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Effect on the Ras/Raf Signaling Pathway of Post-Translational Modifications of Neurofibromin: In Silico Study of Protein Modification Responsible for Regulatory Pathways

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ABSTRACT

Mapping and chemical characterization of post-translational modifications (PTMs) in proteins are critical to understand the regulatory mechanisms involving modified proteins and their role in disease. Neurofibromatosis type 1 (NF-1) is an autosomal dominantly inherited disorder, where *NF1* mutations usually result in a reduced level of the tumor suppressor protein, neurofibromin (NF). NF is a multifunctional cytoplasmic protein that regulates microtubule dynamics and participates in several signaling pathways, particularly the RAS signaling pathway. NF is a Ras GTPase-activating protein (GAP) that prevents oncogenesis by converting GTP-Ras to GDP-Ras. This function of NF is regulated by phosphorylation. Interplay of phosphorylation with *O*-GlcNAc modification on the same or vicinal Ser/Thr residues, the Yin Yang sites, is well known in cytoplasmic and nuclear proteins. The dynamic aspects of PTMs and their interplay being difficult to follow in vivo, we undertook this in silico work to predict and define the possible role of Yin Yang sites in NF-1. Interplay of phosphorylation and *O*-GlcNAc modification is proposed as a mechanism controlling the Ras signaling pathway. J. Cell. Biochem. 108: 816–824, 2009.

KEY WORDS: HUMAN NEUROFIBROMIN; PHOSPHORYLATION; *O*-GLcNAc MODIFICATION; SIGNALING TRANSDUCTION

Neurofibromatosis (NF-1), an autosomal genetic disorder affecting individuals worldwide [Yunoue et al., 2003], has a higher mutational rate compared to most other genetic diseases [Fahsold et al., 2000]. The gene responsible for neurofibromatosis is *NF1* [Cawthon et al., 1990; Wallace et al., 1990], a gene localized on the long arm of chromosome 17 (17q11.2), where it expresses the tumor suppressor protein neurofibromin (NF) [Bollag et al., 1993]. NF is a good example of signal transduction regulator which is controlled by different PTMs. In vivo, experimental verification of short lived PTMs on a single or few amino acids and validation of their functional modulation of transitory states of proteins or of those proteins available in nano or negligible quantities such as NF is a major undertaking. Nevertheless the study of protein modifications involving such few amino acids and resulting in significant functional changes is key to understanding multi-functionality. In this manuscript we have opted to perform studies in silico to study the role of PTMs in NF and evaluate the functional changes resulting from these modifications.

The NF-1 phenotype is highly variable as several organ systems may be affected such as bones, skin, irises, and central and

Abbreviations used: EGF; epidermal growth factor; EGFR; epidermal growth factor receptor; CSRD; cysteine/serine rich domain; CRMP-2; collapsin response mediator protein-2; CTD; C-terminal domain; GRD; Ras-GTPase activating protein (Ras-GAPs) related domain; HIC1; hyper-methylated in cancer 1; Hsp90; heat shock protein 90; LRD; leucine-repeat domain; MAPK; mitogen activated protein kinase; NF; neurofibromin; NF-1; neurofibromatosis 1; OGT; *O*-GlcNAc transferase; PKA; protein kinase A; PKCα; protein kinase C alpha; PP1; protein phosphatase 1; PP2A protein phosphatase 2A; PTM; post-translational modification; Ras-GAPs; Ras-GTPase; SOS; son of sevenless exchange factor; TGFα; transforming growth factor alpha.

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peripheral nervous systems [Rasmussen and Friedman, 2000]. The *NF1* contains 60 exons, with an 11- to 13-kb transcript and an open reading frame coding for 2818 amino acids, which is the most common transcript [Marchuk et al., 1991; Danglot et al., 1995; Li et al., 1995]. Mutations in the *NF1* gene are distributed along the entire coding sequence, and most of the mutations are nonsense, frame shift, or truncating mutations with premature termination codons [Fahsold et al., 2000].

The *NF1* gene is ubiquitously expressed, but most abundantly in the nervous system [Daston et al., 1992] and several tissue-specific splice variants have been recognized [Vandenbroucke et al., 2002; Bottillo et al., 2007]. Truncated isoforms of NF have been detected in the cell, the expression of which leads to NF-1-pathology [Boeddrich et al., 1995].

