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Regulation of Neurofibromin by Post-Translational Modification

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Abstract.- Neurofibromatosis 1 (NF1) is a congenital disease affecting individuals worldwide. NF1 expresses the tumor suppressor protein neurofibromin, and mutations in the NF1 gene can lead to truncated inactive isoform of neurofibromin. Neurofibromin function is to prevent an up-regulation of the Ras signaling cascade by inhibiting the Ras protein. Phosphorylation of neurofibromin in its different domains regulate its ability to inhibit the Ras signaling. *O*-glycosylation of nucleo- and cytoplasmic proteins, like phosphorylation, is an equally dynamic and ubiquitous modification that can occur on the same or neighboring serine/threonine of proteins. Different post-translational modifications induce functional changes in proteins, but assessing these temporary changes is difficult to determine *in vivo*. Computer assisted studies can help to predict the functional changes of proteins. In this study the functional changes induced by structural modifications in mammalian neurofibromin are predicted using computer-assisted techniques. These findings illustrate the basis of different NF1-pathogenesis.

Keywords: Phosphorylation, *O*-GlcNAc modification, Ras-signaling pathway.

INTRODUCTION

Neurofibromin is a tumor suppressor protein that has been associated with different types of cancer such as colon and breast cancers (Ogata *et al.*, 2001; Čačev *et al.*, 2005). It is mainly found in the cytoplasm, displaying different isoforms depending on the cell type. Neurofibromin is transcribed by the *NF1* gene, which is responsible for the pathological condition neurofibromatosis (Cawthon *et al.*, 1990; Wallace *et al.*, 1990). Neurofibromatosis is an autosomal dominant congenital disease that affect individuals worldwide (Yunoue *et al.*, 2003). This genetic disorder of the nervous system primarily affects the development and growth of neural (nerve) cell tissues.

The disease can affect different organs, including bones, skin, irises, and central and peripheral nervous systems (Rasmussen and Friedman, 2000). The most common clinical manifestations are *café-au-lait* macules, neuro-fibromas, Lisch nodules, axillary freckling and learning disabilities (Rasmussen and Friedman, 2000). In humans the *NFI* gene is found on the long arm of chromosome 17 (17q11.2) and contains 60

exons, with an 11- to 13-kb transcript and an open reading frame coding for 2818 amino acids (Marchuk *et al.*, 1991). A central region of neurofibromin (GTPase-activating protein (GAP)-related domain or GRD) constitutes about 10% of the total protein sequence (Dasgupta *et al.*, 2003). This domain regulates the inactivation of Ras proteins (Trovo-Marqui and Tajara, 2006). The Ras protein plays a central role in cellular growth and differentiation, and in malignant tumors. Ras is mostly in the GTP-bound form without functional neurofibromin (Adjei *et al.*, 2001). The GRD of neurofibromin can be controlled by the different domains of neurofibromin. When the *C*-terminal domain (CTD) of neurofibromin is phoshorylated by PKA, it interacts with the protein 14-3-3 (Feng *et al.*, 2004). This interaction negatively regulates the ability of neurofibromin to inhibit Ras (Feng *et al.*, 2004). Furthermore, when neurofibromin becomes phosphorylated on Ser residues in its Cys/Ser rich domain (CSRD) by PKCα, it leads to binding with the actin cytoskeleton (Mangoura *et al.*, 2006) and consequently control of the RAS signaling transduction. These investigations show that neurofibromin's Gap-function is regulated by its different domains, and that phosphorylation and other modifications might play a crucial role in regulation of neurofibromin.

