some RE1-containing genes. How such a compact chromatin environment is maintained is unknown, although clearly it does not require the continued presence of REST, and inhibition of histone deacetylation is not sufficient to disrupt it. One of the original proposals for a function for REST was a role in silencing neuronal-specific genes in non-neuronal cells. Given the absence of widespread expression of neuronal genes in non-neuronal tissue in mice that lack REST, this hypothesis can

be rejected. Several groups have suggested that this lack of widespread expression of neuronal genes is due to the lack of appropriate activators; however, it has been demonstrated that promoters of the *Stmn2* and *Scn2a2* genes are both highly active in non-neuronal cells upon removal of the RE1 site. Although REST expression seems important in developing neural cells, results of studies in which the normal REST function was inhibited have suggested that downregulation of REST might be important for neural stem-cell differentiation, as persistent REST expression prevents differentiation of the neuronal phenotype in mouse neural stem cells [Ballas et al., 2005] and causes axon path-finding errors in developing chick neurons [Paquette et al., 2000].

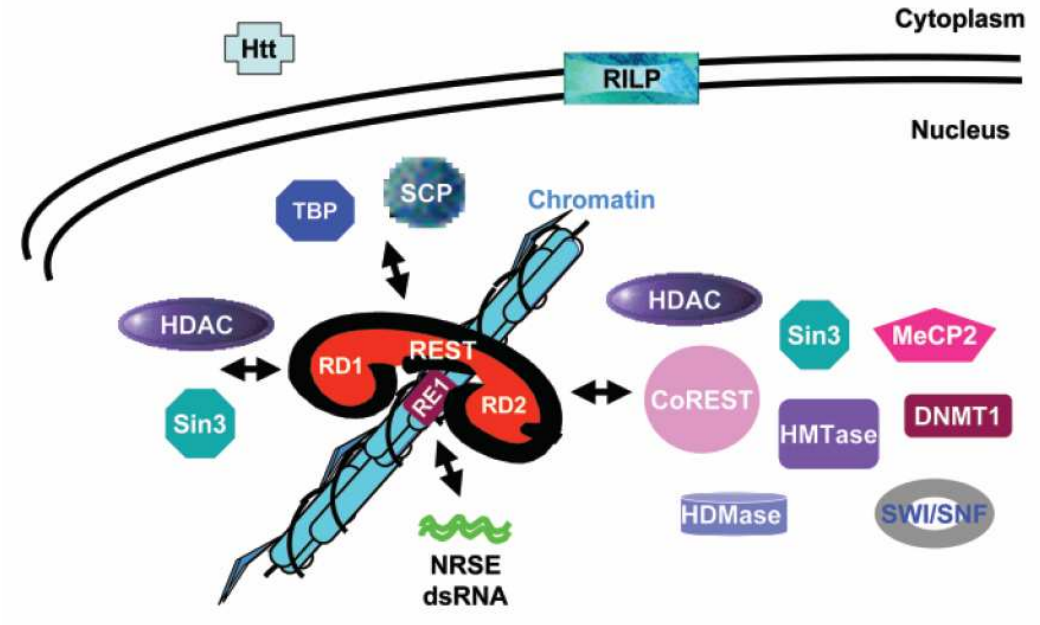


Figure 1.5 *REST is a transcription factor that serves as a giant communication hub for the cell. The activity of REST can be modulated by multiple factors in a cell context-dependent manner.*

The transition from stem or progenitor cell to a post-mitotic neuron requires disarming REST. During cortical differentiation, post-translational degradation of the REST protein precedes both its dismissal from RE1 sites and transcriptional inactivation of the REST gene itself at terminal differentiation (Figure 1.6). In contrast to differentiation during embryogenesis, the differentiation of adult hippocampal stem cells to neurons occur via a small non-coding double stranded RNA (dsRNA) containing RE1 motif that converts REST from a repressor to an activator of neuron genes (Figure 1.6) [Ballas et al., 2005].

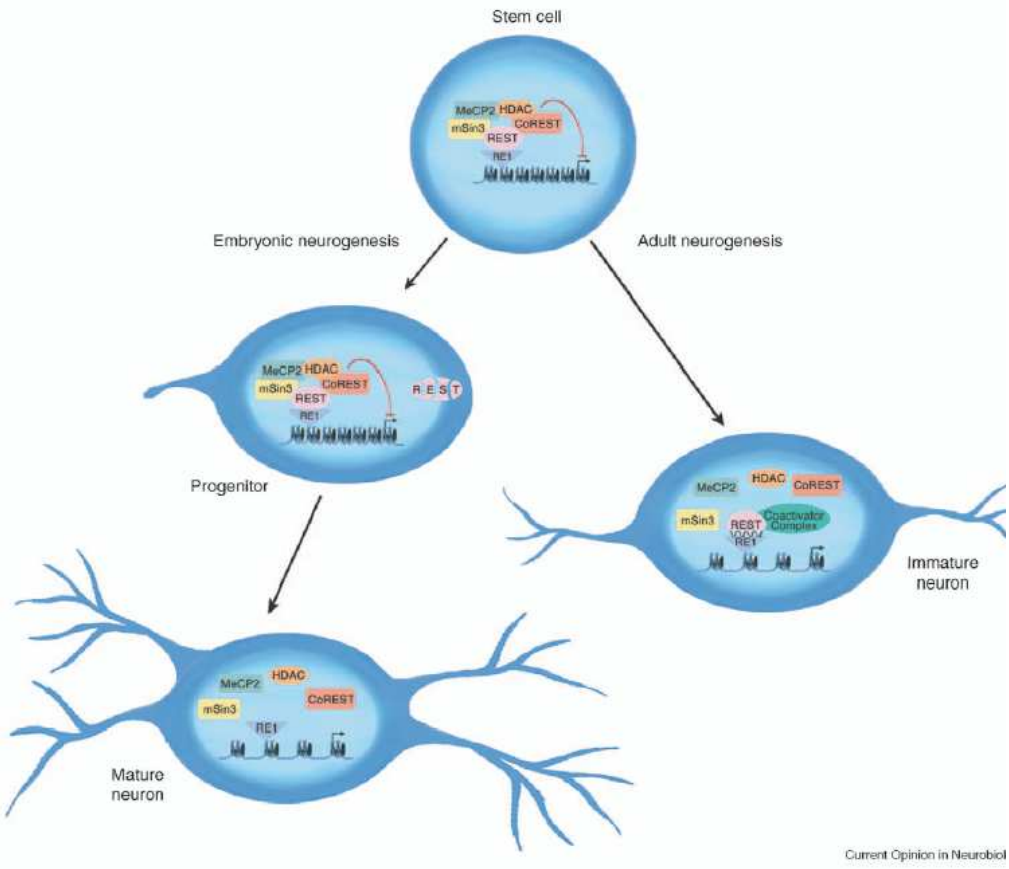


Figure 1.6 *There are two separate models to explain REST regulation of neuronal genes during embryonic and adult neurogenesis. In both the embryonic and the adult neural stem cell, neuronal genes are actively repressed by a REST repressor complex and chromatin is relatively compact. (a) During embryonic differentiation, REST is removed at two distinct stages, first at the dividing progenitor stage by proteosomal degradation (broken pink oval), and then at terminal differentiation (mature neuron) by removal from chromatin and transcriptional repression. In the mature neuron, REST corepressors are dissociated from RE1 but still present, chromatin is relaxed and neuronal genes are expressed. (b) During differentiation of adult neural stem cells (right), REST remains on neuronal gene chromatin, and a small double stranded non-coding RNA containing RE1 (green wavy line between RE1 and REST), converts REST from a repressor to an activator by dismissal of corepressors and recruitment of coactivators.*

Other researchers reported a novel mechanism of synergistic transcriptional regulation seen in non-neuronal MSCs (mesenchymal stem cells) and its differentiated progeny (stroma) in comparison with neurons derived from MSCs. Specifically, they have reported the mechanisms by which the *TAC1* neurotransmitter gene is regulated in these cells. In undifferentiated MSCs and the stroma, REST and NFκB act synergistically to mediate repression of *TAC1* transcription. IL-1α stimulation or 6-day neuronal induction of MSCs suppresses REST and NFκB expression, thereby partially alleviating repression of *TAC1*. IL-1α stimulation of the stroma or 12-day

neuronal induction of MSCs results in undetectable expression of REST and NFκB, thereby eliminating repression of *TAC1* [Greco et al., 2007].

1.4 REST and non-coding RNA

MicroRNAs (miRNAs) are excellent candidates for regulating cellular phenotype. These small, highly conserved regulatory molecules modulate gene expression post-transcriptionally by targeting mRNAs for translational repression or degradation. miRNAs are produced from larger primary transcripts originating from distinct genes. The primary transcript is subjected to two processing steps, generating first a stem-loop precursor transcript that is exported to the cytoplasm and subsequently the ≈21- to 23-nt mature miRNA. One strand of the mature miRNA is preferentially incorporated into the RNA-induced silencing complex. This effector complex then is guided by the miRNA to target mRNAs bearing miRNA recognition elements in their 3'UTR [He and Hannon, 2004]. Since their discovery, miRNAs have been shown to play important roles in diverse biological processes, including developmental timing and patterning in *Caenorhabditis elegans*, apoptosis in *Drosophila melanogaster*, and insulin secretion of pancreatic islet cells. Evidence also supports the idea that miRNAs may be important during late vertebrate development, particularly for lineage differentiation of various tissue types [Conaco et al., 2006].
Genome-wide analysis of RE1 sites revealed that some REST-binding sites are adjacent to non-coding RNA genes (encoding miRNAs). These small RNAs negatively regulate target protein levels by reducing translation efficiency and/or increasing mRNA turnover, and have significant roles in cell-fate determination and tissue development. The hypothesis that REST regulates miRNA expression has been experimentally validated; REST has been shown to regulate the expression of mouse miRNA genes *mir-9*, *mir-124* and *mir-132* [Conaco et al., 2006], all of which promote neuronal differentiation. REST itself might be a target of miRNAs such as *mir-153*, the expression of which it also regulates [Mortazavi et al., 2006]. Such reciprocal repression mechanisms are widespread in biology, and lead to the formation of a bistable switch which would be predicted to result in either high or low, but not intermediate, levels of REST. These postulated interactions create a potential feedforward loop that might have the effect of more quickly or definitively down-regulating REST mRNA, as REST activity begins to fall (Figure 1.7): in stem cells and progenitors REST acts by repressing hundreds of protein-coding genes and a handful of microRNA genes. Upon developmental progression to the differentiated states, REST is down-regulated, first at the protein level and then transcriptionally [Ballas et al., 2005]. Thus, its

targets are freed – perhaps sequentially according to RE1 strenght and number – for induction by various transcription activators. In this model, feedforward connections of microRNA onto CoREST and REST may modulate or accelerate the change from precursor cell to neuron. MicroRNA and splicing factors can go on to down-regulate other target genes not wanted in differentiating neurons [Mortazavi et al., 2006].

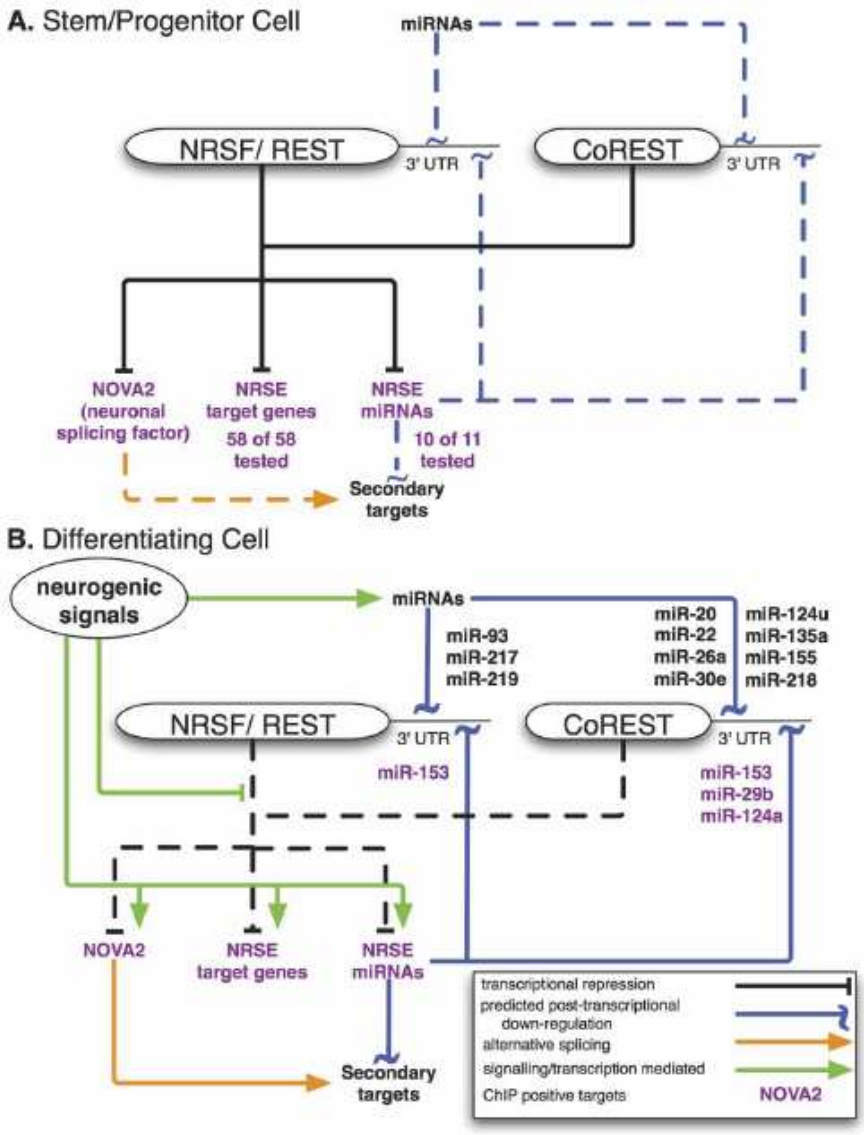


Figure 1.7 *REST gene regulatory network model. (A) REST in conjunction with CoREST and other corepressors prevents the transcription of several hundred targets, including neuronal splicing factors, transcription factors, and microRNAs, as well as many terminal differentiation genes in a stem cell. (B) Upon receiving neurogenic signals to terminally differentiate, the REST protein is degraded, which leads to derepression of its targets, which are now available to activators.*

Other regulatory loops involving REST corepressors have been postulated [Wu and Xie, 2006], and at least one REST corepressor, the small C-terminal domain phosphatase (SCP1), has been shown to be a target of a REST-regulated miRNA, *mir-124*, in the developing mouse brain [Visvanathan et al., 2007].

Non-coding RNAs are known to be important for regulating transcription, mRNA stability and translation. These data suggest that other RNA molecules might also be important in regulating gene transcription by modulating the function of specific transcription factors.

Although REST represses a large number of neuronal genes, including miR-124a, miR-124a inhibits the expression of many nonneuronal transcripts. In non-neuronal cells and neural progenitors in which REST is present, it binds to the gene chromatin of miR-124a and represses its expression such that non-neuronal transcripts can exist. In differentiated neurons, in which REST is no longer present on the miR-124a gene chromatin, miR-124a is expressed, causing rapid degradation of the non-neuronal transcripts. Thus, the combined reciprocal actions of REST and miR-124a provide a robust regulation of neurogenesis [Conaco et al., 2006].

In addition to potential modulation by miRNAs, it has been proposed that REST can be modulated by a dsRNA [Kuwabara et al., 2004]. Kuwabara *et al.* identified a 20-nucleotide dsRNA that contained an RE1 sequence, in adult rat hippocampal neural stem cells. The dsRNA is present at low levels in rat neuronal progenitor cells and at high levels in mature neurons, but absent from astrocytes. Unlike miRNAs, this dsRNA does not function by interacting with REST mRNA; rather, it seems to interact specifically with the REST protein to alter its activity. Ectopic expression of this dsRNA in rat neural progenitor cells led to increased expression of the RE1-containing genes *Gria2* (also known as *GluR2*) and *Stmn2*, and induction of neuronal markers. The mechanism of action of the dsRNA is not entirely clear, although the increase in *Gria2* and *Stmn2* required both the presence of RE1 in the promoter and the dsRNA, suggesting that the dsRNA converts REST from a repressor to an activator. Furthermore, the dsRNA does not alter the amount of REST that is bound to the *Gria2* and *Stmn2* genes; instead, its expression is associated with an increased presence of the histone acetyltransferase CBP and increased histone-acetylation levels at these genes. The implications of the NRSE dsRNA in neuronal differentiation are enormous and suggest a therapeutic potential in neuroregeneration research. It will be interesting to determine whether neurogenic stimulation such as exercise upregulates the expression of the NRSE dsRNA in adult mice.

1.5 REST function and disease

Most of our understanding about the role of REST in normal adult tissue has actually come from studies of disease states in which REST function is altered.

REST has been implicated in diverse diseases, highlighting the importance of REST-mediated regulation to the integrity of the cell. For example, REST was identified as a tumour-suppressor gene in epithelial cells. Deletions of human chromosome 4q12, on which REST is located, are frequent events in colon cancer, and the loss of heterozygosity for REST was identified in one-third of primary human colon tumours and colon cancer cell lines [Westbrook et al., 2005; Coulson et al., 1999; Coulson et al., 2000]. A frameshift mutation in the REST coding region, resulting in a truncated protein that lacks the C-terminal repression domain, was identified in a primary colorectal adenocarcinoma (DLD-1). Finally, reduced REST function caused by increased expression of a splice variant of REST that lacks some of the DNA-binding domain and the C-terminal repression domain is associated with small-cell lung carcinomas: this alternative 50-base exon inserted to produce the truncated protein is not used in non-small cell lung cancer, so the extra exon is a potential clinical marker for small cell lung cancer [Coulson et al., 2000; Majumder et al., 2006]. Thus, the inhibition of REST function seems to be a common event that occurs in diverse tissues during normal physiological responses, as well as in disease. In fact, a reduction in REST mRNA levels is required for both vascular smooth muscle cell proliferation [Cheong et al., 2005] and in cardiac hypertrophy [Kuwahara et al., 2003].

Several human SCLC primary samples, along with established SCLC cell lines, and not normal bronchial epithelial cells or human brain tissue, expressed enormous levels of a truncated isoform of REST [Coulson et al., 1999; Coulson et al., 2000]. This isoform, similar to REST4, is likely to function as a dominant-negative inhibitor of REST activity in these cells, causing the expression of neuroendocrine markers such as arginine vasopressin. Other laboratories showed that the reduced REST activity seen in some other SCLC cell lines was due to the lack of REST protein expression rather than to the expression of the dominant-negative isoform, leading to the concomitant abnormal expression of REST target genes, such as the glycine receptor a1 subunit [Gurrola-Diaz et al., 2003; Neumann et al., 2004]. The tumor-suppressor function of REST was finally proven using an RNAi-based screen for tumor suppressor genes in human mammary epithelial cells; they discovered REST as one such gene. Blockade of REST function in the epithelial cells was found to cause a transformation phenotype, such as anchorage-independent growth. They went on to show that the REST gene was mutated in several primary colorectal cancer samples and established cell lines. Expression of the mutated, dominant-negative form of

REST in epithelial cells caused a stimulated PI(3)K pathway and subsequent transformation [Westbrook et al., 2005] (Figure 1.8).

Increased levels of REST are important in other physiological responses, occurring in rat hippocampal and cortical neurons in response to epileptic seizures and ischaemia. In ischaemia, increased levels of REST are associated with a downregulation of *Gria2*, which leads to increased calcium entry through GluR2-lacking AMPA receptors and subsequent neuronal cell death [Calderone et al., 2003]. In the CA1 region of the hippocampus, increased REST levels directly repress expression of the *Opmr1* (μ-opioid receptor 1) gene in inhibitory interneurons. Repression of *Opmr1* seems to be neuroprotective, possibly because of an increased GABA (γ-aminobutyric acid) release that reduces the level of neuronal activity [Formisano et al., 2007].

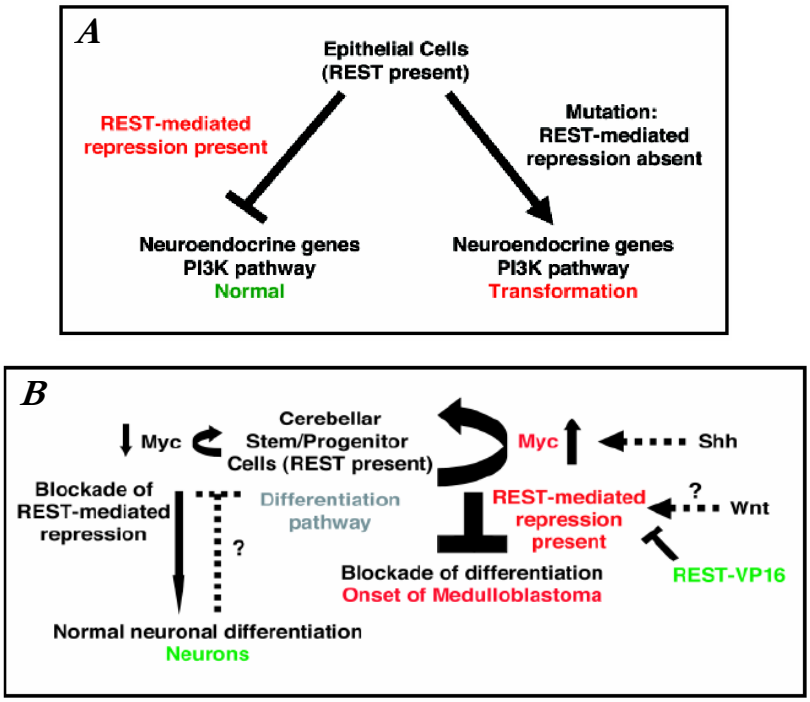


Figure 1.8 *Proposed tumor suppressor function in epithelial cells (A) and oncogenic function of REST in neuronal cells (B).*

Despite its tumour-suppressor function, in some neural cells, REST can actually behave as an oncogene (Figure 1.8). Increased REST expression has been implicated in neuroblastomas and medulloblastomas, in fact many human medulloblastoma samples and established human medullolastoma cell lines overexpressed REST compard with neuronal cells or brain cells [Palm et al., 1999; Fuller et al., 2005]. Moreover, REST alone was not sufficient to cause tumorigenesis:

it has been observed, in fact, that high levels of REST and MYC induce tumour formation in neural stem cells [Su et al., 2006].

Elevated REST expression has also been observed in pheochromocytomas that are associated with von Hippel–Lindau syndrome. Cells in these tumours are derived from neural crest progenitor cells and arrest at an early developmental phenotype. This developmental stage is characterized by reduced expression of REST target genes, reduced regulated but increased constitutive secretion of catecholamine and lack of expression of phenylethanolamine *N*-methyltransferase, the enzyme that converts noradrenaline to adrenaline. The ensuing constitutive release of noradrenaline drives increased blood pressure in patients with von Hippel-Lindau syndrome [Huynh et al., 2006].

Potential roles for REST and its target genes have also been implicated in the pathogenesis of Huntington disease. One of the factors that contributes to the disease phenotype is the inability of mutant huntingtin (HD) protein to interact with REST. Wild-type HD sequesters REST in the cytoplasm of mouse striatal neurons, thereby inhibiting its function. However, the mutant HD protein cannot interact with REST, resulting in higher levels of REST in the nucleus and repression of its target genes. One such target is the neuronal survival factor, BDNF, low levels of which are thought to contribute to neuronal degeneration in Huntington disease [Zuccato et al., 2003]. Moreover, increased binding of REST occurs at multiple genomic RE1 loci in HD cells, in animal models and in postmortem brains, resulting in decrease of RE1-mediated gene transcription. Novel therapeutic strategies could therefore be aimed at restoring the activity of the downstream targets of wild-type huntintin [Zuccato et al., 2007].

Recently, Tahiliani *et al.* showed that SMCX, a protein implicated in X-linked mental retardation and epilepsy, is associated with REST chromatin repressor complex. Although mutations in REST have not yet been linked to neurological disorders, mutations or dysregulated expression of the SMCX/REST-regulated genes SCG10, BDNF and SCN2A are implicated in mental retardation and epilepsy [Lossin et al., 2002; Nelson et al., 2001; Bahn et al., 2002]. Then, it is possible that loss of SMCX activity impairs REST-mediated neuronal gene regulation, thereby contributing to SMCX-associated X-linked mental retardation [Tahiliani et al., 2007].

Understanding the mechanisms that underlie the involvement of REST and its downstream targets in disease should identify putative therapeutic targets. For example, in smooth muscle proliferation and cardiac hypertrophy, changes in the expression of genes that encode ion channel components seem to be particularly important in causing abnormal physiology; therefore, targeting these channel proteins or augmenting REST function should prove beneficial.

These studies suggested that the abnormal overexpression of REST in neuronal cells, in which REST is normally not present, blocks these cells from terminal neuronal differentiation and produces the cancerous phenotype, perhaps by forcing the cells to persist in a stem/progenitor state.

2 INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM AND ITS PLEIOTROPIC FUNCTION

Since the discovery of Salmon and Daughaday in 1957, a considerable body of investigation has been devoted to defining the role of the IGF system in many tissues, including the brain. There is no doubt that these pleiotropic factors, in concert with their receptor and binding proteins, are involved in controlling key processes in brain development and traumatic or degenerative disorders of the nervous system.

The ubiquitous IGFs (Insulin-like growth factors), their cell membrane receptors, and their carrier binding proteins, the IGFBPs (Insulin-like growth factor-binding proteins), are expressed early in the development of the nervous system and are therefore considered to play a key role in these processes. *In vitro* studies have demonstrated that the IGF system promotes differentiation and proliferation and sustains survival, preventing apoptosis of neuronal and brain derived cells. Furthermore, studies of transgenic mice overexpressing components of the IGF system or mice with disruptions of the same genes have clearly shown that the IGF system plays a key role *in vivo*.

2.1 Overview of the IGF System

IGF-I and IGF-II are growth-promoting peptides, members of a superfamily of related insulin-like hormones that includes insulin and relaxin in the vertebrates The IGFs are major growth factors, wheras insulin predominantly regulates glucose uptake and cellular metabolism. They consist of A, B, C, and D domains. Large parts of the sequences within the A and B domains are homologous to the α- and β- chain of the human pro-insulin. This sequence homology is 43% for IGF-I and 41% for IGF-II. The C domain of the IGFs is not removed during prohormone processing; thus the mature IGF peptides are single chain polypeptides. The gene encoding IGF-I is highly conserved. Expression of the Igf-1 gene is affected at many levels including gene transcription, splicing, translation, and secretion [Russo et al., 2005].

The biological actions of the IGFs are mediated by the type I IGF receptor (IGF-IR), a glycoprotein on the cell surface that transmits IGF binding to a highly integrated intracellular signalling system [Kim et al., 2004]. Binding of the IGFs (IGF-I and -II) to the IGF-IR promotes intrinsic tyrosine kinase activity that phosphorylates the insulin receptor substrates (IRS-1 to IRS-

4), which then leads to the activation of two main downstream signaling cascades, the MAPK and the phosphatidylinositol 3-kinase (PI3K) cascades [van der Geer et al., 1994].

Expression of the IGF-IR gene has been detected in many tissues and is constitutively expressed in most cells [Santos et al., 1994]; its promoter is regulated *in vitro* and *in vivo* by transcription factors such as SP1 [Beitner-Johnson et al., 1995] and the transcription factor p53 [Ohlsson et al., 1998]. Various IGF-I receptor subtypes that present distinct structures or binding properties have also been described. Two of these subtypes, namely hybrid and atypical IGF-I receptors, have been particularly investigated in a variety of cell types. The atypical IGF receptors are characterized by their ability to bind insulin as well as IGFs with relatively high affinity [Siddle et al., 1994]. Hybrid insulin/IGF-I receptors have been reported in cells expressing both IGF-I and insulin receptors [Pandini et al., 2002]; however, the physiological significance of hybrid and atypical IGF receptors is unclear.

The IGF-II ligand has greatest affinity for a distinct receptor, the type-II or IGF-II receptor. More recently, high-affinity binding of IGF-II to an insulin receptor isoform (IR-A) has also been reported [Denley et al., 2004], thus suggesting that IGF-II might also signal via the insulin receptor.

A family of six high-affinity IGF-binding proteins (IGFBP-1 through IGFBP-6) coordinate and regulate the biological activity of IGF in several ways: 1) transport IGF in plasma and control its diffusion and efflux from the vascular space; 2) increase the half-life and regulate clearance of the IGFs; 3) provide specific binding sites for the IGFs in the extracellular and pericellular space; and 4) modulate, inhibit, or facilitate interaction of IGFs with their receptors [Jones et al., 1995; Firth et al., 2002]. IGFBP biological activity is regulated by posttranslational modifications such as glycosylation and phosphorylation and/or differential localization of the IGFBPs in the pericellular and extracellular space. It is therefore hypothesized that IGFBPs, in addition to stabilizing and regulating levels of diffusible IGFs, might regulate IGF-I cellular responses by facilitating receptor targeting of IGF-I or modulating IGF-I bioavailability in the pericellular space.

The effects of the IGFBPs are further regulated by the presence of specific IGFBP proteases, which cleave the binding proteins, generating fragments with reduced or no binding affinity for the IGFs [Jones et al., 1995]. Some IGFBPs, including IGFBP-2 and -3, can induce direct cellular effects independent of the IGFs [Firth et al., 2002].

2.2 The IGF-I signal transduction pathway

As previouslt described, the IGFs bind with high affinity to two cell surface receptors, the IGF-I receptor, which has a high degree of homology to the insulin receptor, and the IGF-II receptor. The IGF-IR binds IGF-I with the highest affinity but also binds IGF-II and insulin with lower affinity. Most of the observed IGF biological effects on cell growth, differentiation and survival depend on the activation of the IGF-I receptor signalling pathway, which shares multiple intracellular mediators with the insulin signalling cascade (Figure 2.1).

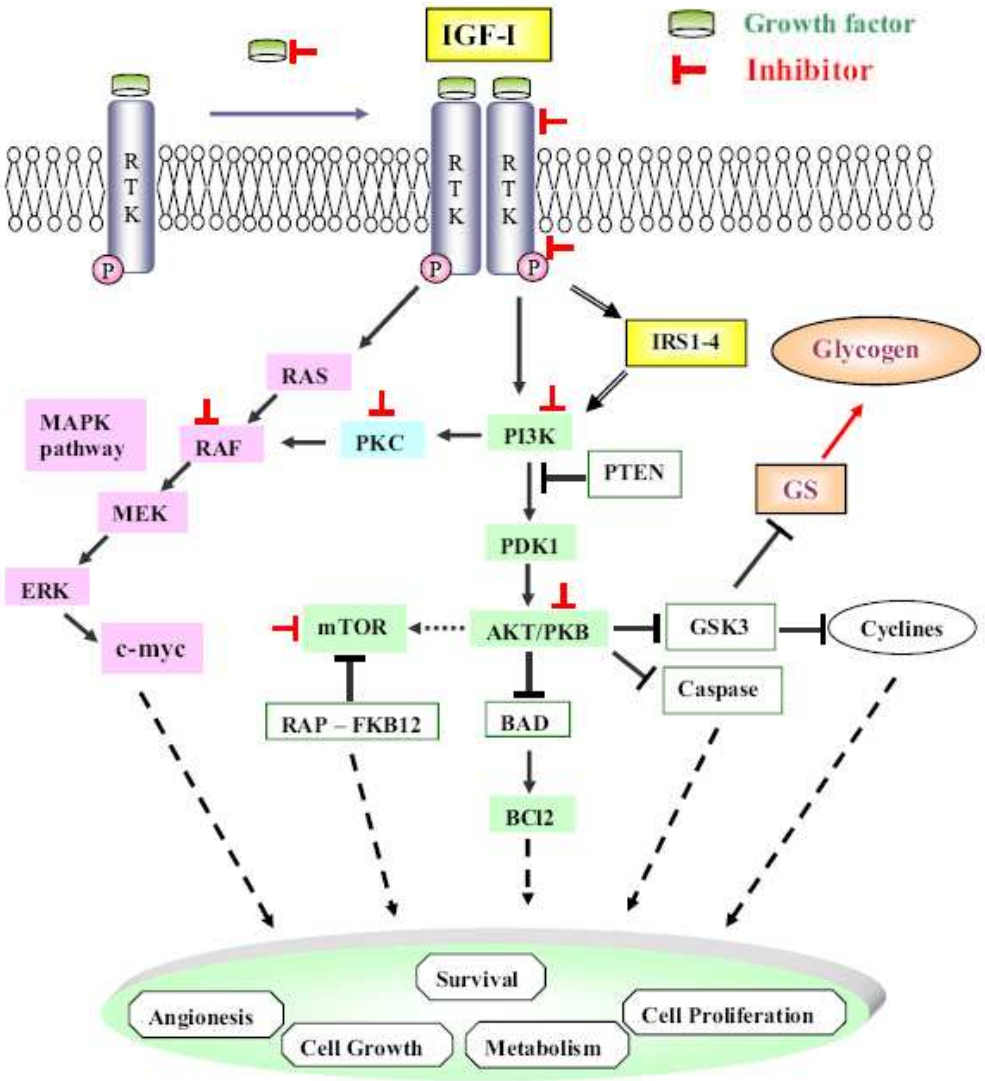


Figure 2.1 *IGF-I signalling pathway.*

The IGF-I receptor is a hetero-tetrameric protein, consisting of two identical extracellular α-subunits containing a cysteine-rich IGF-binding site, and two transmembrane β-subunits, bearing an intrinsic tyrosine kinase activity, which depends on an ATP-binding site and a cluster of three tyrosine residues. Binding of IGF-I to its receptor causes the activation of the tyrosine kinase, leading to autophosphrylation of the intrinsic tyrosines and tyrosine phosphorylation of multiple cytoplasmic substrates. Signalling emanating from the IGF-I receptor to either proliferation or differentiation could be determined at the receptor level by activation of specific tyrosine residues on the β-subunit, recruiting distinct signalling molecules further down the signal transduction pathway [Laviola et al., 2007].

The IGF-I receptor interacts with and signals to adaptor molecules, namely the insulin receptor substrate (IRS)-1/4 and the Shc (Src homology collagen) proteins, which serve as signalling nodes for distinct intracellular pathways. Phosphorilated IRSs recruit and activate class 1a phosphoinositide 3-kinase (PI3-kinase) via the two SH2 domains of the adaptor protein p85, leading to synthesis of membrane-associated phosphorylated inositols. These molecules, in turn, recruit and activate phosphoinosited-dependent kinases (PDKs), which then phosphorylate and activate other protein kinases, including Akt/protein kinase B, p70rsk and protein kinase C [Vanhaesebroeck and Alessi, 2000]. There is a substantial evidence that PI3-kinase activity mediates a wide range of insulin/IGF effects, including stimulation of glucose synthesis, protein synthesis, mitogenesis, inhibition of apoptosis, and regulation of gene transcription [Shepherd et al., 1998].

Another IGF-I-activated signalling pathway, which is coupled mainly to mitogenic and transcriptional responses, involves recruitment to both IRS-1 and Shc of the guanine-nucleotide-exchange factor Sos, *via* the SH2 domain of the adaptor Grb2. This lead to activation of the small G-protein Ras, which in turn activates the protein serine kinase Raf and the extracellular-signal-regulated kinase (ERK) cascade (Figure 2.2). Shc may actually compete with IRS-1 for a limited cellular pool of Grb2, and the extent of Shc/Grb2 binding correlates with the amount of insulin-activated ERK and c-fos transcription [Yamauchi and Pessin, 1994].

IGF-I receptor activation is also coupled to the stimulation of a family of MAP kinases, besides ERK-1/2, including Jun kinase (JNK)-1 and -2 and p38 MAP kinase [Laviola et al., 2007].

IGF-I has also been shown to induce tyrosine phosphorylation of the cytoplasmic protein Crk, which shares homology with Grb2, and may be involved in IGF-I receptor-mediated modulation of the Ras-MAP-kinase signalling pathway, thus enhancing IGF receptor-dependent mitogenesis [Okada and Pessin, 1997; Okada et al., 1998].

Finally, different G-proteins show a specific pattern of association with the insulin of the IGF-I receptor. Gαq has been shown to be a substrate for the insulin receptor, and has been implicated in insulin signalling to glucose transport; in contrast, IGF-I but not insulin receptors appears to be constitutively associated with Gi, and to induce the release of Gβγ subunits following activation [Dalle et al., 2001].

In neurons insulin/IGF-I receptor activation triggers the activation of the PI3-kinase/Akt pathway. The active, phosphorilated form of Akt is concentrated in granular deposits in IGF-I-expressing neuronal process. IGF-I-induced Akt phosphorylation appears linked to both production and translocation of neuronal GLUT4 from intracellular pools to nerve process membranes in the normal developing brain. These data suggest that IGF-I signalling serves an anabolic, insulin-like role in brain metabolism. Another target of insulin/IGF signalling *via* Akt in the brain is glycogen synthase kinase 3β (GSK3β): IGF-I acts in an autocrine manner to promote glucose uptake and storage as glycogen in developing projection neurons [Cheng et al., 2000].