The Ras-GTPase activating protein (Ras-GAPs) related domain (GRD), the central domain of NF-1, is homologous to a family of proteins known as Ras-GAPs, which function as negative regulators for Ras proteins [Trovo'-Marqui and Tajara, 2006]. The Ras-GAPs interact with the Ras protein and mediate hydrolysis of Ras-bound GTP to GDP, resulting in inactivation of Ras [Trovo'-Marqui and Tajara, 2006]. Inhibition of Ras prevents up-regulation of the Ras-mediated signaling transduction [Adjei, 2001]. This very crucial GAP-related domain only constitutes about 10% of the total protein sequence [Dasgupta et al., 2003]. The C-terminal domain (CTD) and Cys/Ser-rich domain (CSRD) are constitutively phosphorylated by the cAMP dependent protein kinase A (PKA) [Izawa et al., 1996]. In the CTD domain, different phosphorylation sites are located, and the PKA-dependent phosphorylation sites include Ser (2576, 2578, 2580, 2813) and Thr2556 [Feng et al., 2004]. Phosphorylation of these residues is important for interaction of NF with the 14-3-3 protein [Feng et al., 2004]. This interaction negatively regulates the ability of NF to hydrolyse Ras-GTP [Feng et al., 2004]. Furthermore, mitogen activated protein kinase (MAPK), and protein kinase C alpha (PKCα), among others, are found to phosphorylate NF [Marchuk et al., 1991]. In epidermal growth factor (EGF)-stimulated cells, NF becomes phosphorylated on Ser residues by PKCα in its CSRD domain and, consequently, interacts with the actin cytoskeleton [Mangoura et al., 2006]. It has been further reported that phosphorylation of NF inhibits its lysosomal degradation [Kaufmann et al., 1999]. It appears that phosphorylation of NF in its different domains by various kinases controls its ability to regulate the Ras signaling pathway.

O-Glycosylation (*O*-GlcNAc) of cytoplasmic and nuclear proteins is a dynamic and regulatory modification [Comer and Hart, 2000]. *O*-GlcNAc-modified tumor suppressor proteins such as p53 [Shaw et al., 1996], the protein hyper-methylated in cancer 1 (HIC1) [Lefebvre et al., 2004], etc. have been observed. This PTM occurs on Ser/Thr residues like phosphorylation and influences protein folding, localization, trafficking, solubility, antigenicity, biological half-life, as well as cell–cell interactions [Love and Hanover, 2005]. However this type of PTM has not been reported in NF. The enzyme catalyzing the addition of *O*-GlcNAc to the hydroxyl function of Ser and Thr residues in proteins is the *O*-GlcNAc transferase (OGT). The transferase enzyme catalyzes diverse substrates or *O*-GlcNAc modification, but a consensus motif has not been defined as yet. Many *O*-GlcNAc attachment sites are found to be identical to those recognized by kinases. Such sites may be the same or neighboring Ser/Thr that alternatively carry a phosphate or an *O*-GlcNAc, and are known as Yin Yang sites [Wells et al., 2004]. This interplay has been observed in several nuclear and cytoplasmic proteins, and is important in signal transduction [Wells et al., 2001; Hart et al., 2007]. This in silico study identifies the most potential sites for phosphorylation and *O*-GlcNAc modification in human NF on evolutionary conserved Ser/Thr residues in other mammals, vertebrates and invertebrate members. Several computational methods have been developed, which are useful for protein function prediction [Zhao et al., 2008].

Based on the results obtained from different in silico methods for predicted Yin Yang sites, it becomes possible to predict signaling events induced by the interplay of the two PTMs on NF from existing information.

MATERIALS AND METHODS

The sequence data used to predict phosphorylation and *O*-glycosylation potential of NF in *Homo sapiens* was retrieved from the Swiss-Prot database [Boeckmann et al., 2003] with primary accession no. P21359-2. BLAST search was performed using NCBI database of non-redundant sequences using all default parameters [Altschul 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 *Mus musculus* (RefSeq. CAI24835.1), *Rattus norvegicus* (RefSeq. NP_036741.1), and *Takifugu rubripes* (RefSeq. AAD15839.1). The sequences selected from invertebrates included those of *Culex pipiens quinquefasciatus* (RefSeq.XP_001862517.1), *Drosophila melanogaster* (RefSeq. AAB58977.1), and *Aedes aegypti* (RefSeq. XP_001653220.1). The chosen sequences were multiply aligned using ClustalW with all default parameters [Thompson et al., 1994].

The potential for phosphorylation and *O*-GlcNAc modification in human NF 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 server [Gupta and Brunak, 2002] produces neural network predictions for *O*-GlcNAc attachment sites in eukaryotic protein sequences. This method can also predict phosphorylation potential and thus, possible "Yin Yang" sites. 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. These methods are neural network-based and are trained to recognize the sequence environment of modified and non-modified amino acids. Furthermore, the neural network-based method YinOYang 1.2 predicts potential Yin Yang sites that can be glycosylated or alternatively phosphorylated. NetPhos 2.0 predicts phosphorylation on the hydroxy-function of Ser, Thr or Tyr residues with a relatively high sensitivity (69–96%) [Blom et al., 1999]. Artificial neural networks receive many inputs and give one output as a result. The results obtained from the neural networks are sigmoidally arranged with values lying in between 0 and 1. Usually a threshold of 0.5 is used and a site with an output of