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The interplay or combinations of different post-translational modifications (PTM) regulate the multifunctionality of proteins (Kaleem *et al*., 2007). *O*-glycosylation (*O*-GlcNAc) of cytoplasmic and nuclear proteins is a very dynamic modification similar to phosphorylation (Comer and Hart, 2000). Although *O*-GlcNAc modification is not known in neurofibromin, it has been reported in other tumor suppressor proteins (Shaw *et al*., 1996; Lefebvre *et al*., 2004). The enzyme *O*-GlcNAc transferase (OGT) catalyze the addition of *O*-GlcNAc to proteins. Mostly *O*-GlcNAc attachment sites are found to be similar to phosphorylation sites in proteins, where a reciprocal relationship between *O*-GlcNAc modification and phosphorylation occurs on the same or neighboring Ser/Thr residues (Wells *et al*., 2004). These interplay sites are known as Yin Yang sites, and are well known in several nuclear and cytoplasmic proteins (Hart *et al*., 2007; Slawson *et al*., 2005; Wells *et al*., 2001). Yin Yang sites can be predicted and investigated, using various bio-informatic tools, which can be helpful to elucidate the multifunctional regulation of proteins.

In this study utilizing *in silico* methods functional role of different modifications in mammalian neurofibromin's GRD is described.

MATERIAL AND METHODS

The sequence data used for the prediction of phosphorylation and *O*-glycosylation potential of neurofibromin in *Mus musculus* was retrieved from the Swiss-Prot database (Boeckmann *et al.*, 2003) with primary accession no. Q04690-2. BLAST search was performed using NCBI database of non-redundant sequences using all default parameters (Altshul *et al.*, 1997). The search results were divided into vertebrates and invertebrates. The sequences selected for multiple alignments from different species of vertebrates were from *Homo sapiens* (RefSeq. NP_001035957), *Rattus norvegicus* (RefSeq. NP_036741) and *Takifugu rubripes* (RefSeq. AAD15839). The sequences selected from invertebrates included those of *Culex pipiens quinquefasciatus* (RefSeq.XP_ 001862517.1), *Drosophila melanogaster* (RefSeq. AAB58975.1), and *Aedes aegypti* (RefSeq. XP_001653220.1). The chosen sequences were multiple aligned using ClustalW2 with all default parameters (Thompson *et al*., 1994).

The potential for phosphorylation and *O*-GlcNAc modification in *Mus musculus* neurofibromin was predicted by Netphos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999) and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) (unpublished), respectively.

The YinOYang 1.2 (unpublished) and the Netphos 2.0 server produces neural network predictions for *O*-GlcNAc and phosphorylation attachment sites in eukaryotic protein sequences, respectively. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential for phosphorylation, while the threshold value used by YinOYang 1.2 is variable, depending upon surface accessibility of the different amino acid residues. False negative sites are identified as well, by coupling conservation status and modification potential of the two methods.

For the prediction of methylation sites in *Mus musculus* neurofibromin the MEMO prediction tool was utilized (a computational method for prediction of methylation modifications in proteins) (Chen *et al.*, 2006).

RESULTS AND DISCUSSION

Proteins function are generally controlled by different PTMs like phosphorylation, acetylation, methylation and similar other modifications. PTMs increase the complexity and dynamics of proteins by inducing structural changes, alter their activities, subcellular localization, stability, and interactions with other proteins and molecules. Furthermore, the interplay between different modifications or a distinct combination of PTMs modulates the function of the protein. In growth factor stimulated cells neurofibromin becomes heavily phoshorylated (Izawa *et al.*, 1996). Phosphorylated neurofibromin leads to interaction with the 14-3-3 protein and regulate its GAP-activity (Feng *et al.*, 2004), which shows that neurofibromin is regulated by PTMs. The *in silico* phosphorylation sites in neurofibromin was predicted by Netphos 2.0 (Blom *et al.*, 1999), and showed a high potential for phosphorylation

(Fig. 1, Table I), which is in agreement with the already known experimental data.