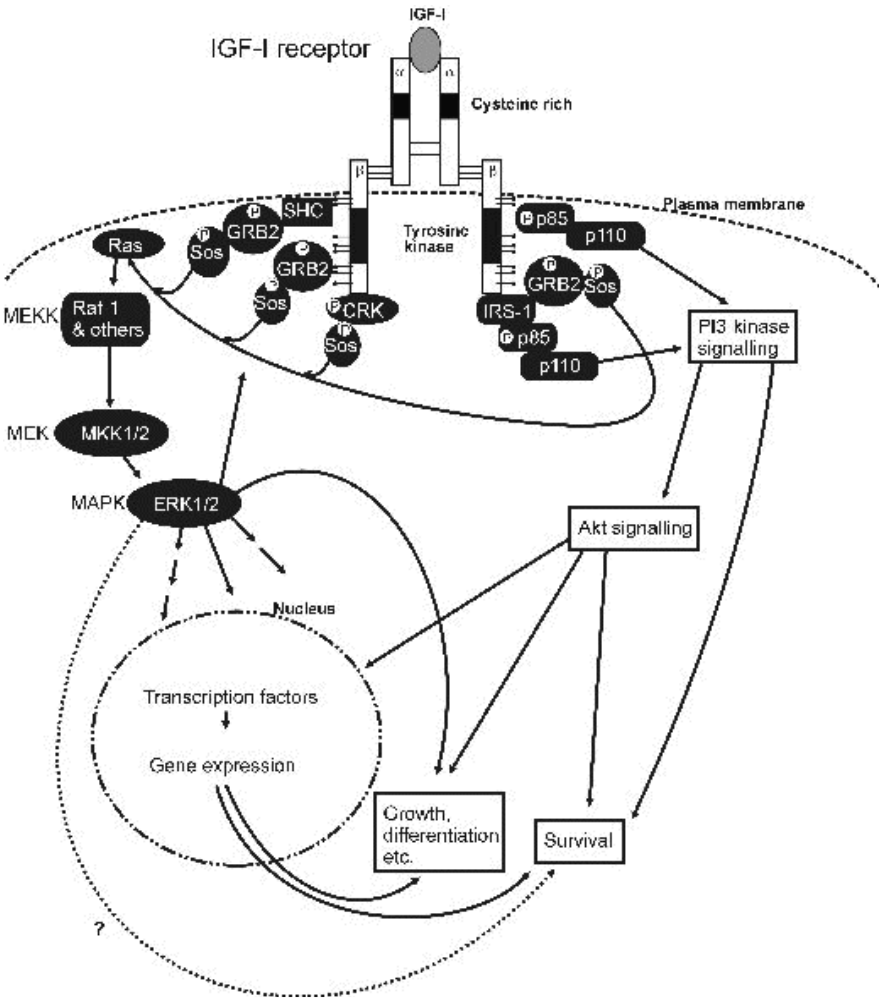


Figure 2.2 *IGF-I can activate multiple signalling pathway mediating several biological effects, like cell growth, differentiation and survival.*

2.3 IGF expression in the nervous system

IGF-I plays a key role in the development of the nervous system, with demonstrated effects on many stages of brain development including cell proliferation, cell differentiation, and cell survival [D'Ercole et al., 1996; Bondy et al., 2004]. Central nervous system (CNS) development begins in the embryo with the formation and closure of the neural tube, followed by the rapid division of pluripotential cells (stem cells), which then migrate to the periphery of the neural tube and differentiate into neural or glial cells. These cells form special structures such as nuclei, ganglia, and cerebral cortical layers, and develop a network with their cytoplasmic extensions, neurites. These complex processes are regulated by a number of growth factors including the IGFs. Although recent reports have demonstrated that postnatal circulating IGF-I might exert neurogenic/survival activity [Torres-Aleman et al., 1994; Carro et al., 2003], systemic IGF-I is not readily transported through the bloodbrain-barrier, and therefore local production of IGF-I is considered the primary source of the ligand (autocrine and paracrine action) for brain cells. During embryogenesis, IGF-I mRNA expression is detectable in many brain regions, with its expression being particularly high in neuronal rich regions such as the spinal cord, midbrain, cerebral cortex, hippocampus, and olfactory bulb. More precisely IGF-I is found expressed in neuronal cells with large soma and complex dendritic formations, including sensory and projecting neurons such as the Purkinje cells. In most neurons, IGF-I transcription decreases significantly postnatally, a decrease that correlates with the degree of cell maturation and reaches very low levels in the adult. IGF-II mRNA is abundantly expressed in the embryonic rat CNS. IGF-II is the most abundantly expressed IGF in the adult CNS, with the highest level of expression found in myelin sheaths, but also in leptomeninges, microvasculature, and the choroid plexus, all nonneuronal structures that enable diffusion of growth factors to their sites of activity [Russo et al., 2005].

The IGF receptors are widely expressed throughout the CNS, with high levels of expression found in specific regions and located to specific cell types. Given that IGF receptors are expressed from early stages of embryogenesis and throughout life, and that their ligands also show similar a "temporal-spatial" pattern of expressions, it is evident that the local brain IGF circuits are crucial modulators of the processes activated during brain development. The level of IGF-IR decreases to adult levels soon after birth [Baron-Van Evercooren et al., 1991] but remains relatively high in the choroid plexus, meninges, and vascular sheaths. It is thus not surprising that knockout of the IGF-I receptor gene [Liu et al., 1993] produced, in addition to *in utero* growth retardation, a strong brain phenotype, namely a small brain. [Couce et al., 1992].

Whether the IGFs are produced locally or reach brain cells systemically, these molecules exert potent neurotrophic, neurogenic, and neuroprotective/antiapoptotic activities. A large number of *in vitro* studies have demonstrated that IGF-I promotes mitogenesis and differentiation in glial cells, oligodendrocytes, neuronal cells, adult stem cells, and brain explants, and regulates axon myelination. IGF-I enhances growth cone motility and promotes neurite outgrowth [Russo et al., 2005].

Studies using the IGF-I -/- mice models [Cheng et al., 1998] have clearly demonstrated that most of the IGF functions determined *in vitro* also apply to the *in vivo* situation, affecting a wide range of brain cells. The IGF-I null mice have reduced brain size and altered brain structures and show alteration of myelination processes. This appears to be the result of reduced oligodendrocyte proliferation and maturation, which is also associated with reduction of axonal growth [Ye et al., 2002]. A study from Vicario-Abejon *et al.* suggests that reduction of stem cell proliferation/differentiation in the IGF-I null mice might be the cause of reduced plasticity/maturation normally occuring in some brain regions (*i.e.*, olfactory bulb) postnatally [Vicario-Abejon et al., 2003]. However, actions of IGF are not limited to fetal life, but extend into postnatal and adult life, with effects on proliferation, neuronal differentiation, and maturation maintained in some regions of the adult brain. Furthermore, alterations in levels of IGF-I have been reported in the brains of aging rats, alterations that also correlate with deterioration of cognitive functions observed in elderly patients with low circulating IGF-I. In this regard, the IGFs have been shown to be neurotrophic factors, *i.e.*, they promote the survival and differentiation of neuronal cells, including sensory, sympathetic, and motor neurons (MNs). In fact, the IGFs are the only known growth factors that support both sensory and motor nerve regeneration in adult animals [Rabinovsky et al., 2003]. IGF-I is involved in brain plasticity processes, and it specifically modulates synaptic efficacy by regulating synapse formation, neurotransmitter release, and neuronal exicitability [Anderson et al., 2002].

IGF-I also provides constant trophic support to neuronal cells in the brain and in this way maintains appropriate neuronal function. Alteration of this trophic input may lead to brain disease as seen in neurodegenerative disorders such as Alzheimer's disease (AD), Ataxia telangiectasia, Huntington's disease, and Parkinson's disease, all variably responsive to IGF-I treatment. The ability of IGF-I (and IGF-II) to promote neuronal survival is associated with the ability of these factors to prevent apoptosis, and IGF-I appears to be a potent agent for rescuing neurons from apoptosis. [Russo et al., 2005].

2.4 IGF-I and neurogenesis

Neurogenesis in the adult brain is regulated by many factors including aging, chronic stress, depression and brain injury. In most brain regions of highly developed mammals, the generation of neurons is confined to a discrete developmental period. Thus, the majority of neurogenesis is terminated soon after birth [McKay, 1989]. However, exceptions are found in specific areas that retain their ability to generate new neurons well into adulthood. In the adult mammalian brain, new granule neurons are continuously generated from progenitors cells located in the subgranular zone of the hippocampal dentate gyrus. Moreover, new neurons can also be generated from a second pool of progenitor cells residing in the subventricular zone. These neurogenic regions have been found in the brains of all mammalian species investigated, including the human brain [Eriksson et al., 1998; Roy et al., 2000].

During development, regulation of proliferation and neural cell fate in the central nervous system (CNS) is determined by complex interactions between a number of growth factors and neurotransmitters. Neurogenic regions in the adult brain must retain some of these features of early development to enable the proliferation, differentiation and migration of progenitor cells. Furthermore, many of the proteins that are upregulated following brain injury in adults have demonstrated effects on normal growth and development of the neonatal and early postnatal brain. Some of these promote cell survival during development and therefore their upregulation in tissue surrounding the injury may be an attempt by the CNS to protect cells and thus minimize the impact of the damage. These factors may also mediate the induction of neurogenesis and extensive rearrangement of surviving pathways seen following injury to the CNS in the adult rodent [Anderson et al., 2002].

The influence of IGF-I on the developing nervous system is well described, with demonstrated effects on many stages of brain development including cell proliferation, differentiation and survival. On the other hand, the influence of IGF-I on neurogenesis in the adult nervous system is only now just emerging. Research to date indicates that IGF-I has many of the same effects on neural progenitor cells in the adult CNS as it does in the developing nervous system. Mice that overexpress IGF-I display augmented brain size, characterized by a greater number of neurons. Conversely, disruption of the gene for either IGF-I or its receptor leads to pronounced brain growth retardation in both animals and humans. These profound effects of IGF-I mutations suggest that IGF-I modulates neuronal development [Anderson et al., 2002].

IGF-I drives proliferation of embryonic CNS progenitors as well as promoting neuronal lineage from these precursor cells. Moreover, IGF-I affects many aspects of neuronal maturation

including neurotransmitter production and electrical activity. The effects of IGF-I on proliferation and neuronal differentiation appear to be conserved in neurogenic regions during adulthood. Adult rat hippocampal progenitors incubated with IGF-1 not only proliferated faster, but also exhibited a greater rate of neurogenesis than control cultures. Given that IGF-I induces proliferation, neurogenesis and cell survival, it is difficult to definitively determine the mechanism behind the observed increase in newborn neurons with the information available. Further studies are needed to clarify whether it is an effect on cell survival, neurogenesis or a combination or both. Although neurogenesis has been demonstrated in the hippocampal dentate gyrus of the adult human brain, the consequences of this have yet to be determined. Furthermore, the factors that control in vivo neurogenesis in the adult mammal under normal conditions are only just beginning to be elucidated.

Interestingly, the rate neurogenesis in the adult brain can be affected by aging, stress and injury and all of these conditions can be associated with changes in IGF-I levels. In rodents there is a clear association between the rate of hippocampal neurogenesis and performance in spatial memory tests, suggesting that hippocampal neurogenesis is involved in spatial learning and memory [Shors et al., 2001].

The observed decline in memory with increasing age may be explained in part by age-related reductions in hippocampal neurogenesis as described in animal models [Cameron and McKay, 1999]. In rodents, aging is associated with reductions in the expression of IGF-I and its receptor in particular brain regions, including the hippocampus and reductions in serum IGF-I are observed in aging humans [Ghigo et al., 1996]. Interestingly, imaging studies have demonstrated decreased hippocampal volume in patients with stress-related neuropsychiatric disorders such as post-traumatic stress disorder, Cushing's syndrome, recurrent depressive illness and schizophrenia; this may be due to stress-induced reductions in granule cell proliferation. Thus, it is possible that suppression of neurogenesis may contribute to depression.

Neurogenesis may also play a part in the improvements in functional recovery that are commonly observed following adult brain injury. Until recently, these improvements were solely attributed to the rearrangement of surviving neuronal pathways. Although some of the improvement is probably mediated by this mechanism, we now need to consider the role that neurogenesis may play in regeneration. Excitingly, recent studies have demonstrated increased rates of neurogenesis in both the subventricular zone and the dentate gyrus in response to injury to the adult rodent brain. Furthermore, newborn neurons have reportedly been found in the otherwise non-neurogenic regions such as the periinfarct cortex [Zhang et al., 2001].

The presence of new neurons in regions such as the cortex following injury to the brain indicates that the environment surrounding the damage must alter to support either neurogenesis from local, otherwise quiescent progenitors or the migration of newborn neurons from regions such as the SVZ. Given that injury to the adult brain is associated with the accumulation of IGF-I and IGFBPs in the brain parenchyma as well as an increase in the number of IGF-I receptors, it is possible to speculate that IGF-I may take part in supporting neurogenesis following injury. The finding that neurogenesis is induced by injury suggests that it may also contribute to functional recovery, however this has yet to be determined [Anderson et al., 2002].
IGF-I induces neurogenesis from adult-derived neural precursors in vitro and has similar effects on the hippocampal progenitor population in vivo. Therefore, stimulating neurogenesis via the IGF-I system may represent an attractive treatment strategy in some situations. This may be achieved directly by infusing IGF-I, or indirectly, using simple protocols involving exercise training.

2.5 The IGF system and neuroendocrine cross-talk in the nervous system

In vivo, growth factors such as IGF-I do not exist in isolation. Hence, the presence of other growth factors (Figure 2.3) may further modulate the biological activity and cellular responses of IGFs. This modulation can be seen in the neurogenic activity of IGF, which in many cases requires the presence of basic FGF or FGF-2 to function. Increased interest has recently been devoted to the interaction of IGF-I with other "classic" neurotrophic factors. A study by Corse et al. [Corse et al., 1999] has investigated the potential effects of neuroprotective factors including IGF-I, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin (NT)-4 and -3 (NT-4/5, NT-3), and ciliary neurotrophic factor on mature motor neurons. Only IGF-I, glial cell line-derived neurotrophic factor, and NT-4/5 were found to be potently neuroprotective, pointing to the potential combined use of these neuroprotective factors in treatment of neurodegenerative diseases. Subsequently, the results from a number of phase III clinical trials with nerve growth factor, brain-derived neurotrophic factor, and IGF-I [Yuen, 2001] indicate that these neurotrophic factors may find an application in degenerative disorders or injury of peripheral nerves and motor neurons.

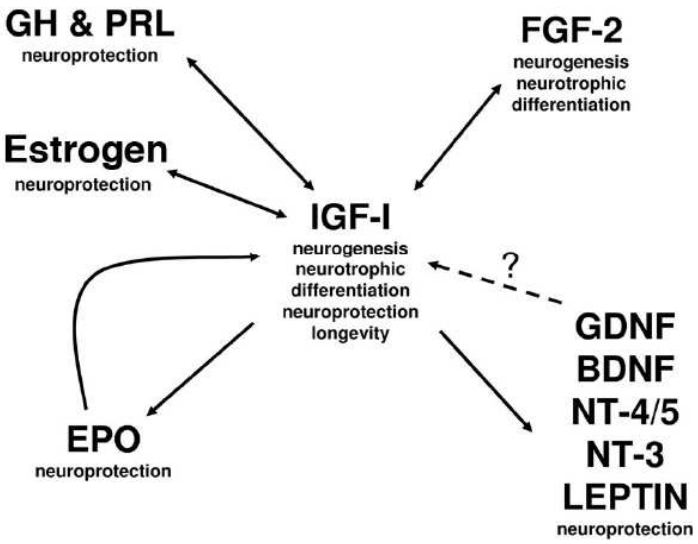


Figure 2.3 *Neuroendocrine cross-talk. In vivo, growth factors such as IGF-I do not exist in isolation.*

Erythropoietin (EPO) is traditionally known as a hematopoietic cytokine produced by the fetal liver and adult kidney in response to hypoxia. However, the expression of EPO and EPO-receptors in the CNS and the up-regulation of EPO by hypoxic-ischemic insult [Ghezzi et al., 2004] suggest that this cytokine is an important mediator of the brain's response to injury. These findings suggest the presence of a potential complex synergistic cross-talk between the IGF and the EPO system involving both activation of common intracellular signalling pathways and regulation of gene expression. The presence of synergistic cross-talk between the IGF and the EPO system in neuronal cells has recently been demonstrated: in fact EPO can exert a more immediate neuroprotective action when administered in concert with IGF-I. The neuroprotective mechanism, after coadministration of EPO and IGF-I, involved synergistic activation of the PI3K–Akt pathway [Digicaylioglu et al., 2004]. These findings further support the concept that the coadministration of synergistic neuroprotective agents rather than a single agent might provide improved therapeutic outcome. Thus, treatment with appropriate combinations of EPO and IGF-I could be a future therapeutic strategy for a variety of acute neurological events [Digicaylioglu et al., 2004].

2.6 Altered expression of the IGF-I system in malignancies of the nervous system

Within the CNS, the IGF family is critical for normal development: aberrant expression and/or activation may be associated with CNS malignancy. IGF-IR plays a crucial role in the induction and maintenance of the malignant phenotype. Increased expression of IGF-I, IGF-II, and IGF-IR is present in a wide range of human cancers, including lung, breast, thyroid, and prostate carcinomas, rhabdomyosarcomas, leukemias, and the peripheral nervous system tumor neuroblastoma and in numerous CNS tumors, including low-grade gliomas, glioblastomas, medulloblastomas, astrocytomas, ependymomas, and meningiomas [Zumkeller and Westphal, 2001]. IGF-IR overexpression leads to cellular transformation, tumor cell proliferation,and growth, whereas disruption of IGF-IR expression reverses the transformed phenotype [Adams et al., 2004].

Expression of the IGF family in CNS malignancy has been most thoroughly investigated in glioblastoma. IGF-IR expression is higher in glioblastomas than in normal brain, and glioblastoma cell lines exhibit a dose-dependent IGF-I stimulation of both receptor autophosphorylation and thymidine incorporation, indicating a functional receptor [Merrill and Edwards, 1990]. Both ligands for the IGF-IR, IGF-I and IGF-II, have increased mRNA transcript formation in glioblastoma compared with normal brain, and the major IGF-II transcript of 6.0 kb found in glioblastoma is similar to that found in fetal brain. The IGF ligands are also secreted into the CSF of glioblastoma patients. Most of the IGF binding proteins, including IGFBP-1, -2, -3, -5, and -6, are expressed in glioblastomas, with the most abundant being IGFBP-3. In addition, glioblastomas express membrane-bound forms of the binding proteins, and IGFBP-3 is secreted into culture media [Zumkeller et al., 1998]. Much of the *in vitro* work on the role of IGF-IR in transformation has been performed in rat glioblastoma cell lines. In this work, researchers have found through mutational analyses that distinct regions of IGF-IR are critical for the biological actions of the IGF ligands, including mitogenesis, transformation, and differentiation, through the induction of specific downstream targets.

IGF ligands acting through the IGF-IR are known to affect several aspects of glioblastoma tumor formation in addition to growth and transformation. IGF-I stimulates the migration and invasion of glioblastoma cell lines in culture, prerequisites for tumor cell metastasis. IGF-I acts as a potent survival factor in glioblastoma cell lines, even overcoming proapoptotic stimuli. Glioblastoma cell treatment with IGF-I triple helix-forming DNA, antisense IGF-IR, kinase-defective IGF-IR, or additional IGF-IR mutant dominant-negative constructs all induce growth suppression and/or apoptosis, resulting in decreased tumor growth. This results have led the way

for future work on IGF-IR inhibition and ligand interference in glioblastoma patient therapy [Zumkeller, 2002].

Although IGF ligand levels are increased in the majority of CNS tumors, ligand increases are not detected in the CSF, indicating that IGFs act locally in an autocrine and paracrine fashion to control tumor cell proliferation. This idea is supported by studies in both primary intracranial tumors and brain metastases from non-small cell lung tumors that show that interference with the IGF-I/IGF-IR autocrine signaling prevents cell growth [Patti et al., 2000].

The IGF system is also important in neuroblastomas, which typically occur peripherally but are occasionally found intracranially. IGF expression is present in all stages of primary neuroblastoma tumours [Sullivan et al., 1995]. IGF-I or IGF-II coupled to IGF-IR promotes both autocrine and paracrine growth and survival of human neuroblastoma cell lines [El-Badry et al., 1991; Meghani et al., 1993]. IGF-IR over-expression protects neuroblastoma from apoptosis [Kim et al., 2004], whereas inhibition of IGF-IR using antisense strategies blocks tumour growth and induces regression of neuroblastoma tumours in mice. Because neuroblastoma is also thought to arise through improper differentiation, these studies coupled with the CNS tumour reports suggest a critical role for the IGF family in nervous system tumour development and growth.

2.6.1 Targeting the IGF/IGF-IR signalling in cancer therapy

Interference with IGF ligand and IGF-IR autocrine or paracrine signalling provides a novel therapeutic target for CNS malignancies [Reiss, 2004]. In fact, targeting IGF-IR expression and signalling is a new therapeutic approach to cancer treatment in numerous tumor types. A truncated IGF-IR that acts as a dominant negative receptor or antisense RNA to IGF-IR prevents metastatic breast carcinoma [Dunn et al., 1998;Chernicky et al., 2000]. A kinase-defective IGF-IR dominant negative receptor and α-IR3, the IGF-IR blocking antibody, inhibit glioblastoma tumor growth in mice [Seely et al., 2002]. A dominant negative IGF-IR introduced using retroviral infection decreases pancreatic tumor burden, particularly when used in combination with chemotherapeutic drugs. Antisense IGF-IR strategies also enhance the susceptibility of Ewing's sarcoma to doxorubicin-induced apoptosis, implying a use for IGF-IR disruption in combinatorial drug therapy. Other cancers for which IGF-IR interference is effective as a treatment strategy include melanoma, lung carcinoma, ovarian carcinoma, and rhabdomyosarcoma [Baserga, 1996]. One problem with these treatment strategies in the CNS is

delivery, although direct injection into brain tumors or implantation of antisense. However, gene therapy approaches will likely be more advantageous and less traumatic for IGF-I inhibition treatment paradigms in CNS tumors.

2.7 IGF-I therapy in nervous system disease

A role for endogenous IGFs in the injured brain is suggested by a number of studies showing the induction of components of the IGF system after transient unilateral hypoxic/ischemic injury and stroke in the rat model [Breese et al., 1996]. Local IGFBPs (i.e., IGFBP-2) play a key role in IGF-I delivery to the injured site (Figure 2.4). These findings might point to a role for cell-associated IGFBP-2 to modulate local IGF-I bioavailability. Nevertheless, these data might point to a potential role for IGFBP-3 as carrier/transporter of vascular IGF-I into the brain tissue in the early phases of the injury response, when local IGF-I availability and expression might not be sufficient to trigger the neuroprotective mechanisms.

IGF-I has also been explored in chronic neurological disease. Clinical trials of IGF-I have been conducted in MN (motor neuron) disease [Mitchell et al., 2002] based on preclinical *in vitro* and *in vivo* observations in a variety of rodent models. The results have been conflicting. A recent systematic review concluded that although IGF-I therapy may have a modest effect, the current data are insufficient for definitive conclusions [Mitchell et al., 2002]. It is also difficult in such studies to separate effects on the CNS from peripheral effects, for example, on muscle.

Similarly, it has been suggested that chronic IGF-I administration may have some therapeutic role in AD (Alzheimer's disease) [Dore et al., 2000]. IGF-I in vitro inhibits amyloid induced neuronal death, induces choline acetyl-transferase, and affects CNS amyloid-ß levels. Studies of this kind are limited by the poor transfer of IGF-I across the intact blood-brain barrier. However, IGF-I will cross the compromised blood-brain barrier, as in multiple sclerosis or other acute syndromes, and new delivery approaches are in development [Carrascosa et al., 2004]. Accumulating data support the therapeutic use of IGF-I in the treatment of ALS (amytrophic lateral sclerosis). As discussed earlier, IGF-I is essential for normal development and is the only known neurotrophic factor capable of supporting both sensory and motor nerve regeneration in adult animals. The ability of IGF-I to promote neuronal survival is directly related to its potent antiapoptotic properties. IGF-I prevents apoptosis in MN, glial cells, and muscle cells, all cell types affected by ALS. IGF-I serum levels are decreased in ALS patients could contribute to the development of disease [Torres-Aleman et al., 1998].

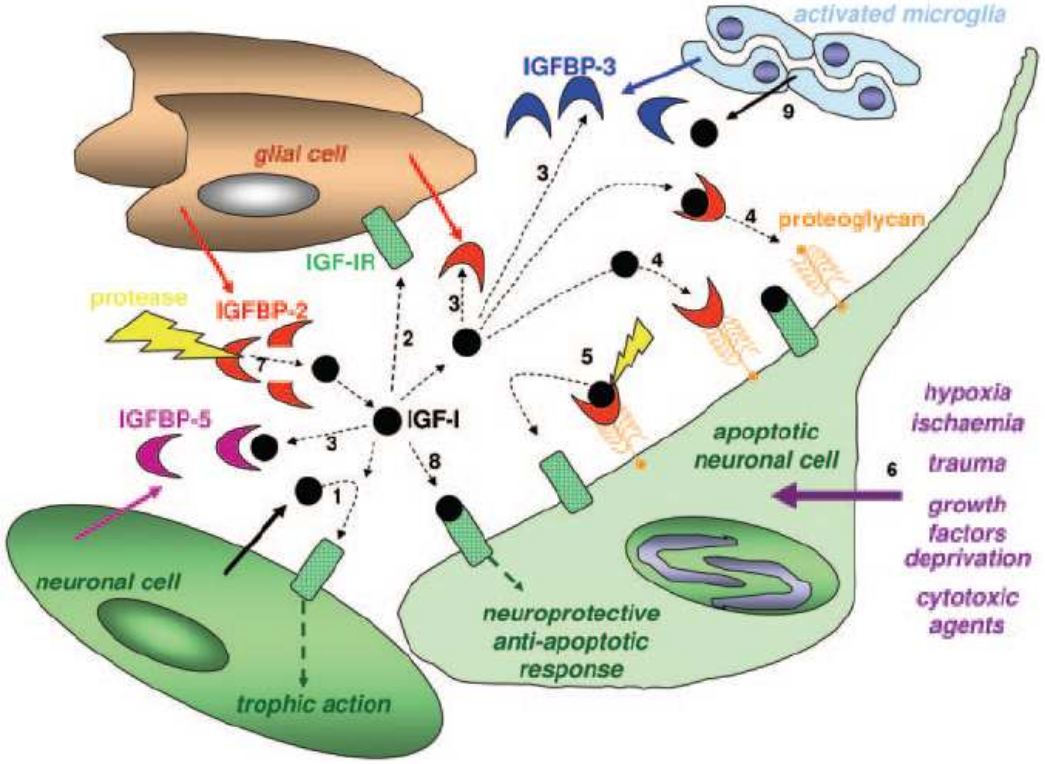


Figure 2.4 *A model for neurotrophic and neuroprotective actions of IGF-I in brain: after injury, IGF-I may act as endogenous neuroprotective agents and limit the degree of neuronal and glial loss.*

Evidence suggests that altered levels of growth factors and/or their receptors, which exert trophic and survival function in the nervous system, may underlie neuronal degeneration as seen in AD brain. Metabolic and hormonal functions decline in a variety of severe age-associated pathologies, including those associated with alterations in the insulin and IGF systems. AD patients show changes in circulating levels of both insulin and IGF-I, often associated with abnormal responses to insulin. Compelling evidence indicates that insulin and IGF-I have a direct effect on the metabolism and clearance of the Aß (ß-amyloid peptide). In neuronal cells, insulin inhibits Aß degradation, directly increases Aß secretion, and decreases the intracellular level of Aß peptides by stimulating their intracellular trafficking. Decreased circulating insulin and IGF-I might influence the development of neurofibrillary tangles, another AD "marker". This process appears to be a consequence of altered tau phosphorylation, a major component of neurofibrillary tangles. These findings indicate a potential key role for insulin and IGF-I in regulating tau protein phosphorylation, a failure of which augments the onset of neurofibrillary-tangle AD pathology. The role of other IGF system components, such as the IGFBPs, in AD

pathology remains unclear. The data discussed above point to insulin and IGF-I signaling as a potential therapeutic target in AD. The effects of IGF-I on key AD markers (i.e., amyloid toxicity, tau phosphorylation) suggest the potential usefulness of this growth factor in the treatment of neurodegenerative diseases. The primary challenge in translating successful use of IGF-I in laboratory research models to growth factor therapy in the clinic is delivery of IGF-I to the brain in sufficient concentrations to influence neuronal functions [Dore et al., 2000; Russo et al., 2005].

3 PROTEIN KINASE C (PKC)

The PKC (protein kinase C) family of serine/threonine protein kinases is widely recognized as a central element of signal transduction pathways. Conversion of extracellular signals into intracellular messages is ultimately realized by PKC through phosphorylation, and consequent modulation of activity, of a wide range of proteins implicated in cellular functions such as regulation of cell growth, cell proliferation, apoptosis and malignant transformation.

The protein kinase C (PKC) family represents a large group of phospholipid dependent enzymes catalyzing the covalent transfer of phosphate from ATP to serine and threonine residues of proteins. Phosphorylation of the substrate proteins induces a conformational change resulting in modification of their functional properties. The PKC family consists of at least ten members, divided into three subgroups. The specific cofactor requirements, tissue distribution, and cellular compartmentalization suggest differential functions and fine tuning of specific signaling cascades for each isoform. Thus, specific stimuli can lead to differential responses via isoform specific PKC signaling regulated by their expression, localization, and phosphorylation status in particular biological settings. PKC isoforms are activated by a variety of extracellular signals and, in turn, modify the activities of cellular proteins including receptors, enzymes, cytoskeletal proteins, and transcription factors.

3.1 Basic structure and properties of the protein kinase C family

Protein kinase C (PKC) comprises a multigene family of phospholipid-dependent, serine/threonine protein kinases. In mammals, 12 PKC isoforms (Figure 3.1) have currently been identified, with various PKC isoforms being usually co-expressed in the same neurons. Based on their molecular structures and sensitivity to activators, PKC isoforms are divided into three subfamilies: classical PKC (cPKC: α, βI, βII and γ); novel PKC (nPKC: δ, ε, η and υ); and atypical PKC (aPKC: ζ and λ/ι). All PKC family members share a structural backbone, mainly consisting of a regulatory domain at the N-terminus and a catalytic domain at the C-terminus. The regions are categorized as conserved regions (C1–C4) and regions that vary between isoforms (V1–V5) [Nishizuka et al., 1988; Kikkawa et al. 1989]. Moreover, in common is a pseudosubstrate domain in the regulatory region, closely resembling the substrate recognition motif, which blocks the recognition site and prevents activation [Blumberg, 1991; House and

Kemp, 1987]. The cPKC isoforms require both Ca^{2+} and phosphatidylserine, diacylglycerol or other activators for activation. They contain four homologous domains: two regulatory (the activator-binding C1 domain and the cofactor Ca^{2+}-binding C2 domain) and two catalytic (the C3 domain, containing the ATP-binding site, and the C4 domain, containing the substrate-binding site), interspaced with the isoforms-unique (variable, or V) regions. The nPKC isoforms lack the C2 domain and are Ca^{2+}-independent in activation. The cPKC and nPKC isoforms can thus be activated by diacylglycerol, phorbol esters and bryostatins, with cPKCs requiring Ca^{2+} as the cofactor for activation. The aPKC isoforms lack both the C2 domain and half of the C1 homologous domains and are insensitive to Ca^{2+}, diacylglycerol, phorbol esters or other PKC activators. All the PKC isoforms contain, near the C1 domain, an N-terminal pseudosubstrate motif, which binds to the catalytic domain in the inactive state, and functions as an autoinhibitory domain of PKCs. Each of the PKC isoforms is encoded by a separate gene, with the exception of the βI and βII isoforms, which are alternative splice variants [Alkon et al., 2007]. Consistent with their different biological functions, PKC isoforms differ in their structure, tissue distribution, subcellular localization, mode of activation and substrate specificity.

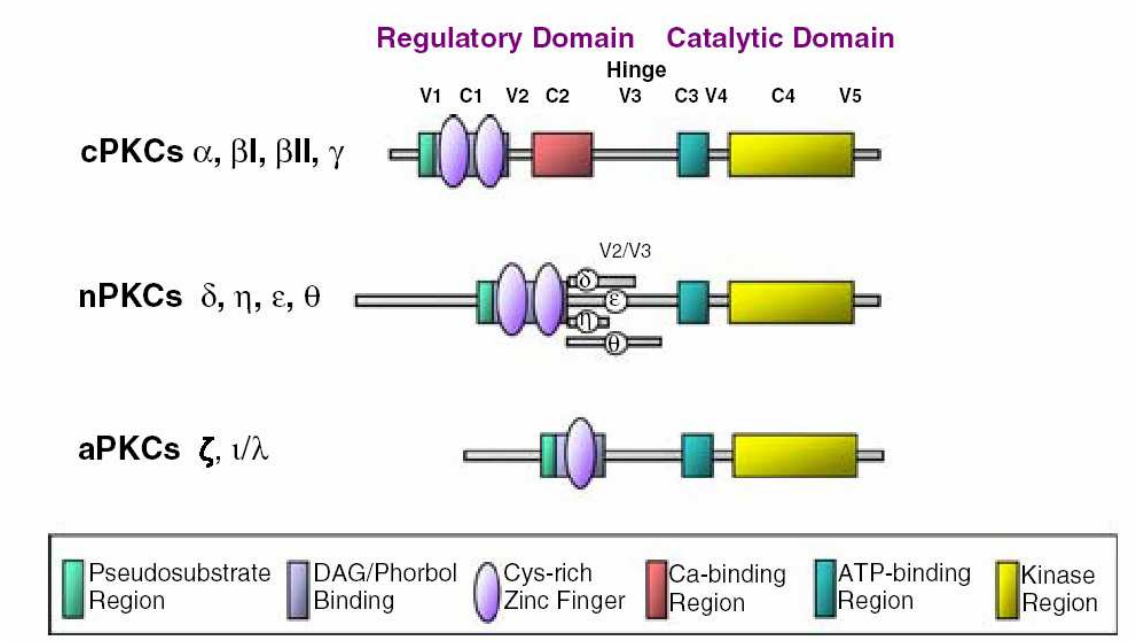


Figure 3.1 *Protein kinase C (PKC) family isoforms.*

3.2 Substrate specificity

One of the major regulatory mechanisms implicated in specific activities of PKC isoforms in cellular signalling is associated with phosphorylation of distinct target substrates. For example,

PKC α, β and γ are potent kinases for histones, myelin basic protein (MBP), and protamine [Hofmann et al., 1997], whereas PKC δ, ε and η do not exhibit this activity. Crucial for substrate recognition is the relief of the inhibitory pseudosubstrate region within the regulatory domain. This issue was studied using chimeras as well as by mutational analysis, which confirmed the role of the pseudosubstrate region in the selectivity of PKC α, ε and η [Dekker and Parker, 1994]. Representative examples for typical PKC substrates are STICKs (substrates that interact with C kinase), which are phospholipid-binding proteins. Phosphorylation of STICKs by PKC modifies their activity, reducing their binding to calmodulin and actin [Jaken and Parker, 2000]. Nevertheless, although various substrates have been linked to distinct PKC isoforms, it became clear that most PKC isoforms phosphorylate similar sequences.

As reported in several studies, substrate selectivity is quantitative in nature, where the affinity of each PKC to specific substrates is determined by Km values. Thus, other mechanisms must exist to direct these PKC isoforms to distinct signalling pathways. The levels of control include the multiplicity and quantity of PKC isoforms expressed in specific cellular settings, distinct tissue distribution, intracellular compartmentalization mediated by various adapter or scaffolding proteins, and modification of the phosphorylation state of the PKC isoforms and those adapters. Together, these regulatory processes provide the fine tuning of PKC action in numerous ways, depending on the particular stimuli and the cellular or tissue context [Breitkreutz et al., 2007].

3.3 Activation and degradation of protein kinase C

Activation of PKC depends on its catalytic competence and its targeting to membrane compartments (Figure 3.2). Catalytic competence is achieved by three sequential phosphorylation steps involving the activation loop, the turn motif, and the hydrophobic motif [Liu et al., 2002]. Targeting to membrane compartments is promoted by second messengers such as diacylglycerol and arachidonic acid and is stabilized by specific anchoring proteins (e.g. RACK1) [Schechtman and Mochly-Rosen, 2001]. For PKC isoforms α and ε, proteolytic degradation begins when dephosphorylation occurs at the three 'priming' sites required for activation. Phosphorylated PKC enters the ubiquitin-proteasome degradation cascade [Leontieva and Black, 2004].

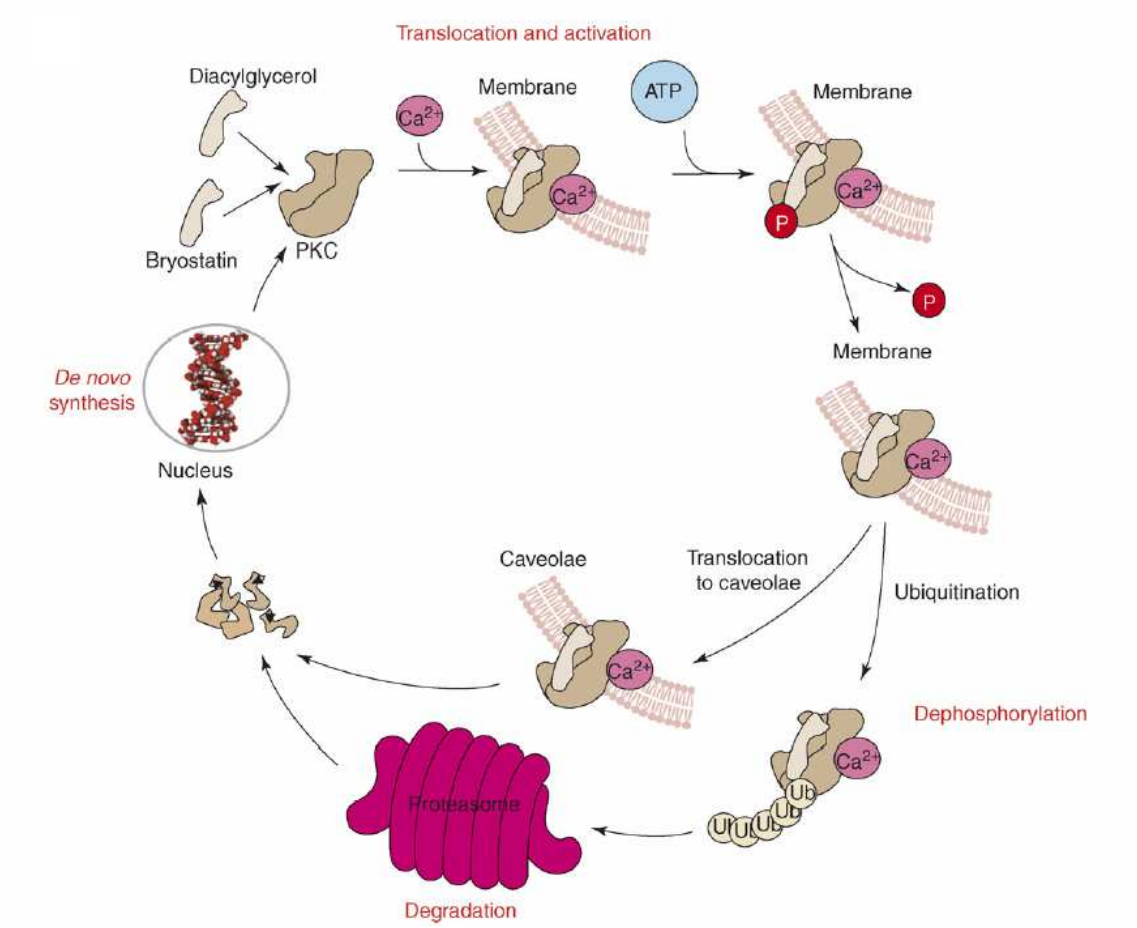


Figure 3.2 *Activation and down-regulation cycle of PKC.*

Furthermore, there is evidence that another degradation pathway involves caveolae-dependent trafficking of the active enzyme and subsequent proteasome-independent degradation. Bryostatin, a potent PKC activator, triggers both proteasome-dependent and proteasome-independent pathways for PKC α and ε degradation following PKC activation. Degradation (i.e. 'downregulation') of PKC isoforms is then followed by a prolonged increase of PKC isoform synthesis. Bryostatin and phorbol esters bind to the same site on PKC, the diacylglycerol (DAG)-binding site. However, the DAG-binding site is complex, and marked differences are observed among the PKC isozymes. Bryostatin is more effective than phorbol esters in downregulating PKC. Both conventional and novel isoforms of PKC are activated by the binding of the natural PKC activator diacylglycerol to the cysteine-rich C1A and C1B domains of PKC. Bryostatin or phorbol esters also produce activation by binding to the same activation site. In the presence of calcium, activation results in rapid translocation to the membrane fraction, where PKC undergoes autophosphorylation.

