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ARTICLES

Phosphorylation and Glycosylation Interplay: Protein Modifications at Hydroxy Amino Acids and Prediction of Signaling Functions of the Human β_3 Integrin Family

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Abstract Protein functions are determined by their three-dimensional structures and the folded 3-D structure is in turn governed by the primary structure and post-translational modifications the protein undergoes during synthesis and transport. Defining protein functions in vivo in the cellular and extracellular environments is made very difficult in the presence of other molecules. However, the modifications taking place during and after protein folding are determined by the modification potential of amino acids and not by the primary structure or sequence. These post-translational modifications, like phosphorylation and O-linked *N*-acetylglucosamine (*O*-GlcNAc) modifications, are dynamic and result in temporary conformational changes that regulate many functions of the protein. Computer-assisted studies can help determining protein functions by assessing the modification potentials of a given protein. Integrins are important membrane receptors involved in bi-directional (outside-in and inside-out) signaling events. The β_3 integrin family, including, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, has been studied for its role in platelet aggregation during clot formation and clot retraction based on hydroxyl group modification by phosphate and GlcNAc on Ser, Thr, or Tyr and their interplay on Ser and Thr in the cytoplasmic domain of the β_3 subunit. An antagonistic role of phosphate and GlcNAc interplay at Thr758 for controlling both inside-out and outside-in signaling events is proposed. Additionally, interplay of GlcNAc and phosphate at Ser752 has been proposed to control activation and inactivation of integrin-associated Src kinases. This study describes the multifunctional behavior of integrins based on their modification potential at hydroxyl groups of amino acids as a source of interplay. J. Cell. Biochem. 99: 706–718, 2006.

Key words: integrins; post-translational modifications; glycosylation; phosphorylation; Yin Yang sites; multifunctional proteins

Multifunctional proteins are involved in diverse and often unrelated functions and may perform different tasks in different biological environments [Jeffery, 1999]. It is usual for proteins to perform multiple functions in the context of their intra- and extra-cellular interactions. Reversible phosphorylations induce changes in the secondary and tertiary structures and control the modular interactions of proteins [Li et al., 2004]. In addition, phosphorylation and alternatively glycosylation control the functional behavior of a growing list of proteins [Cheng and Hart, 2000]. For instance promotion of proteolysis may result from phosphorylation of serine/threonine or tyrosine residues [Elorza et al., 2003]. Alternatively, resistance to proteolysis may result from the presence of carboxyl or sulfate groups in glycoproteins [Nasir-ud-Din et al., 2003]. Evolutionarily conserved motifs and residues are often involved in performing vital functions of

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the proteins [La et al., 2005]. Cytoplasmic domains of the β_3 chain contains seven Thr and one Ser residues that are generally conserved. Thus the cytoplasmic domain of integrin β_3 chain is important in integrin signaling because of modifications occurring at conserved residue(s).

The recognition events and functions of proteins depend on their precise three-dimensional shape as well as on the presence of specific modifications [Bork et al., 1998; Attwood, 2000]. The knowledge of the 3-D structure of proteins is a prerequisite for the full understanding of their involvement in biological processes. However, determination of the 3-D structure in vivo is difficult, as a given configuration is constantly modified by intra- and inter-molecular interactions occurring with proteins present in body fluids or in the cell. Most of the structural data available in protein databases have been determined by X-ray crystallography or NMR, but these structural determination methods provide information that is only partially relevant to the dynamic behavior of proteins in vivo. The study of the molecular interactions between multifunctional proteins in vivo is likely to be facilitated by computer-assisted techniques that assess the modification potential of the proteins involved.

Integrins are a family of cell adhesion molecules, which act as two-way signaling receptors to promote the attachment of cells to the extracellular matrix and for cell–cell interactions [Ugarova et al., 1998; Yokoyama et al., 2000]. These functions are implicated in many cell-cell and cell-matrix adhesion processes in the context of immune responses, tumor metastasis, atherosclerosis, and thrombosis. The integrin family is composed of over 18 α and 8 β subunits expressed in at least 24 different $\alpha\beta$ heterodimeric combinations. Integrins facilitate cellular adhesion and migration on extracellular matrix proteins located within the intercellular spaces and basement membranes. The heterodimeric combination of β_3 integrin takes place only with α_{IIb} and α_v subunits. The family of β_3 integrins thus results in $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ complete integrins.