Table I.- *In silico* Yin Yang and methylation sites in *Mus musculus* neurofibromin

		Vertebrates	Invertebrates
Yin Yang sites	Ser620	Conserved[1]	Not conserved
	Ser859	Conserved	Conserved
	Ser1815	Conserved	Conserved
	Ser2578	Conserved	Not conserved
	Ser2741	Conserved	Conserved
Methylation sites	Arg366	Conserved	Conserved
	Arg664	Conserved[1]	Not conserved
	Arg1132	Conserved	Conserved
	Arg1134	Conserved	Conserved
	Arg1139	Conserved	Conserved[2]
	Arg1278	Conserved	Conserved
	Arg1418	Conserved	Conserved
	Lys195	Conserved[1]	Not conserved
	Lys478	Conserved	Conserved[2]
	Lys513	Conserved	Conserved[2]
	Lys662	Conserved[1]	Not conserved
	Lys1706	Conserved	Conserved
	Lys1917	Conserved	Conserved[2]
	Lys1958	Conserved	Conserved
	Lys2030	Conserved	Conserved
	Lys2335	Conserved[1]	Not conserved
	Lys2479	Conserved[3]	Not conserved

[1]Not conserved in pisces
[2]Conserved substitution (Arg is substituted by Lys in Invertebrates)
[3]Conserved in mammals (Lys is substituted by Arg in humans)

Neurofibromin binds to the family of syndecan transmembrane heparan sulfate proteoglycans via its GRD and CTD predominantly in the brain (Hsueh, 2007; Hsueh *et al.*, 2001). Neurofibromin also interacts with collapsing, response mediator proteins (CRMPs) in neurites (Lin and Hsueh, 2008; Patrakitkomjorn *et al.*, 2008), and play a role in synaptic signaling and neural development. CRMPs are developmentally regulated (Lin and Hsueh, 2008). Like neurofibromin is the expression of CRMP-2 highest during embryonic and neonatal stage in mammals (Lin and Hsueh, 2008). The active non-phoshorylated CRMP-2 binds to neurofibromin's CTD (Patrakitkomjorn *et al.*, 2008). It co-localizes with neurofibromin in neurites in the distal tips and branches, and the neurofibromin-CRMP-2 complex regulate neurite outgrowth and extension (Patrakitkomjorn *et al.*, 2008). When CRMP-2 is phosphorylated, it cannot bind to neurofibromin, and neurite extension is retracted. Phosphorylation of CRMP-2 is regulated by neurofibromin via is GAP-function, and take place in a specific order. First CRMP-2 is phosphorylated by Cdk5 on S522, followed by GSK-3 on T514 (and T509 or T518) and finally by Rho-kinase on T555 (Patrakitkomjorn *et al.*, 2008). When non-phoshorylated CRMP-2 binds to neurofibromin, it leads to inhibition of the GAP-activity of neurofibromin and an up-regulation of the Ras signal transduction. This leads to activation of cdk5, GSK-3 and Rho-kinase via different signaling pathways, and eventually phosphorylation of CRMP-2. The *in silico* predicted Yin Yang sites in neurofibromin are shown in Table I and illustrated in Figure 2. In the CTD of neurofibromin two Yin Yang sites were found S2578 and 2741. It can be speculated that the interaction between CRMP-2 and neurofibromin can be regulated by PTMs. The first Yin Yang site (S2578) is known to serve as a binding site for 14-3-3, and thereby regulates the GAP-funtion of neurofibromin (Feng *et al.*, 2004). The other Yin Yang site S2741 might play a role in neurofibromin-CRMP-2 interaction. Moreover, when Yin Yang sites in CRMP-2 were predicted by the same procedure as with neurofibromin, S518, 522 and T555 were found (data not shown). CRMP-2 is a highly *O*-GlcNAc-modified protein in synaptosomes, and *O*-GlcNAc is found to be a major regulator in neurodegenerative diseases (Cole and Hart, 2001). Also, recently it has been found that CRMP-2 is dephosphorylated by protein phosphatase 1 (PP1) on residues phosphorylated by GSK-3 in neuroblastoma cells and primary cortical neurons (Cole *et al.*, 2008). OGT is known to make complex wit PP1, and is active in converting a phosphorylated substrate to an *O*-GlcNAc-modified product (Wells *et al.*, 2004). This suggests that OGT can *O*-GlcNAc modify neurofibromin and CRMP-2 and regulate neurite outgrowth in mammals.