TABLE I. In Silico Predicted Phosphorylation Sites in Human NF

Ser	15,35, 137, 155, 158, 259, 302, 365, 368, 387, 474, 488, 521, 592, 620, 641, 644, 665, 666, 727, 807, 818, 821, 829, 858, 871, 876, 879, 882, 883, 892, 1030, 1072, 1135, 1140, 1234, 1282, 1329, 1331, 1354, 1355, 1399, 1449, 1503, 1545, 1546, 1578, 1630, 1733, 1745, 1765, 1813, 1847, 1914, 1916, 1917, 1992, 2004, 2043, 2096, 2100, 2121, 2131, 2160, 2165, 2167, 2170, 2239, 2334, 2430, 2433, 2439, 2446, 2463, 2475, 2479, 2494, 2500, 2502, 2509, 2540, 2564, 2565, 2576, 2578, 2649, 2730, 2739, 2750, 2758, 2767, 2770, 2781, 2805, 2808, 2813
Thr	101, 123, 159, 165, 260, 317, 467, 685, 750, 889, 1127, 1178, 1191, 1195, 1273, 1577, 1609, 1669, 2013, 2101, 2175, 2179, 2201, 2388, 2412, 2423, 2466, 2513, 2544, 2554, 2560, 2729
Tyr	80, 182, 333, 408, 1614, 1650, 1671, 1968, 2171, 2455, 2464, 2482, 2556, 2677

more than 0.5 is predicted as a potential glycosylation and/or phosphorylation site. Furthermore back-propagation is used for optimizing the prediction results. The performance and sensitivity of these neural networks has been cross-validated by using the Matthews correlation coefficients [Matthews, 1975].

In many instances Ser and Thr residues show a very high potential for either *O*-GlcNAc modification or phosphorylation, or show a potential very close to the specific threshold value predicted by the existing methods. These sites are termed false-negative Yin Yang sites, when they additionally are conserved sites, as on these sites OGT and kinases may have an equal accessibility for modification.

RESULTS

PREDICTION OF O-LINKED PHOSPHORYLATION SITES IN HUMAN NF

The predictions of phosphorylation sites in human NF performed by Netphos 2.0 are given in Table I, and graphically presented in Figure 1. These results suggest that NF is a potentially highly phosphorylated protein.

TABLE II. In Silico Predicted *O*-GlcNAc Modification and Yin Yang Sites in Human NF

O-GlcNAc modification	Ser: 637, 674, 892, 1373, 1380, 1468, 1813, 1843, 2576, 2680, 2739, 2763 Thr: 1467, 1744, 2489, 2493
Yin Yang sites	Ser: 892, 1813, 2576, 2739
False negative Yin Yang sites	Ser: 821, 871, 1399, 2475, 2500, 2502, 2509, 2578 Thr: 2423, 2560

PREDICTION OF O-LINKED GLYCOSYLATION AND YIN YANG SITES IN HUMAN NF

The prediction results of *O*-GlcNAc modification and Yin Yang sites in human NF by YinOYang 1.2 are given in Table II and illustrated in Figure 2. The positively predicted Yin Yang sites Ser 1813 (data not shown) and Ser 2739 (Fig. 3) were fully conserved in vertebrates and invertebrates. Ser 892 is conserved in vertebrates but not in pisces (*T. rubripes*), where it is mutated to Gly. In invertebrates, the Ser residue is substituted by Thr (data not shown for both observations). Ser 2576 is fully conserved in vertebrates, but is mutated to Asn in invertebrates (Fig. 3). The predicted Yin Yang sites are of functional importance as these Ser/Thr residues can be modified by kinases as well as by OGT. The false-negative Yin Yang sites are conserved residues showing a very high potential for phosphorylation and a potential for *O*-GlcNAc modification very close to the threshold value. On these sites, OGT and kinases may have an equal accessibility to modify a specific site. Ser 821 and 871 are fully conserved in vertebrates and invertebrates, with the exception in pisces (*T. rubripes*), where there is a deletion gap and a substitution to Ala, respectively (data not shown). Ser 1399, 2502, and 2509 are fully conserved in vertebrates and invertebrates, and Ser 2475, 2500,