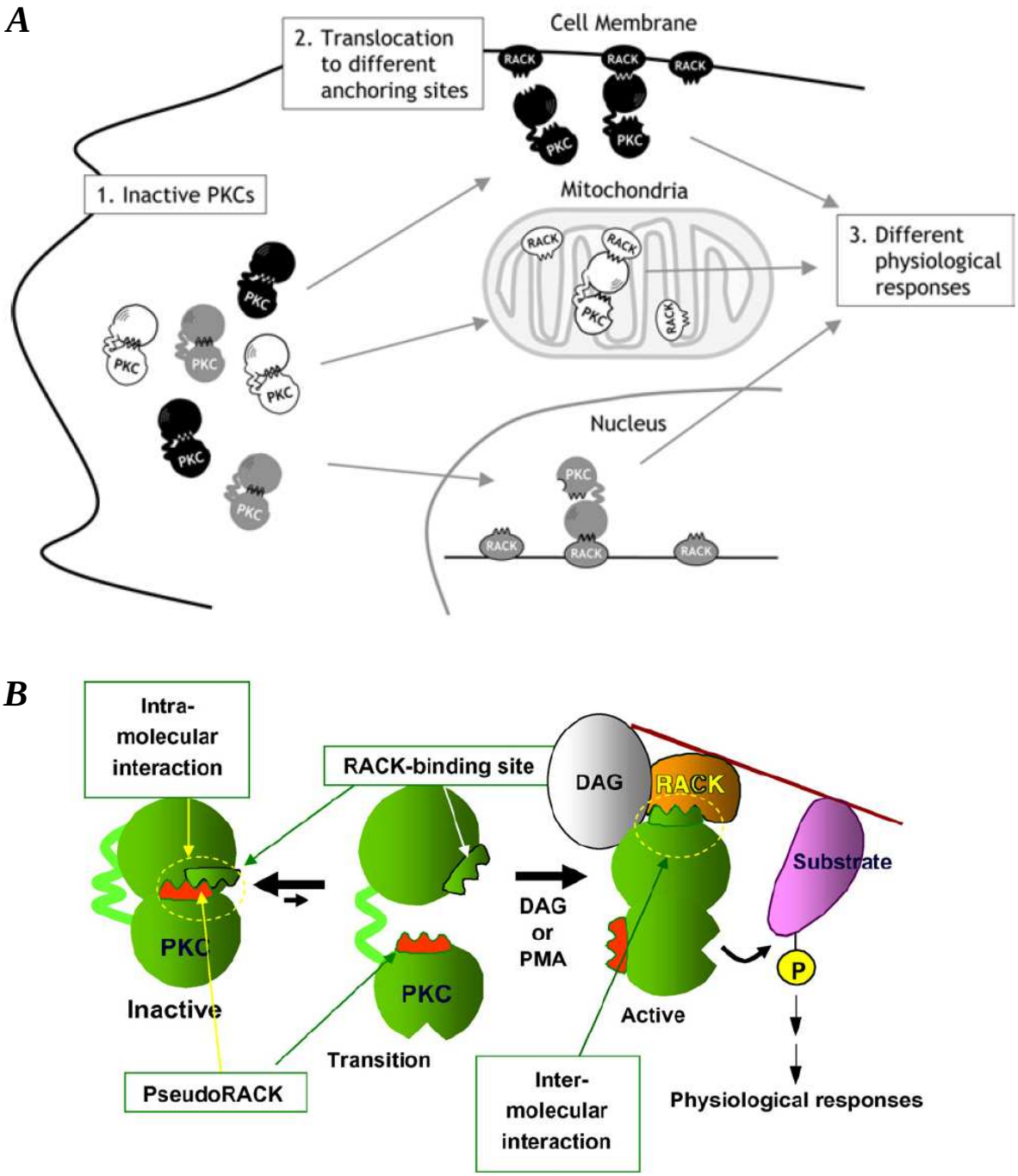


Figure 3.3 A. *Upon stimulation, inactive PKC translocates to its corresponding isoenzyme-specific subcellular location to phosphorylate location-specific downstream substrates. The destination of subcellular translocation is determined by the location of the RACKs, which provide the isoenzyme-specific anchor and scaffold for the catalytic function. B.PKC contains several intra-molecular interactions that keep the enzyme in the inactive state. These intra-molecular interactions can be disrupted spontaneously to create an open transition state. However, most of the time, the inter-molecular interactions are maintained. When there is a rise in diacylglycerol (DAG) (and calcium for the calcium-sensitive PKCs), or when cells are treated with the tumor promoter phorbol ester (PMA), the open state is stabilized by binding of the enzyme to membranes and the corresponding RACK, resulting in its anchoring nearby a particular substrate and away from others. The numerous intra- and inter-molecular interactions can be disrupted to yield selective regulators of PKC functions.*

Endogenous phosphatases eventually dephosphorylate the PKC, initiating the downregulation phase, which might occur by ubiquitination and degradation by the proteasome, or by translocation of PKC to caveolae and delivery to endosomes, where it undergoes proteolytic degradation [Alkon et al., 2007].

Multiple PKC isozymes can be present in the same cell, and can translocate to different subcellular localizations in response to the same stimuli [Disatnik et al., 1994]. In order to explain this phenomenon, it was hypothesized that each individual PKC isoform might have an isoform-selective anchoring protein to which each PKC isoform binds upon activation. These anchoring proteins, termed receptors for activated C kinase (RACKs), are hypothesized to anchor specific PKC isoforms at unique subcellular locations [Mochly-Rosen, 1995]. Thus, anchoring of a specific PKC isoform to its respective RACK localizes that PKC isoform in close proximity to its isoform-specific protein substrates. Subcellular translocation and binding to isoform-selective RACKs can therefore bestow functional specificity for each PKC isoform (Figure 3.3).

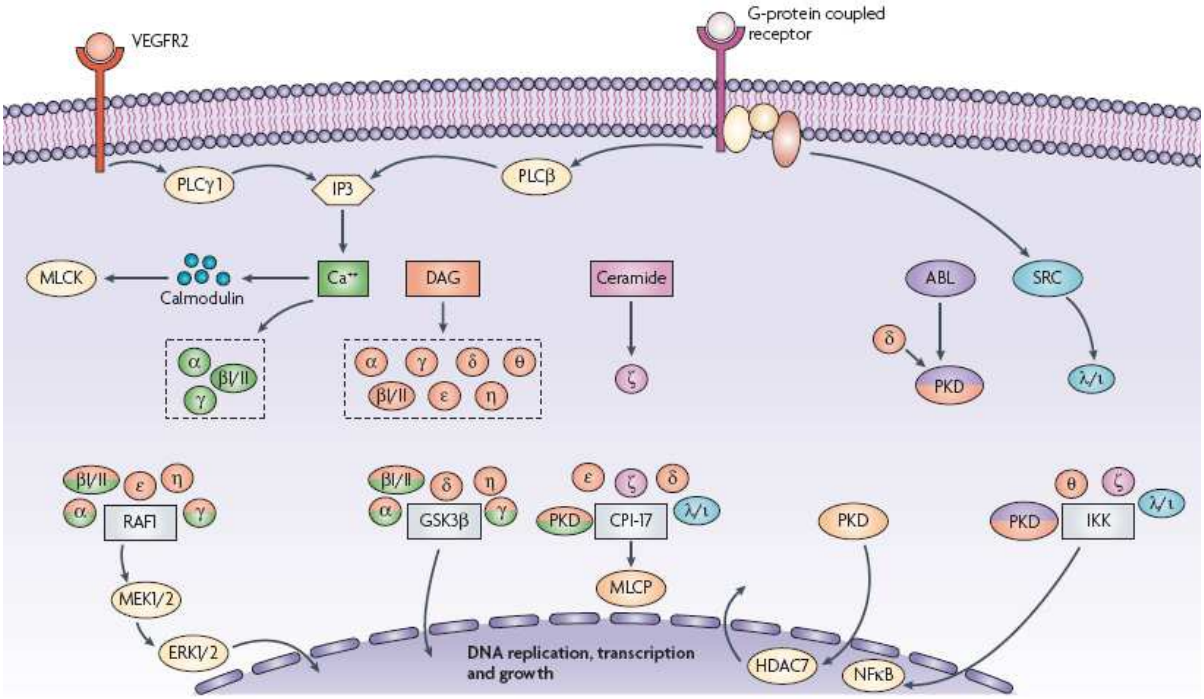
Two RACKs have been identified to date: theRACK for PKC βII, known as RACK1 [Ron et al., 1994], and the RACK for PKC ε, known as RACK2 [Csukai et al., 1997]. The specificity of PKC-RACK interaction is thought to be mediated by the C2 and the V5 domains.

PKC isoforms have a serine/threonine kinase activity, but conversely require serine/threonine phosphorylation for their own activation [Le Good and Brindley, 2004]. However, they are also found to be regulated by tyrosine phosphorylation, which initially has been linked to PKC δ inhibition. According to several publications, phosphorylation of tyrosines can also positively regulate the activation of PKC δ as well as other isoforms, including PKC α, β, ε, and ζ. Thus, the specific effects of tyrosine phosphorylation on the activation of the various PKC isoforms are currently still somewhat controversial. A likely mechanism is that phosphorylation of distinct tyrosine residues induces specific conformational changes depending on the particular PKC isoform [Joseloff et al., 2002; Le Good and Brindley 2004]. As another explanation, it has been proposed that tyrosine phosphorylation directs PKCs towards specific substrates thereby addressing diverse signalling routes [Jaken and Parker, 2000; Tapia et al., 2003].

The downstream events following PKC activation are little understood, although the MEK–ERK (mitogen activated protein kinase kinase–extracellular signal-regulated kinase) pathway is thought to have an important role (Figure 3.4) [Marshall, 1996; Ueda et al., 1996].

There is also evidence to support the involvement of PKC in the PI3K–Akt pathway [Balendran et al., 2000]. PKC isozymes probably form part of the multiprotein complexes that facilitate cellular signal transduction.

The protein kinase C family influence many cellular pathways. Some isozymes can be activated by several different pathways, such as release of calcium (Ca^{2+}), or production of diacylglycerol (DAG). Others are activated by one pathway, such as ceramide. Many of the isozymes display overlapping substrate specificities in vitro, and may interact to control a number of signalling



pathways that regulate cell-cycle control, proliferation, apoptosis, cellular adhesion and metastasis (Figure 3.5).

Figure 3.4 *Proposed effects of protein kinase C activation.*

3.4 Intracellular distribution

Another important feature of PKC activation involves the association of PKC isoforms with phospholipids to form stable membrane complexes. In addition, some PKC isoforms were found to localize to distinct cellular compartments. This includes preferential localization of PKC α at the keratin cytoskeleton, tight junctions, caveolae and desmosome complexes, PKC γ within the Golgi apparatus, and of PKC η throughout the perinuclear rough endoplasmic reticulum (RER) [Cardell et al., 1998].

Furthermore, specific translocation of activated PKCs participates in determining of the functional outcome such as induction of transcription. For example, translocation of PKC α, β, δ, ε and ζ to mitochondria, the Golgi apparatus, nuclear or perinuclear regions results in

regulation of mitosis, apoptosis, and cell survival pathways [Denning et al., 2002; DeVries et al., 2002]. Moreover, PKC activation in the plasma membrane leads to serine phosphorylation and endocytosis of various transmembrane proteins and receptors. This includes internalization of fibroblast growth factor receptor (FGFR) by PKC α and of insulin receptor by PKC δ. In addition, PKC δ phosphorylates insulin receptor substrate-1 (IRS-1) in response to insulin, serving as negative feedback regulation of IR function and further the extracellular matrix (ECM) receptor integrin α6β4 [Alt et al., 2004], thus destabilizing firm adhesion of epidermal basal cells.

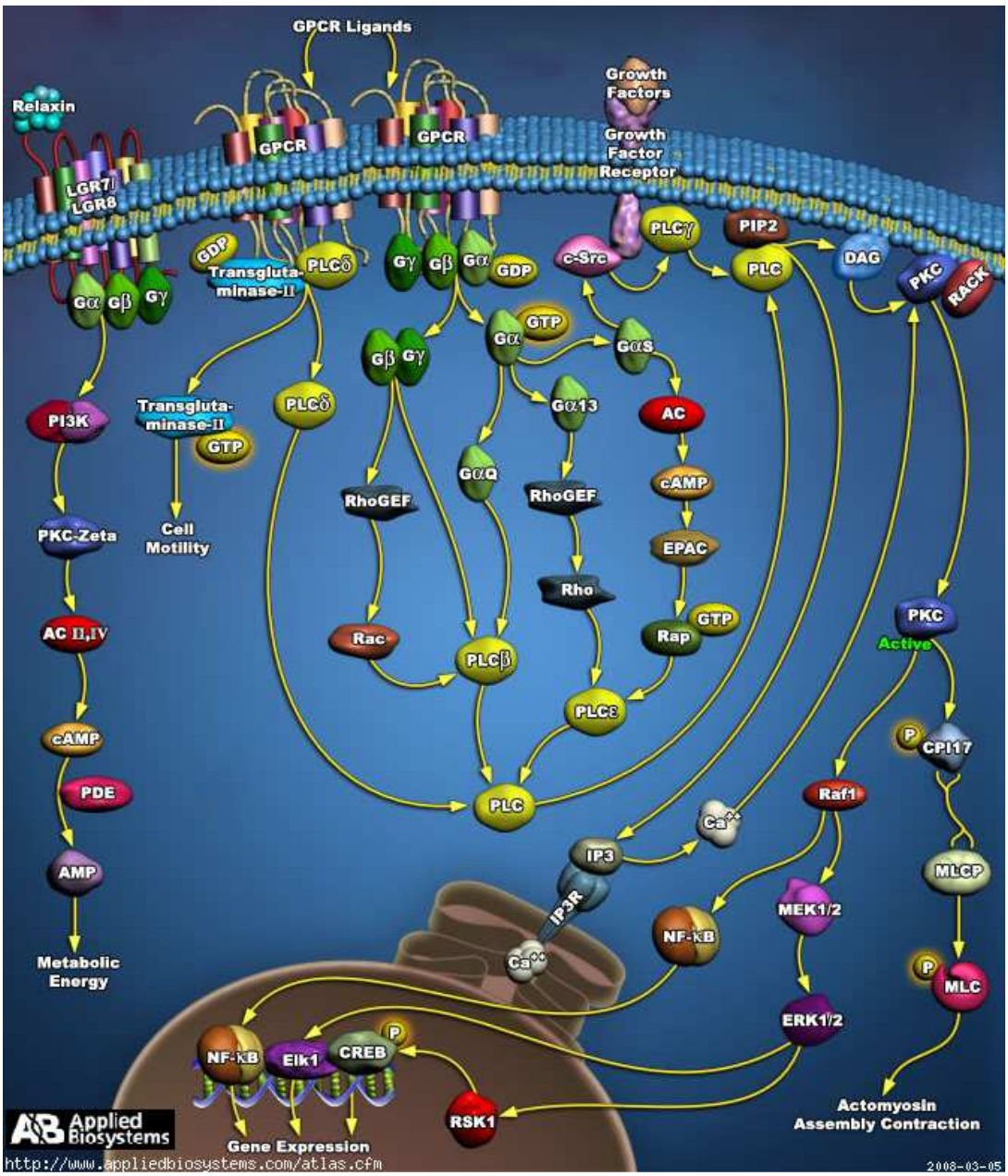


Figure 3.5 *PKC may interact to control a number of signalling pathways that regulate cell-cycle control, proliferation, apoptosis, cellular adhesion and metastasis.*

3.5 Tissue distribution

PKC isoforms are ubiquitous, but while some (PKC α, β and ζ) are widely expressed in all tissues, other isoforms are expressed in a tissue-specific manner [Mellor and Parker, 1998]. PKC γ for example is largely confined to brain and neuronal tissue [Cardell et al., 1998] and a shortened transcript of PKCζ, PKMζ to brain as well. According to earlier reports PKCι was restricted to testis and insulin secreting cells [Selbie et al., 1993] and PKCθ to skeletal muscle and T cells [Czerwinski et al., 2005]. However, later on both isoforms were also detected in epidermis; so PKCι for example is involved in the formation of tight junction [Helfrich et al., 2007]. The activity of distinct PKC isoforms in various tissues directs their function in a tissue-specific manner. For instance, PKCδ was shown to control both proliferation and apoptosis in various cell models [Wertheimer et al., 2001]. On the other hand, in the "classical" insulin responsive tissues or cells, muscle, liver, and adipocytes, PKCδ regulates glucose transport and metabolism. Finally, in C6 glioma cells, PKCδ is involved in the stimulation of the Na^+/H^+ exchanger [Chen and Wu, 1995].

3.6 The complexities of PKC and cancer

The function of PKC in cancer is complex, primarily because much of the data indicate that the isozymes subtly regulate many pathways involved in cellular transformation. Overall, increased PKC levels have been associated with malignant transformation in several cell lines including breast, lung and gastric carcinomas. *In vivo*, however, the relationship is less clear. For example, the expression of PKCβ in colon tumours has been shown to be increased, the same or decreased compared with normal epithelium. Not surprisingly, this relationship becomes even more complex when considered at the level of individual isozymes [Mackay and Twelves, 2007].

Adding to this complexity is the fact that PKC is activated in response to many signals - it functions in many pathways that are important in tumorigenesis.

The PKC family also has a role in cellular adhesion, and is therefore important for cancer cell invasion mediated by integrin binding, the activation of matrix metalloproteinases and the expression of extracellular matrix proteins. Inhibition of PKC activity produces a reduction in invasiveness paralleled by the inhibition of cell motility in some cell lines. The interplay between PKC isozymes is complex, making it hard to predict the impact of inhibiting one isozyme in the

clinic. For example, stable overexpression of PKCα in a breast cancer cell line increases the expression of PKCβ accompanied by decreased levels of PKCη and PKCδ [Ways et al., 1995].
Given their many cellular roles, PKC isozymes are undoubtedly attractive targets for therapeutic intervention. However, the very factors that make them attractive targets also pose significant problems in designing an inhibitory strategy. The complexity of their interactions and the many secondary messenger systems involved coupled with their cellular and tissue-specific variability, renders selective drug action difficult. The principal agents under investigation are listed in Table 3.1.

Drug	Class	Route of administration	Specificity/selectivity	Comments
PMA (TPA)	Phorbol ester	Intravenous	Non-specific	PKC activator; in a phase I trial in haematological malignancy
Staurosporine	Indolocarbazole	Intravenous	Poor specificity, also inhibits other serine/threonine kinases and tyrosine kinases	Preclinical
PKC412 (midostaurin)	Indolocarbazole	Oral	PKCs α, β, γ, δ, ε, η; also inhibits tyrosine kinase pathways	Potentiates treatment with doxorubicin or vinblastine; in phase II trials
UCN01	Indolocarbazole	Intravenous	cPKCs > nPKCs; check point kinase inhibitor	Potentiates treatment with cisplatin, mitomycin C, camptothecin or 5FU; in phase II trials
Go6976	Indolocarbazole	Intravenous	cPKCs > nPKCs	
Bryostatin 1	Macrocyclic lactone	Intravenous	Activator of cPKC and nPKCs, in presence of activating ligands acts as an antagonist	Potentiates treatment with cytosine arabinoside, paclitaxel, tamoxifen or vincristine
Tamoxifen	Nonsteroidal anti-oestrogen	Oral	PKCs α, β, γ, non-selective	
Bisindolylmaleimide (LY333531)	Indolocarbazole	Oral	PKCβ	Used in the treatment of diabetic retinopathy
LY317615 (enzasautaurin); Ro31-8220; Ro32-0432; GF109203X	Indolocarbazole	Oral	PKCβ	Potentiates treatment with gemcitabine, 5FU, cisplatin or radiotherapy
ISIS3521 (aprinocarsen)	Antisense oligonucleotide; 19-mer phophorothioate oligodeoxynucleotide	Intravenous	PKCα	In phase II and phase III trials
ISIS9606	Antisense oligonucleotide; 19-mer phophorothioate oligodeoxynucleotide	Intravenous	PKCα	Not developed further in the clinic

Table 3.1 *A list of the main protein kinase inhibitors (PMA: phorbol 12-myristate 13-acetate; TPA: 12-o-tetradecanoyl-phorbol-13-acetate; 5-FU: 5-fluorouracil).*

4 PROTEIN GLYCOSYLATION

The molecular complexity that defines different cell types and their biological responses occurs at the level of the cell's proteome. The recent increase in availability of genomic sequence information is a valuable tool for the field of proteomics. While most proteomic studies focus on differential expression levels, post-translational modifications such as phosphorylation, glycosylation, and acetylation, provide additional levels of functional complexity to the cell's proteome. There are many ways a cell can increase the complexity of its proteome when moving from gene to functional gene products (Figure 4.1). In eukaryotes, the process of going from DNA to functional protein usually consists, at the most basic level, of at least six discreet steps: transcription, splicing, polyadenylation, transport, translation, and post-translational modification. At the level of RNA, alternative transcriptional start sites and alternative mRNA splicing may increase complexity. mRNA levels do not always correspond to protein levels due to translational regulation and differential protein stability. Cell type specific expression, and different expression patterns in response to specific external stimuli also contribute to the uniqueness of a cell's proteome. Finally, post-translational modifications have the potential to exponentially increase the variety of protein molecular species in a cell. Virtually all proteins are known to contain some form of post-translational modification.

While only 20 amino acids are used in protein synthesis, as many as 140 different amino acids are found in proteins due to covalent post-translational modification. In the context of multiple posttranslational modifications of the same protein, the combinatorial possibilities become very complex [Vosseller et al., 2001].

4.1 Glycosylation

Glycosylation, the enzymatic addition of carbohydrates to proteins or lipid, is the most common and the most complex form of post-translational modification of proteins in eukaryotic cells. This is illustrated by the estimation that 1% of human genes are required for this specific process [Lowe and Marth, 2003]. Furthermore, more than one half of all proteins are glycosylated, according to estimates based on the SwissProt database [Apweiler et al., 1999].

These glycoproteins are involved in a wide range of biological functions such as protein sorting, receptor binding, cell signaling, immune recognition, inflammation, pathogenicity, metastasis, and other cellular processes.

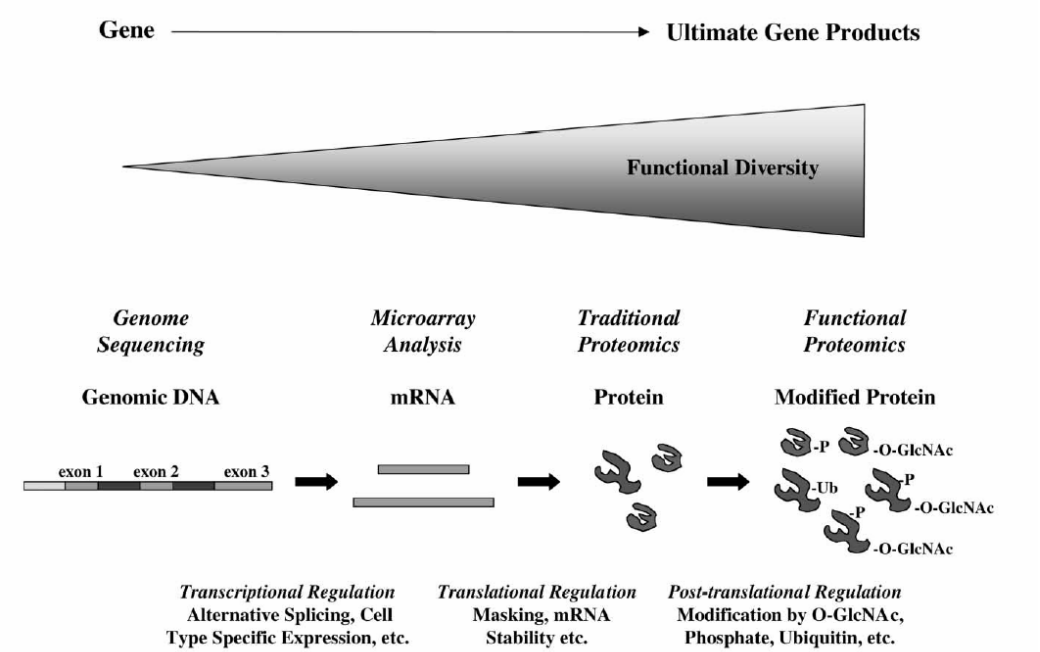


Figure 4.1 *A single gene can give rise to multiple functional gene products. The image represents the increasing complexity as you go from genomic DNA to modified proteins, listing three main transitions that are regulated and the four types of analysis that can be performed in genomic/proteomic experiments.*

Mammalian glycoproteins contain three major types of oligosaccharides (glycans): N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors. N-Linked glycans are attached to the protein backbone via an amide bond to an asparagine residue in an Asn-Xaa-Ser/Thr motif, where X can be any amino acid, except Pro. O-Linked glycans are linked via the hydroxyl group of serine or threonine. O-Linked glycans often have lower mass than N-linked structures, but can be more abundant and heterogeneous. Variation in the degrees of saturation at available glycosylation sites results in heterogeneity in the mass and charge of glycoproteins.

N-Glycans share a common protein–glycan linkage and have a common biosynthetic pathway that diverges only in the late Golgi stage.

4.2 N-Linked Glycans

All eukaryotic cells express N-linked glycoproteins. Protein glycosylation of N-linked glycans is actually a co-translational event, occurring during protein synthesis. N-linked glycosylation requires the consensus sequence Asn-X-Ser/Thr. Glycosylation occurs most often when this consensus sequence occurs in a loop in the peptide. Oligosaccharide intermediates destined for

protein incorporation are synthesized by a series of transferases on the cytoplasmic side of the endoplasmic recticulum (ER) while linked to the dolichol lipid. Following the addition of a specific number of mannose and glucose molecules, the orientation of the dolichol precursor and its attached glycan shifts to the lumen of the ER where further enzymatic modification occurs. The completed oligosaccharide is then transferred from the dolichol precursor to the Asn of the target glycoprotein by oligosaccharyltransferase (OST). Further processing includes trimming of residues such as glucose and mannose, and addition of new residues via transferases in the ER and, to a great extent, in the Golgi. In the Golgi, high mannose N-glycans can be converted to a variety of complex and hydrid forms which are unique to vertibrates. The diverse assortment of N-linked glycans are based on the common core pentasaccharide, $Man_3GlcNAc_2$ (Figure 4.2).

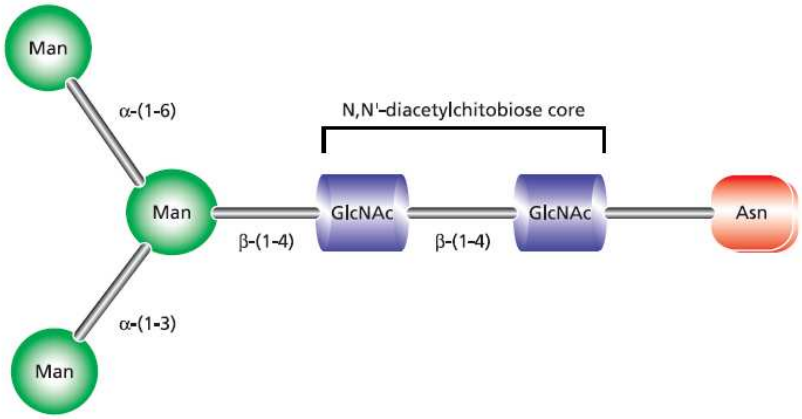


Figure 4.2 *Basic N-linked structure.*

The enzymes involved in modification of N-linked glycosylation of glycoproteins are found in the ER and the Golgi. Processing of oligosaccharides begins in the ER by a-glycosidases I and II and a collection of processing mannosidases both in the ER and Golgi complex. The endoplasmic reticulum contains a unique pathway for the folding and retention of glycoproteins, central to which is calnexin which assists in the initial folding of glycoproteins, retains transport-incompetent misfolded glycoproteins, and may function in assembling of oligomeric proteins in the ER. Each glycosylation site may further exhibit variable occupancy, in that a site may be fully, partially or totally unoccupied. Each occupied site in turn exhibits heterogeneity in the attached glycan structures. Site heterogeneity is reproducible although the glycans at each site may or may not belong to the same structural class. The composition and structure of the carbohydrate attached to a glycoprotein are determined by the glycosylation machinery available in a specific

cell type, changes in which can significantly alter the structure of the protein. Variable sequon occupancy – glycoforms structure of the oligosaccharide. The term glycoform essentially describes this structural diversity which is a result of cell specific biosynthesis, and leads to functional diversity as seen in the case of ribonuclease [Devasahayam, 2007].

4.3 O-Linked Glycans: biosynthesis and functions

The O-glycosylation process produces an immense multiplicity of chemical structures. Each monosaccharide has 3 or 4 attachment sites for linkage of other sugar residues and can form a glycosidic linkage in an α or β configuration, allowing glycan structures to form branches. Glycans therefore have a larger structural diversity in contrast to other cellular macromolecules such as proteins, DNA, and RNA, which form only linear chains. Theoretically, the 9 common monosaccharides found in humans could be assembled into more than 15 million possible tetrasaccharides, all of which would be considered relatively simple glycans [Dove, 2001].
O-Linked glycans are classified on the basis of the first sugar attached to a Ser, Thr, or hLys residue of a protein. The mucin-type O-glycan, with *N*-acetylgalactosamine (GalNAc) at the reducing end, is the most common form in humans. A frequently occurring O-linked glycan is the single GlcNAc linked to nuclear and cytosolic proteins. For most O-glycosylation types, a recognition consensus sequence for the attachment of the first sugar residue remains unknown; the exceptions are the O-Glc and O-Fuc modifications, for which putative consensus sites have been described. The lack of a consensus sequence can arise from the coexistence of multiple transferases with overlapping but different substrate specificities, as seen, e.g., in mucin-type O-glycosylation, or is the result of a nonlimited consensus sequence, as seen, e.g., in O-GlcNAc modifications. Statistical studies yielded some general rules for mucin-type O-glycans and O-GlcNAc modifications, leading to the development of algorithms for the prediction of these 2 O-glycan types. These O-glycosylation prediction sites are available on the Internet. The NetOglyc 3.1 prediction server correctly predicts 76% of the glycosylated residues and 93% of the nonglycosylated residues in any protein [Julenius et al., 2005].
The main pathway for the biosynthesis of complex N- and O-linked glycans is located in the endoplasmic reticulum (ER) and Golgi compartments, the so-called secretory pathway. Glycosylation is restricted mainly to proteins that are synthesized and sorted in this secretory pathway, which includes ER, Golgi, lysosomal, plasma membrane, and secretory proteins. There is one exception; nuclear and cytosolic proteins can be modified with a single O-linked GlcNAc.

Proteins synthesized by ribosomes and sorted in the secretory pathway are directed to the rough ER by an ER signal sequence in the NH_2 terminus. After protein folding is completed in the ER, these proteins move via transport vesicles to the Golgi complex. The biosynthesis of O-glycans is initiated after the folding and oligomerization of proteins either in the late ER or in one of the Golgi compartments. Intriguingly, for the biosynthesis of glycans, no template is involved; whereas DNA forms the template for the sequence of amino acids in a protein, there is no such equivalent for the design of glycans. The biosynthesis of glycans can be divided into 3 stages. In the first stage, nucleotide sugars are synthesized in the cytoplasm. In the second stage, these nucleotide sugars are transported into the ER or the Golgi. In the third stage, specific glycosyltransferases attach the sugars to a protein or to a glycan in the ER or Golgi. An additional prerequisite for proper glycosylation is Golgi trafficking. Recently, it was discovered that a defect in a protein involved in Golgi traffic secondarily caused abnormal N- and O-glycans in patients with congenital disorders of glycosylation [Wopereis et al., 2006].

Monosaccharides used for the biosynthesis of nucleotide sugars derive from dietary sources and salvage pathways. Glucose (Glc) and fructose (Fru) are the major carbon sources in humans from which all other monosaccharides can be synthesized. Series of phosphorylation, epimerization, and acetylation reactions convert them into various high-energy nucleotide sugar donors, whose biosynthesis takes place in the cytosol. Several steps in the biosynthesis of nucleotide sugars require ATP; therefore, the metabolic state of the cell influences the availability of the nucleotide sugars. The tight regulation of the biosynthesis of nucleotide sugars means that alterations in a single nucleotide sugar can significantly impair glycosylation.

As the nucleotide sugars are biosynthesized in the cytosol their monosaccharides must be translocated into the lumen of the ER and/or Golgi before they can be used for the glycosylation process. Because nucleotide sugars cannot cross the membrane lipid bilayer, specific transport mechanisms are responsible for their translocation. Two transport mechanisms for the generation of monosaccharide donors in the ER/Golgi can be distinguished. The first mechanism is the entrance of Man and Glc through binding to the lipid carrier dolichol phosphate (Dol-P). The second mechanism is the transport of nucleotide sugars through specific nucleotide sugar transporters (NSTs). These NSTs are antiporters in which nucleotide sugar entry into the ER/Golgi is coupled to equimolar exit of the corresponding nucleoside monophosphate from the ER/Golgi lumen [Hirschberg et al., 1998]. After entrance of the nucleotide sugar into the ER/Golgi lumen, a glycosyltransferase will transfer the monosaccharide to a glycan by cleaving off the nucleotide part. The nucleoside diphosphates are converted to dianionic nucleoside monophosphates (used for the antiporter) and inorganic phosphate by a nucleoside

diphosphatase. Nucleoside di- and monophosphates can inhibit the nucleotide sugar transport process and the activity of glycosyltransferases. In general, the transport of a nucleotide sugar occurs in the organelle in which the corresponding glycosyltransferase is localized. Some nucleotide sugars enter only the lumen of Golgi vesicles, others enter the lumen of ER-derived vesicles, and a few enter both.

The activity of glycosyltransferases can be influenced by different factors. It is known, for example, that some of the glycosyltransferases require divalent cations, such as Mn^{2+} and/or Mg^{2+}, for optimal action. Furthermore, it was recently discovered that human core 1 β3-galactosyltransferase (core 1 β3-Gal-T), which is involved in the formation of core 1 (and core 2) mucintype O-glycans, requires a molecular chaperone for its functioning. This molecular chaperone is called core 1 β3-Gal-T-specific molecular chaperone (Cosmc) and is an ER-localized type II transmembrane protein that appears to be required for the proper folding of the core 1 β3-Gal-T enzyme.

A third factor that might influence glycosyltransferase activity is the structure of the protein substrate. It is thought that the protein structure contains information for the action of specific transferases. However, how proteins are recognized by glycosyltransferases remains largely unknown. Finally, glycosyltransferase activity can be dependent on heterocomplex formation. Golgi transferases can recognize a single sugar residue, a sugar sequence, or a peptide moiety, leading to variable specificity. With very few exceptions, each type of transferase is regio- and stereospecific. Glycosyltransferases involved in the linkage of monosaccharides to the protein backbone and those involved in the core processing of mucin-type O-glycans are specific and not involved in other classes of glycoconjugates, whereas most glycosyltransferases involved in the elongation, branching, and termination of glycans are not specific for one glycoconjugate class. Only Ser and Thr residues that are exposed on the protein surface will be glycosylated, as O-glycosylation is a postfolding event. Therefore, O-glycosylation takes place mainly in coil, turn, and linker regions. Furthermore, all attachment sites have high Ser, Thr, and Pro content [Julenius et al., 2005].

Given the sequential and competing nature of glycosyltransferases, the precise localization of these enzymes within the Golgi is of great importance. It is thought that glycosyltransferases are arranged in an assembly line in the Golgi, whereas early-acting transferases are localized in the cis-Golgi, intermediate-acting transferases in the medial-Golgi, and terminating transferases in the trans-Golgi. A signal targeting glycosyltransferases to a specific Golgi localization has not yet been described. Studies have indicated that glycosyltransferases from a certain Golgi compartment form high–molecular-mass complexes. The presence of multienzyme complexes is

likely to be functionally relevant in the regulation of glycosylation and contribute to the maintenance of the steady-state localization of the Golgi glycosyltransferases [Opat et al., 2001]. Not all glycosyltransferases form complexes; in particular, those found in the trans-Golgi network seem to be unbound. Another factor that is likely to play a role in the targeting of glycosyltransferases is the thickness of the lipid bilayer, which increases en route to the plasma membrane.

In general, O-linked glycans have been found to function in protein structure and stability, immunity, receptor-mediated signaling, nonspecific protein interactions, modulation of the activity of enzymes and signaling molecules, and protein expression and processing. The biological roles of oligosaccharides appear to span the spectrum from those that are trivial to those that are crucial for the development, growth, function, or survival of an organism. A particular glycan may mediate diverse functions at distinct locations at specific times within a single organism. Just like N-glycans, O-glycans can influence the secondary protein structure: the glycan can break the α-helicity of peptides; can have a role in the tertiary protein structure and in the quaternary protein structure and protein aggregation. Subsequently, O-linked glycans maintain protein stability, heat resistance, hydrophilicity, and protease resistance by steric hindrance [Wopereis et al., 2006].