Integrin $\alpha_v\beta_3$ binds a wide variety of extracellular matrix proteins including vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand factor, osteopontin etc. [Eliceiri and Cheresh, 2000]. Despite its promiscuous ligand binding behavior, $\alpha_v\beta_3$ is mainly expressed in vascular, intestinal, and uterine smooth muscle cells [Brem et al., 1994]. This receptor has also been found on activated leukocytes, macrophages, and osteoclasts, where it regulates bone resorption [McHugh et al., 2000]. Most prominently, $\alpha_v\beta_3$ becomes upregulated on endothelial cells exposed to hypoxia and cytokines such as VEGF-A [Suzuma et al., 1998; Walton et al., 2000] and was found to be overexpressed in tumor-associated vessels and atherosclerotic plaques [Hoshiga et al., 1995].

The integrin $\alpha_{IIb}\beta_3$ mediates platelet adhesion, spreading, and aggregation and thus plays a critical role in thrombosis and hemostasis [Ferrara, 2000]. In normal circulating platelets, the integrin $\alpha_{IIb}\beta_3$ is in a resting state with low affinity for fibrinogen and von Willebrand factor (vWF). At sites of vascular injury, exposure of platelets to soluble agonists (such as thrombin and ADP) or to matrix-bound adhesive proteins (such as collagen and vWF) induces platelet activation. A frequent consequence of platelet activation is the concomitant activation of ligand binding by the integrin $\alpha_{IIb}\beta_3$ [Byzova et al., 2000]. Under high shear flow rate, such as in stenotic atherosclerotic arteries, initial platelet adhesion, and activation are dependent on the interaction between subendothelium-bound vWF and its receptor, the glycoprotein Ib-IX (GPIb-IX) complex [McHugh et al., 2000; Yokoyama et al., 2000]. GPIb-IX not only mediates the physical adherence of platelets to the site of vascular injury but also initiates signal transduction, leading to the activation of the platelet integrin $\alpha_{IIb}\beta_3$ [Kirk et al., 2000; Walton et al., 2000]. In addition, GPα_{IIb} binds thrombin and is required for the low-dose thrombin-induced integrin activation and platelet aggregation [Gadek et al., 2002].

Involvement of hydroxyl group phosphorylation of cytoplasmic serine, threonine, and tyrosine has been described in signaling via α and β integrin subunits [Kirk et al., 2000; Woodside et al., 2001; Fagerholm et al., 2002]. The arginine-glycine-aspartic acid (RGD)-motif has been shown to be the core recognition sequence for many integrins, including $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ [Hynes, 1992], and is present in a variety of integrin ligands, including collagen, fibronectin, and other extracellular matrix proteins, blood-borne adhesive proteins, viral coat proteins, bacterial membrane proteins, proteins from the IgG superfamily, snake venom proteins, and other integrins [Clark

and Brugge, 1995]. Binding of the ligand RGD-motif to an integrin may induce cytoplasmic phosphorylation, conformational change, and signaling via the conformationally modified integrin molecule [Erb et al., 2001]. Besides tyrosine phosphorylation, integrins have also been reported to be phosphorylated on serine and threonine in cytoplasmic domains of both α and β subunits [Kirk et al., 2000; Fagerholm et al., 2002; Han et al., 2003].

Modification of the β_3 cytoplasmic domains by tyrosine phosphorylation is known to be involved in signaling events [Blystone et al., 1996, 1997]. Threonine phosphorylation in the β_3-cytoplasmic domain is also known to block outside-in signaling [Blystone et al., 1997]. Involvement of Thr758 in the cytoplasmic domain of the β_3 subunit of $\alpha_{IIb}\beta_3$ in controlling bi-directional signaling events through interplay of GlcNAc and phosphate is proposed. In addition, we suggest that an interplay of phosphate and GlcNAc at Ser752 of the β_3 subunit could control the regulation of Src kinase activation.