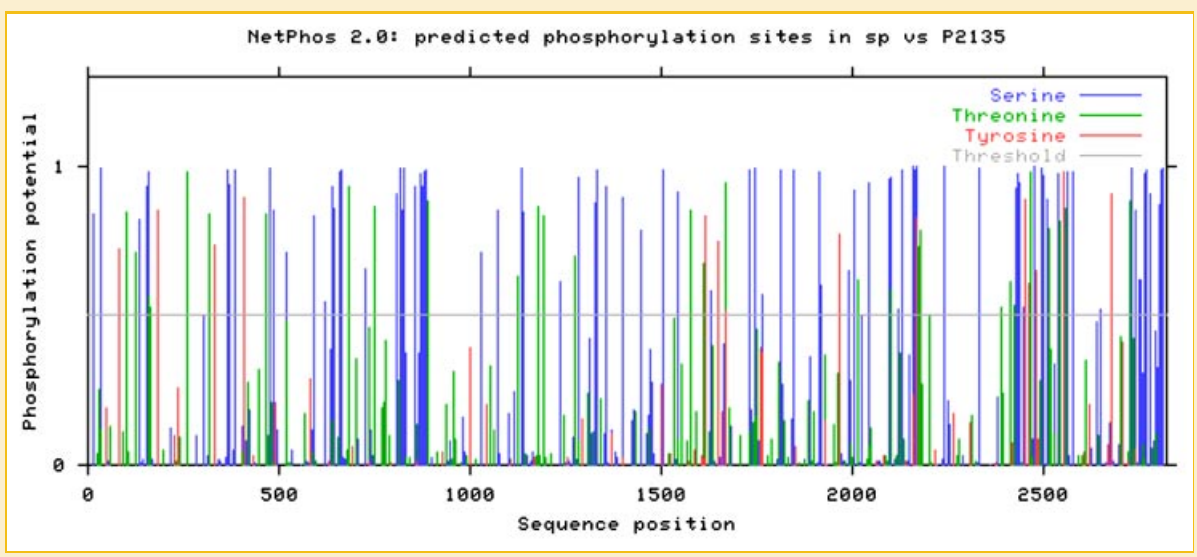

Fig. 1. Predicted potential sites for phosphate modification on Ser, Thr, and Tyr residues in human NF. The blue vertical lines show the potential phosphorylated Ser residues; the green lines show the potential phosphorylated Thr residues; the red line show the potential phosphorylated Tyr residues. The light gray horizontal line indicates the threshold for modification potential. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

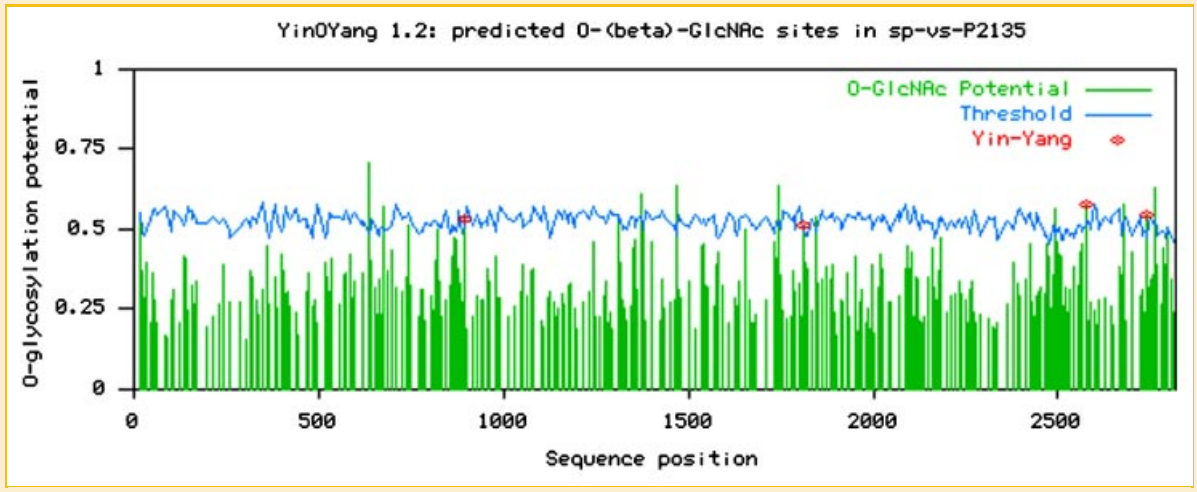

Fig. 2. Predicted potential sites for both *O*-GlcNAc modification and phosphorylation (the Yin Yang sites) in human NF. The positively predicted Yin Yang sites are shown with red asterisk at the top. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and the light blue horizontal wavy line indicates the threshold for modification potential. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2578 and Thr 2423, 2560 are fully conserved in vertebrates (Fig. 3, conservation status for Ser 1399 not shown).

DISCUSSION

Different PTMs of proteins regulate their multifunctionality by inducing temporary changes in their structure/conformation resulting in increased or decreased activities for protein–protein and protein–ligand interactions regulating different cellular processes, and often one PTM may enhance or prevent another PTM resulting in their interplay regulating diverse molecular processes. The interplay between phosphorylation and *O*-GlcNAc modification on the same or neighboring Ser/Thr residues has been reported to occur on many proteins such as c-Myc [Hart et al., 2007]. A number of other proteins have also been predicted and screened for *O*-GlcNAc modification and proposed to intervene in diverse functional modulations [Ahmad et al., 2006; Kaleem et al., 2007, 2009].