Another important function of O-linked sugars is to mediate recognition between proteins. Glycan structures can be substrates for nonenzymatic sugar-binding proteins, known as lectins. By interacting with lectins, glycans influence the targeting of the proteins to which they are attached. Moreover, O-linked glycans are known to have an effect on immunologic recognition; for example, the ABO blood group antigens and recognition of glycopeptides by the MHC complex or by antibodies. The effects of O-linked glycosylation on the bioactivity of many signaling molecules, particularly hormones and cytokines, and a relatively small number of enzymes, have been described. Another example is the dynamic O-GlcNAc modification that seems to have an important role in a variety of signaling pathways, such as transcriptional regulation, proteasome-mediated protein degradation, insulin, and cellular stress signalling.

Finally, O-linked glycosylation is essential for the expression and processing of particular proteins. The influence of O-glycans in the processing of proteins is, for example, seen in pro-insulin-like growth factor II, which is cleaved into IGF-II only when Thr75 contains an O-linked sugar [Daughaday et al., 1993].

The most common type of O-linked glycans contain an initial GalNAc residue (or Tn epitope), these are commonly referred to as mucin-type glycans. Other O-linked glycans include glucosamine, xylose, galactose, fucose, or manose as the initial sugar bound to the Ser/Thr

residues. O-Linked glycoproteins are usually large proteins (>200 kDa) that are commonly bianttennary with comparatively less branching than N-glycans. Glycosylation generally occurs in high-density clusters and may contribute as much as 50-80% to the overall mass. One notable core structure is the Gal-β(1-3)GalNAc (Figure 4.3) sequence that has antigenic properties. Termination of O-linked glycans usually includes Gal, GlcNAc, GalNAc, Fuc, or sialic acid. By far the most common modification of the core Gal-β(1-3)-GalNAc is mono-, di-, or trisialylation. A less common, but widely distributed O-linked hexasaccharide structure contains β(1-4)-linked Gal and β(1-6)-linked GlcNAc as well as sialic acid.

Figure 4.3 *Core Gal-β(1-3)GalNAc.*

4.4 Enzymatic deglycosylation strategies

Analysis of the glycan structure of glycoproteins normally requires enzymatic or chemical methods of deglycosylation. Removal of carbohydrates from glycoproteins is useful for a number of reasons:

- To simplify analysis of the peptide portion of the glycoprotein;
- To simplify the analysis of the glycan component;
- To remove heterogeneity in glycoproteins for X-ray crystallographic analysis;
- To remove carbohydrate epitopes from antigens;
- To enhance or reduce blood clearance rates of glycoprotein therapeutics;
- To investigate the role of carbohydrates in enzyme activity and solubility;
- To investigate ligand binding;
- For quality control of glycoprotein pharmaceuticals.

Sequential hydrolysis of individual monosaccharides from glycans can be useful for the elucidation of the structure and function of the glycan component. Due to the restraints of the specificity of glycolytic enzymes currently available, sequential hydrolysis of individual monosaccharides is also required in many instances in order to completely remove a glycan

component enzymatically. This is particularly true in the enzymatic deglycosylation of many O-linked glycans.

Use of the enzyme PNGase F is the most effective method of removing virtually all N-linked oligosaccharides from glycoproteins. The oligosaccharide is left intact and, therefore, suitable for further analysis (the asparagine residue from which the sugar was removed is deaminated to aspartic acid, the only modification to the protein). A tripeptide with the oligosaccharide-linked asparagine as the central residue is the minimal substrate for PNGase F (figure 4.4).

Steric hindrance slows or inhibits the action of PNGase F on certain residues of glycoproteins. Denaturation of the glycoprotein by heating with SDS and 2-mercaptoethanol greatly increases the rate of deglycosylation.However, N-glycans with fucose linked α(1,3) to the Asn-bound N-acetylglucosamine are resistant to the action of PNGase F.

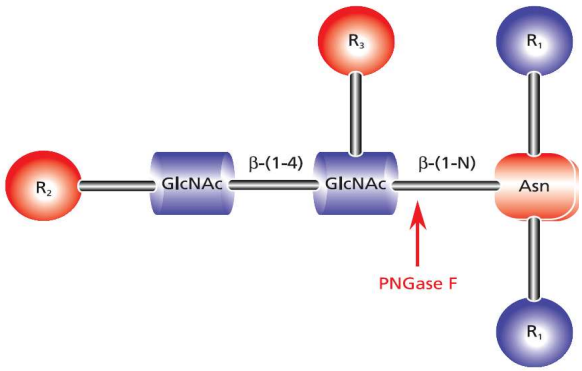


Figure 4.5 *PNGase F has like the minimal substrate a tripeptide with the oligosaccharide-linked asparagine as the central residue.*

There is no enzyme comparable to PNGase F for removing intact O-linked sugars. Monosaccharides must be sequentially hydrolyzed by a series of exoglycosidases until only the Gal-β(1-3)-GalNAc core remains. The O-glycosidase endo-α-N-Acetilgalactosaminidase, can then remove the Gal-β(1-3)-GalNAc core witohut modifying the protein backbone (Figure 4.5).

O-Glycosidase can then remove the core structure intact with no modification of the serine or threonine residue. Denaturation of the glycoprotein does not appear to significantly enhance O-deglycosylation. Any modification of the core structure can block the action of O-Glycosidase. The most common modification of the core Gal-β(1-3)-GalNAc is a mono-, di-, or tri-sialylation. These residues are easily removed by α(2-3,6,8,9)-Neuraminidase since only this enzyme is capable of efficient cleavage of the NeuNAc-α(2-8)-NeuNAc bond (Figure 4.6).

Another commonly occurring O-linked hexasaccharide structure contains β(1-4)-linked galactose and β(1-6)-linked N-acetylglucosamine as well as sialic acid. Hydrolysis of this glycan will require, in addition to neuraminidase, a β(1-4)-specific galactosidase and an N-acetylglucosaminidase (Figure 4.6). A non-specific galactosidase will hydrolyze β(1-3)-galactose from the core glycan and leave O-linked GalNAc that cannot be removed by O-Glycosidase. β(1-4)-Galactosidase and β-N-acetylglucosaminidase can be used for the hydrolysis of these and any other O-linked structures containing β(1-4)-linked galactose or β-linked N-acetylglucosamine such as polylactosamine. Fucose and mannose directly O-linked to proteins cannot presently be removed enzymatically.

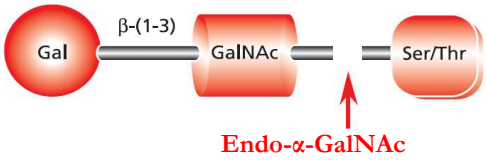


Figure 4.5 *The enzyme endo-α-N-Acetilgalactosaminidase can remove the Gal-β(1-3)-GalNAc core witohut modifying the protein backbone.*

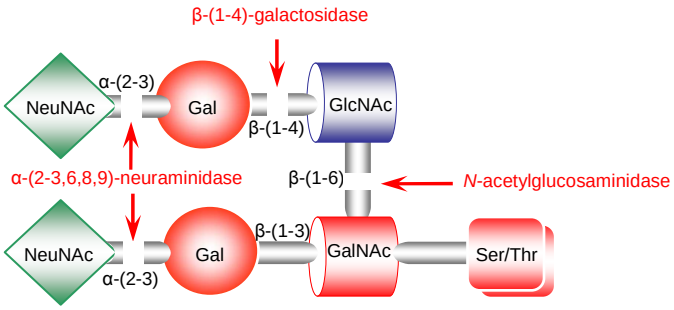


Figure 4.6 *Removal of several modifications of Gal-β(1-3)-GalNAc core.*

4.5 Nucleocytoplasmic O-glycosilation: O-GlcNAc

In 1984, Torres and Hart described a new post-translational modification [Torres and Hart, 1984], which is abundant on cytoplasmic and nuclear proteins, a carbohydrate now known as O-linked β-N-acetylglucosamine (O-GlcNAc). The monosaccharide N-acetylglucosamine is

attached in a β-linkage to serine and threonine hydroxyl group of many nuclear and cytoplasmic proteins.

O-GlcNAc was considered "novel" for three reasons: (1) O-GlcNAc was not elongated into more complex structure; (2) it had a nucleocytoplasmic distribution, whereas "traditional glycoproteins" had previously been localized to the cell surface and to the lumen of membranous intracellular compartments, such as the endoplasmic reticulum and Golgi apparatus; (3) *O*-GlcNAc cycles (Figure 4.7) by means of mechanisms and on a timescale similar to those of phosphorylation and quite different from the cycling of complex extracellular glycans. In terms of high-energy compounds, the intracellular concentration of the direct donor for O-GlcNAcylation, UDP-GlcNAc, is second only to that of ATP, with 2–5% of all glucose being used to generate this sugar nucleotide.

O-GlcNAc has a predominantly nuclear distribution. The greatest concentration of O-GlcNAc modified proteins are found in the nuclear pore complex and associated with chromatin. O-GlcNAc has also been found on many cytoskeletal proteins, RNA polymerase II, transcription factors, hnRNOs, proto-oncogenes, tumour suppressors, hormone receptros, phosphatases and kinases, mitochondrial, and membrane-associated proteins (Figure 4.8) [Zachara and Hart, 2006]. O-GlcNAc is an inducible and dynamically cycling posttranslational modification.

4.5.1 Enzymes regulating O-GlcNAc cycling

Enzymes that catalyze the addition and removal of O-GlcNAc have been cloned and characterized. Unlike protein phosphorylation, where genetically distinct enzymes regulate the addition and removal of phosphate, just two catalytic polypeptides catalyze the turnover of O-GlcNAc; a uridine diphospho-N-acetylglucosamine: peptide β-N-acetylglucosaminyl transferase (OGT) and a neutral β-N-acetylglucosaminidase (OGlcNAcase). However, these two catalytic subunits are non-covalent components of many different multiple component holoenzyme complexes, which appear to regulate both the targeting and rates of O-GlcNAc cycling [Yang et al., 2002].

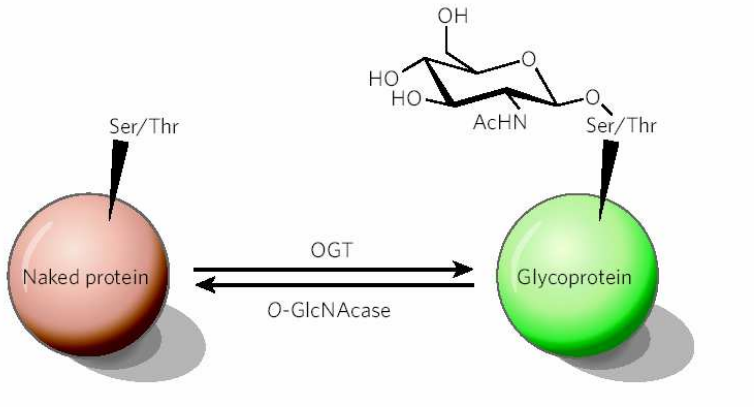


Figure 4.7 *Cycling of O-GlcNAc on serine or threonine residues of nuclear and cytoplasmic proteins is controlled by two highly conserved enzymes, OGT and O-GlcNAcase. O-GlcNAc cycles at rates similar to that of O-phosphate in response to various cellular stimuli.*

OGT is expressed in all tissues studied, although it appears to be particularly rich in the pancreas, brain, and thymus. OGT is a unique glycosyltransferase as it is a soluble protein rather than a type II membrane protein and has little or no homology to other glycosyltransferases. OGT contains two domains, an N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal catalytic domain (Figure 4.9). These domains are separated by a bipartite nuclear localization sequence (NLS). Not surprisingly, OGT is located predominantly within the nucleus as determined by immunofluorescence and subcellular fractionation. In mammalian cells, splicing of OGT mRNA leads to alternative transcripts of which two are well characterized, mitochondrial OGT (mOGT) and nucleocytoplasmic OGT (ncOGT).

ncOGT is the most comprehensively studied of the known splice variants. This 1037-amino-acid protein (110 kDa) was thought to exist predominantly either as a homotrimer comprised of identical 110-kDa subunits or as a heterotrimer comprised of 2 identical 110-kDa subunits coupled with a 78kDa subunit [Lubas et al., 1997].

The activity of OGT is thought to be in the C-terminus, the sequence of which belongs to the glycogen phosphorylase superfamily, with two predicted Rossman folds, and a predicted UDP-GlcNAc binding site. The mechanism(s) by which "one" OGT specifically modifies myriad proteins are unclear, although it appears that UDP-GlcNAc concentration, protein–protein interactions, glycosylation, and phosphorylation may all play a role. Several proteins have been identified that interact with OGT, and while these interactions are not known to change the activity of OGT, they do act to anchor and/or target OGT to signaling and transcriptional complexes. These interactions may modulate the activity of OGT by affecting its localization, modulating the binding of substrate proteins, or targeting to complexes where it is specifically

activated by signal transduction events. Notably, OGT is both O-GlcNAc modified and tyrosine phosphorylated [Kreppel et al., 1997]. The coordinate regulation of substrate binding, substrate preference, association with target complexes, and post-translational modification may mediate OGT in such a way that it specifically modifies different proteins in response to diverse signals, appropriately regulating cellular function. OGT's overall catalytic activity is controlled by the concentration of its donor substrate, UDP-GlcNAc, over a broad range. Concentrations of UDP-GlcNAc are highly sensitive to fluxes in nutrients, energy and metabolic nitrogen, making it an ideal metabolic sensor. Rapid changes in UDP-GlcNAc concentration serve as a sensor by directly affecting the extent of O-GlcNAcylation of regulatory proteins.

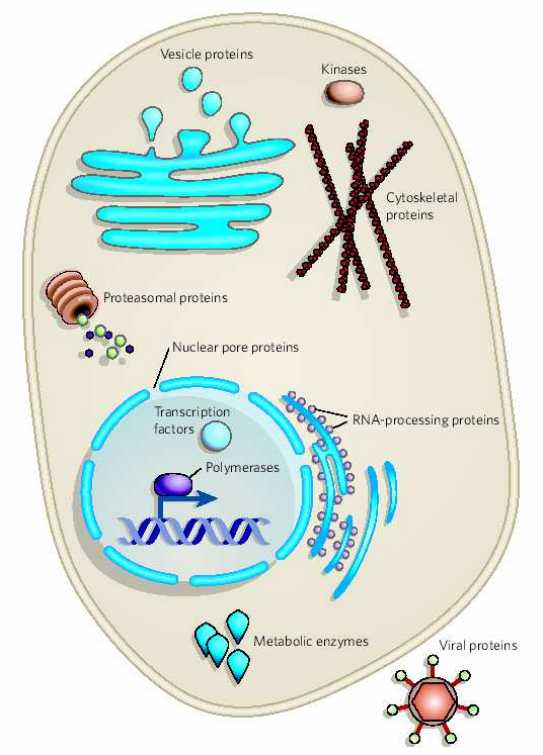


Figure 4.8 *Cellular distribution of O-GlcNAc. Many proteins from almost all functional classes are O-GlcNAcylated. Many viral proteins are also O-GlcNAcylated.*

O-GlcNAcase is a soluble, cytosolic β-N-acetylglucosaminidase (Figure 4.9) expressed in all tissues examined and predominantly in brain [Wells et al., 2002].

O-GlcNAcase is specific for β-N-acetylglucosamine, is not inhibited by N-acetylgalactosamine. O-GlcNAcase is a 917-amino-acid protein, with at least two functional domains, an N-terminal hexosaminidase domain, and a C-terminal histone acetyltransferase (HAT) domain. O-GlcNAcase active site is within the N-terminal half of this protein. The C-terminal domain of O-

GlcNAcase has homology to HATs at residues AA 772–899 and was recently shown to acetylate both free core histones and nucleosomal histone proteins [Toleman et al., 2004].

4.5.2 O-GlcNAc functions

OGT is known to associate with histone deacetylase complexes and to promote transcriptional silencing. Together, these data suggest that dynamic modification of transcriptional complexes by O-GlcNAc and histone acetylation play key roles in transcriptional regulation.

Addition of O-GlcNAc might be a dynamic regulatory modification. Support for this hypothesis was lent by studies showing that multiple signals result in dynamic changes in O-GlcNAc levels within the cell.

The dynamic addition of O-GlcNAc to proteins has been implicated in modulating protein behavior via different mechanisms that include (1) regulating protein phosphorylation and thus protein function; (2) altering protein degradation; (3) adjusting the localization of proteins; (4) modulating protein–protein interactions; and (5) mediating transcription.

Early site-mapping studies highlighted the similarities between O-GlcNAc modification sites and proline-directed kinase sites. In a handful of cases, the sites of attachment of O-GlcNAc and O-phosphate were mapped to the same residue. Such data led to the hypothesis that O-GlcNAc and O-phosphate are reciprocal and compete for the same Ser/Thr residue. Thus, by altering the pattern of available Ser/Thr sites available for phosphorylation, O-GlcNAc would alter phosphorylation patterns and protein function. In support of this model, Wells and co-workers have shown that OGT and protein phosphatase 1 (PP1) β and γ exist together in a complex [Wells et al., 2004].

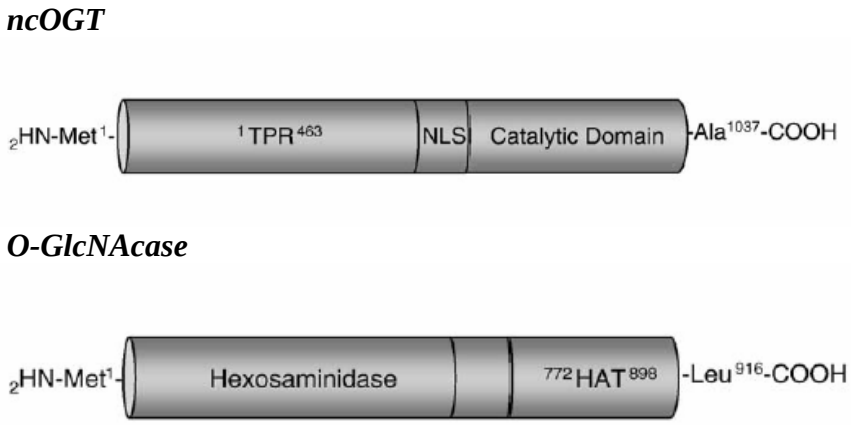


Figure 4.9 *The domain structure of OGT and O-GlcNAc.*

Additional support for this model comes from studies that have determined the effects of kinase and phosphatase inhibitors on the levels of O-GlcNAc on some proteins. In these studies, activation of PKC and PKA kinase reduced glycosylation in a detergent insoluble cytoskeletal and cytoskeleton-associated protein fraction. Conversely, inhibition of PKC and PKA increases O-GlcNAc protein modification in this fraction.

In neuroblastoma, Cos-7, Hela, and Kelly cells, the phosphatase inhibitor okadaic acid decreases glycosylation, although this effect is predominantly observed on nuclear proteins [Griffith and Schmitz, 1999]. Finally, on some proteins when O-GlcNAc levels are reduced, phosphorylation levels are increased suggesting a "reciprocal relationship" between O-GlcNAc and O-phosphate.

The reciprocal glycosylation and phosphorylation of RNA Pol II may regulate transcription. A large number of transcription factors are modified by O-GlcNAc as well as the C-terminal domain (CTD) of RNA polymerase II (Figure 4.10). RNA Pol II is hypophosphorylated (IIa form) when it assembles with other transcription factors to form the pre-initiation complex. Subsequently, RNA Pol II becomes hyperphosphorylated (IIo form) and transcription begins. In this model O-GlcNAc could prevent premature transcriptional initiation [Zachara and Hart, 2006].

Glycosylation of the CTD induces a conformational change in the CTD that could have a variety of functional consequences. Recent data have shown that in vitro glycosylation of the CTD of RNA polymerase II prevents the required phosphorylation for elongation. Thus it has been proposed that O-GlcNAc may modify RNA polymerase II that is in the pre-initiation complex or a storage form. The O-GlcNAc modification of eukaryotic initiation factor (eIF)2α-p67, Sp1 and oestrogen receptor (ER)-β prolongs the half-life of these proteins. In the cases of eIF2α-p67, O-GlcNAc modification prevents protein degradation by an unknown mechanism. When eIF2α-p67 is degraded, eIF2α becomes phosphorylated, resulting in translational inhibition.

Protein degradation plays a key role in regulating cellular function, through the timed destruction of cell-cycle regulators, anti-apoptotic proteins, transcription factors and tumor suppressors. O-GlcNAc has been shown to alter protein degradation by two different mechanisms, first by altering the targeting of some proteins to the proteasome and secondly by altering the activity of the proteasome.

There is no obvious consensus sequence for O-GlcNAc. However, about 50% of the known sites have a Pro-Val-Ser motif similar to that recognized by proline-directed kinases. Many of the known *O*-GlcNAc sites have high "PEST" scores — PEST (Pro-Glu-Ser-Thr) being a sequence that is associated with rapid degradation — which suggests that O-GlcNAcylation at these sites might slow or prevent degradation [Rechsteiner and Rogers, 1996].

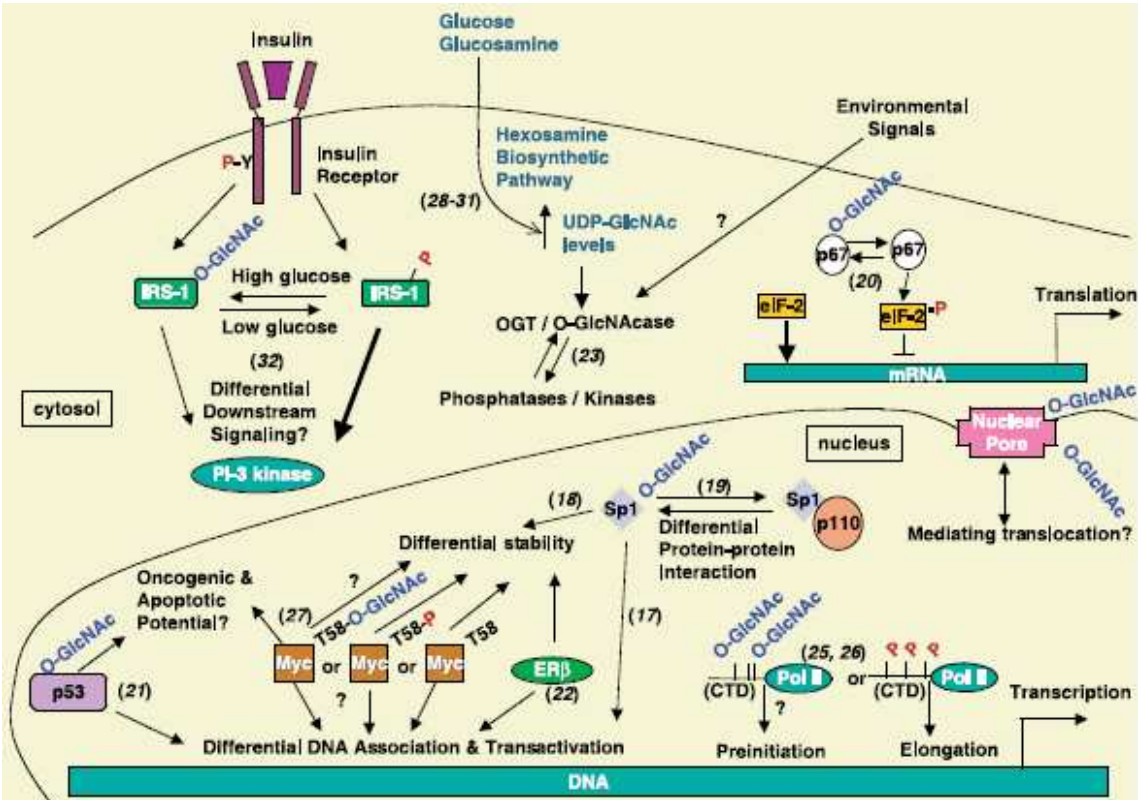


Figure 4.10 *Cellular processes in which O-GlcNac has been implicated.*.

Several O-GlcNAc sites are in sequences that have high "PEST" scores. These amino acid motifs are rich in Pro, Glu, Ser, and Thr residues and have been shown to mediate protein degradation. While some PEST sequences are constitutive, many require activation by mechanisms such as phosphorylation.

Modification of the proteasome in vitro by O-GlcNAc reduces degradation, seemingly by inhibition of ATPase activity, particularly of the ATPase Rpt2.

Notably, in mammals, the glycosylation state of the proteasome responds to the nutritional state of the cell [Zhang et al., 2003]. The authors suggest that at times of nutritional depravation, the proteasome is deglycosylayed and activated to provide the cell with energy. For proteins such as c-Myc, Tau, Stat5a, Pax-6, ELF-1, Sp1, and the mTOR α4 phosphoprotein, the nuclear forms have been shown to be proportionally more O-GlcNAc modified than the cytoplasmic forms of these proteins. In support of O-GlcNAc playing a role in nuclear localization, treatment of neuronal cells with okadaic acid results in an increase in phosphorylation of Tau and a subsequent decrease in nuclear localization. If O-GlcNAc plays a role in nuclear localization, it may not be as a NLS signal but rather as a nuclear retention signal.

In addition to altering the nuclear localization and the halflife of the transcription factor Sp1, O-GlcNAc also modulates the protein–protein interactions of Sp1 downregulating transcription in some experimental models.

Numerous transcription factors are O-GlcNAc modified, and the transcription of multiple genes is up- and downregulated when extracellular glucose/glucosamine concentrations are changed. O-GlcNAc modification of Sp1 targets it to specific areas/genes in the nucleus, where it is subsequently deglycosylated and phosphorylated, resulting in transcriptional activation. In this model, both increasing and decreasing glycosylation could result in transcriptional inhibition.

OGT/O-GlcNAc has also been shown to negatively regulate Sp1 by altering its protein–protein interactions, recently another mechanism of O-GlcNAc-dependent Sp1 repression has been identified, the association of OGT with transcriptional repressors [Yang et al., 2001].

In both in vitro and in vivo binding assays, OGT and the repressor mSin3a were shown to interact. The interaction between mSin3a and OGT was mapped to TPR repeats 1–6 of OGT and the PAH4 domain (AA 888–967) of mSin3a [Yang et al., 2002]. mSin3a and OGT were subsequently shown to cooperatively repress transcription.

OGT interacts with a histone deacetylase complex by binding to the corepressor mSin3A. Functionally, OGT and mSin3A cooperatively repress transcription in parallel with histone deacetylation. mSin3A targets OGT to promoters to inactivate transcription factors and RNA polymerase II by O-GlcNAc modification, which acts in concert with histone deacetylation to promote gene silencing in an efficient and specific manner. The interaction between mSin3A and OGT represents a mode of HDAC-independent repression by mSin3A [Yang et al., 2002].

Finally, the glycosylation state of estrogen responsive genes was examined. It was found that the silenced pS2, EB1 and CatD (but not p21) promoters were bound to proteins that were hyperglycosylated and also associated with mSin3a. These data suggest a model in which mSin3a recruits OGT to promoters, and subsequently glycosylation aids in the repression of these promoters. However, given the contrasting data on the role of O-GlcNAc in mediating Sp1 and other transcription factors, it is likely that O-GlcNAc will act in a gene-specific manner.

4.5.3 O-GlcNAc's complex interplay with O-phosphate

Several studies have found a 'yin-yang' relationship between O-GlcNAc modification and phosphorylation. Globally, it has been shown that phosphatase inhibitors and kinase activators decrease overall levels of O-GlcNAc and conversely that kinase inhibitors increase levels of O-

GlcNAc [Griffith et al., 1999]. Furthermore, on a select few proteins the mapped site for glycosylation and phosphorylation are identical, such as c-Myc, the estrogen receptor, and SV-40 large T antigen. This apparent reciprocity again emphasizes that proteins exist in a variety of forms through combinatorial post-translational modification.

O-GlcNAc and *O*-phosphate site-mapping studies suggest that there are at least four different types of dynamic interplay between *O*-GlcNAc and *O*-phosphate (Figure 4.11). First, there is competitive occupancy at the same site, for example that which occurs in the transcription factor c-Myc and oestrogen receptor, and on the oncoprotein SV-40 large T-antigen and endothelial nitric oxide synthase. Second, competitive and alternative occupancy occur at adjacent sites, such as that observed in the tumour suppressor p53 and synapsin I. Third, there is a complex interplay whereby some *O*-phosphate attachment sites on a given protein are the same as some *O*-GlcNAc sites, whereas others are adjacent to, or even distant from, each other, such as on the C-terminal domain of RNA polymerase II and on cytokeratins. The final type of interplay involves proteins in which this relationship has yet to be clearly defined. The interplay between *O*-GlcNAc and *O*-phosphate is also underscored by the recent finding that OGT transiently forms complexes containing the catalytic subunit of protein phosphatase 1 (PP1c).

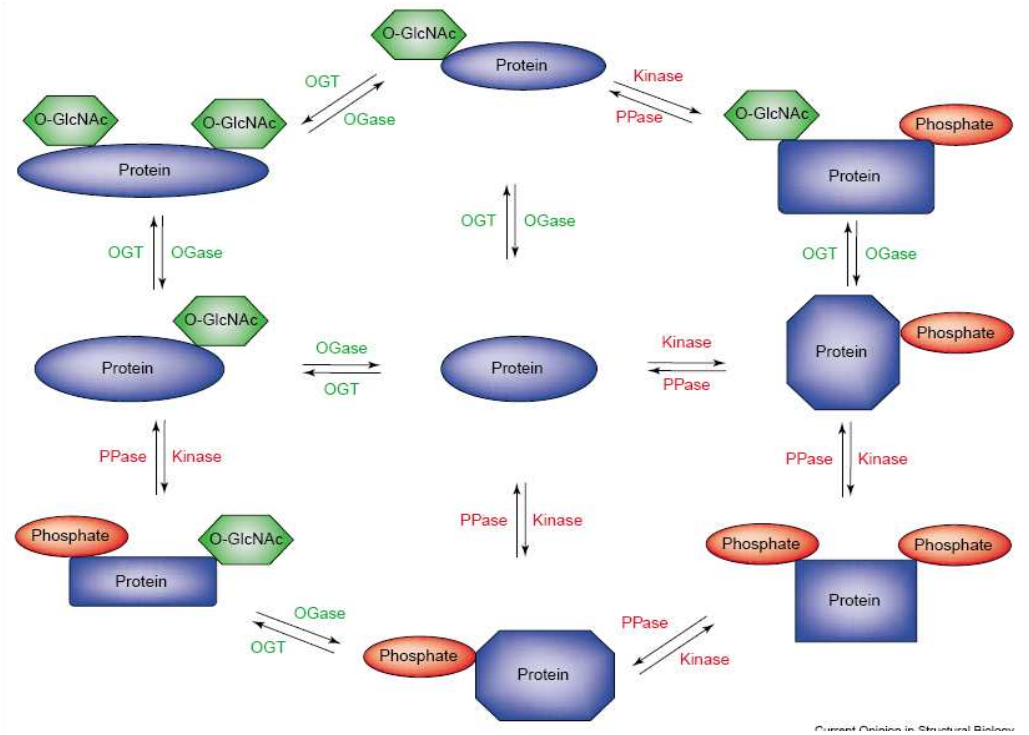


Figure 4.11 *There is a complex and dynamic interplay between O-GlcNAc and O-phosphate.*

Adjacent occupancy of a protein region by either *O*-GlcNAc or *O*-phosphate can affect protein function by each modification influencing the cycling of the other, or by their affect on other post-translational modifications. For example, *O*-GlcNAc at Ser 149 of p53 reduces phosphorylation at Thr 155, and also reduces ubiquitin-mediated degradation of p53 [Yang et al., 2006].

The dynamic and complex interplay between *O*-GlcNAc and *O*-phosphate requires modification of the current dogma with respect to cellular signalling. Clearly, the system is not binary with an 'on' or 'off ' state for each signalling molecule controlled by phosphate occupancy alone. Rather, the combination of modifications, which are differentially regulated, creates enormous molecular diversity.

4.6 Glycosylation and disease

4.6.1 Congenital disorders of glycosylation

Initially, the study of glycoproteins and their role in human congenital diseases focused on N-linked glycans. The diseases in this pathway have collectively been referred to as congenital disorders of glycosylation (CDG).

CDG form a group of autosomal recessive metabolic disorders caused by defects in the biosynthesis of protein-linked glycans. To date, mainly genetic defects in N-glycan biosynthesis have been classified as CDG. It is becoming increasingly evident that the primary defect of these disorders is not necessarily localized in one of the glycan-specific transferases, but can likewise be found in the biosynthesis of nucleotide sugars, their transport to the ER/Golgi, and in Golgi trafficking. The clinical variations within a disorder and among the different inborn errors of O-glycan metabolism are enormous. Defects can lead to a severe autosomal recessive multisystem syndrome with neurologic involvement, whereas some defects do not produce a clinical phenotype.

As O-glycans are involved in numerous processes, it is inevitable that defects in O-glycan biosynthesis might lead to severe abnormalities for cellular functioning. As O-glycosylation biosynthesis is a very complex process with an enormous number of genes involved, it is obvious that the disorders described to date are just the tip of the iceberg. Defects in the biosynthesis of protein-linked O-glycans lead to a highly heterogeneous group of diseases. The majority of patients with "classical CDG" have a defect in N-glycan biosynthesis. They have common

symptoms such as muscle hypotonia, central nervous system abnormalities, growth delay, feeding problems, coagulation defects, and liver disease, and frequently show specific signs such as abnormal fat distribution and inverted nipples, which help with the early clinical diagnosis. In contrast, patients with O-glycosylation disorders commonly have involvement of only one organ or one organ system and do not have the general symptoms that are suggestive for an inborn error of metabolism. Most of the disorders of O-glycan biosynthesis seem to have very specific tissue expression, whereas N-glycans are expressed ubiquitously. Another remarkable difference between N- and O-glycan deficiencies is that N-glycan deficiencies generally have recessive inheritance, whereas in some of the O-glycan biosynthesis diseases inheritance is autosomal dominant.

Patients with combined defects in protein N- and O-glycosylation often have a phenotype that is a mixture of the features of inborn errors in combination with congenital malformations.

In summary, the phenotypes of patients with a congenital defect in O-glycosylation are a continuum. This ranges from patients who have a defect in the biosynthesis of O-glycans affecting only a few proteins and therefore have only one or two tissues involved to patients who have a defect in the biosynthesis of O-glycans disturbing many proteins and thus have more than one organ/organ system involved to patients who have combined defects in the biosynthesis of N- and O-glycans, who have a typical multisystem disease [Wopereis et al., 2006].

4.6.2 O-GlcNAc and diabetes

O-GlcNAcylation not only has an important role in many fundamental cellular processes, but also its dysregulation contributes to the aetiology of important human diseases, particularly diabetes and neurological disorders (Table 1).

There is growing evidence of a link between anomalous O-GlcNAc modification and diabetes. Hyperglycemia is associated with insulin resistance or 'glucose toxicity' in type II diabetes, although the underlying molecular mechanisms for these effects are still largely unknown. O-GlcNAc transferase (OGT) uses the donor sugar UDP-GlcNAc in catalyzing the formation of O-GlcNAc. Concentrations of UDP-GlcNAc (the end product of the hexosamine pathway) in the cell are highly sensitive to ambient glucose levels. Increased flux through the hexosamine pathway under hyperglycemic conditions leads to elevated levels of O-GlcNAc modified proteins in skeletal muscle and in pancreatic beta cells. In the case of muscle cells, reduced insulin receptor substrate (IRS) –1 and –2 signaling are associated with their increased O-GlcNAc modification

and decreased phosphorylation. High concentrations of extracellular glucose, which leads to an increase in O-GlcNAc modified proteins, causes attenuated insulin stimulated signaling. For example, the ability of the p85 subunit of PI 3-kinase and Grb2 to bind IRS-1 are attenuated. Also, hyperglycemic conditions increase O-GlcNAc levels on the transcription factor Sp1 and this correlates with increased Sp1 dependent TGFβ1 expression. Thus, altered O-GlcNAc levels under hyperglycemic conditions appear to perturb normal signaling events required for insulin-mediated homeostasis [Vosseller et al., 2001].

Table 1 | Pathologies associated with aberrant *O*-GlcNAcylation

Tissue	Proposed mechanism	Pathology
Muscle, fat	Decreased GLUT4 trafficking Decreased insulin signalling Decreased glycogen synthesis	Insulin resistance
Vasculature	Decreased eNOS activity Increased SP1 activation	Vascular disease Erectile dysfunction Retinopathy
Kidney	Increased TGF-β expression	Glomerular sclerosis
Cardiac muscle	Decreased SERCA2a expression	Cardiomyopathy
Brain	Increased tau phosporylation Decreased tau *O*-GlcNAcylation	Alzheimer's disease

Table 1 *Pathologies associated with aberrant O-GlcNAcylation.*

O-GlcNAc has a key role in the regulation of insulin signalling and as a mediator of glucose toxicity [Buse, 2006]. Increasing O-GlcNAcylation in adipocytes or muscle blocks insulin signalling at several points. Elevated O-GlcNAcylation on insulin receptor substrate seems to reduce its interactions with phosphatidylinositol-3-OH kinase, thus blocking insulin signalling at an early stage. Many aspects of "glucose toxicity" require its metabolism into glucosamine, which, in turn, elevates UDP-GlcNAc and increases O-GlcNAcylation. Hyperglycaemia, hyperlipidaemia and/or hyperinsulinaemia all result in abnormal increases in O-GlcNAcylation, which disturbs the normal dynamic balance between O-GlcNAcylation and O-phosphorylation that controls signalling, transcription and other cellular functions.

Evidence is mounting that dysregulation of O-GlcNAcylation is a major mechanism underlying the molecular basis of glucose toxicity and insulin resistance, the two main hallmarks of type 2 diabetes.

4.6.3 Neurodegenerative disease

O-GlcNAc represents a key regulatory modification in the brain, contributing to transcriptional regulation, neuronal communication and neurodegenerative disease. Several studies have highlighted the importance of *O*-GlcNAc glycosylation in the nervous system. However, several lines of evidence suggest that *O*-GlcNAc has crucial roles in both neuronal function and dysfunction. The *O*-GlcNAc modification is abundant in the brain and present on many proteins important for transcription, neuronal signaling and synaptic plasticity, such as cAMP-responsive element binding protein (CREB), synaptic Ras GTPase-activating protein (synGAP) and β-amyloid precursor protein (APP). An intriguing interplay between *O*-GlcNAc glycosylation and phosphorylation has been observed in cerebellar neurons, wherein activation of certain kinase pathways reduces *O*-GlcNAc levels on cytoskeleton-associated proteins. Finally, recent studies suggest that *O*-GlcNAc is dynamically regulated by excitatory stimulation of the brain *in vivo* [Rexach et al., 2008].