In the GRD another important amino acid residue is R1276. It's evaluation in the NF1 International Mutation Database (http://www.nfmutation.org/) revealed that the mutation of R

1276 (nonsense and missense), was among the most widespread mutation in NF1. Mutation of R1276

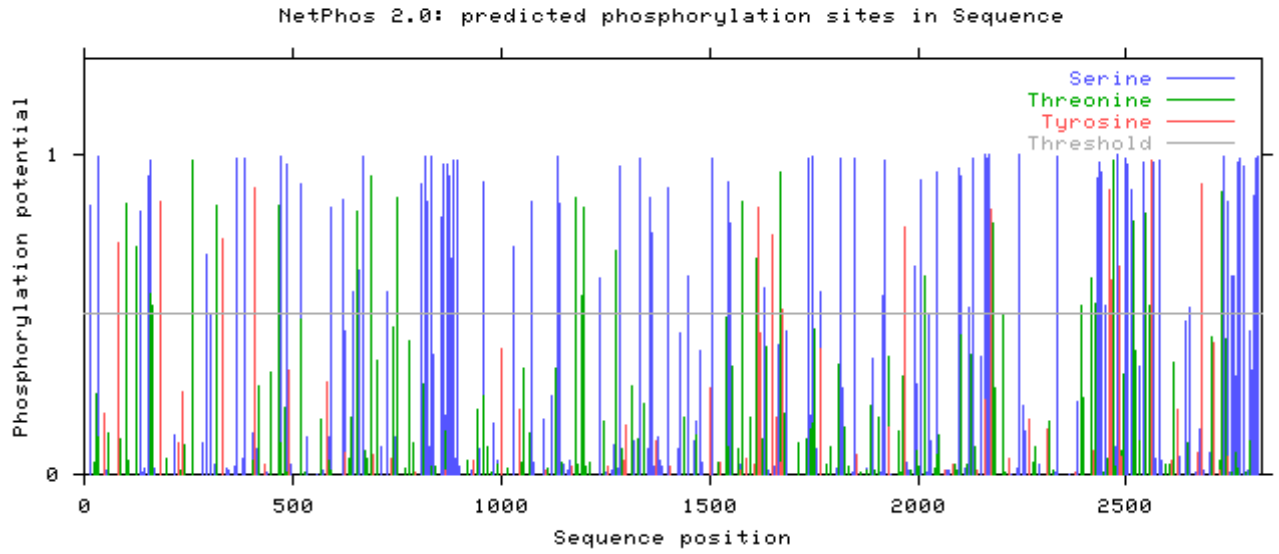

Fig. 1. Graphic presentation of the potential for phosphorylation in *mus musculus* neurofibromin. The green lines show the potential for phosphorylation in neurofibromin and the blue line present the treshold value.