NF regulates Ras signaling pathway by preventing epidermal growth factor receptor (EGFR) mediated cell proliferation and differentiation and SOS mediated activation of Ras by inducing GTP-binding [Downward, 1996]. Specific interactions of these proteins maintain the integrity of the Ras signaling cascade, and prevent tumorigenesis. NF is a highly phosphorylated protein with highest phosphorylation potential in its CTD followed by its CSRD. Recently it has been suggested that the CTD of NF is crucial for functional regulation of NF, because several important proteins such as CRMP-2 [Patrakitkomjorn et al., 2008] and 14-3-3 protein [Feng et al., 2004] bind to NF through this domain.

The 14-3-3 scaffolding protein family influences diverse biological activities by regulating the sub-cellular localization of proteins and recruiting downstream effector proteins [Muslin and Xing, 2000; Brunet et al., 2002]. The 14-3-3 proteins are a group of highly conserved acidic proteins that interact with many diverse signaling proteins such as Raf kinases [Tzivion et al., 2001]. When PKA phosphorylates NF on Ser (2576, 2578, 2580, 2813) and Thr2556 in rat, the 14-3-3 protein (14-3-3η) binds to CTD of NF [Feng et al., 2004]. This interaction between NF and 14-3-3 inhibits the ability of NF to inactivate the signaling protein Ras [Feng et al., 2004]. The majority of the predicted Yin Yang sites in human NF (Table II and Fig. 4) were in the CTD, suggesting that OGT might play a role in functional regulation of NF by *O*-glycosylating its CTD. Amongst the predicted Yin Yang sites, Ser 2578 (false-negative Yin Yang site) (Table II) may serves as a binding site for 14-3-3η, when phosphorylated by PKA. This residue is fully conserved in vertebrates. In invertebrates, Ser 2578 (human) is substituted by Asn (Fig. 3), suggesting that in invertebrates, either the binding site for 14-3-3 protein on NF is different or the binding pattern could in some manner regulate its GAP-activity. Feng et al. [2004] showed that phosphorylated Ser (2576, 2578, 2580, 2813) and Thr2556 in rat are crucial for binding of the 14-3-3η protein to NF. It is suggested when Ser 2578 in human NF is dephosphorylated, it becomes available for alternative *O*-GlcNAc modification. This sugar modification prevents the 14-3-3η from binding to NF. A Yin Yang relationship between PKA (or PKC) and OGT is already known to exist in proteins [Griffith and Schmitz, 1999]. Furthermore, *O*-GlcNAc modification is already known to be important for signal transduction, where several signaling proteins such as the protein kinase B/Akt are known to be *O*-GlcNAc modified [Wells et al., 2001; Soesanto et al., 2008]. Therefore, a possible interplay between phosphorylation and *O*-GlcNAc modification on Ser 2578 in CTD of human NF is suggested to regulate its GAP-activity. Binding of 14-3-3 to Raf (the Ras effector kinase), also contributes to signal transduction [Tzivion et al., 2001]. The Raf-1 signaling protein in the cytoplasm exists as a protein complex consisting of the heat shock protein 90 (Hsp90) and dimeric 14-3-3 [Beeram et al., 2005; Chen and Siddiqui, 2007]. Two phosphorylation sites, Ser 259 and 621,