Proteomic studies of *O*-GlcNAc–modified proteins from the brain have also underscored the importance of *O*-GlcNAc in regulating transcription. Approximately one-quarter of the neuronal *O*-GlcNAc–modified proteins known to date are transcriptional regulatory proteins. This includes numerous transcription factors (for example, SRY box–containing gene 2 (SOX2), activating transcription factor 2 (ATF2) and EGR1), as well as transcriptional coactivators (steroid receptor coactivator-1 (SRC1)), repressors (p66β and BRAF-HDAC complex 80 (BHC80)) and corepressors (transducin-like enhancer of split 4 (TLE4), carbon catabolite repression 4-negative on TATA-less (CCR4–NOT)) [Rexach et al., 2008].

There is considerable indirect evidence that O-GlcNAc may play a role in neurodegenerative disorders. There are a variety of O-GlcNAc modified proteins that are enriched in brain neurons including tau, β-amyloid precursor protein, neurofilaments, microtubule-associated proteins, clathrin assembly proteins (AP3 and AP180), synapsin I, collapsin response mediator protein-2 (CRMP-2), ubiquitin carboxyl hydrolase-L1(UCH- L1), and βsynuclein. Interestingly, OGT maps to the X-linked Parkinson dystonia locus and O-GlcNAcase maps to the Alzheimer's disease locus on chromosome 10 [Wells et al., 2003].

Tau is required for microtubule polymerization and stability in neurons. In neurons affected by Alzheimer's disease, tau becomes hyperphosphorylated, which, in turn, causes it to aggregate into the paired helical filaments that constitute the visible neurofibrillary tangles characteristic of the disease. In the healthy adult brain, tau is extensively O-GlcNAcylated at more than 12 sites. O-GlcNAcylation of human tau negatively regulates its O-phosphorylation in a site-specific manner

both *in vitro* and *in vivo* [Liu et al., 2004]. Neuron glucose metabolism declines with age, and the decline is more marked in Alzheimer's disease. One normal function of *O*-GlcNAc seems to be the prevention of excessive phosphorylation at sites at which this would be deleterious. Most proteins involved in the pathology of Alzheimer's disease are O-GlcNAcylated. Comparisons between brain tissue from healthy individuals and those with Alzheimer's disease have shown that global O-GlcNAcylation is reduced in Alzheimer's disease. The amyloid-β precursor protein (APP) is O-GlcNAcylated and its cytoplasmic tail is phosphorylated, which is known to affect its proteolytic processing. Abnormal proteolysis of APP gives rise to the toxic β1–42 peptide fragment, which forms amyloid plaques that are found in neurons in Alzheimer's disease. Neurofilaments are hypo-O-Glc-NAcylated in neurons in a rat model of amyotrophic lateral sclerosis (Lou Gehrig's disease). O-GlcNAcylation of the clathrin assembly proteins AP-3 and AP-180 declines in Alzheimer's disease, suggesting that reduced O-GlcNAcylation contributes to the loss of synaptic vesicle recycling [Hart et al., 2007].

Finally, *O*-GlcNAc glycosylation has been demonstrated to inhibit the proteasome, thus providing a mechanism to couple ubiquitin-mediated protein degradation to the general metabolic state of the cell. Blocking the removal of *O*-GlcNAc from the proteasome leads to increased protein ubiquitination and possibly neuronal apoptosis. Proteasomal dysfunction and ubiquitinated inclusion bodies are found in the diseased tissue of individuals with ALS, Parkinson's disease, Huntington's disease and Alzheimer's disease [Ciechanocer and Brundin, 2003].

In the brain, phosphorylation serves as a central mechanism for neuronal communication by regulating ion channels, neurotransmitter receptors, gene transcription and synaptic vesicle release. Protein kinases and phosphatases work together to coordinate different forms of synaptic plasticity, and they are necessary for the induction and maintenance of postsynaptic long-term potentiation and long-term depression. Thus, the potential interplay between *O*-GlcNAc glycosylation and phosphorylation has exciting implications for many neuronal functions. Early studies showed that activation of protein kinase C (PKC) or cAMP-dependent protein kinase (PKA) significantly decreased overall *O*-GlcNAc glycosylation in the cytoskeletal protein fraction of cultured cerebellar neurons. Conversely, inhibition of PKC, PKA, cyclin-dependent protein kinases or S6 kinase increased overall *O*-GlcNAc levels in these fractions [Rexach et al., 2008].

Current data suggest that *O*-GlcNAc has a significant role in normal neuronal function, and in the pathology of neurodegenerative disease. The interplay between *O*-GlcNAc and *O*-phosphate seems to be critical to the aetiology of neurodegenerative disease, as well as to the functions and turnover of oncoproteins or tumour suppressors.

4.6.4 O-GlcNAc and Cancer

As noted above, O-GlcNAc is present on many transcription regulatory proteins. Mutations of many of these proteins contribute to the oncogenic phenotype. Some of these mutations may exert their effects in part by disruption of O-GlcNAc-mediated regulation of these proteins. For example, the c-myc oncogene is glycosylated in its transactivation domain at Thr-58, which is also the mutational hotspot found in a large percentage of Burkitt's lymphomas from human patients. Interestingly, this glycosylation site is also an important phosphorylation site that regulates c-myc transcriptional activity, but it is not yet possible to distinguish between the biological importance of O-phosphate and O-GlcNAc at these sites of reciprocal modification. There also appears to be a reciprocal relationship between the O-phosphorylation and O-GlcNAc modification of the tumor-associated SV-40 Large T antigen. The tumor suppressor, p53, which is the most commonly mutated gene in a wide range of human cancers, is also O-GlcNAc-modified. There is preliminary evidence that the O-GlcNAc on p53 regulates its binding to DNA. Many essential regulators of cellular function are subject to complex phosphoregulation pathways. O-GlcNAc modification adds another level of regulation, which could allow for exquisite control of cell regulatory mechanisms. Disruptions of either of these post-translational modifications may interfere with critical control mechanisms, leading to the transformed phenotype [Comer and Hart, 2000].

4.7 Therapeutic glycoprotein production

Glycosylation patterns of recombinant proteins are relevant for the immunogenicity, the pharmacological activity, pharmacokinetic profile, solubility and stability of the protein.
Processing of oligosaccharides is specified by the processing enzymes present in the cell. Hence the glycoform populations for a glycoprotein are both species and cell specific. Thus for cells in stasis a characteristic oligosaccharide profile of the glycoprotein is obtained.
The importance of therapeutic proteins has grown rapidly since the emergence of the biotechnology industry more than 30 years ago. Currently, 64 products have been approved by European and US regulatory agencies with some 500 product candidates in clinical and preclinical development. Of these, approximately 70% are glycoproteins. As of 2003, the global market for biopharmaceuticals (recombinant therapeutic protein and nucleic acid based products) was estimated at more than $30 billion [Walsh, 2003].

Therapeutic proteins were initially derived from human sources; for example, blood clotting factors and human serum albumin from plasma, insulin from pancreas, and glucocerebrosidase from placenta. However, concerns over product purity and consistency, the potential for viral contamination and, importantly, the emergence of genetic engineering tools has shifted therapeutic protein production into recombinant expression systems. Mammalian cell lines, bacteria, yeast and insect cells have evolved as the major recombinant protein expression hosts, although these expression systems vary widely with regard to their ability to incorporate the post-translational modifications found on native human proteins. For example, only mammalian cell lines have the inherent capacity to carry out N-linked glycosylation of proteins during secretion; bacteria lack the N-linked glycosylation machinery and are thus not suitable hosts for glycoprotein production. Because many proteins of therapeutic importance require N-glycosylation for biological activity, expression systems with N-glycosylation capability have become essential for therapeutic glycoprotein production.

Both the function and efficacy of proteins are affected by the presence and composition of N-glycosylation structures. For example, non-human glycoforms can adversely affect pharmacokinetic (PK) properties and raise immunogenicity and safety concerns. Thus, the therapeutic use of glycoproteins derived from expression systems that do not perform human glycosylation could lead to rapid clearance, complement activation, and enhanced immunogenicity by targeting to antigen-presenting cells [Sethuraman and Stadheim, 2006].

PK plays a major role in determining the efficacy of many therapeutic proteins. The circulating half-life of proteins is affected by many factors, including molecular size and net charge, both of which are affected by the presence and composition of glycan structures. Glycans contribute significantly to the hydrodynamic volume and charge of glycoproteins. In particular, the content of sialic acid contributes to the net negative charge, and improves the PK of glycoproteins such as erythropoietin (EPO) [Macdougall, 2002]. Although glycosylation can improve plasma half-life, the presence of certain carbohydrate moieties can trigger lectin-mediated clearance, thereby reducing plasma half-life. Carbohydrate-binding proteins, or lectins, are differentially expressed and thus different tissues have varying affinities for specific glycoforms. Therapeutic proteins with distinct N-glycans can therefore be developed for cell- and tissue-specific targeting. N-linked glycosylation has been shown to affect the efficacy of some therapeutic glycoproteins by modulating the interaction with specific receptors.

The most effective production of pharmaceutically active glycoproteins is using mammalian expression systems like Chinese hamster ovary cells (CHO), mouse fibroblast cells (C127-BPV) and transgenic animals expressing the cDNA or genomic DNA encoding the protein of interest.

Various recombinant molecules have been expressed in CHO cell lines. CHO-K1 cells in common with human cells contain a large repertoire of oligosaccharide processing enzymes and can therefore process a wide variety of glycans.

Chinese hamster ovary (CHO) cell lines are currently the preferred host for the production of therapeutic glycoproteins. These cell lines offer several advantages, including extensive know-how of this system and an established infrastructure in the biotechnology industry, process scalability, and the capacity to produce proteins with N-glycans similar to those found on human proteins. However, CHO cell-based expression systems also have several disadvantages: a relatively high cost of goods; the potential for propagating infectious agents, such as viruses and prions; a long development time from gene to production cell line; and the inability to adequately control N-glycosylation. For example, whereas humans lack the pathway for the synthesis of N-glycolylneuraminic acid, EPO produced from CHO cells contains both N-glycolylneuraminic acid and N-acetylneuraminic acid. Glycoconjugates that contain N-glycolylneuraminic acid might be subject to clearance by anti-N-glycolylneuraminic acid antibodies present in human serum [Zhu and Hurst, 2002]. In addition, glycoproteins produced in CHO cells display inherent glycan heterogeneity, resulting in a mixture of molecules with varying efficacy profiles. Moreover, this heterogeneity is sensitive to culture conditions making batch-to-batch reproducibility a process development challenge.

An expression system that allows for control over the glycosylation of therapeutic proteins has, until recently, been unavailable. Through the customization of glycan profiles, such as that recently demonstrated in glycoengineered yeast, it is now possible to systematically probe for glycosylation-dependent therapeutic effects. Increased availability of glycoengineered expression systems will enable a greater understanding of structure-function relationships among glycoforms, which should lead to the development of novel therapeutics [Sethuraman and Stadheim, 2006].

5. AIM OF THE RESEARCH

The repressor element 1-silencing transcription factor (REST) was first identified as a protein that binds to a 21-bp DNA sequence element (known as repressor element 1 (RE1)) resulting in transcriptional repression of the neural-specific genes [Chong et al., 1995; Schoenherr and Anderson, 1995]. The original proposed role for REST was that of a factor responsible for restricting neuronal gene expression to the nervous system by silencing expression of these genes in non-neuronal cells. REST is usually expressed in nonneuronal cells and is rarely expressed in neurons *in vivo* [Ballas et al., 2005]. However, REST was also found to be expressed in certain maure neurons in adults [Shimojo et al., 2004; Griffith et al., 2001], suggesting that it is either re-expressed or that its expression is maintained in a specific type of neurons. Although it was initially thought to repress neuronal genes in non-neuronal cells, the role of REST is complex and tissue dependent.

In support of this view, it was subsequently observed that REST is transcribed in NSCs but its transcription is blocked as the cells undergo neuronal differentiation [Ballas et al., 2005; Paquette et al., 2000]. Although REST repression is required to block the transcription of multiple neuronal differentiation genes, its absence alone is not sufficient to activate these multiple target genes. From this observation, the expression of multiple neuronal differentiation genes requires both the absence of REST function and the presence of other promoter/enhancer-specific positive activators [Mortazavi et al., 2006].

Mandel's group also found that the REST gene is transcribed in both ES cells and NSCs. In ES cells, the REST protein is expressed at high levels, and the REST-corepressor complex occupies and strongly represses its target genes. As the ES cells transition into NSCs, most of the REST protein is degraded by the proteasomal pathway. In NSCs, as the level of the REST protein decreases, the REST-corepressor complex still occupies and represses its target genes but less strongly than in ES cells, causing the target genes to be poised for transcription. Then, as the NSCs exit the cell cycle and differentiate into mature neurons, REST gene transcription is blocked. In the absence of active REST transcription, the REST protein level diminishes, resulting in the expression of class I genes. Proper physiological stimulus will then activate the class II genes and contribute to neuronal plasticity [Ballas et al., 2005].

Extracellular signalling molecules regulating REST have been investigated. Growth factors are among the environmental signals implicated in the control of neurogenesis and neuronal differentiation [Cameron et al., 1998]. Insulin-like growth factor I (IGF-I) and its receptors are expressed early in the development of the nervous system and play a key role in the development

of the nervous system, with demonstrated effects on many stages of brain development including cell proliferation, cell differentiation, and cell survival [D'Ercole et al., 1996; Bondy et al., 2004].
In vitro, IGF-I acts as a mitogen, stimulates neurite outgrowth and inhibits apoptosis in a variety of neuronal cells, including neuroblastoma-derived cell lines [Kurihara et al., 2000; van Golen and Feldman, 2000]. Moreover, several studies have demonstrated that the SH-SY5Y neuroblastoma cell line can be induced to differentiate in presence of serum following activation of protein kinase C (PKC) with phorbol esters [Påhlman et al., 1981], at concentration that activate but do not down-regulate PKC.
Neuroblastoma cell lines are a suitable model for investigating the mechanisms that induce neuronal differentiation. Continuously dividing neuroblastoma cells can, in fact, be induced to differentiate into cells that are morphologically, biochemically and electrophysiologically similar to neurons after exposure to chemicals and⁄or neurotrophic factors and have reported that IGF-I and phorbol esters induce neurite outgrowth in the human neuroblastoma cell lines SH-SY5Y [Påhlman et al., 1995; Seiler et al., 2001].
In this study I investigated any role played by REST in the induction and patterning of differentiation of SH-SY5Y human neuroblastoma cells exposed to IGF-I. and phorbol 12-myristate 13-acetate (PMA) To down-regulate REST expression we developed an antisense (AS) strategy based on the use of phosphorothioate oligonucleotides (ODNs). To evaluate REST mRNA levels, we developed a real-time PCR technique and REST protein levels were evaluated by western blotting.
REST levels were further related to:

(i) cell proliferation;
(ii) morphological differentiation;
(iii) expression of synapsin I and βIIItubulin, two neuronal genes containing an RE-1 motif in the promoter and regulated by REST [Schoch et al., 1996; Lunyak et al., 2002];
(iv) expression of growth cone-associated protein 43 (GAP-43), a neuronal marker gene not directly regulated by REST [Bahn et al., 2002; Lunyak et al., 2002].

In order to relate the decreased REST expression with a progressive neurite extension, I investigated any possible involvement of the ubiquitin–proteasome system (UPS), a multi-enzymatic pathway which degrades polyubiquinated soluble cytoplasmic proteins [Pickart and Cohen, 2004]. For this purpose, SH-SY5Y cells are concomitantly exposed to PMA and the proteasome inhibitor MG132.

Finally, the glycosylation pattern of the REST protein was analysed, moving from the observation that the molecular weight calculated on REST sequence is about 116 kDa but using western blotting this transcription factor appears to have distinct apparent molecular weight (see Table 1.1): this difference could be explained by post-translational modifications of the proteins, like glycosylation. In fact recently, several studies underlined the importance of O-glycosylation in modulating transcriptional silencing, protein phosphorylation, protein degradation by proteasome and protein–protein interactions [Julenius et al., 2005; Zachara and Hart, 2006].

6. MATERIALS AND METHODS

6.1 Materials

Cell culture media and fetal calf serum (FCS) were from Invitrogen (Carlsbad, CA, USA). Recombinant human IGF-I was purchased from Pepprotech Inc. (Rocky Hill, NJ, USA). The monoclonal antibody 12C11-IBII was raised against the N-terminal region of REST [Schoenherr & Anderson, 1995] and was a kind gift of Dr D.J. Anderson (Caltech, Pasadena, CA, USA); monoclonal GAP-43, β-actin, βIII-tubulin and synapsin I and FLAG antibodies, Phorbol 12-myristate 13-acetate (PMA) and MG132 were from Sigma (Steinheim, Germany). Polyclonal anti-histone H1 antibody was purchased from ProSci (Poway, CA, USA). Polyclonal antibodies for the active form of the MAPK enzymes and the MAPK enzymes were obtained from Promega (Madision, WI, USA). The monoclonal anti-human IGF-I receptor antibody was from R & D Systems (Minneapolis, MN, USA). The peroxidase-conjugated secondary antibodies goat anti-mouse IgG and goat anti-rabbit IgG were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The secondary fluorescent antibodies Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR, USA). EXGEN 500 Transfection Reagent was from Fermentas (Hanover, MD, USA). Methyl-[^{3}H]-thymidine (5Ci/mmol) was provided by Amersham Biotec (Milan, Italy). Phosphorothioate and phosphodiester oligonucleotides were from Invitrogen or Sigma-Genosys (Pampisford, UK). PD 98059 was from Alexis Italia (Vinci, Italy). GF109203X, Gö6976 and Glycoprotein deglycosylation kit were from Calbiochem (San Diego, CA). All other reagents were of analytical grade or of the highest purity available, purchased from Sigma or Roche-Boehringer (Mannheim, Germany).

6.2 Cell culture

SH-SY5Y cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and routinely grown as monolayers in MEM and Ham's F12 (1:1) medium supplemented with 15% (v/v) heat-inactivated FCS (65°C, 30min), L-glutamine (2mM), pyruvic acid (1mM), 1× non-essential amino acids and 1× antibiotic-antimycotic solution (defined as FCS medium) in a humidified environment containing 5% CO_2 and 95% air. Cells were grown in 75-cm^2 flasks,

detached with a cell scraper and viability was determined using propidium iodide staining. For IGF-I experiments, cells were first seeded at 5×10^5/75-cm^2 flask in FCS medium for 24h and subsequently cultured in serum-free medium (SFM) composed of Dulbecco's modified Eagle's medium and Ham's F12 (1:1) supplemented with glucose (4.5g/L), transferrin (100μg/mL), sodium selenite (5ng/mL), HEPES (3.36mg/mL), sodium bicarbonate (1.2mg/mL) and 1× antibiotic-antimycotic solution (defined as SFM). The change from serum-containing medium to SFM was considered day 0; 24h later, the cells were treated with IGF-I in SFM and controls were maintained in SFM alone. Cells were exposed to IGF-I for 48h, 3 or 5days. In these latter experiments, the cells received fresh medium containing IGF-I every 2days. For PMA experiments FCS medium was used. Cells were used over a minimum of 12 passages to minimize intersubline differentiation. To assess the effect of the exposure to IGF-I and PMA on neurite outgrowth, the cells were analysed in an inverted phase contrast microscope (Eclipse TS100; Nikon) and photographed with a 35-mm camera (Nikon). Processes that were at least two cell diameters in length were considered neurites and the results are expressed as percentage of cells bearing neurites. The length of the neurites was also scored. The length of neurite processes of cells was taken as the distance from the tip of a process to the middle of the cell soma. Neurites were quantified by counting at least 100 cells in randomly chosen fields (20× magnification) for each treatment group. At least three independent experiments were conducted.

HEK293 cells were obtained from European Collection of Cell Cultures (Salisbury, UK) and routinely grown as monolayers in EMEM (EBSS) supplemented of 2mM glutamine, 1% non essential amino acids, 10%FCS and 1× antibiotic-antimycotic solution in a humidified environment containing 5% CO_2 and 95% air. HEK293 cells were plated in 100mm dishes and at 50–60% confluence were transiently transfected with pCMV-Tag2+REST plasmid (10μg/dish) using the EXGEN 500 Transfection Reagent (Fermentas, Hanover, MD, USA). After 72h from transfection cells were collected and nuclear and cytoplasmic proteins were extracted. pCMV-Tag2B+REST plasmid contains REST coding sequence fused to FLAG epitope.

6.3 Antisense oligonucleotide experiments

The AS phosphorothioate ODN used in this study was complementary to nucleotides 390–411 of REST mRNA: (5'-TGTTGCCACTGCTGGTAAACAG-3', GenBank™ Accession no. NM_005612). A mismatch (MS) ODN was also designed for use as negative control :

(5'-GGTTACCTCTCCTAGCCATCGG-3').

A GenBank™ search indicated that these ODNs were not complementary to mRNA sequences in any other human gene so far entered in the database. ODNs were reconstituted in sterile, nuclease-free Tris-EDTA buffer (10mM Tris HCl, 1mM EDTA, pH7.4) and stored at −20 C. ODNs were diluted to the desired concentration and added to SH-SY5Y cells as a complex with oligofectamine (Invitrogen) according to the manufacturer's protocol. When required, IGF-I or FCS (15%, v/v) was added 2h later. Cultures were maintained up to 5days in the presence of ODNs during which time they received fresh medium containing ODNs every 2days.

6.4 [^{3}H]Thymidine incorporation

Cells were seeded in 24-well plates (5×10^3 cells/well) and grown in FCS medium for 24h. The cells were then starved by culturing in SFM and treated as reported in the Results. [^{3}H]Thymidine (2μCi/mL) was added during the final 5h of incubation; the medium was then removed and the cells fixed with 10% trichloroacetic acid, washed in methanol and lysed in 0.1M NaOH, followed by 0.1M HCl and liquid scintillation counting.

6.5 Semiquantitative real-time polymerase chain reaction

For reverse transcription-polymerase chain reaction (PCR) experiments, cells were collected from tissue culture flasks, centrifuged (500***g*** for 5min) and rinsed with phosphate-buffered saline (PBS). Total cellular RNA was extracted using Trizol® reagent (Invitrogen) and digested with RNAse-free DNAse (Invitrogen) for 15min at 25°C according to the manufacturer's instructions. A 5-μg aliquot was reverse transcribed using oligo(dT) primers (300ng), 1μL RNAse inhibitor (40U/μL) and 1μL Maloney murine leukemia virus-reverse transcriptase (50U/μL) (Stratagene, La Jolla, CA, USA) in a final volume of 50μL. The reaction mixture was incubated for 60min at 37°C and the reverse transcriptase was inactivated by heating at 94°C for 5min. A real-time PCR was employed for relative quantification of REST transcripts using the Light Cycler Instrument and the LightCycler-Faststart DNA Master SYBR Green I kit (Roche Diagnostics, Milan, Italy). This ready-to-use 'hot start' reaction mix contains *Taq* DNA polymerase, dNTP mix and the fluorescent dye SYBR Green I for real-time detection of double-stranded DNA. Reactions were set up in 20μL including 100nM of each primer, 150ng of target cDNA and a convenient $MgCl_2$

concentration (4mM). To amplify the 3' cDNA REST region, a sense primer (5'-TCACATGGAGCCAATTTCCA-3') and an AS primer (5'-TTTCCTTTGGCAGTGGTGGT-3') amplifying a 197-bp fragment (from 2762 to 2958bp) were used. As control, a 169-bp fragment of the L19 ribosomal protein gene was amplified using a sense primer (5'-CTAGTGTCCTCCGCTGTGG-3') and an AS primer (5'-AAGGTGTTTTTCCGGCATC3') (amplifying a fragment from 62 to 230bp; GenBank™ Accession no. BC062709). Amplification was performed using the following profile: 95 C for 10min followed by 45 cycles of 95°C for 2s, 63°C for 5s and 72 C for 8s. Following PCR, the temperature was lowered to 40°C for 30s and the specificity of the reaction was then verified by melting the curve analysis obtained by increasing the temperature from 55 to 95 C (0.1°C/s). Optical data were collected over the duration of the temperature drop, with a dramatic increase in fluorescence occurring when the strands reanneal. This was done to ensure that only one PCR product was amplified per reaction. Relative expression of the reverse transcription-PCR products was determined using the $\Delta\Delta C_t$ method [Winer etal., 1999]. This method calculates relative expression using the equation: fold induction=$2^{-\Delta\Delta Ct}$ where C_t is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta C_t$=[C_t gene of interest (unknown sample)−C_tL19 (unknown sample)]− [C_t gene of interest (calibrator sample)−C_tL19 (calibrator sample)]. One of the control samples was chosen as the calibrator sample and used in each PCR. Each sample was run in triplicate and the mean C_t was used in the $\Delta\Delta C_t$ equation. L19 was chosen for normalization because this gene showed consistent expression relative to other housekeeping genes among the treatment groups in our experiments. In a separate set of experiments, the DNA sequences of reverse transcription-PCR products were confirmed by DNA sequencing (Macromolecular Resources, Colorado State University, Fort Collins, CO, USA) and found to be identical to the corresponding sequences of human REST and L19 cDNA (data not shown).

6.6 Western blotting

SH-SY5Y and HEK293 cells were scraped off in 10mL of cold PBS, pelleted and resuspended in 200μL of CER I buffer (NE-PER™ Extraction Reagent; Pierce, Rockford, IL, USA). After 10min incubation on ice, 11μL of CER II buffer (NE-PER™ Extraction Reagent; Pierce) was added and the suspension was resuspended by a vortex, incubated on ice for 1min and then resuspended. The cytoplasmic fraction was separated by centrifugation at 16000***g*** for 5min. To

obtain the nuclear extract, the cell pellet was resuspended in 100μL of NER (NE-PER™ Extraction Reagent; Pierce) buffer and incubated on ice for 40min. The soluble proteins in the lysate were separated by centrifugation at 16000***g*** for 10min at 4 C. The protein content was quantified using a BCA protein assay (Pierce). Proteins of the cytoplasmic extract (20μg) were separated by 7.5 or 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins of the nuclear extract (70μg) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We used a Cruz marker (sc-2035; Santa Cruz Biotechnology, Inc.) as molecular weight standard. Proteins were then transferred to Protran™ nitrocellulose membranes (Schleicher & Schuell Italia, Legnano, Italy) which were blocked with 5% non-fat milk in TBS (10mM Tris-HCl, pH8, containing 150mM NaCl) plus 0.1% Tween 20 for 1.5h at room temperature (25°C). The blots were then probed for 1.5h at room temperature in TBS containing 0.1% Tween 20, 5% non-fat milk and antibodies with a dilution of 1:50 for REST monoclonal antibody, 1:8000 for GAP-43, 1:2500 for synapsin I, 1:2000 for βIII-tubulin, 1:5000 for β-actin antibody (used as loading control for cytoplasmic lysates) and 1:1000 for histone H1 antibody (used as loading control for nuclear lysates) and FLAG antibody. The membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:8000. Blots were finally developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce).

6.7 Electrophoretic mobility shift assays

Nuclear extracts were prepared using the NE-PER™ extraction kit (Pierce). The protein concentration of nuclear fractions was determined by a BCA protein assay (Pierce) and all extracts were stored at −70°C. Electrophoretic mobility shift assays were performed using 15μg of nuclear protein extract obtained from cells cultured in SFM and treated with IGF-I. The probe was a 60-bp biotin end-labeled, duplex oligonucleotide containing the 21-bp RE-1 sequence upstream of the human vescicular acetylcholine transporter and choline acetyltransferase gene:
(sense sequence,
5'CTTGGTTGCGGTGGGGCCGGGGCCAGCACCCTGGACAGCTCCCGGCGGCTGGC
GGGGAGA-3';
complementary sequence,
5'TCTCCCCGCCAGCCGCCGGGAGCTGTCCAGGGTGCTGGCCCCGGCCCCACCGCA
ACCAAG-3')

[Shimojo et al., 2001]. DNA binding reactions were set up employing the LightShift™ Chemiluminescent electrophoretic mobility shift assay kit (Pierce), the nuclear extract and 62.5fmol of biotin-labeled oligonucleotide duplex. For reactions containing the specific competitor oligonucleotide, a 200-fold molar excess of unlabeled double-stranded oligonucleotide (the sequence is reported above) was added before the biotin end-labeled probe and incubated for 20min at room temperature (22–24°C). The reactions were performed in the standard binding buffer supplied (10mM Tris-HCl, 50mM KCl, 1mM DTT, pH7.5) plus 50ng/μL poly(dI-dC) non-specific competitor DNA and were incubated for 20min at room temperature. In some experiments, 15μg of nuclear protein extract was pre-incubated at room temperature with or without a monoclonal antibody to REST (12C11-IBII) diluted 1:2 for 30min before adding the probe. The reaction mixture was loaded onto a 4% non-denaturating polyacrylamide gel with 0.5× TBE buffer and electrophoresed for 60min at 100V. The gel was transferred to a nylon membrane (Nytran™; Schleicher & Schuell Italia) in 0.5× TBE at ≈350mA for 60min. The transferred DNA was cross-linked (120mJ/cm^2 for 1min at 254nm) using a UV cross-linker and the detection was performed by a chemiluminescent procedure as indicated by the manufacturer.

6.8 Enzymatic removal of O-linked Oligosaccarides

In order to characterize the presence of any carboydrate chain on REST protein we performed an enzymatic removal of O-linked oligosaccarides using Glycoprotein Deglycosylation kit following manufacturer instruction (Calbiochem). Briefly, reaction mixtures contained 0.5μl each of several enzyme (different combinations of the following enzymes: α2-3,6,8,9-Neuraminidase, Endo-α-N-acetylgalactosaminidase, β1-4-galactosidase and β-N-acetylglucosaminidase), 100μg of proteins, and 5X reaction buffer (250mM sodium phosphate buffer, pH 7.0). The mixtures were incubated at 37°C for 3 days. Then mixtures were analyzed using western blotting.

6.9 Immunocytochemistry

SH-SY5Y were grown (10^{4}cells/mL) on poly-L-lysine-coated coverslips in six-well plates and cultured, as previously described, in FCS for 24h and then in SFM. After treatments, the cells

were washed three times with SFM and fixed using 4% paraformaldehyde (w/v) in PBS (pH7.4) for 10min and then with methanol for 20min at room temperature. Fixed cell cultures were blocked in PBS containing 2% bovine serum albumin for 1h at 4 C. The same blocking buffer was used for incubation with primary antibodies to βIII-tubulin (1:1000), synapsin I (1:1000) and GAP-43 (1:2500) that was carried out overnight at room temperature in the dark. Following washing in PBS, coverslips were incubated in Alexa Fluor 488 anti-mouse IgG secondary antibody or Alexa Fluor 568 anti-rabbit IgG secondary antibody at room temperature for 1h in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dilactate (Sigma). Coverslides were mounted with Prolong antifade kit (Molecular Probes). The slides were examined using an Eclipse E800 fluorescence microscope (Nikon) with a digital camera attachment. Composites were prepared using Adobe Photoshop. Controls, treated with the secondary antibodies alone, showed only non-specific background staining. Stained cells were counted in a minimum of six parallel fields per group. Data are the results of three or four independent experiments.

6.10 Data analysis

To quantify the intensity of the bands, membranes were scanned and analysed by an imaging densitometer (GS-700; Bio-Rad, Hercules, CA, USA). The bands were quantified with the Molecular Analyst™ image analysis software (Bio-Rad). All data are presented as mean ± SEM for the indicated number of experiments. Statistical significance was determined by Newman-Keuls test after ANOVA using GraphPad Prism (version 3.0; GraphPad Software, Inc., San Diego, CA, USA). *P*-values<0.05 were considered to be significant.

7. RESULTS

7.1 Insulin-like growth factor I regulates repressor element 1 silencing transcription factor expression in SH-SY5Y cells

To evaluate REST mRNA levels, we developed a semiquantitative real-time PCR technique by amplifying a cDNA sequence of 197bp located in the 3'-end region of mRNA coding for this transcription factor. This strategy allowed us to quantify only REST transcripts of the entire sequence of mRNA avoiding any interference of truncated forms which have been already described in SH-SY5Y cells [Palm et al., 1999]. In neuroblastoma cells maintained in SFM for 24h and then in FCS (15% v/v) for 24h, concentrations of REST mRNA were significantly higher (P<0.01) than in cells cultured in SFM for 48h (Figure 7.1A).

Exposure of SH-SY5Y cells to IGF-I (10 and 100nM) for 24h induced a significant (P<0.01) increase in steady-state mRNA levels of REST in comparison to cells maintained in SFM. REST mRNA levels with IGF-I started to rise at a concentration of 10nM and showed a maximal increase in cells exposed to 100nM IGF-I (Figure 7.1A). Neutralization of the IGF-I receptor with a specific monoclonal antibody (20μg/mL; added to the cells 60min before the trophic agent) significantly reduced (P<0.05) the action of IGF-I on REST mRNA (Figure 7.1A). Thus, it can be suggested that the action of IGF-I on REST mRNA is mediated by the IGF-I receptor.

The effect of IGF-I (100nM) appeared by 12h of treatment and reached a peak in cells exposed to this trophic factor for 48h; mRNA levels afterward declined in cells exposed to IGF-I for 3days ($2^{-\Delta\Delta C_t}$: 16±2; n=4) or 5days (Figure 7.1B).

We used PD 98059, a non-competitive blocker of MAPKs [Woodgett, 2000], to evaluate whether this pathway participates in IGF-I-induced neuron-restrictive silencer factor/REST expression. In SH-SY5Y cells exposed for 24h to 100nM IGF-I in the presence of PD 98059 (50μM), a significant reduction (P<0.05) of steady-state mRNA coding for REST was found (Figure 7.1B).

The levels of the corresponding REST protein in nuclear cell extracts were evaluated by western blotting (Figure 7.1C and D) and followed a pattern similar to mRNA expression. In agreement with other studies [Shimojo et al., 1999; Grimes et al., 2000; Calderone et al., 2003; Martin et al., 2003], we observed only a single band of ≈ 200kDa. Adopting the technique described in Materials and methods, no truncated form corresponding to REST4 or REST5 was detected (these truncated proteins should have an apparent molecular weight of ≈ 53kDa; Lee et al., 2000).

REST levels were significantly elevated (P<0.01) in cells exposed to FCS or IGF-I (100nM). The

protein content rose more with 48h exposure to IGF-I and was then reduced in cells exposed to IGF-I for 5days, thus suggesting a timing expression of this protein in cells exposed to IGF-I.

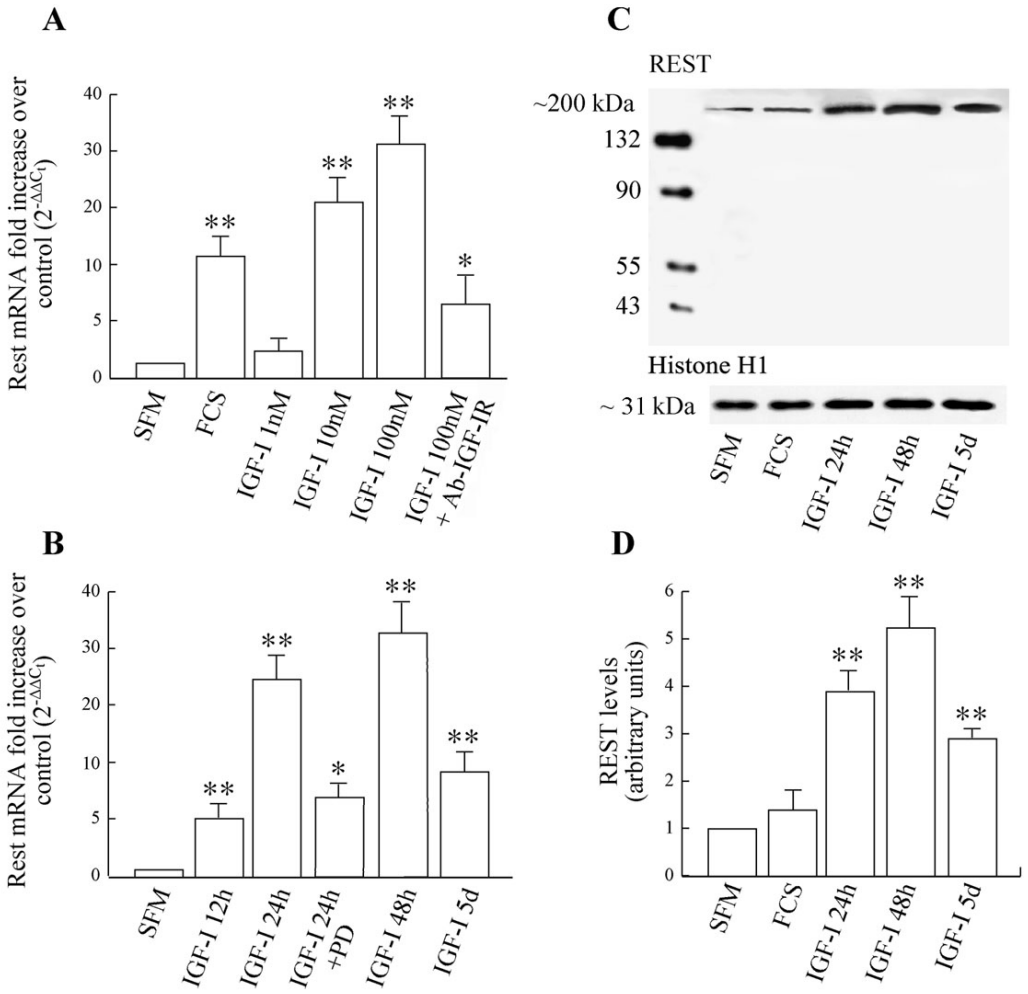


Figure 7.1 *IGF-I and fetal calf serum (FCS) up-regulate steady-state levels of REST mRNA (A and B) and nuclear protein levels (C and D) in SH-SY5Y cells maintained in serum-free medium (SFM) for 24 h. Relative fold induction of the REST gene following various treatments was calculated using the $\Delta\Delta C_t$. Exposure to FCS or IGF-I (10-100 nM) for 24 h increases REST mRNA levels (A) or REST protein levels (C and D) in comparison to cells maintained in SFM for 48 h. Time-dependent increase of REST mRNA levels (B) and REST nuclear protein levels (D) in cells exposed to IGF-I (100 nM) for 24, 48 h and 5 days is shown. The action of IGF-I on REST mRNA was reduced by neutralization of the IGF-I receptor with a specific monoclonal antibody (Ab-IGF-IR) [20 μg/ mL; added 60 min before 100 nM IGF-I; (A) IGF-I 100 nM + Ab-IGF-IR] or in the presence of PD 98050 (50 μM; added 60 min before 100 nM IGF-I; (B) IGF-I 24 h + PD). Histone H1 was evaluated in the same samples and used for loading control. Values are the mean ± SEM of at least six independent experiments carried out in triplicate. *P < 0.05 vs. IGF-I 100 nM; **P < 0.01 vs. SFM (Newman-Keuls test after ANOVA).*

7.2 Antisense inhibition of repressor element 1 silencing transcription factor expression in SH-SY5Y cells

In order to investigate any role played by REST on effects elicited by IGF-I in neuroblastoma cells, we developed an AS strategy based on the administration of ODNs to down-regulate the expression of this transcription factor. To confirm the specificity and efficacy of the AS-ODN complementary to REST mRNA, this was added to cells cultured in FCS for 48h as, in cells cultured in SFM, the levels of this protein were too low to quantify any appreciable reduction (see Figure 7.2A). As ascertained by western blotting analysis, exposure to AS-ODN (1μM) resulted in a significant decrease ($P<0.01$) of nuclear REST levels whereas, in cells cultured in the presence of an MS-ODN (1μM) or 0.1μM AS-ODN, REST levels were similar to control cells maintained in FCS alone (Figure 7.2A), thus indicating a sequence-specific down-regulation of this protein by AS-ODN. In nuclear extracts of cells exposed to 1μM AS-ODN the reduction of REST levels was ≈ 70% of the control cells; the exposure to 10μM AS-ODN induced a decrease of REST approaching 80% whereas higher concentrations of ODNs (>15μM) affected cell viability (data not shown).