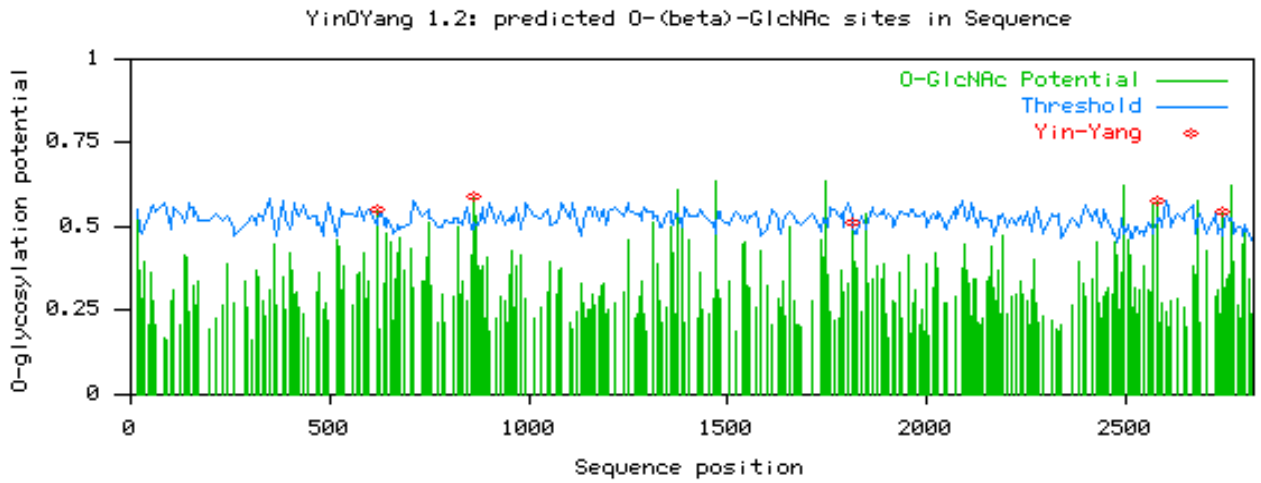

Fig. 2. Graphic presentation of the potential for *O*-GlcNAc modification in *mus musculus* neurofibromin. The green lines show the potential for *O*-GlcNAc modification in neurofibromin and the blue line present the treshold value.

reduces neurofibromin's GAP-activity (Klose *et al.*, 1998; Sermon *et al.*, 1998). The MeMo prediction results showed a potential of methylation on both Lys and Arg residues in neurofibromin (Table I). Two arginine residues (1276 and 1391) are important in the regulation of neurofibromin (Sermon *et al.*, 1998). It has been suggested that Arg situated in the GRD loop (Arg finger) of GAP-activating proteins, are essential for the regulation of their GAP function (Iwashita *et al.*, 2007). R1276 is a predicted methylation site (Table I), and is fully conserved in vertebrates and invertebrates. Methylation of R1276 might affect the protein 3D-structure and inhibit neurofibromin function, but

more work is needed to elucidate the role of methylation in neurofibromin. The interplay between the different modifications such as methylation, phosphorylation and *O*-glycosylation is known to occur in histone protein H3 (Kaleem *et al.*, 2008), and it is possible that the interplay between phosphorylation and *O*-GlcNAc modification in the CTD of neurofibromin affect the methylation of the GRD and thereby regulate the interaction between neurofibromin and Ras. The regulation of neurofibromin by PTMs will help to understand the abnormal development of neurons and neural networks in children, which can lead to learning disabilities and cognitive deficits.

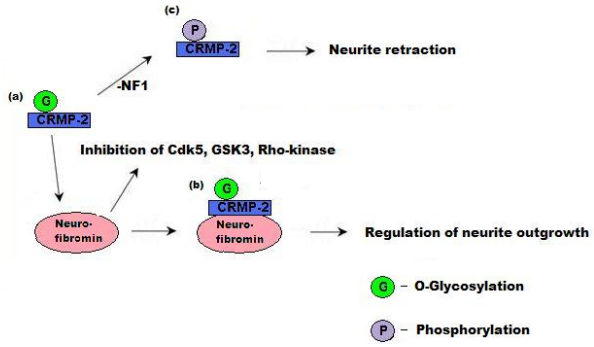

Fig. 3. Developmental control of neurites is regulated by the interaction between neurofibromin and CRMP-2. Free or unbound CRMP-2 is *O*-GlcNAc modified by OGT (a) and interact with neurofibromin in the cytoplasm (b). Furthermore neurofibromin can inhibit different kinases (Cdk5, GSK-5 and Rho-kinase) activity in the cell, and leads to regulation of neurite outgrowth. When the NF1 gene is depleted CRMP-2 becomes phosphorylated by Cdk5, GSK-5 and Rho-kinase that are activated via different alternate signaling pathways. Phosphorylation of CRMP-2 inactivates the protein (c). This inactivation leads to retraction of neurites.

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