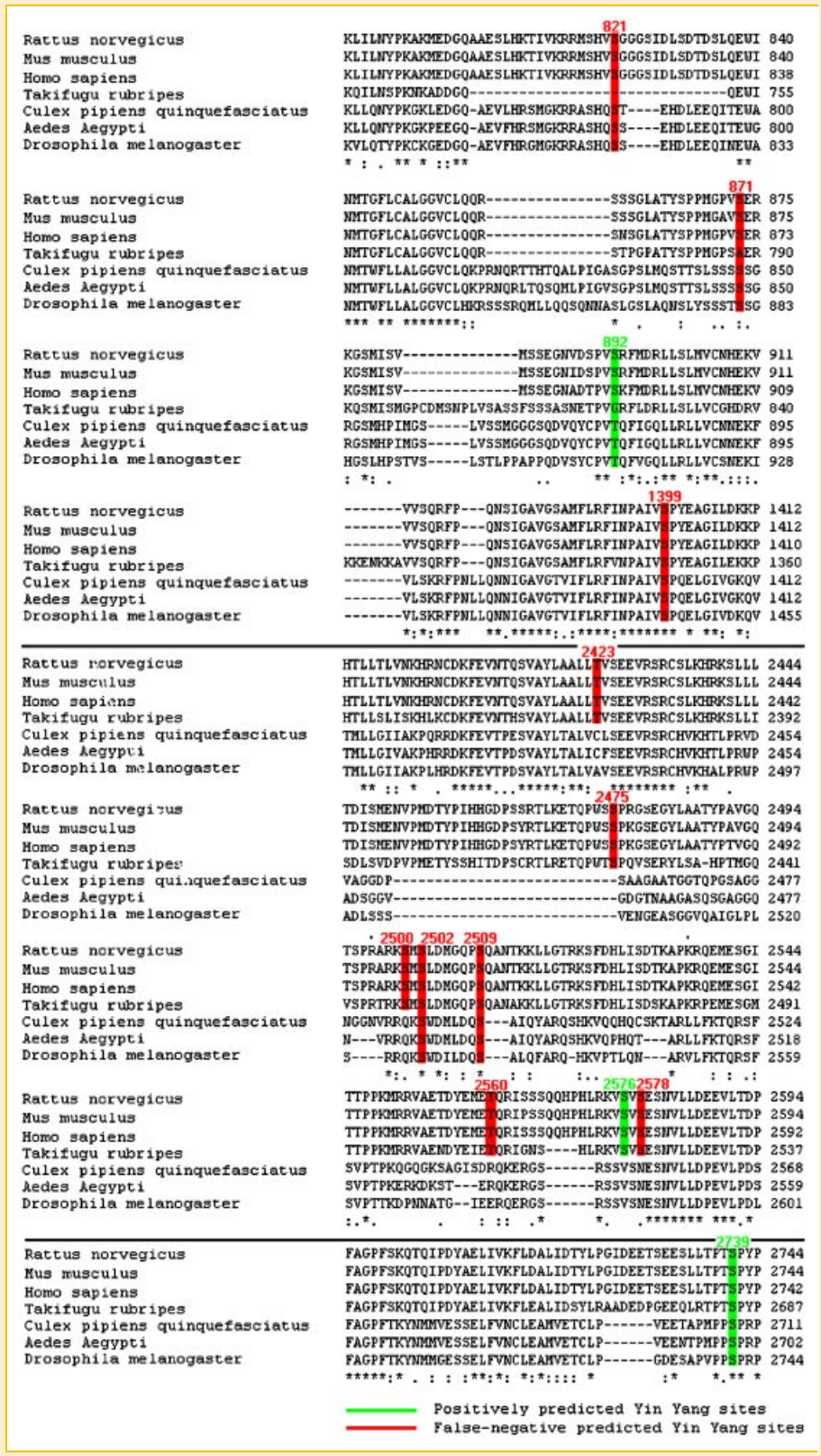

Fig. 3. Multiple alignments of three vertebrates sequences (*Takifugu rubripes*, *Mus musculus*, *Rattus norvegicus*) and three invertebrates (*Culex pipiens quinquefasciatus*, *Aedes aegypti*, *Drosophila melanogaster*). The consensus sequence is marked by an asterisk, conserved substitution by a double dot and semiconserved substitution by a single dot. The different sequences are ordered as in aligned results from CLUSTALW. The positively predicted Yin Yang sites are highlighted in green, as predicted by the YinOYang 1.2 server. The negatively predicted Yin Yang sites (false-negative sites) are highlighted in red. These sites are predicted by Netphos 2.0 and showed a potential very close to the threshold value of the YinOYang 1.2 server. Thus these sites may act as Yin Yang sites and OGT and kinases may have an equal accessibility to modify a specific site. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

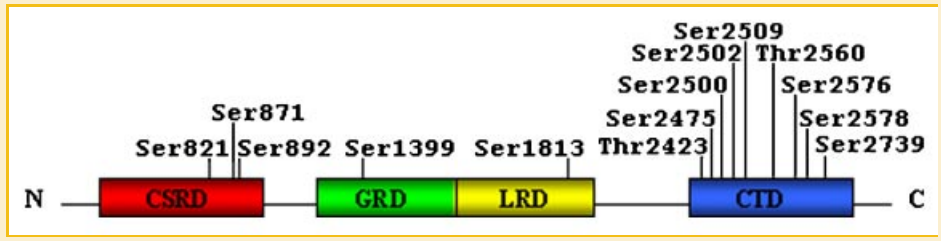

Fig. 4. The distribution of the predicted Yin Yang sites in human NF. It is observed that the CTD of NF contains the highest number of Yin Yang sites (reference Table II). CSRD, Cys/Ser-rich domain (543–909); GRD, Ras-GTPase activating protein (Ras-GAPs) related domain (1168–1545); LRD, leucine-repeat domain (1530–1950); CTD, C-terminal domain (2262–2818). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

have been identified as 14-3-3 binding sites in Raf-1 [Jaumot and Hancock, 2001]. The initial step is recruitment of inactive Raf-1 from the cytosol to the plasma membrane. The 14-3-3 protein plays a role in recruitment of Raf-1 to the plasma membrane, where it becomes displaced from Raf-1 upon interaction between Raf-1 and GTP-Ras [Jaumot and Hancock, 2001]. This step is followed by dephosphorylation of Raf-1 on Ser 259 by protein phosphatase 1 (PP1) and 2A (PP2A) [Jaumot and Hancock, 2001; Kubicek et al., 2002]. *O*-GlcNAc transferase is known to make a functional complex with PP1, and this OGT-PP1 protein complex can dephosphorylate and