In SH-SY5Y cells exposed for 24h to SFM and then to IGF-I (100nM) for 5days, the concomitant exposure to AS-ODN (1μM) but not to MS-ODN (1μM) produced a noteworthy reduction of REST nuclear levels, as evaluated by western blotting analysis (Figure 7.2B).

Electrophoretic mobility shift assays were used to evaluate any change of the DNA binding activity of REST to the RE-1 element as a consequence of the exposure of SH-SY5Y cells to IGF-I and with the aim of relating these changes to nuclear levels of this transcription factor evaluated by western blot analysis. As shown in Figure 7.3A, the extracted nuclear proteins bound to the 60-bp duplex oligonucleotide containing the RE-1 sequence to form a retarded band (indicated by an arrow) which was elevated in intensity by FCS and IGF-I treatment (100nM) and it followed a pattern similar to western blotting experiments shown in Figure 7.1C and D. A competitive electrophoretic mobility shift assays with a 200-fold excess of unlabeled, double-strand oligonucleotide confirmed that DNA–protein interaction was specific (Figure 7.3B). Incubation of nuclear extracts with a REST antibody significantly decreased and super-shifted the protein–oligonucleotide complex (Figure 7.3B). In SH-SY5Y cells exposed to IGF-I (100nM) for 5days, the concomitant exposure to AS-ODN (1μM) but not to MS-ODN (1μM) produced a relevant reduction of the protein-oligonucleotide complex in comparison to cells treated with IGF-I (100nM) alone (Figure 7.3C).

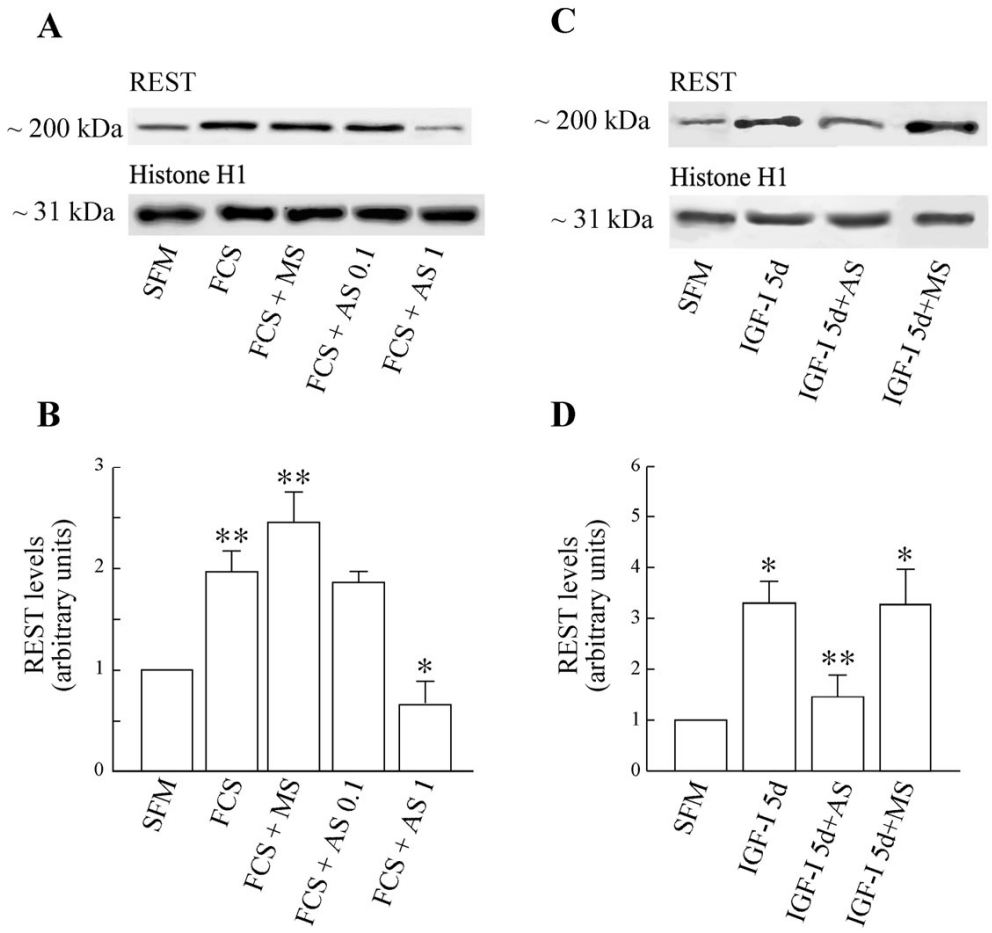


Figure 7.2 *Antisense (AS) inhibition of REST expression in SH-SY5Y cells. (A and B) AS oligonucleotide complementary to REST mRNA down-regulates REST nuclear protein levels in SH-SY5Y cells maintained in fetal calf serum (FCS). Cells were exposed for 48 h to AS (0.1 and 1 μM) or to a mismatch (MS) oligonucleotide. Control cells were cultured in FCS alone. (A) Representative western blot analysis. (B) Densitometric analysis of the bands (mean ± SEM; n = 6). (C and D) Effect of insulin-like growth factor I (IGFI) and AS oligonucleotide exposure on nuclear protein levels in SH-SY5Y cells maintained in serum-free medium (SFM). Cells grown for 6 days in SFM alone or for 24 h in SFM and for 5 days in the presence of IGF-I (100 nM); IGF-I and AS (1 μM) or an MS oligonucleotide (1 μM). (C) Representative western blot analysis. (D) Densitometric analysis of the bands (mean ± SEM; n = 6). Histone H1 was evaluated in the same samples and used for loading control. *P < 0.05; **P < 0.01 vs FCS (Newman-Keuls test after ANOVA).*

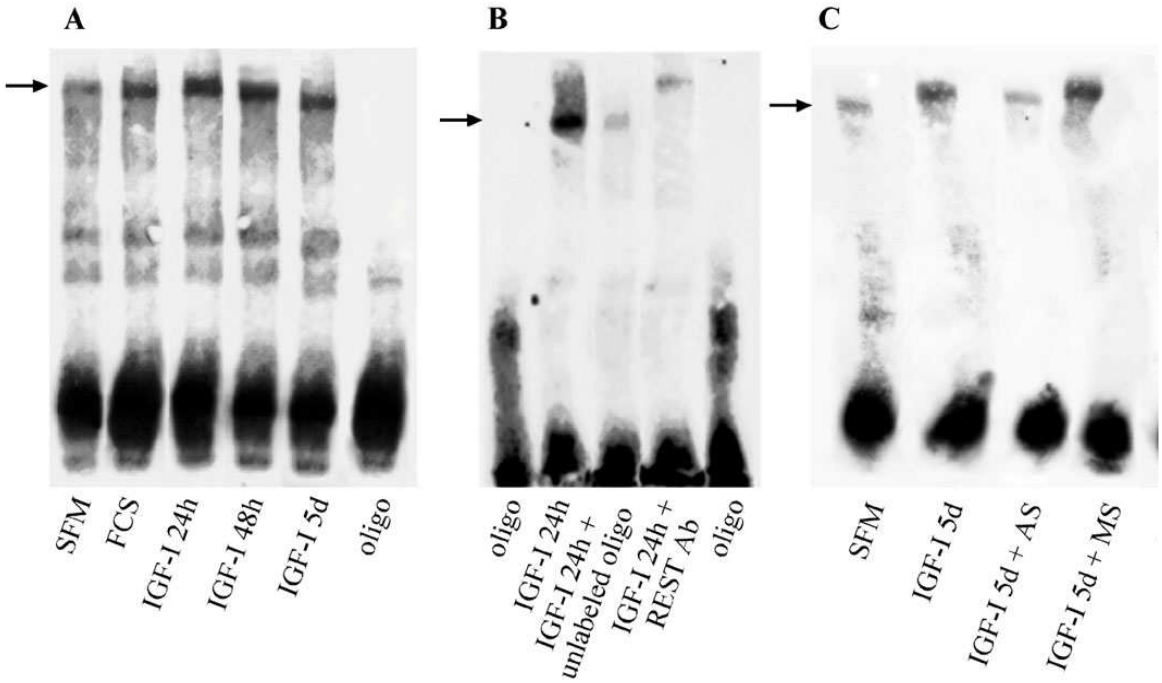


Figure 7.3 *REST binding to RE-1 element is increased in nuclear extracts obtained from cells maintained in serum-free medium (SFM) and exposed for 24 h to fetal calf serum (FCS) or insulin-like growth factor I (IGF-I) (100 nM) for 24, 48 h or 5 days. (A) Representative EMSA analysis of an experiment repeated five times with similar results. Control cells were cultured in SFM for 48 h. (B) Specificity of the complexes was examined in nuclear extracts of cells exposed to IGF-I (100 nM; 24 h) (IGF-I 24 h) and incubated with a 200-fold molar excess of unlabeled double-strand oligonucleotide containing the RE-1 sequence (IGF-I 24 h + unlabeled oligo); this treatment decreased the formation of the biotin-labeled oligonucleotide / protein complex. Incubation with the monoclonal REST antibody 12C11-IBII (IGF-I 24 h + REST Ab) reduced and super-shifted the REST–oligonucleotide complex (as described in Bai et al., 2003). (C) Effect of IGF-I and antisense (AS) oligonucleotide exposure on nuclear protein levels in SH-SY5Y cells. Cells grown for 6 days in SFM alone or for 24 h in SFM and for 5 days in the presence of IGF-I (100 nM), IGF-I and AS (1 μM) or a mismatch (MS) oligonucleotide (1 μM); a representative experiment, repeated at least four times with similar results, is shown. In each panel, the line indicated as oligo refers to the biotin-labeled 60-bp duplex bearing the RE-1 binding sequence incubated in the reaction mixture lacking the nuclear protein extract. The arrow indicates the REST-related specific complex.*

In a separate set of experiments we investigated the viability and proliferation of SH-SY5Y cells exposed to AS-ODN or MS-ODN (1μM). Exposure to ODNs for up to 6days, in the presence of 15% FCS, did not affect cell proliferation (evaluated as change of [^{3}H]thymidine incorporation) or cell viability evaluated by propidium iodide staining (data not shown).

In time-course experiments we investigated the viability and proliferation of SH-SY5Y cells exposed to SFM, IGF-I and ODNs. SH-SY5Y cells remained viable in SFM for a limited period; viability was only moderately reduced in cells maintained in these conditions for 6days (viable cells were 72.7±2.4 vs. 91.2±1.8% in the presence of FCS; n=24). However, exposure to SFM for

up to 7days tended to detach the cells and significantly increased cell death (data not shown). Therefore, experiments were carried out on cells maintained in SFM alone up to 6days. In these conditions cells survive and allow analysis of the effects of IGF-I as this factor increases survival in the absence of other growth factors [Kim et al., 1997]. The cells enter a quiescent state when serum deprived whereas exposure to IGF-I for 48h restored the proliferation of SH-SY5Y cells. IGF-I-induced cell proliferation was time dependent and was reduced in cells exposed to this agent for 3days (% vs. SFM at day 0, 160±4%; n=5) or 5days (Figure 7.4). Therefore, by prolonging the exposure to IGF-I, SH-SY5Y cells decreased proliferation and became more differentiated as described later. IGF-I-induced cell proliferation was not affected by 1μM AS-ODN and MS-ODN (Figure 7.4).

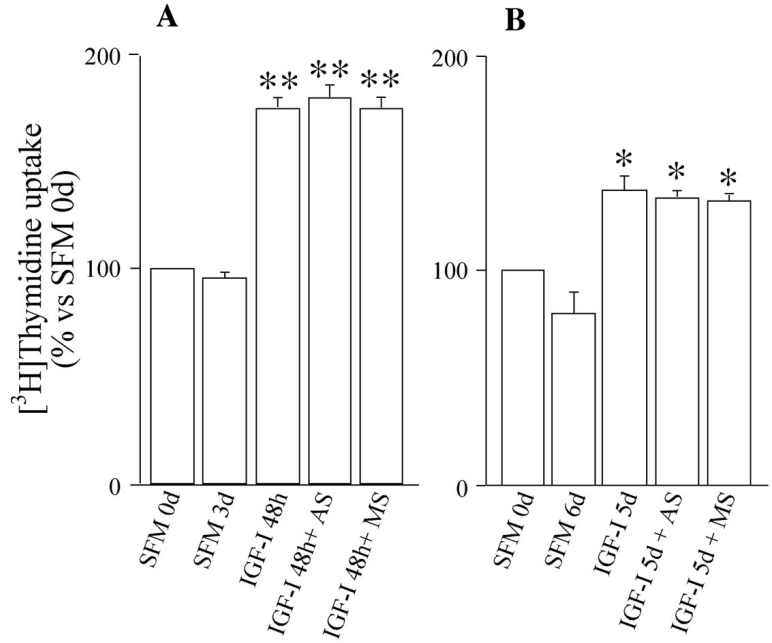


Figure 7.4 *Effects of antisense (AS) oligonucleotide complementary to REST mRNA (AS) on DNA synthesis and proliferation of SH-SY5Y cells maintained in serum-free medium (SFM) and exposed to insulin-like growth factor I (IGF-I). Cells were exposed to SFM for 24 h and then to IGF-I (100 nm) for 48 h (A) or 5 days (B) alone or in the presence of AS (1 lm) or a mismatch (MS) oligonucleotide (1 μM). [³H]Thymidine (2 μCi / mL) was added to the culture medium 5 h before harvesting. Control cells were grown in SFM alone for 3 or 6 days. The results are expressed as percent change from cells grown in SFM for 24 h and maintained for 5 h in the presence of [³H]thymidine (SFM 0 d). This technique is described in Materials and methods. Each value represents the mean ± SEM of six experiments performed in triplicate. *P < 0.05; **P < 0.01 vs. SFM 0 d (Newman-Keuls test after ANOVA).*

7.3 Insulin-like growth factor I-induced mitogen-activated protein kinase activation

In agreement with previous studies [Kim et al., 1997, 2004; Seiler et al., 2001], IGF-I-mediated MAPK activation was observed in SH-SY5Y cells cultured for 24h in SFM and treated for 10min with IGF-I (100nM); MAPK phosphorylation was maintained in cells exposed to IGF-I (100nM) for 5days (Figure 7.5). A more marked MAPK phosphorylation was detected in cells treated for 5days with IGF-I, washed and incubated for 90min in fresh SFM and further exposed to IGF-I (100nM) for 10min (Figure 7.5).

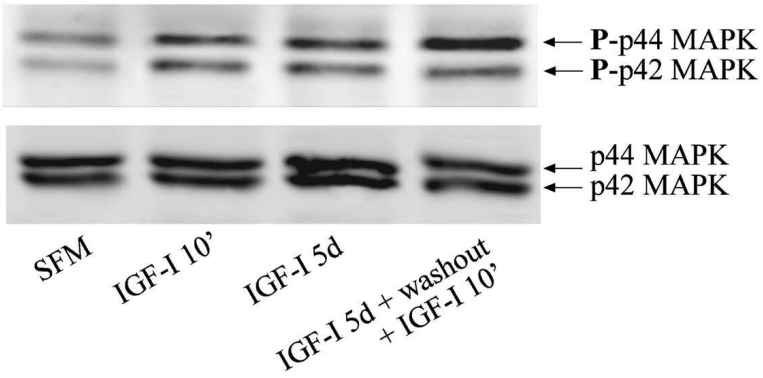


Figure 7.5 *Effect of IGF-I on p42 / 44 MAPK phosphorylation in SH-SY5Y cells. Cells were cultured for 24 h in serum-free medium (SFM) and treated for 10 min with 100 nM IGF-I (IGF-I 10'); MAPK phosphorylation was maintained in cells exposed to 100 nM IGF-I for 5 days (IGF-I 5 d). A more marked MAPK phosphorylation was detected in cells treated for 5 days with IGF-I, washed and incubated for 90 min in fresh SFM and further exposed to 100 nM IGF-I for 10 min (IGF-I 5 d + washout + IGF-I 10'). Phosphorylated p42 / 44 MAPK (upper panel) and total MAPK (lower panel) were evaluated by western. A representative experiment repeated three times with similar results is shown.*

7.4 Antisense inhibition of repressor element 1 silencing transcription factor increases insulin-like growth factor I-induced effects on neurite outgrowth in SH-SY5Y cells

Visual microscopic analysis showed that SH-SY5Y cultured in SFM for 6days displayed a fibroblast-like morphology with few, short neuritic processes (Figure 7.6A). SH-SY5Y cells grown in SFM for 24h and then in the presence of IGF-I (10 nM) for 5days underwent a sequence of morphological changes which has already been described [Påhlman et al., 1990, 1995; Perez-Juste & Aranda, 1999; Kurihara et al., 2000; van Golen & Feldman, 2000]. The cells showed an extensive network of discernible neuritic processes. Approximately 74% of cells cultured in the presence of IGF-I had the majority of neurites reaching only a limited length (they were at least two cell diameters in length) whereas the occurrence of longer neuritic processes (exceeding twice the diameter of the cell soma in length) was scarce (Figure 7.6A). In cells exposed to IGF-I (100nM) and AS-ODN or MS-ODN (1μM) for 5days, the number of cells bearing neurites (77 and 73%, respectively) was similar to that found in cells exposed to IGF-I alone. Interestingly, exposure to IGF-I in the presence of AS-ODN increased the appearance of longer neurites combined with a prominent branching. Thus, a significant increase ($P<0.01$) in the percentage of cells bearing neuritic processes exceeding twice the length of the cell soma was observed in cells treated with AS-ODN but not with MS-ODN; these latter, in fact, displayed a pattern similar to cells exposed to IGF-I alone (Figure 7.6B). Moreover, the average length of neurites (only those exceeding twice the diameter of the cell soma in length were measured) was significantly higher ($P<0.01$) in AS-ODN-treated cells (Figure 7.6C). A similar pattern was observed in cells exposed to IGF-I (100μM) and a higher concentration of AS-ODN (10μM; data not shown). In SH-SY5Y cells exposed to IGF-I (100nM) for 48h, only short neuritic processes, less than two cell diameters in length, were observed and, on the basis of visual microscopic analysis, these early events were not modified by AS-ODN treatment (data not shown). In some experiments, cells were exposed to IGF-I (100nM) for 48h and then for 3days to IGF-I in the presence of AS-ODN or MS-ODN (1μM). In cells exposed to AS-ODN for the last 3days, the percentage of cells bearing longer neurites and the mean neurite length were similar to cells treated with AS-ODN from the beginning (Figure 7.6). In cells exposed to IGF-I and to MS-ODN for the last 3days, the above parameters were similar to those of cells treated with IGF-I alone (Figure 7.6).

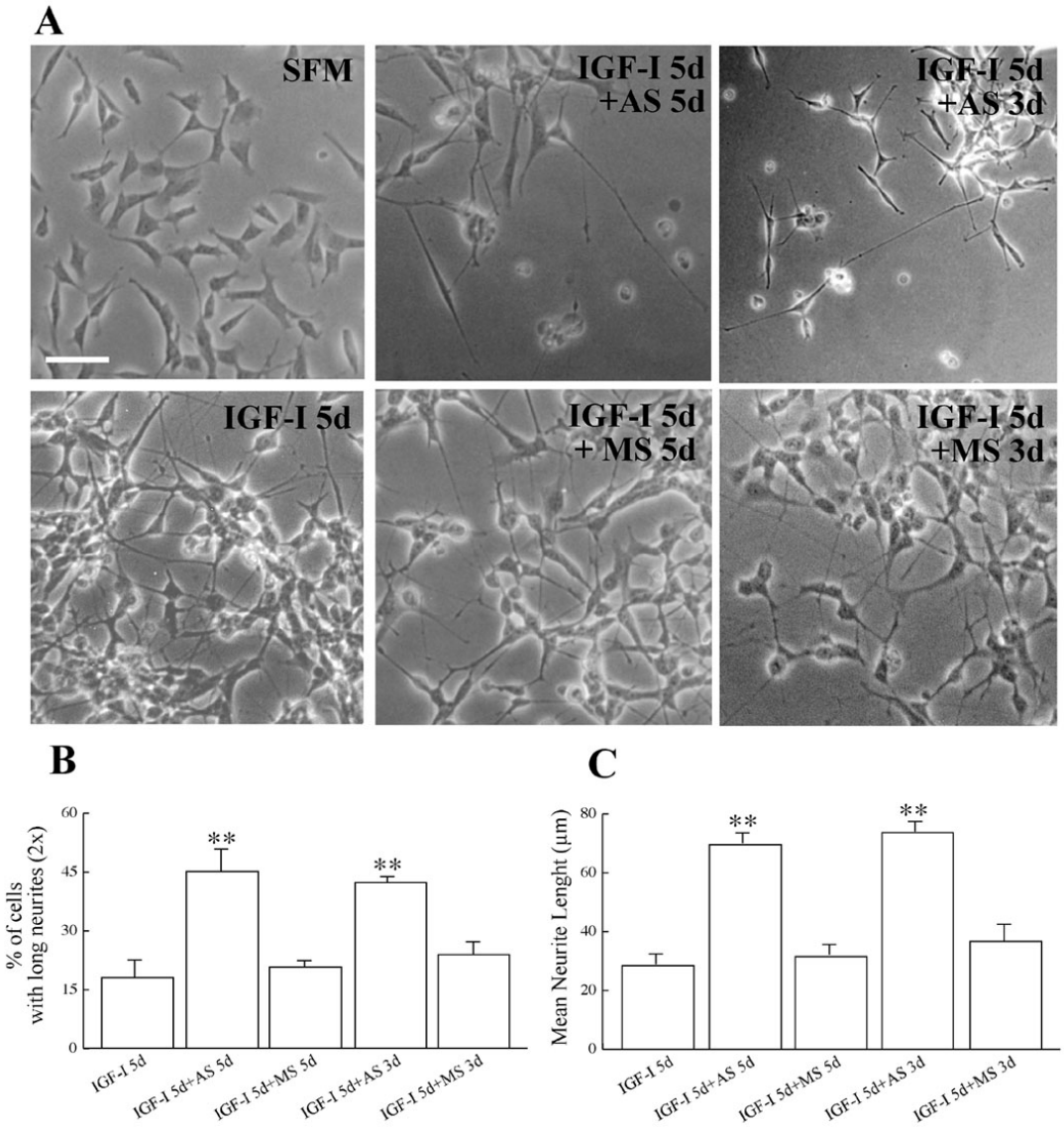


Figure 7.6 *Effect of IGF-I and antisense (AS) oligonucleotide on morphology and neurite outgrowth of SH-SY5Y cells. (A) Phase-contrast photomicrographs of cells cultured in serum-free medium (SFM) for 6 days or in SFM alone for 24 h and then exposed to 100 nM IGF-I for 5 days alone or in the presence of AS (1 µM) or a mismatch (MS) oligonucleotide (1 µM) for 3 or 5 days. Scale bar, 20 µm. (B and C) Morphometric analysis of neurite length performed on captured images. Cells with neurites longer than two cell body lengths were scored as long neurite-bearing cells and reported as percentage of cells with long neurites in (B) whereas (C) shows the mean neurite length (neurites longer than two cell body lengths). Each value represents the mean ±SEM of at least 300 cells for each treatment counted from randomly selected fields of three independent experiments. **P < 0.01 vs. IGF-I (Newman-Keuls test after ANOVA).*

7.5 Western blotting and immunocytochemical analysis of repressor element 1 silencing transcription factor-regulated genes in response to insulin-like growth factor I and antisense oligonucleotide exposure

The gene promoters of synapsin I and βIII-tubulin contain an RE-1 sequence motif and appear to be repressed by REST [Schoch et al., 1996; Lunyak et al., 2002]. Lietz et al. (1998) have shown that the expression pattern of REST and synapsin I displays an inverse relationship in neuroblastoma cells. We observed that differentiation of SH-SY5Y cells by IGF-I was accompanied by a significant increase ($P<0.05$) of cytoplasmic levels of βIII-tubulin and synapsin I, as evaluated by western blotting analysis (Figure 7.7), an effect that was concomitant with REST down-regulation (Figure 1C and D). In cells cultured in SFM for 24h and then exposed to IGF-I and AS-ODN for 5days, both proteins were further elevated as a consequence of the reduction of REST expression by AS treatment (Figure 7.7).

Previous studies have reported that, in neuroblastoma cells, IGF-I can induce the appearance of neurite-like processes and these cells can express markers consistent with a neuronal phenotype [Påhlman et al., 1990, 1991; Perez-Juste & Aranda, 1999; Kurihara et al., 2000; van Golen & Feldman, 2000]. Among these proteins, we have chosen to evaluate the levels of the neuronal protein GAP-43 which is up-regulated by IGF-I during the differentiation process [Påhlman et al., 1995] whereas it is not included among the genes transcriptionally regulated by REST as its promoter lacks any RE-1 sequence [Bahn et al., 2002; Lunyak et al., 2002]. Expression of GAP-43 was examined by western blotting and, as expected, exposure of SH-SY5Y cells to IGF-I (100nM) for 5days significantly increased cytoplasmic levels of this protein. GAP-43 was significantly more elevated ($P<0.01$) in cells exposed to IGF-I and AS-ODN but not to MS-ODN (Figure 7.7). Cytoplasmic levels of β-actin, chosen as housekeeping gene, were not modified by these treatments (Figure 7.7).

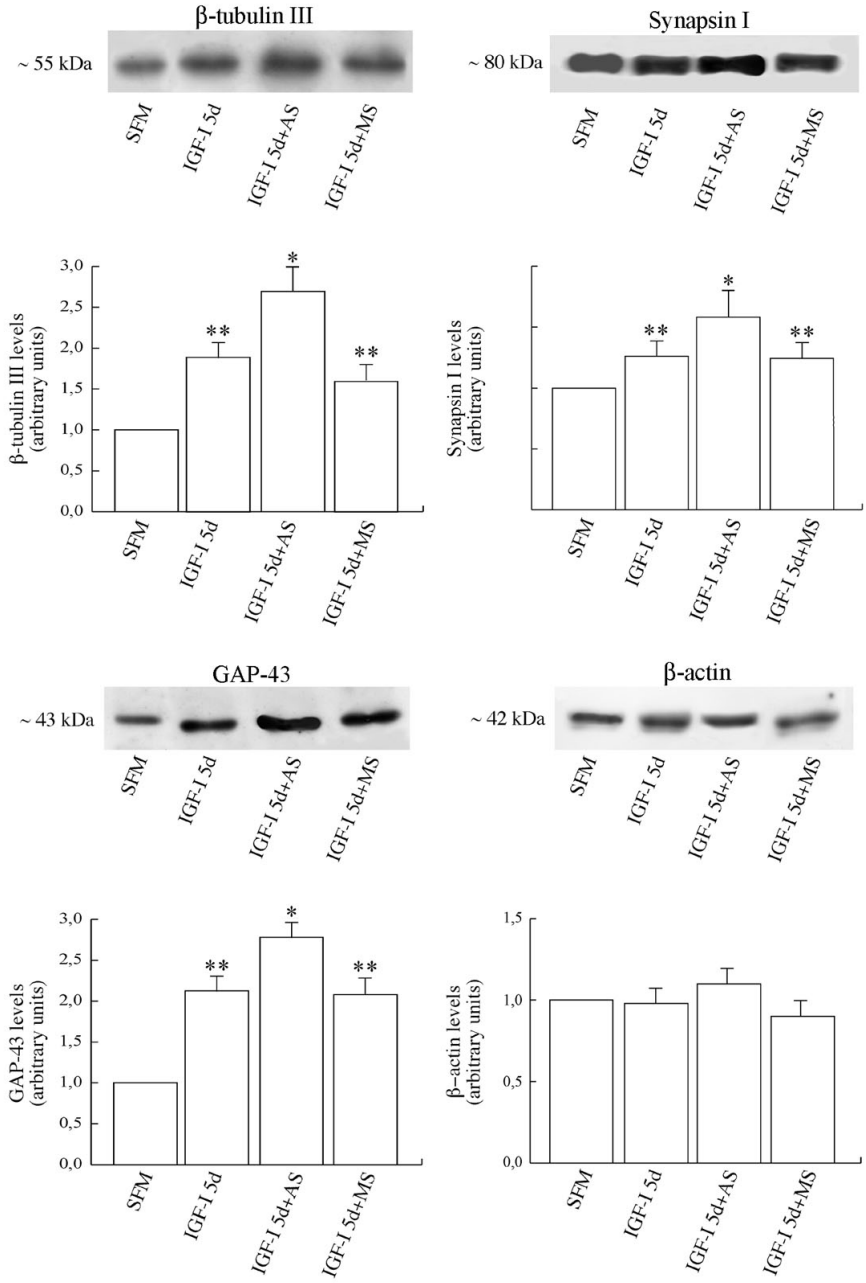


Figure 7.7 *Antisense (AS) inhibition of REST up-regulates βIII-tubulin (≈ 55 kDa), synapsin I (≈ 80 kDa) and growth cone-associated protein 43 (GAP-43) (≈ 43 kDa) but not β-actin (≈ 42 kDa) detected by western blotting analysis in SH-SY5Y cells cultured in serum-free medium (SFM) alone for 24 h and then exposed to 100 nM insulin-like growth factor I (IGF-I) for 5 days. Control cells were cultured in SFM for 6 days. IGF-I exposure raises synapsin I, βIII-tubulin and GAP-43. Interestingly, expression of these proteins is further increased in cells exposed to IGF-I in the presence of an AS oligonucleotide (1 μM) but not in the presence of a mismatch (MS) oligonucleotide (1 μM). Representative autoradiograms and quantitative densitometric analysis of western blot experiments performed as described in Materials and methods are shown. Results shown are the mean ±SEM of five independent experiments. *P < 0.05 vs. SFM; **P < 0.01 vs. IGF-I (Newman-Keuls test after ANOVA).*

Immunocytofluorescence analysis confirmed that synpasin I and GAP-43 expression started to increase in cells exposed to IGF-I for 48h and they were expressed to a greater extent in AS-ODN-treated cells. In fact, a more marked staining was observed at cytoplasmic level; however, exposure to AS-ODN did not modify the appearance of neuritic processes (Figure 7.8).

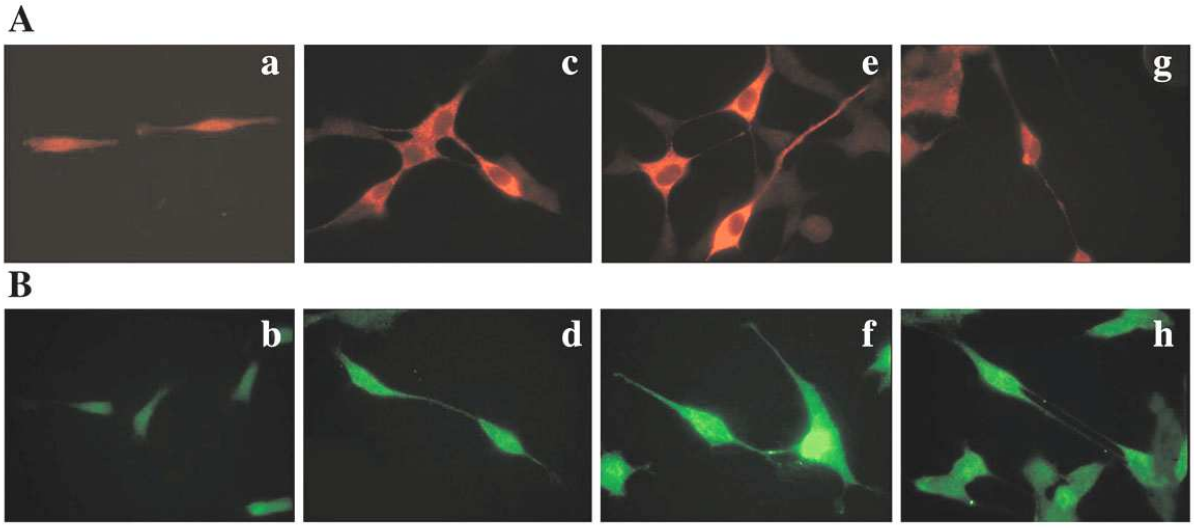


Figure 7.8 *Antisense (AS) inhibition of REST up-regulates synapsin I (A) and growth cone-associated protein 43 (GAP-43) (B), detected by immunocytochemistry, in SH-SY5Y cells cultured in serum-free medium (SFM) alone for 24 h and then exposed to 100 nM IGF-I for 2 days. Cells were cultured: (a and b) in SFM for 3 days; (c and d) in the presence of IGF-I (100 nM); (e and f) in the presence of IGF-I and an AS oligonucleotide (1 μM); (g and h) in the presence of IGF-I and a mismatch (MS) oligonucleotide (1 μM). Cells were fixed as described in Materials and methods. Synapsin I immunostaining (in red with fluorescein-conjugated secondary antibody) and GAP-43 immunostaining (in green with fluorescein-conjugated secondary antibody) are increased in cells exposed to IGF-I (c and d) in comparison to cells cultured in SFM (a and b). Interestingly, a more intense staining was observed in cells exposed to the AS complementary to REST mRNA (e and f) but not in cells exposed to MS (g and h). This is representative of at least three independent experiments.*

To better relate synapsin I and GAP-43 expression to AS-induced down-regulation of REST at single cell level, we carried out immunocytochemical studies by double staining of the same cells with antibodies raised against GAP-43 and synapsin I. As shown in Figure 7.9, synapsin I and GAP-43 (Figure 7.9a–c) staining was weaker in cells maintained in SFM for 6days than in cells treated with IGF-I (Figure 7.9d–f). In these latter, synapsin I was visible in the cytoplasm and in neuritic processes. GAP-43 showed a clear pattern of staining which was indicative of an accumulation of this protein at the cell pole and inclusions were observed within neurites, suggesting its transport along them. Both synapsin I and GAP-43 were expressed more in SH-SY5Y cells treated with IGF-I and AS-ODN (Figure 7.9g and h) but not with MS-ODN (Figure

7.9l–n). This was observed particularly in the regions marked by the arrows in Figure 7.9g–i where a typical cell bearing long neurites is shown. These results suggest that a precocious REST down-regulation during neuronal differentiation could induce a more marked cell differentiation characterized by an increased expression of markers, not directly regulated by this transcription factor, and consistent with a neuronal phenotype.

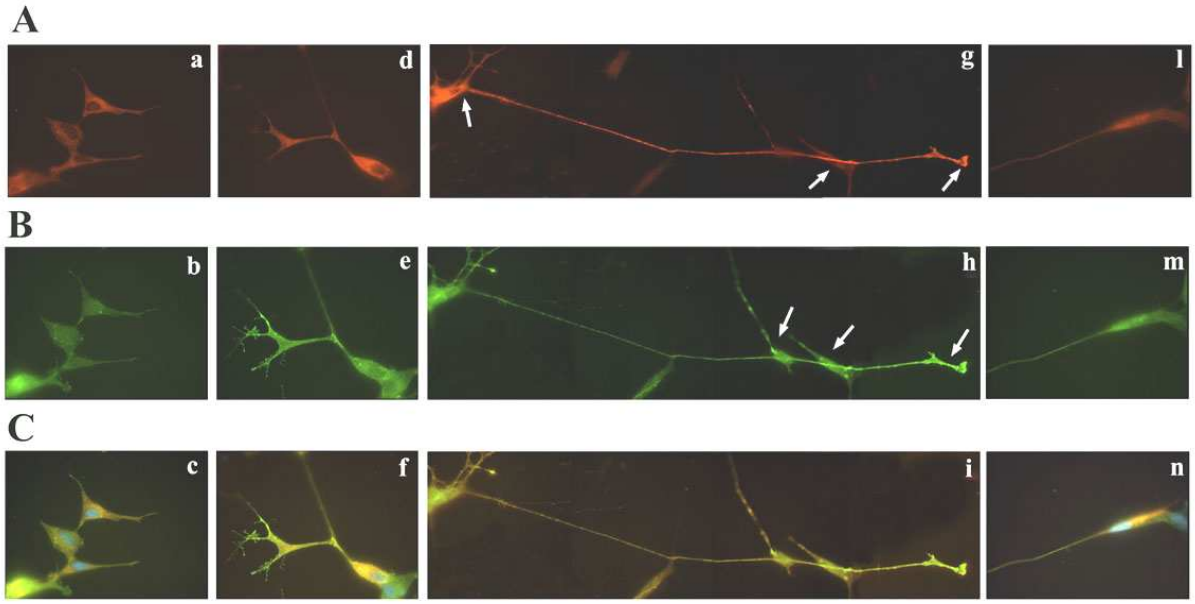


Figure 7.9 *Antisense (AS) inhibition of REST up-regulates synapsin I and growth cone-associated protein 43 (GAP-43), detected by immunocytochemistry, in SH-SY5Y cells cultured in serum-free medium (SFM) for 24 h and then exposed to 100 nm insulin-like growth factor I (IGF-I) for 5 days. Cells were cultured: (a–c) in SFM; (d–f) in the presence of IGF-I (100 nM); (g–i) in the presence of IGF-I and an AS oligonucleotide (1 μM); (l–n) in the presence of IGF-I and a mismatch (MS) oligonucleotide (1 μM). Cells were fixed as described in Materials and methods and double labeled with antibodies against synapsin I and GAP-43. (A) Synapsin I immunostaining (in red with fluorescein-conjugated secondary antibody). (B) GAP-43 immunostaining (in green with fluorescein-conjugated secondary antibody). (C) Double exposure of both stainings. Note that synapsin I and GAP-43 staining are increased in cells exposed to IGF-I (d–f) in comparison to cells cultured in SFM (a–c). Interestingly, a more intense staining for both proteins was observed in cells exposed to the AS complementary to neuron-restrictive silencer factor / REST mRNA (g–i; arrows indicate a more intense staining in cell bodies and neurites) but not in cells exposed to MS (l–n). Note that AS-treated cells extend longer neurites in comparison to cells exposed to IGF-I alone or in the presence of the MS. This is representative of at least three independent experiments.*

7.6 PKC contributes to regulate REST expression in SH-SY5Y neuroblastoma cells

To evaluate REST mRNA levels, we developed a semiquantitative real-time PCR technique by amplifying a cDNA sequence of 197bp located in the 3'-end region of REST mRNA, as previously described.

Exposure of SH-SY5Y cells to PMA (16nM) for 24h induced a significant (P<0.01) increase in steady-state mRNA levels of REST in comparison to control cells, maintained in . The effect of PMA (16nM) appeared by 24h of treatment and reached a peak in cells exposed to this trophic factor for 48h; mRNA levels afterward declined in cells exposed to PMA for 3days ($2^{-\Delta\Delta Ct}$: 3.3±0.2; n=4) (Figure 7.10A).

We used two PKC inhibitors GF109203X, which inhibits classical and novel PKC isoforms, and Gö6976, which only inhibits classical isoforms, to evaluate whether PKC pathway participates in PMA-induced REST expression. In SH-SY5Y cells exposed for 72h to 16nM PMA in the presence of GF109203X (2μM) or Gö6976 (2μM), a significant reduction (P<0.05) of steady-state mRNA coding for REST was found (Figure 7.10B).

The levels of the corresponding REST protein in nuclear cell extracts were evaluated by western blotting (Figure 7.10 C and D) and followed a pattern similar to mRNA expression. In agreement with other studies [Shimojo et al., 1999; Grimes et al., 2000; Calderone et al., 2003; Martin et al., 2003], we observed only a single band of ≈ 200kDa. REST levels were significantly elevated (P<0.01) in cells exposed to PMA (16nM). The protein content rose more with 48h exposure to PMA and was then reduced in cells exposed to PMA for 6days, thus suggesting a timing expression of this protein in cells exposed to phorbol ester.

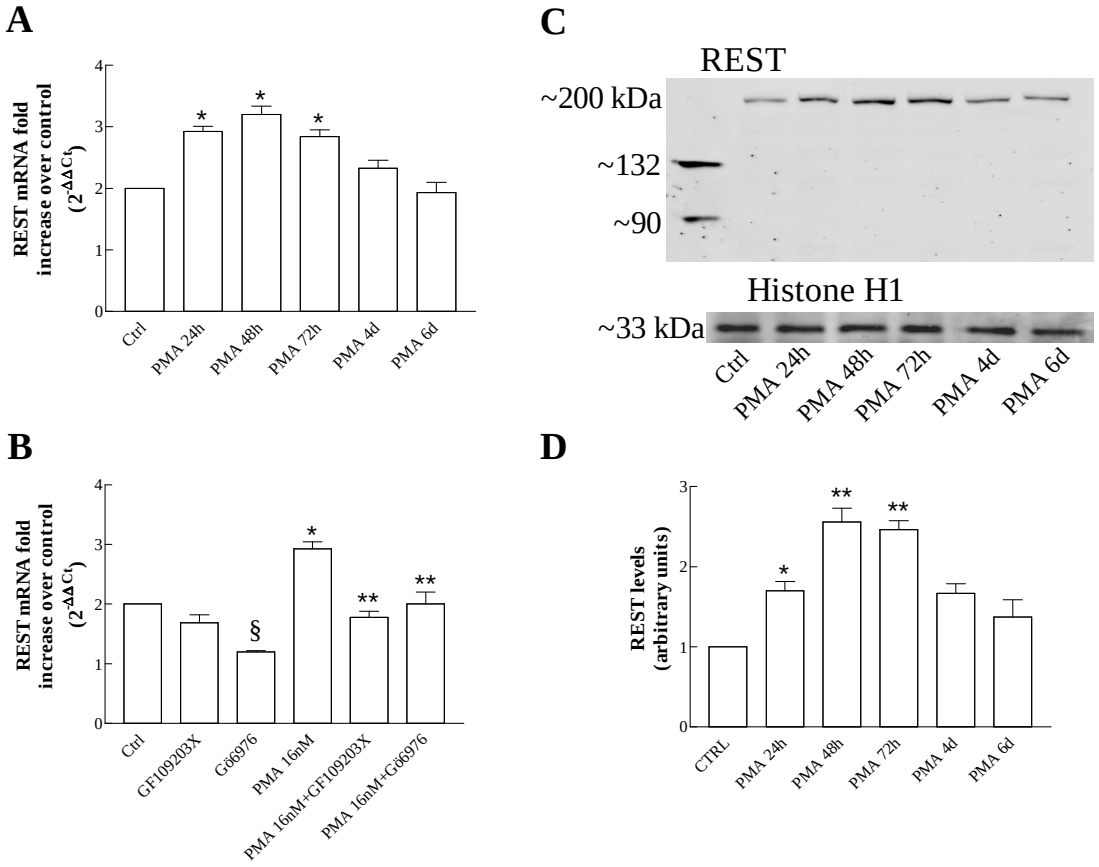


Figure 7.10 *PMA up-regulate steady-state levels of REST mRNA (A and B) and nuclear protein levels (C and D) in SH-SY5Y cells maintained in FCS. Relative fold induction of the REST gene following various treatments was calculated using the* $\Delta\Delta C_t$. *Exposure to FCS (Ctrl) or PMA (16nM) for 24 h increases REST mRNA levels (A) or REST protein levels (C and D) in comparison to cells maintained in FCS. Time-dependent increase of REST mRNA levels (B) and REST nuclear protein levels (D) in cells exposed to PMA (16nM) for 24, 48 h and 72h is shown. The action of PMA on REST mRNA was reduced or in the presence of GF109203X and Gö6976 (2*μ*M; added 15 min before 16nM PMA). Histone H1 was evaluated in the same samples and used for loading control. Values are the mean ± SEM of at least six independent experiments carried out in triplicate. *P < 0.05 vs. Ctrl; **P < 0.01 vs. PMA16nM; §P<0.05 vs Ctrl (Newman-Keuls test after ANOVA).*

7.7 Western blotting analysis of REST-regulated genes in response to the phorbol ester PMA

As previously described, the gene promoters of synapsin I and βIII-tubulin contain an RE-1 sequence motif and appear to be repressed by REST [Schoch et al., 1996; Lunyak et al., 2002]. Lietz et al. (1998) have shown that the expression pattern of REST and synapsin I displays an inverse relationship in neuroblastoma cells. We observed that differentiation of SH-SY5Y cells by PMA was accompanied by a significant increase ($P<0.05$) of cytoplasmic levels of βIII-tubulin and synapsin I, as evaluated by western blotting analysis (Figure 7.11), an effect that was concomitant with REST down-regulation (Figure 7.10).

Previous studies have demonstrated that the SH-SY5Y neuroblastoma cell line can be induced to differentiate in the presence of serum following activation of protein kinase C with phorbol ester [Påhlman et al., 1981]. This differentiation process is accompanied by a decreased growth rate, increased neurite outgrowth and enhanced expression of the differentiation markers, for exmple growth-associated protein 43 (GAP-43) [Bjelfman et al., 1990]. Expression of synapsin I and βIII-tubulin was examined by western blotting and exposure of SH-SY5Y cells to PMA (16nM) for 6days significantly increased cytoplasmic levels of this protein (Figure 7.11).

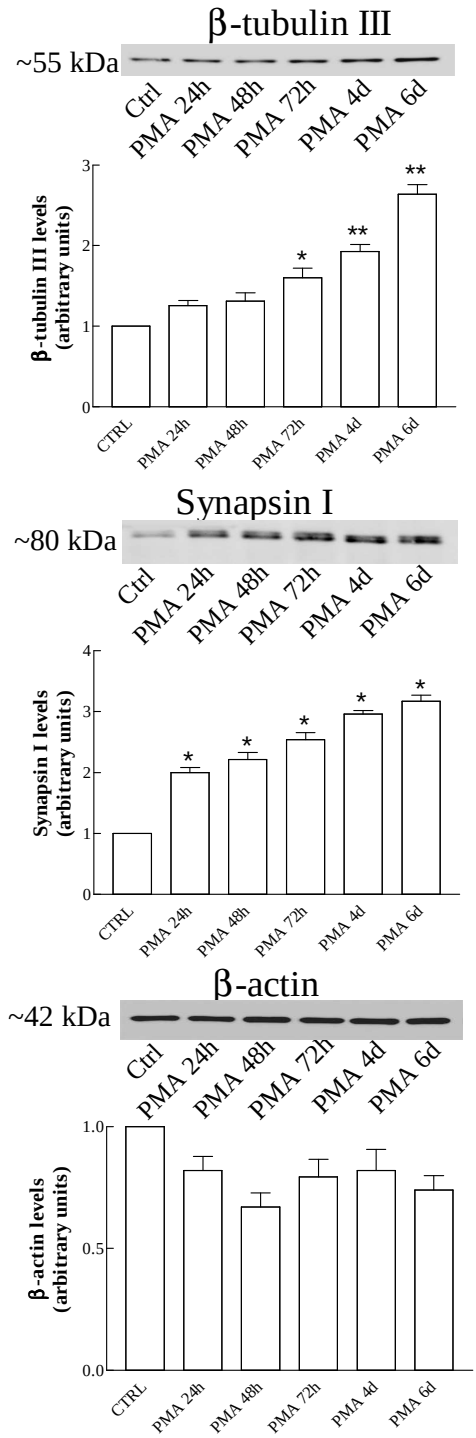


Figure 7.11 *Effect of PMA (16nM) on REST-regulated genes βIII-tubulin (≈ 55 kDa), synapsin I (≈ 80 kDa) detected by western blotting analysis in SH-SY5Y cells. Control cells were cultured in FCS medium for 6 days. PMA exposure raises synapsin I, βIII-tubulin till 6d. Representative autoradiograms and quantitative densitometric analysis of western blot experiments are shown. βactin was evaluated in the same samples and used for loading control.Results shown are the mean ±SEM of five independent experiments. *P < 0.05 vs. Ctrl; **P < 0.01 vs. Ctrl (Newman-Keuls test after ANOVA).*

7.8 Antisense inhibition of REST expression in SH-SY5Y cells exposed to PMA

In order to investigate any role played by REST on effects elicited by protein kinase C in neuroblastoma cells, we used an AS-ODN to down-regulate the expression of this transcription factor. In SH-SY5Y cells exposed to PMA (16nM) for 72h, the concomitant exposure to AS-ODN (5μM and 10μM) but not to MS-ODN (10μM) produced a noteworthy reduction of REST nuclear levels, as evaluated by western blotting analysis (Figure 7.12).

The concomitant exposure to 5μM AS-ODN and PMA (16nM) reduced augmented REST levels 25% of the control cells, whereas the exposure to 10μM AS-ODN induced a decrease of REST approaching 50%

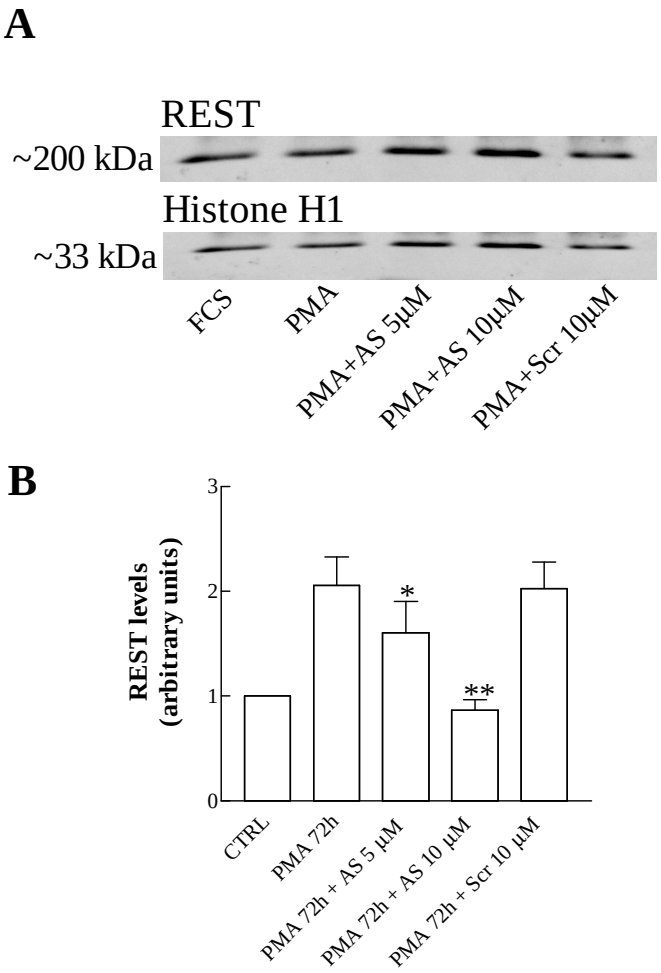


Figure 7.12 *Effect of PMA and AS oligonucleotide exposure on nuclear protein levels in SH-SY5Y cells maintained in FCS medium. Cells grown for 72h in FCS alone or in the presence of PMA (16nM); PMA and AS (1 and 10μM) or an MS oligonucleotide (10μM). (A) Representative western blot analysis. (B) Densitometric analysis of the bands (mean ± SEM; n = 6). Histone H1 was evaluated in the same samples and used for loading control. *P < 0.05; **P < 0.01 vs FCS (Newman-Keuls test after ANOVA).*

7.9 Antisense inhibition of REST increases PKC effects on neurite outgrowth and REST-regulated genes in SH-SY5Y cells

Visual microscopic analysis showed that SH-SY5Y cultured in FCS medium for 72h displayed a fibroblast-like morphology with few, short neuritic processes (Figure 7.13A). SH-SY5Y cells grown in FCS medium and in the presence of PMA (16nM) for 72h underwent a sequence of morphological changes which has already been described [Parrow et al., 1992; Zeidman et al., 1999]. The cells form processes with axonal growth cones wich are enriched in the GAP-43 protein, a set of events in which PKC has a suggested role.

In cells exposed to PMA (16nM) and AS-ODN or MS-ODN (5μM) for 72h, the number of cells bearing neurites was similar to that found in cells exposed to PMA alone. Interestingly, exposure to PMA in the presence of AS-ODN (10μM) increased the appearance of longer neurites combined with a prominent branching. In fact, the average length of neurites (only those exceeding twice the diameter of the cell soma in length were measured) was significantly higher (P<0.01) in AS-ODN-treated cells (Figure 7.13B).

Previous studies have reported that, in neuroblastoma cells, phorbol ester can induce the appearance of neurite-like processes and thee cells can express markers consistent with a neuronal phenotype [Parrow et al., 1992; Zeidman et al., 1999]. Among these proteins, we have chosen to evaluate the levels of the neuronal protein GAP-43 which is up-regulated by phorbol ester during the differentiation process [Zeidman et al., 1999] whereas it is not included among the genes transcriptionally regulated by REST as its promoter lacks any RE-1 sequence [Bahn et al., 2002; Lunyak et al., 2002].

Immunocytofluorescence analysis confirmed that synpasin I and GAP-43 expression increased in cells exposed to PMA for 72h and they were expressed to a greater extent in AS-ODN-treated cells. In fact, a more marked staining was observed at cytoplasmic level; however, exposure to AS-ODN did not modify the appearance of neuritic processes (Figure 7.14). Both markers were expressed more in SH-SY5Y cells treated with PMA and AS-ODN (Figure 7.14 panel e,g,f,h) but not with MS-ODN (Figure 7.14 panel i,j). Moreover, it is possible to observe a more pronounced differentiation, i.e. an higher number of neurites and longer process in neuroblastoma cells exposed to PMA and AS-ODN 10μM than in cells treated with PMA alone or combined with AS-ODN 5μM or MS-ODN (Figure 7.14 panel g and h).

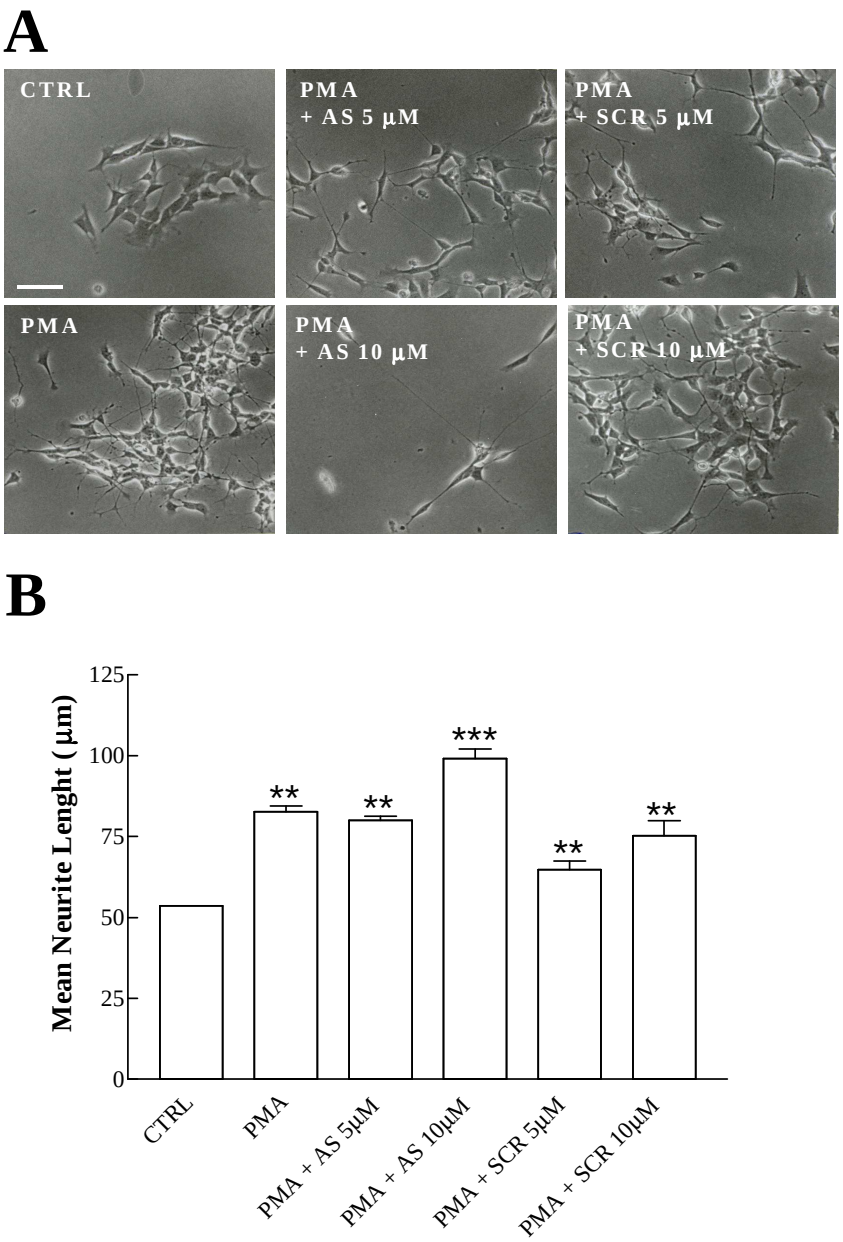


Figure 7.13 *Effect of PMA and antisense (AS) oligonucleotide on morphology and neurite outgrowth of SH-SY5Y cells. (A) Phase-contrast photomicrographs of cells cultured in FCS medium (CTRL) for 72h or exposed to 16nM PMA for 72h alone or in the presence of AS (5 and 10 µM) or a mismatch (MS) oligonucleotide (10µM). Scale bar, 20 µm. (A) Morphometric analysis of neurite length performed on captured images. Cells with neurites longer than two cell body lengths were scored as long neurite-bearing cells. (B) Shows the mean neurite length (neurites longer than two cell body lengths). Each value represents the mean ±SEM of at least 300 cells for each treatment counted from randomly selected fields of three independent experiments. **P < 0.01 vs. CTRL, ***P<0.01 vs PMA (Newman-Keuls test after ANOVA).*

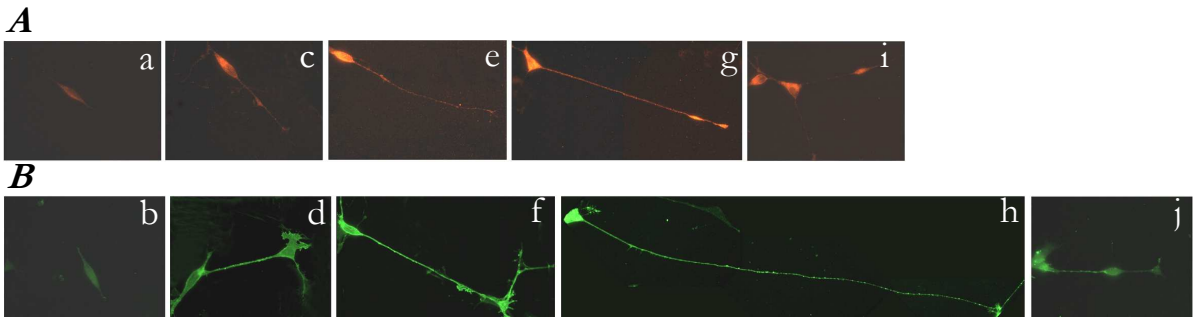


Figure 7.14 *Antisense (AS) inhibition of REST up-regulates synapsin I (A) and growth cone-associated protein 43 (GAP-43) (B), detected by immunocytochemistry, in SH-SY5Y cells. Cells were cultured: (a and b) in FCS for 72h; (c and d) in the presence of PMA (16nM); (e and f) in the presence of PMA and an AS oligonucleotide (5μM); (g and h) the presence of PMA and an AS oligonucleotide (10μM); (I and j) in the presence of PMA and a mismatch (MS) oligonucleotide (10μM). Cells were fixed as described in Materials and methods. Synapsin I immunostaining (in red with fluorescein-conjugated secondary antibody) and GAP-43 immunostaining (in green with fluorescein-conjugated secondary antibody) are increased in cells exposed to PMA (c and d) in comparison to cells cultured in FCS (a and b). Interestingly, a more intense staining was observed in cells exposed to the AS complementary to REST mRNA (e, f, g and h) but not in cells exposed to MS (i and j). This is representative of at least three independent experiments.*

7.10 REST proteasomal degradation contributes to neuronal differentiation in SH-SY5Y cell line

To evaluate any involvement of ubiquitin-proteasome system in decreased REST levels during neuronal differentiation, SH-SY5Y cells were exposed to the proteasome inhibitor MG132.

In cells exposed to PMA (16nM) for 4days, the concomitant exposure to MG132 (1μM) restores REST nuclear protein levels down-regulated by the phorbol ester, as determined by western blotting analysis (Figure 7.15).

The blockade of REST degradation is accompanied by a significant reduction of neuronal differentiation markers and arrest of neurite extension. Visual microscopic analysis showed that SH-SY5Y exposed concomitantly to PMA and MG132 for 4 days displayed a fibroblast-like morphology with few, short neuritic process, if compared to neuroblastoma cells treated with PMA alone (Figure 7.15 C).

Moreover, in cells exposed to PMA and MG132, it was possible to observed an inverse pattern of expression of synapsin I and GAP-43, two neuronal differentiation markers. Synapsin I cytoplasmic levels were reduced when compared to cells exposed to PMA alone, as evaluated by western blotting analysis (Figure 7.15 D and E).

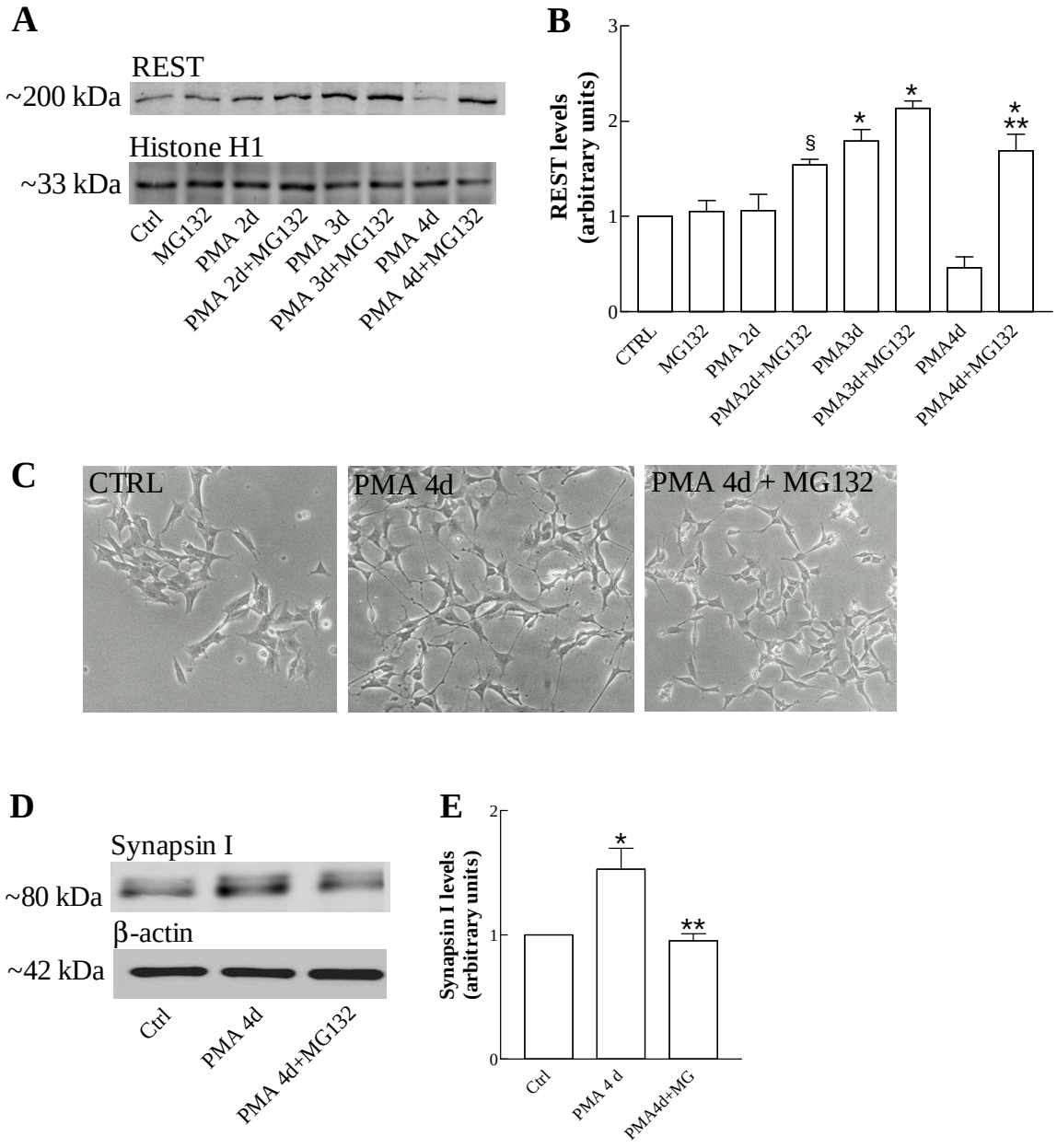


Figure 7.15 *Effect of PMA and MG132 exposure on SH-SY5Y cells maintained in FCS medium. Cells grown for 4d in FCS alone or in the presence of PMA (16nM); PMA and MG132 (1μM). (A) and (D) Representative western blot analysis. (B) and (E) Densitometric analysis of the bands (mean ± SEM; n = 6). (C) Morphology of SH-SY5Y cells. Histone H1 was evaluated in the same samples and used for loading control. *P < 0.05; **P < 0.01 vs FCS (Newman-Keuls test after ANOVA).*

Immunofluorescence analysis confirmed that synapsin I expression was reduced when REST was still present after 4 days PMA and MG132 treatment (Figure 7.16 g). Interestingly, a weaker intense staining was observed analyzing GAP-43 expression (Figure 7.16 h), a neuronal marker not regulated by REST, supporting the idea of a reduced global neuronal differentiation.

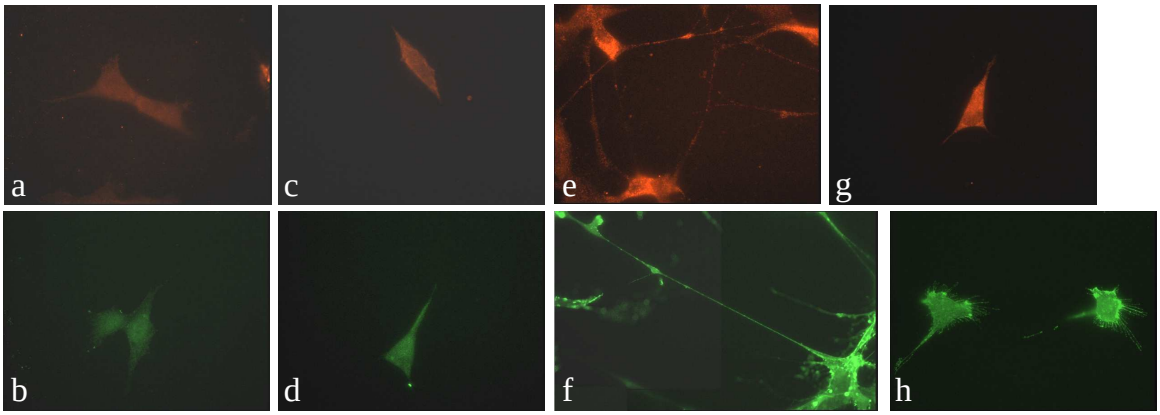


Figure 7.16 *Effect of the proteasome MG132 on expression of synapsin I(A) and growth cone-associated protein 43 (GAP-43) (B), detected by immunocytochemistry, in SH-SY5Y cells. Cells were cultured: (a and b) in FCS for 4d; (c and d) in the presence of MG132 (1μM); (e and f) in the presence of PMA (16nM) for 4d; (g and h) the presence of PMA and MG-132 (1μM). Cells were fixed as described in Materials and methods. Synapsin I immunostaining (in red with fluorescein-conjugated secondary antibody) and GAP-43 immunostaining (in green with fluorescein-conjugated secondary antibody) are increased in cells exposed to PMA (e and f) in comparison to cells cultured in FCS (a and b). Interestingly, a weaker intense staining was observed in cells exposed to the PMA and MG132 (g and h). This is representative of at least three independent experiments.*

7.11 Analysis of glycosylation pattern of REST protein in SH-SY5Y cell line

Moving from the observation that the molecular weight calculated on REST sequence is about 116 kDa but using western blotting this transcription factor appears to have distinct apparent molecular weight (see Table 1.1), we analyzed the pattern of REST protein expression in SH-SY5Y neuroblastoma cells.

In nuclear extract it was possible to identify three bands, which had an apparent molecular weight of about 220, 150 and 120 kDa (Figure 7.17A). In the cytoplasmic compartment it was possible to recognize only two bands, of about 150 and 120 kDa (Figure 7.17B). These several bands could be due to REST's post-translational modifications (for example glycosylation).

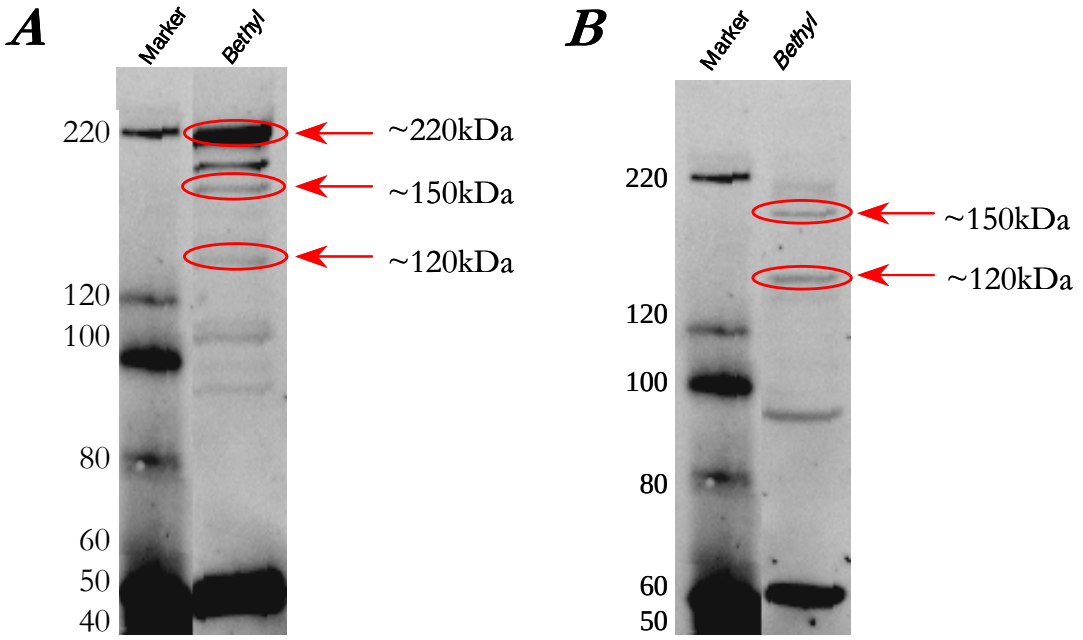


Figure 7.17 *Western blotting analysis of REST expression in SH-SY5Y cells, in both nuclear (A) and cytoplasmic (B) compartment.*

7.11.1 O-glycosylation prediction on REST protein using O-GLYCBASE

In order to identify probable glycosylation sites on REST protein, O-GLYCBASE database was used. O-GLYCBASE is a revised database of glycoproteins with O-linked glycosylation site. I has 242 glycoprotein entries. The criteria for inclusion in that database are at least on experimentally verified O- or C-glycosylation site. The database is non-redundant in the sense that it contains no identical sequences. O-GLYCBASE is available via internet at http://www.cbs.dtu.dk/databases/OGLYCBASE.

Examination of REST sequence with O-GLYCBASE found out several putative O-glycosylation sites (Figure 7.18 and Table 7.1). The sequence analysis generated a graph in which the probability of glycosylation was represented (Figure 7.18). O-GLYCBASE analysis predicted on REST protein sequence one probable site modified with O-GalNAc and eleven probable sites modified with O-GlcNAc.

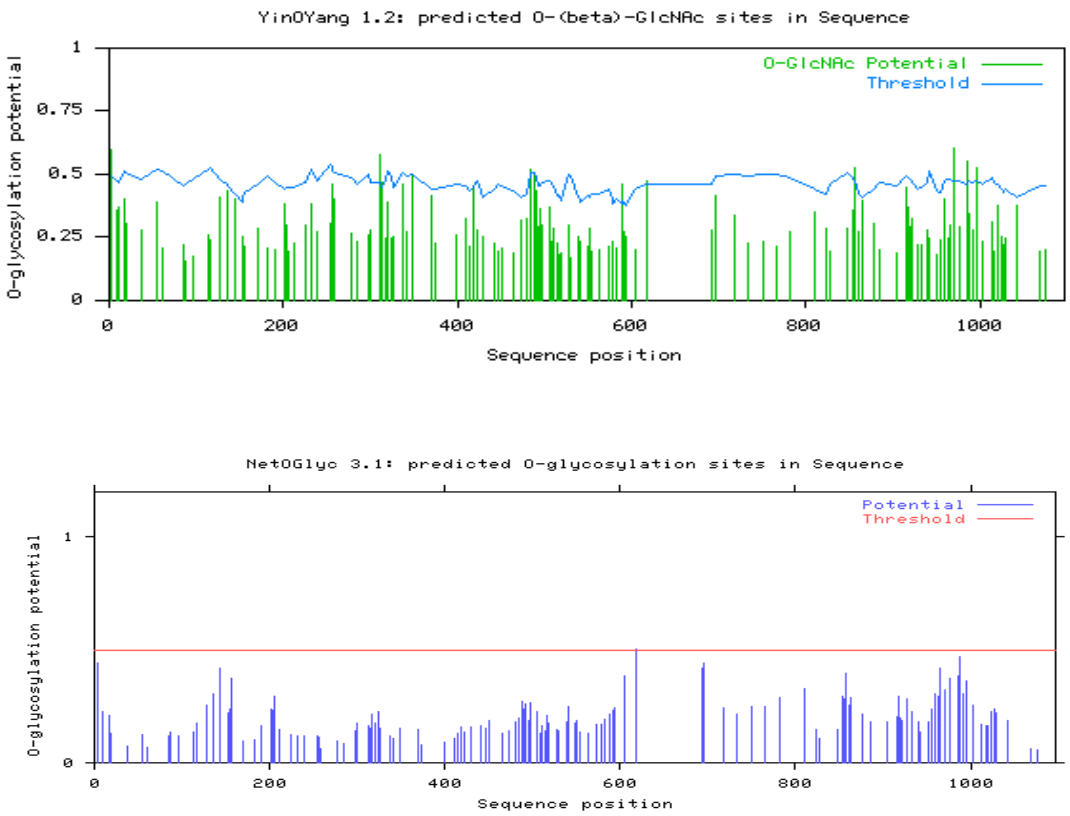


Figure 7.18 *Prediction of putative glycosylation sites on REST sequence: O-β-NAcetylGlucosamine (upper panel) and N-acetylgalactosamine (lower panel).*

GLYCOSILATION	POSITION	AMINOACID	PROBABILITY
O-GalNAc	618	Threonine	
O-β-GlcNAc	3	Threonine	+
	312	Serine	+
	313	Serine	+
	484	Serine	+
	588	Serine	+
	589	Serine	+
	618	Threonine	+
	857	Threonine	+
	971	Serine	++
	986	Serine	+
	996	Threonine	+

Table 7.1 *List of putative glycosylation sites on REST sequence with high probability.*

7.11.2 Enzymatic deglycosylation of REST protein in SH-SY5Y cells

Starting from the identification of REST several isoforms (Figure 7.17), the glycosylation pattern of the REST protein was analysed as these different forms of the transcription factor could be explained by post-translational modifications of the proteins, like glycosylation.