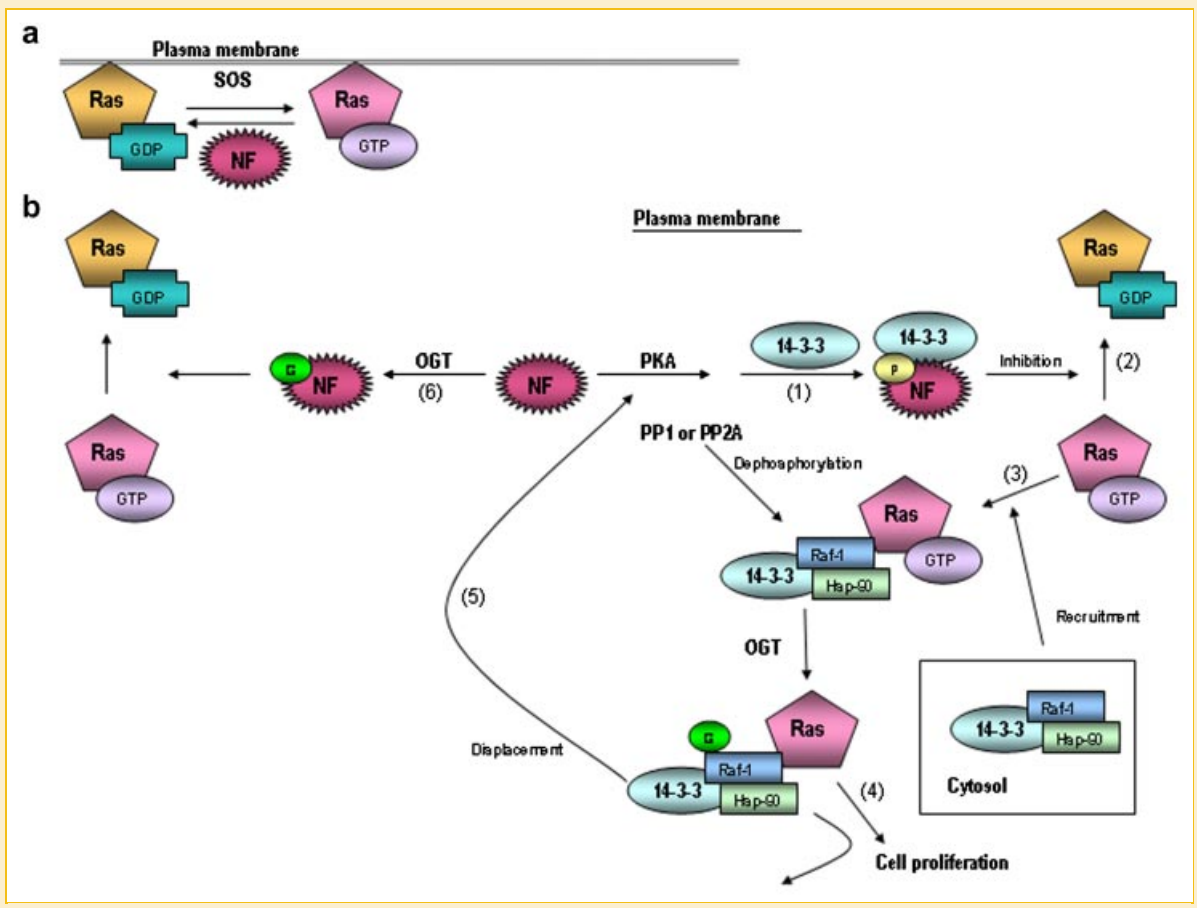

Fig. 5. The regulation of the Ras/Raf signaling pathway can be controlled by the interplay between phosphorylation and *O*-glycosylation in NF. a: NF's function is to convert active Ras (Ras-GTP) to inactive Ras (Ras-GDP), whereas SOS controls the reverse process. b: When NF becomes phosphorylated in its CTD by PKA the 14-3-3 protein binds to NF (1). The interaction between 14-3-3 and NF prevents the inactivation of Ras and enhances cell proliferation (2). The active Ras binds to the Raf-1/14-3-3/Hsp-90 protein complex at the plasma membrane. Binding between active Ras and Raf-1 leads to dephosphorylation of Raf-1 and displacement of Hsp-90 and 14-3-3. Consequently Raf-1 becomes *O*-glycosylated by OGT (3). The Ras/Raf complex leads to activation of downstream effector molecules leading to cell proliferation (4). The displaced 14-3-3 now binds to phosphorylated NF, and thereby maintains the Ras/Raf signaling cascade by preventing NF to inactivate the Ras protein (5). When NF is *O*-glycosylated on Ser 2578, the 14-3-3 protein is unable to bind to NF, and NF is capable of inactivating the Ras protein (6). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

glycosylate a phosphorylated substrate [Wells et al., 2004]. Furthermore cytosolic O-GlcNAc is abundantly found in nerve terminals, where neuron specific proteins such as CRMP-2 are major *O*-GlcNAc modified proteins [Cole and Hart, 2001; Khidekel et al., 2007]. When the interplay between phosphorylation and *O*-GlcNAc modification in Raf-1 was predicted, the Ser 259 was predicted as positive Yin Yang site (Table III). Thus it is proposed that, when Raf-1 is recruited to the plasma membrane, the 14-3-3 protein is released from the Raf-1 protein, which consequently becomes dephosphoylated, and alternatively glycosylated on Ser 259 by OGT (Fig. 5).

Glycosylated Raf-1 binds to GTP-Ras at the plasma membrane. When 14-3-3 is released from Raf-1, it binds to phosphorylated NF and thereby upholds the Ras signal transduction (Fig. 5). On the other hand, when NF is *O*-GlcNAc modified in its CTD, 14-3-3 does not bind NF, and 14-3-3 remains as a complex with phosphorylated Raf-1 in the cytosol, which can lead to suppression of the Ras signaling and eventually cell cycle arrest (Fig. 5).

NF is a multifunctional protein. It inhibits the MAPK signaling via its GAP-activity, whereas it is activated via CSRD. In the CSRD of NF, three Yin Yang sites are predicted (Fig. 4). In this domain and in the CTD, the cellular NO/NOS regulator *NG,NG*-dimethylarginine dimethylaminohydrolase binds to NF and modulates the PKA signaling [Tokuo et al., 2001]. Binding of *NG,NG*-dimethylarginine dimethylaminohydrolase to NF occurs in the region, where the in silico predicted Yin Yang sites (Fig. 4) and PKA phosphorylation sites [Izawa et al., 1996; Jaiswal et al., 1996] are located. Therefore it has been suggested that phosphorylation and alternatively *O*-GlcNAc modification in the CSRD might modulate NF regulated PKA signaling.

Another cAMP activating protein kinase, Akt/PKB has also been shown to phosphorylate Raf-1 on Ser 259 [Filippa et al., 1999]. Akt/PKB co-localizes with OGT at the plasma membrane, and is also known to become *O*-GlcNAc modified [Wells et al., 2001; Foley, 2008; Soesanto et al., 2008]. Taken together, these results suggest that NF via its CSRD domain can regulate the binding of the 14-3-3 protein to its CTD, thus controlling GRD, and consequently tumorigenesis. In this domain, PKCα is shown to phosphorylate NF [Mangoura et al., 2006]. The inverse relationship between PKC and OGT indicates that the interplay between phosphorylation and *O*-GlcNAc modification in the CSRD of NF controls its GAP-activity and hence the Ras signaling pathway. In neurons and astrocytes, phosphorylation of NF increases its GAP-activity and promotes association with the actin cytoskeleton [Mangoura et al., 2006]. During differentiation in neurons and astrocytes, the activity of NF is up-regulated, and NF in the nucleus is co-localized with F-actin in the first phase of differentiation, and with microtubules during the second phase of differentiation [Li et al., 2001]. The PKC phosphorylation of NF in CSRD regulates its association with F-actin [Mangoura et al., 2006]. Among the three Yin Yang sites Ser (821, 871, 892) (Fig. 5), Ser 871 and 892 have vicinal basic amino acid residues, which is a good recognition motif for PKCα [House et al., 1987], indicating that PKCα might be able to phosphorylate these residues. A decrease in the activity of PKCα in response to elevated levels of *O*-GlcNAc is known to exist [Matthews et al., 2005]. This suggests that, when NF becomes phoshorylated in the CSRD by PKCα, NF associates with F-actin, whereas glycosylated NF prevents this association.

The NF emerges to be a multifunctional protein, performing diverse functions in its different domains through the interplay of phosphorylation and *O*-GlcNAc modification on specific Ser/Thr. An important role of NF in regulatory/signaling pathways of growth and differentiation involves a wide range of normal and pathological conditions including Ras/Raf signaling through GAP activity of NF protein. Failure of the GAP activity of NF is the main and critical element of NF-1 pathology. Experimental verification studies of the interplay of PTMs in NF-1 will be helpful to understand the various physiological and/or pathological implications of NF-1 with different set of PTMs in its various domains. Additionally, it will also be useful to develop inhibitors for kinases and/or OGT to regulate the signaling pathways controlling phosphorylation and/or *O*-GlcNAc modification of NF involved in the process of growth and differentiation of the nervous system. This will eventually lead to the treatment and prevention of NF-1.

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