For this purpose several deglycosylating enzymes were used (Figure 7.19) in different combination. The enzymes used were: Endo-α-N-acetylgalactosaminidase (Endo-α), α2,3,6,8,9-Neuraminidase, β1,4-Galactosidase and β-N-Acetylglucosaminidase (β-GlcNAcase). Deglycosylated proteins were subjected to SDS-PAGE to see the effect of carbohydrate chain removal on the apparent molecular weight.

Deglycosylating analysis showed that REST protein in SH-SY5Y nuclear extract was O-glycosylated and not N-glycosylated. Moreover, using several combination of deglycosilating enzymes it was possible to hypothesize the presence of Gal-β(1-3)-GalNAc residues on the endogenous REST as in western blotting analysis a new band (about 200kDa as apparent molecular weight) appeared (Figure 7.20). The same band was always present after using different combinations of deglycosylating enzymes.

In cytoplasmic extract it was not possible to identify any new band.

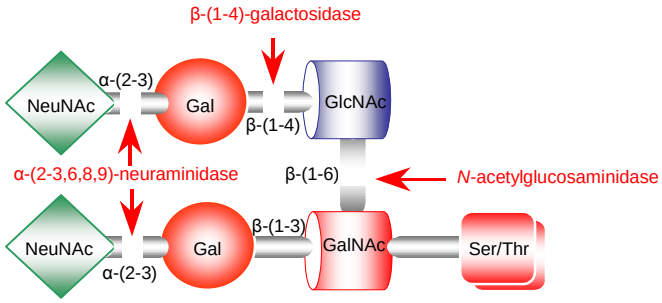


Figure 7.19 *Schematic representation of O-linked residual removal from Gal-β-(1-3)-GalNAc core.*

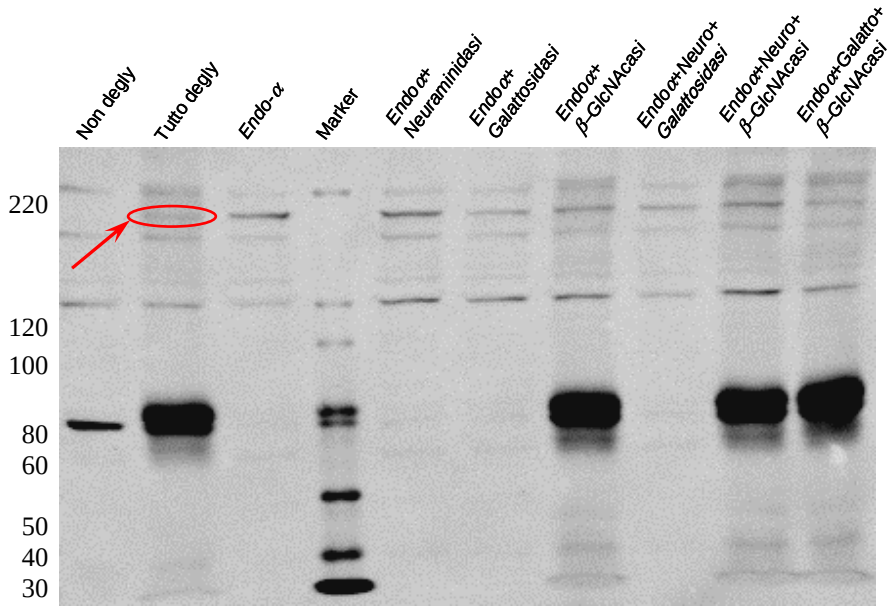


Figure 7.20 *Analysis of O-linked oligosaccharides in REST nuclear protein in SH-SY5Y cells.*

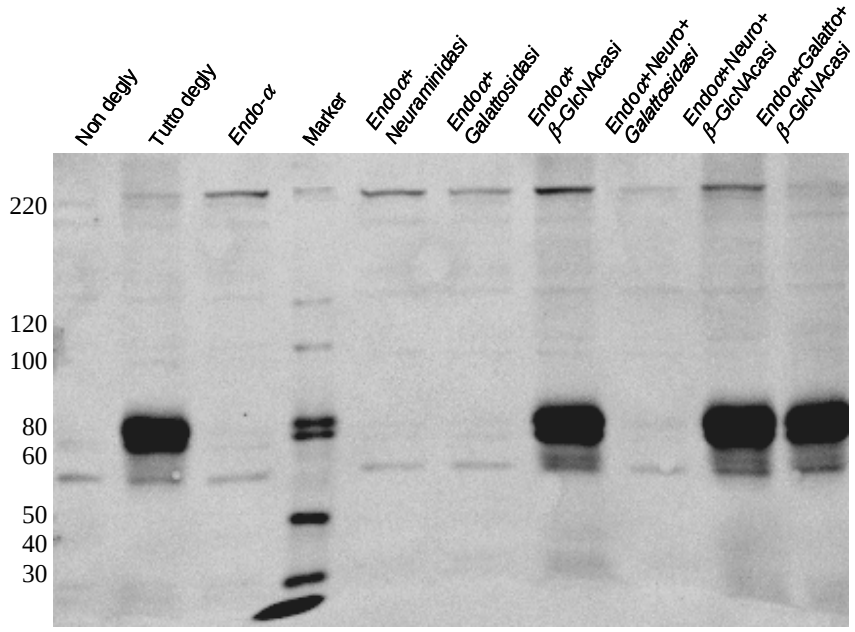


Figure 7.21 *Analysis of O-linked oligosaccharides in REST protein in cytoplasmic extract in SH-SY5Y cell line.*

7.12 Analysis of glycosylation pattern of recombinant REST protein in HEK293 cell line

HEK293 cell line was transiently transfected with a plasmid containing the coding sequence of REST fused with FLAG tag (pCMV-Tag2+REST). In this way it was possible to analyze REST protein using anti-REST and anti-FLAG antibody.

In nuclear extract of transfected cells it was possible to identify three bands, which had an apparent molecular weight of about 220, 150 and 120 kDa (Figure 7.22), with both antibody. In the cytoplasmic compartment it was possible to recognize only two bands, of about 150 and 120 kDa using anti-REST antibody (Figure 7.23A), while using anti-FLAG antibody it was not possible to identify any new band (Figure 7.23B).

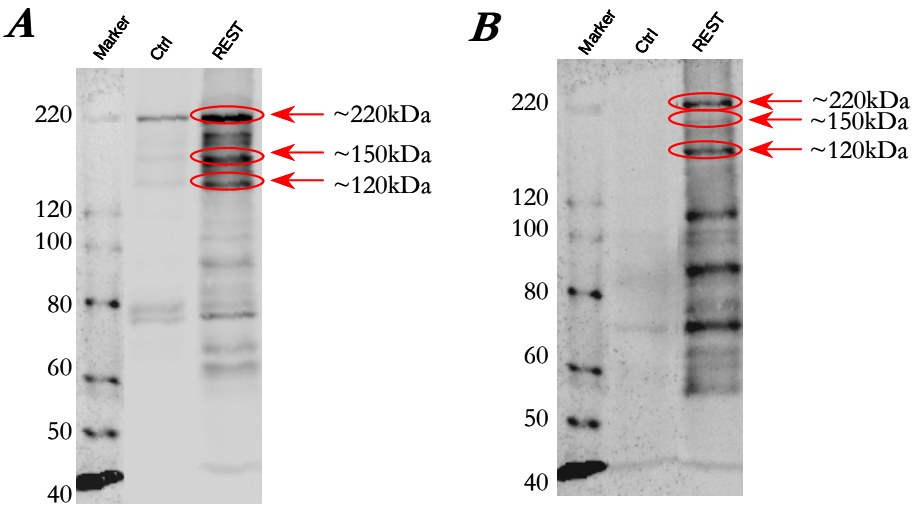


Figure 7.22 *Western blotting analysis of REST nuclear expression in transfected HEK293 cells detected with both anti-REST (A) and anti-FLAG (B) antibodies.*

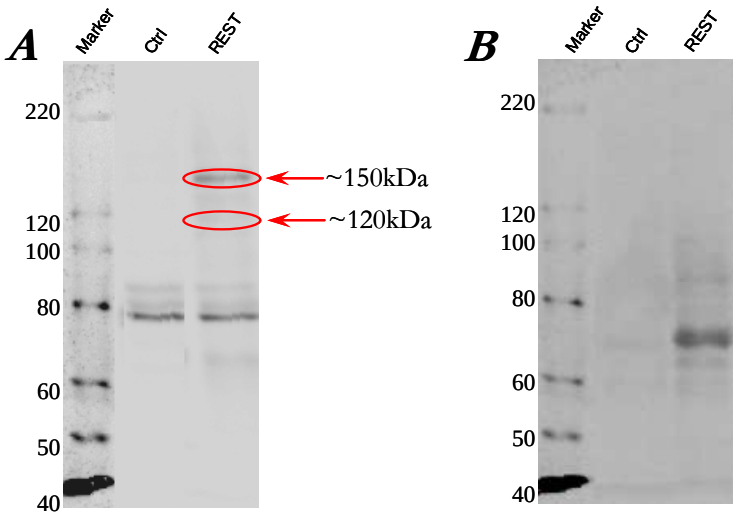


Figure 7.23 *Western blotting analysis of REST cytoplasmic expression in transfected HEK293 cells detected with both anti-REST (A) and anti-FLAG (B) antibodies.*

7.12.1 Enzymatic deglycosylation of recombinant REST protein in HEK293 cells

Treatment of nuclear proteins with all deglycosylating enzymes resulted in a reduction of the 200 and 150 kDa bands and in the appearance of a 120kDa band (Figure 7.24). Analogously results were obtained with the other combinations of enzymes. Moreover, with the combinations of enzymes *Endo+Galacto* and *Endo+Neuro+Galacto* the 200kDa band disappeared, 150kDa band was weaker, and the 100kDa band (probably the unglycosylated form of REST) seemed more intense. These results suggested the presence of oligosaccharide chains containing galactose.

Analogous results were obtained using anti-FLAG antibody (Figure 7.24B).

Treatment of cytoplasmic proteins with several combinations of deglycosylating enzymes failed to change the molecular weight of REST, as determined by SDS-PAGE (Figure 7.25), using both antibodies. These data suggest a nuclear localization of glycosylated REST protein in HEK293 cells.

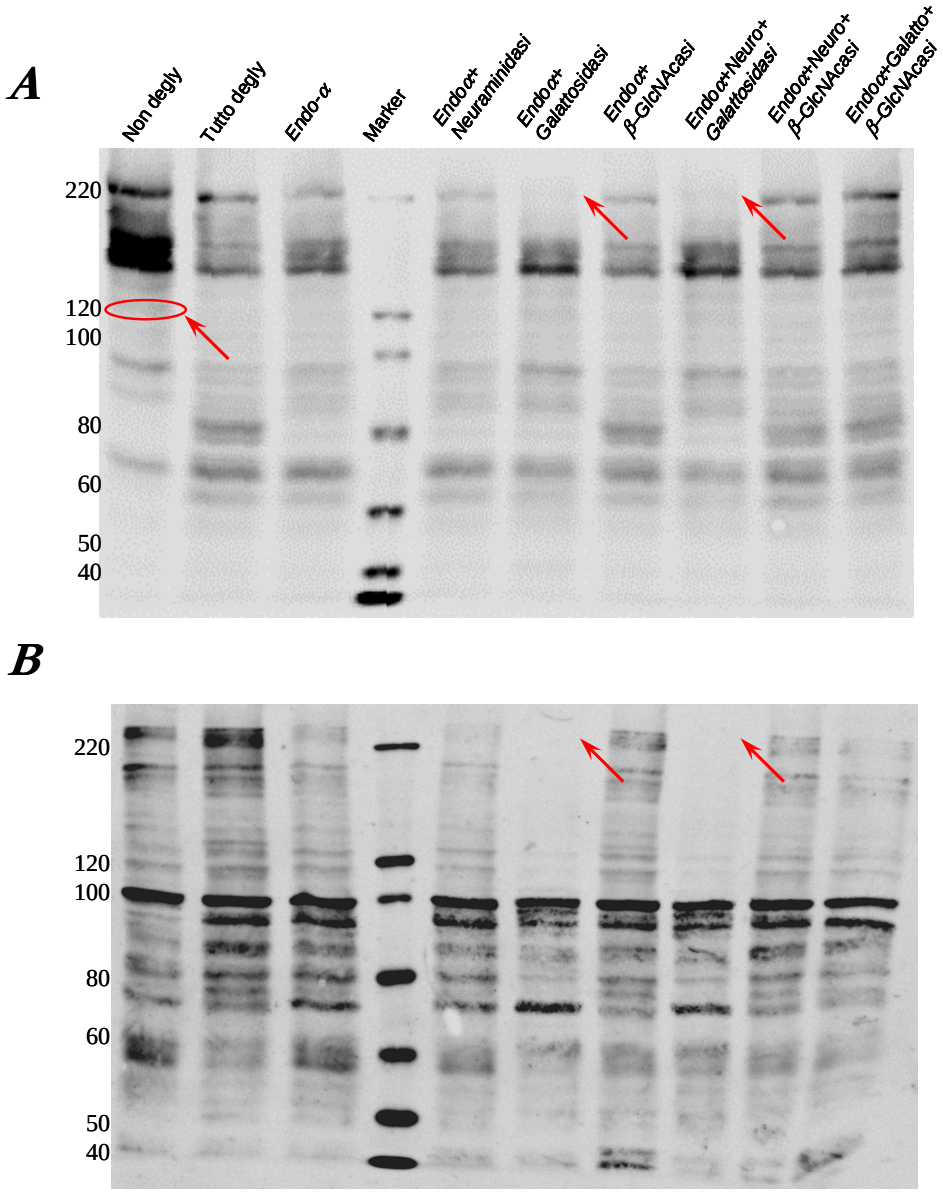


Figure 7.24 *Analysis of O-linked oligosaccharides in RESTnuclear protein in SH-SY5Y cell line, using anti-REST antibody (A) and anti-FLAG antibody (B).*

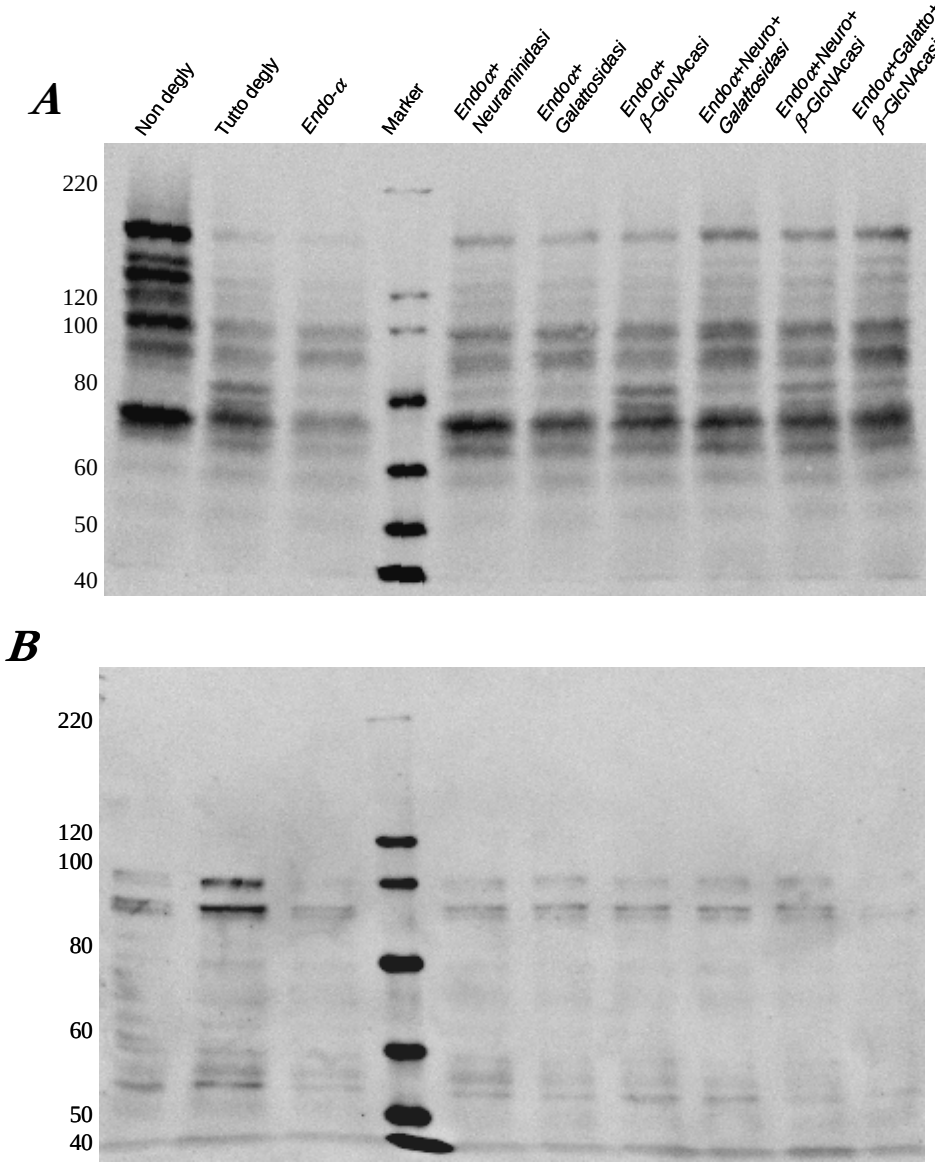


Figure 7.25 *Analysis of O-linked oligosaccharides in REST protein in SH-SY5Y cytoplasmic extract, using anti-REST antibody (A) and anti-FLAG antibody (B). No changes of the molecular weight of REST were observed.*

8. DISCUSSION

In this study we report that nuclear REST is increased in SH-SY5Y neuroblastoma cells cultured in SFM and exposed to IGF-I for 2-days and it then declines in 5-day-treated cells concomitant with a progressive neurite extension. Also the phorbol ester PMA was able to increase nuclear REST levels after 3-days treatment concomitant to neuronal differentiation of neuroblastoma cells. REST expression is also elevated by FCS; however, this effect cannot be clearly explained as it depends on numerous factors acting concomitantly on cellular processes [reviewed in Winkles, 1998].

IGF-I stimulates the increase in neuron number by mechanisms that involve both proliferation and promotion of survival [Arsenijevic & Weiss, 1998; Morrione et al., 2000; Arsenijevic et al., 2001; Anderson et al., 2002; D'Ercole et al., 2002]. Interestingly, several laboratories have reported that IGF-I induces neurite outgrowth and is a mitogen for the human neuroblastoma cell line SH-SY5Y [Mattsson et al., 1986; Påhlman et al., 1991; Kim et al., 1997, 1998; Kuo & Chen, 2002]. Most of the effects of IGF-I are mediated by its binding to the type I IGF receptor which is related to the insulin receptor in structure and function [reviewed by Anlar et al., 1999; D'Ercole et al., 2002]. In this study we have ascertained that the action of IGF-I on induction of REST mRNA involves the IGF-I receptor and requires the activation of the MAPK cascade. This signalling pathway is implicated in the effects of IGF-I on neuroblastoma differentiation [Kim et al., 1997, 2004]. Kim et al. (1997) have reported that, in SH-SY5Y cells, a prolonged MAPK phosphorylation was observed in cells exposed for 24h to IGF-I and was parallel to IGF-I-induced neurite outgrowth; thus, as suggested by this study, the MAPK pathway can regulate neuronal differentiation by modulating gene expression. REST could be included among the genes increased during early cell differentiation induced by IGF-I through the MAPK pathway.

Cellular mechanisms contributing to the decline of REST expression in IGF-I-treated cells remain to be investigated. Interestingly, this decrease was parallel to the reduction of IGF-I-induced cell proliferation observed in cells exposed to this agent for 3 or 5days. It could be hypothesized that, following a prolonged exposure of cells to IGF-I, both effects may be influenced by the expression of novel, unknown factors which may contribute to down-regulate them. On the contrary, GAP-43, synapsin I and βIII-tubulin, taken as markers of the neuronal differentiation, are up-regulated in the presence of IGF-I. Our observation that a prolonged MAPK phosphorylation is maintained in cells exposed for 5days to IGF-I would exclude any

relevant down-regulation of this signaling pathway, whereas it could be hypothesized that a contribution to REST decline may also be made by a differential regulation of signalling pathways, not yet explored, which are involved in differentiation and proliferation events regulated by IGF-I [Hernández-Sánchez et al., 1997; Morrione et al., 2000; Bostedt et al., 2001; Olsson & Nånberg, 2001].

The expression pattern of REST could be consistent with the theory that, during early neuronal differentiation induced by IGF-I, it may help to repress the expression of several genes not yet required by the differentiation program and then it declines later. Interestingly, the observation that REST expression is progressively reduced in parallel with cell proliferation seems to indicate that the role of this transcription factor could also be related to cell survival or to counteract apotosis events [Lawinger et al., 2000] although, as shown by AS-ODN experiments, it does not seem to be directly involved in cell proliferation. Therefore, the decline of REST expression is a comparatively later event during maturation of neuroroblasts *in vitro*.

The patterning and specificity of the nervous system requires an accurate system of neurite elongation [reviewed in Ferreira & Paganoni, 2002]. Therefore, REST down-regulation may be related to elongation and maturation of neurites allowing the interconnection of different brain areas and the proper function of the nervous system.

In the differentiation model adopted in this study, neuroblastoma cells remain viable in the presence of IGF-I alone for a limited period of time (6days) whereas treatment with other differentiating agents, such as retinoids or phorbol esters, may be prolonged over exposure time [reviewed in Påhlman et al., 1990, 1995; Brodeur et al., 2000]. These latter compounds induce a more dramatic morphological change, marked by the elaboration of an extensive network of long neuritic processes, which could require a decisive REST down-regulation.

Nishimura et al. (1996), in fact, reported a marked decrease in REST mRNA content in differentiated neuroblastoma cells exposed for at least 7days, but not for shorter periods, to staurosporine plus cAMP, with a concomitant increase in mRNA levels of synapsin I. Interestingly, transcription of synapsin I and type II sodium channels is silenced by REST and their expression is increased when this transcription factor is down-regulated [Okamoto et al., 1999; Ballas et al., 2001; this study]. Bai et al. (2003) have reported that nuclear levels of REST protein were largely reduced after differentiation for 4days in the presence of retinoic acid whereas no significant change was found in neuroblastoma cells exposed for a shorter period of time to this agent.

SH-SY5Y neuroblastoma cells develop a neuronal sympathetic phenotype when grown in serum-containing medium supplemented with low concentrations (16nM) of TPA (12-O-tetradecanoylphorbol-13-acetate) [Påhlman et al., 1981, 1983]. 16nM TPA is not sufficient for inducing differentiation under serum-free conditions, but do so if combined with one of several defined peptide growth factors e.g., members of the PDGF, IGF, and fibroblast growth factor families [Lavenius et al., 1994]. Differentiated SH-SY5Y cells show increased expression of markers such as GAP-43 and NPY and extend neurites with varicosities and growth cones [Fagerström et al., 1996].

PKC is a family of serine-threonine kinases and the different isoforms are divided into three groups. In addition to different sensitivity to activating signals, PKC isoforms show variations in susceptibility to down-regulation, in tissue-specific distribution, and in substrate specificity. It is therefore likely that they have specific cellular functions [Fagerström et al., 1996]. It has been shown previously that SH-SY5Y cells express PKC-α, PKC-ε and PKC-ζ protein and that treatment with 16nM TPA and serum for 96 h slightly down regulates PKC-α and PKCε, while PKC-ζ remains unaffected [Parrow et al., 1995]. During the differentiation process, a sustained PKC activity is seen [Parrow et al., 1992]. Treatment with 16μM TPA in the presence of serum down regulates PKC-α totally and PKC-ζ partially. These cells still proliferate and show a poor differentiation response. Taken together, previous data have suggested a role primarily for PKC-α, but also for PKC-ζ during differentiation [Parrow et al., 1995]. PKC-α and PKC-ε are enriched in growth cones prepared from SH-SY5Y cells treated either with 16nM TPA and serum or bFGF-IGF-l. This growth cone enrichment of these two isoforms suggests a functional role for these isoforms in growth cone activity [Parrow et al., 1995].

To investigate any possible role of PKC (a target of PI3K pathway) in the regulation of REST expression, SH-SY5Y cells were exposed to PMA (phorbol 12-myristate 13-acetate), a well known activator of PKC. In this study I showed that PKC regulates REST expression during SH-SY5Y neuronal differentiation: this transcription factor is up-regulated in cells exposed for 3days to PMA whereas, at later stages, it is down-regulated. Supporting these data, the exposure to PKC inhibitors (GF10923X and Gö6976) and PMA (16nM) reverted the effects observed with PMA alone.

A detailed understanding of the role played by REST in neuronal development, under the influence of positive inducers such as IGF-I and protein kinase C, would provide a better knowledge of molecular mechanisms regulating these events. Therefore, in this study we

employed an AS strategy to down-regulate REST expression in neuroblastoma cells. Adopting this approach, we observed that, in AS-ODN-treated cells, the effects elicited by IGF-I on cell proliferation were not influenced. On the contrary, a marked decrease of REST expression significantly increased neurite elongation elicited by IGF-I and PKC exposure without any gross perturbation of neurogenesis. This effect was maintained in cells exposed to the AS-ODN added at the peak of REST induction by IGF-I (48h) and it was preceded by increased cell levels of GAP-43, a marker not directly regulated by this transcription factor which is located in growth cones and plays an important role in neuronal sprouting, growth cone migration and pathfinding to target cells [Örtoft et al., 1993; Benowitz & Routtenberg, 1997]. Thus, the experiments performed with AS-ODN seem to suggest that a precocious REST down-regulation in neuroblasts may produce a more rapid neurite outgrowth without preventing overt neurogenesis. In agreement with this hypothesis, Chen et al. (1998) observed that the expression of a dominant-negative form of REST in the developing chick spinal cord resulted in the early presence of neuronal target genes concomitant with embryonic lethality at embryonic day 9.5–10; the embryos showed abnormal patterns of cell proliferation and migration while gross neurogenesis was not influenced. Bahn et al. (2002) have suggested a link between dysregulation of REST and some of the neurological deficits seen in Down's syndrome in which the gross neurogenesis is not affected [Becker et al., 1991].

Studies of overexpression of REST in SH-SY5Y cells have confirmed that this transcription factor plays an essential role in neuroblastoma differentiation. We have observed that REST overexpression (obtained by transfection of SH-SY5Y cells with a plasmid containing the cDNA of human REST) significantly reduced the process of neurite outgrowth and the appearance of neuronal markers induced by IGF-I and PMA (S. Spampinato, unpublished observations). Moreover, Ballas et al. (2001) have reported that, in PC 12 (rat pheocromocytoma) cells, REST overexpression reduces neurite length and blocks sodium channel induction in response to nerve growth factor. In agreement with this idea, Paquette et al. (2000) have found that a prolonged overexpression of REST in neurons may perturb axon pathfinding and repress some, but not all, of the target genes. Thus, it could be suggested that down-regulation of REST in neuronal cells is required for a full elaboration of the terminally differentiated phenotypes.

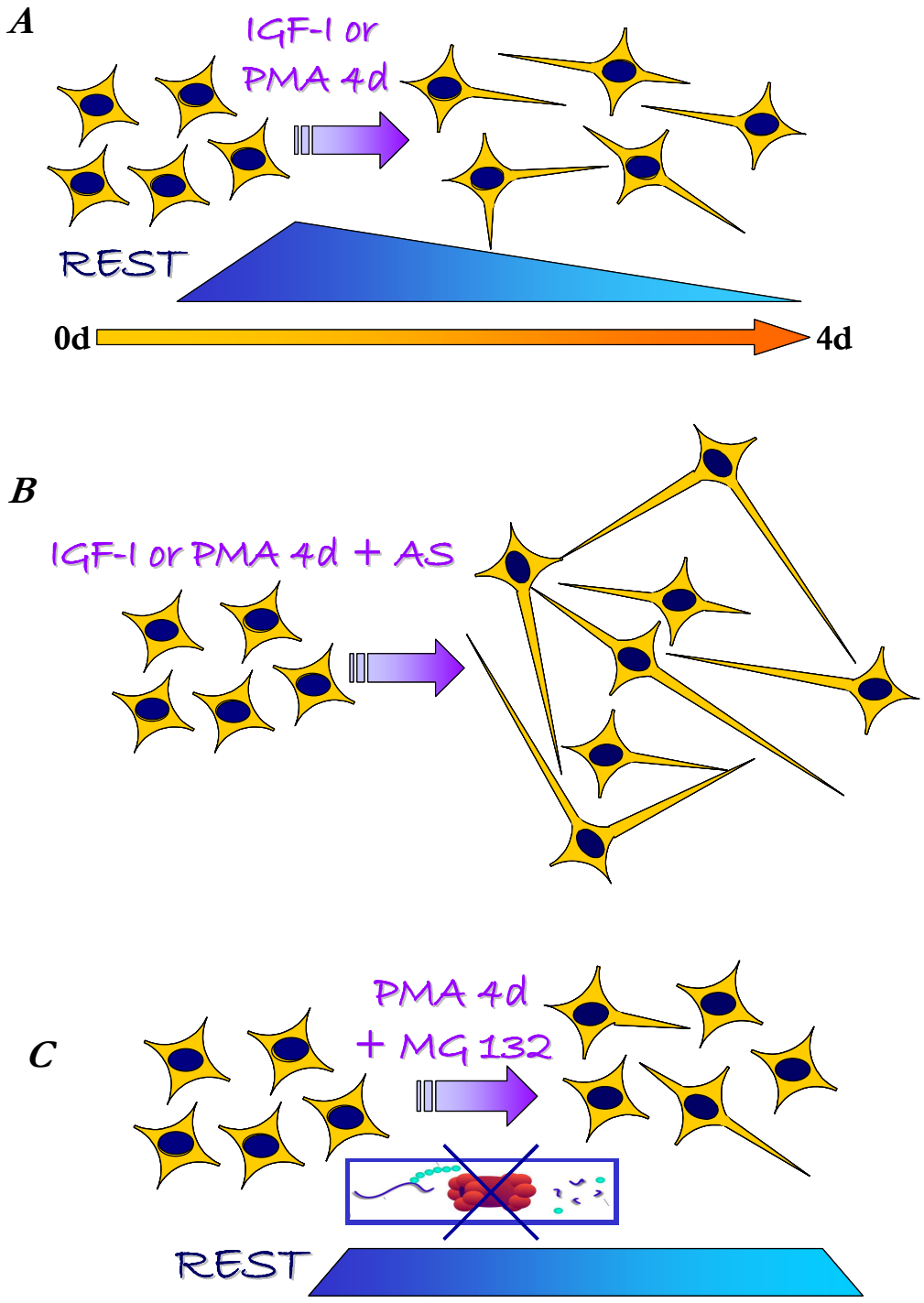


Figure 8.1 ***A.*** *REST may act as a regulator, rather than as a silencer, of neuronal genes, contributing to the coordinated expression of specific batteries of genes.* ***A.*** *In differentiating SH-SY5Y cells, REST is regulated by IGF-I and PKC in a time-dependent manner; it is elevated during early stages of neuronal induction and then declines later.* ***B.*** *A precocious REST down-regulation due to AS in neuroblasts may produce a more rapid neurite outgrowth without preventing overt neurogenesis;* ***C.*** *The blockade of REST degradation is accompanied by a significant reduction of neuronal differentiation markers and arrest of neurite extension.*

Finally, it should be considered that REST may function in different ways at different stages of cell differentiation as a result of changing expression of associated proteins or other signals as it may form complexes with other cellular factors, such as CoREST [Andres et al., 1999; Grimes et al., 2000], Sin3A and histone deacetylase [Huang et al., 1999; Ballas et al., 2001; Belyaev et al., 2004] which contribute to modulate REST-mediated repression. An N-terminal expression domain of REST interacts with Sin3A [Roopra et al., 2000; Naruse et al., 1999] whilst the C-terminal recruits a complex that contains CoREST and a component of the chromatin-remodeling complex [Andres et al., 1999; Battaglioli et al., 2002].

Kubawara et al. (2004) have described a novel mechanism through which REST can act as a flexible mediator of neuron-restrictive silencer element regulatory elements by interacting with a small non-coding double-stranded RNA defined as neuron-restrictive silencer element double-stranded RNA. This latter can trigger gene expression of neuron-specific genes. Therefore, REST expression during neuronal differentiation appears to be time-, gene- and cell-type-dependent.

Taken together, these observations indicate that REST may act as a regulator, rather than as a silencer, of neuronal genes, contributing to the coordinated expression of specific batteries of genes. In differentiating SH-SY5Y cells, REST is regulated by IGF-I and PKC in a time-dependent manner; it is elevated during early stages of neuronal induction and then declines later. The precocious down-regulation of this transcription factor may increase neurite elongation and accelerate cell differentiation. We propose that REST is elevated during early steps of neural induction by IGF-I and PKC activator PMA to decline later for the acquisition of neural phenotypes. Future experiments are needed to better distinguish between an effect of REST that selectively affects neurite outgrowth and a global effect upon the differentiation state of the cell.

Members of the PKC family have been suggested to have important roles in the regulation of cell survival and programmed cell death [Maher, 2001]. Proteasome is responsible for non-lysosomal, energy-dependent degradation of proteins. The cylindrical complex recognize poly-ubiquitinated proteins, unfold them and cleaves he peptide chains in smaller fragments [Bochtler et al., 1999]. It has been suggested that the ubiquitin- and proteasome-dependent protein degradation system is critical for neuronal differentiation, neuronal survival, and prevention of neurodegenerative diseases [Bossy-Wetzel et al., 2006]. For example, it was reported that the levels of proteasome subunits are upregulated during neuronal differentiation and that pretreatment of primary neurons with proteasome inhibitors prevents axon formation, elongation and branching in vitro [Klimaschewski et al., 2006]. Moreover, the rat pheocromocytoma (PC12) cell line and Neuro-2A

cells respond to inhibition of the proteasome with withdrawal from the cell cycle and shifting cellular metabolism from the proliferative to the differentiative state [Omura et al., 1991; Klimaschewski et al., 2006].

In order to relate the decreased REST expression with a progressive neurite extension, we investigated any possible involvement of the ubiquitin–proteasome system (UPS), a multi-enzymatic pathway which degrades polyubiquinated soluble cytoplasmic proteins [Pickart and Cohen, 2004]. In SH-SY5Y cells exposed to PMA 16nM for 4 days, the concomitant exposure to the proteasome inhibitor MG132 (1μM) restores REST nuclear protein levels down-regulated by the phorbol ester, as ascertained by western blotting analysis. The blockade of REST degradation is accompanied by a significant reduction of neuronal differentiation markers and arrest of neurite extension. In SH-SY5Y exposed to PMA and MG 132, we observed an inverse pattern of expression of synapsin I and βIII tubulin, two neuronal differentiation markers regulated by REST. Their cytoplasmic levels are reduced when compared to cells exposed to PMA alone, as a consequence of the increase of REST expression by proteasome inhibitor. The majority of proteasome substrates identified to date are marked for degradation by polyubiquitinylation; however, exceptions to this principle, are well documented [Hoyt and Coffino, 2004]. Interestingly, REST degradation seems to be completely ubiquitin-independent as we observed using two different experimental approaches. Affinity chromatography for polyubiquitinated proteins was not able to identify any REST protein; these data were confirmed by immunoprecipitation experiments performed with a specific antibody against REST (S. Spampinato, unpublished observations). Thus, we propose that REST is regulated by PKC activators in a time-dependent manner: it is elevated during early steps of neural induction by PMA and could contribute to down-regulate genes not yet required by the differentiation program while it declines later for the acquisition of neural phenotypes, concomitantly with a progressive neurite extension. This later decline is regulated by the proteasome system activation in an ubiquitin-indipendent way and adds more evidences to the hypothesis that REST down-regulation contributes to differentiation and arrest of proliferation of neuroblastoma cells.

Ballas et al., (2005) found that REST protein is present at highest level in nuclei of pluripotent embryonal stem (ES) cells. As ES cells differentiate to progenitors, REST mRNA levels stay relatively constant, but REST protein is downregulated posttranslationally to minimal levels. The downregulation of REST by the proteasomal pathway likely occurs in an ubiquitin-indipendent manner, similar to several other proteins such as c-Jun and IkBα [Hoyt and Coffino, 2004]. In

this way, the decrease in REST concentration plays two roles: it facilitates a rapid transition to terminal differentiation as REST leaves the chromatin of neuronal genes.

Moving from the observation that the molecular weight calculated on REST sequence is about 116 kDa but using western blotting this transcription factor appears to have distinct apparent molecular weight (see Table 1.1), the glycosylation pattern of the REST protein was analysed as this difference could be explained by post-translational modifications of the proteins, like glycosylation. Deglycosylating analysis showed that REST protein in SH-SY5Y and HEK293 cells is O-glycosylated and not N-glycosylated. Moreover, using several combination of deglycosylating enzymes it is possible to hypothesize the presence of Gal-β(1-3)-GalNAc residues on the endogenous REST, while β(1-4)-linked galactose residues may be present on recombinant REST protein expressed in HEK293 cells. However, the O-glycosylation process produces an immense multiplicity of chemical structures and monosaccharides must be sequentially hydrolyzed by a series of exoglycosidase. Further experiments are needed to characterize all the post-translational modification of the transcription factor REST.

Recently, several studies underlined the importance of O-glycosylation in modulating transcriptional silencing, protein phosphorylation, protein degradation by proteasome and protein–protein interactions. Nuclear and cytosolic proteins are glycosylated on serine or threonine residues by O-linked β-N-acetylglucosamine (O-GlcNAc). O-GlcNAc is not elongated beyond the monosaccharide unit, takes place at the same sites as does phosphorylation, or at adjacent residues and it is involved in signal transduction [Julenius et al., 2005; Zachara and Hart, 2006]. The addition or removal of O-GlcNAc on proteins has been shown to function in parallel with HDACs to repress transcription of oestrogen-responsive genes [Zhang et al., 2003]. Many transcription factors as well as the proteasome are directly modified by O-GlcNAc to affect their activity. Increased O-GlcNAc levels suppress transcriptional expression of coactivators and of the nuclear hormone receptor, and are also associated with increased transcription of genes encoding corepressor proteins NCoR and SMRT. O-GlcNAc appears to govern the switch from transcriptional repression to activation by integrating proteasome function with direct regulation of transcription cofactors [Bowe et al., 2006].

In this picture, growth factors like IGF-I, PKC, the proteasome and O-GlcNAc may contribute to regulate REST expression and its functions toward neuronal differentiation.

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