This study was undertaken to explain the multifunctional behavior of integrins based on the modification potential of the protein, particularly with reference to modifications at hydroxyl groups of serine, threonine, and tyrosine by phosphorylation and *O*-GlcNAc modification. Computer-assisted studies are therefore useful in determining protein function by assessing the modification potential of a given protein. Several programs based on artificial neural networks have been developed to predict glyco-sylation and phosphorylation sites in proteins with reliable accuracy [Blom et al., 2004]. In most cases the prediction accuracy is very high except when the modification potential of a protein is marginally affected by false negative prediction sites, that is, for example, a Ser residue may have very high predicted potential for phosphorylation and a slightly lower potential than the threshold for *O*-GlcNAc. This is indeed a false negative Yin Yang site since both kinase and OGT may be able to access a Ser to modify it by phosphate or by *O*-GlcNAc, respectively.

MATERIALS AND METHODS

The sequence data used to predict phosphorylation and glycosylation sites of the three subunits of the human β_3 integrin family were obtained from the SWISS-PROT sequence database. The sequences of the platelet integrin subunit α_{IIb} [Charo et al., 1986; Poncz et al., 1987] (SWISS-PROT entry name ITAB_HUMAN and primary accession number P08514), of subunit α_v [Suzuki et al., 1986; Xiong et al., 2001] (SWISS-PROT entry name ITAV_HUMAN and primary accession number P06756), and of subunit β_3 (SWISS-PROT entry name ITB3_HUMAN and primary accession number P05106) were established in the late eighties and early nineties [Fitzgerald et al., 1987; Jiang et al., 1992]. All three sequences in the entry contain a signal peptide, which is not part of the mature expressed integrin. Different subunits of integrins contain a signal peptide of different length. In α_v the signal peptide is 30 amino acids long. α_{IIb} contains a signal peptide of 31 amino acids, while the β_3 chain contains a signal peptide of 26 amino acids in length. The predictions were carried out on full precursor sequence but the number of amino acids described in the results correspond to the actual integrin length without signal peptide. Graphs of prediction results include the signal peptide region. BLAST search was made using NCBI database of non-redundant sequences [Altschul et al., 1997] for human β_3 integrin. The search was made for all organisms' sequences with expect value set to 10 using blosum 62 matrix and low-complexity filter selecting nr database. A total of 1,298 hits were found. Eleven β_3 sequences with highest bits score and zero expect value were selected. Of the 11 sequences, 7 were from mammals including those of human, mouse, rat, pig, dog, horse, and chimpanzee (with more than 90% sequence similarity), 1 from chicken, 1 from frog, and 1 from fish. All the 11 sequences were multiply aligned using CLUSTALW [Thompson et al., 1994]. All 11 proteins selected for multiple alignment from BLAST search results are listed in Table I.

Glycosylation Prediction Methods

The methods used for predicting potential glycosylation sites involved prediction of *O*-linked and *N*-linked sites. The three methods for predicting *O*-linked glycosylation sites include *NetOGlyc* 2.0, 3.0, and 3.1 [Hansen et al., 1998; Julenius et al., 2005], for *O*-glycosylation sites in mucin type proteins (i.e., for *O*-GalNAc sites), *DictyOGlyc* 1.1 [Gupta et al., 1999] for *O*-α-GlcNAc sites in eukaryotic proteins and YinOYang 1.2 [Gupta et al., http://www.cbs.dtu.dk/services/YinOYang/], that pre-

TABLE I. Sequences Selected for Multiple Alignments From Blast Search Results

Serial number	Species	Database	Sequence ID/ accession number	Blast results		
				E-value	Bits score	Identity (%)
1	Homo sapiens	SWISSPROT	ITB3_HUMAN	0.00	1,554	100
2	Pan troglodytes	RefSeq	XP_523684.1	0.00	1,490	93
3	Oryctolagus cuniculus	GenBank	AAD51955.1	0.00	1,478	94
4	Canis familaris	GenBank	AAD13680.1	0.00	1,475	95
5	Equus caballus	GenBank	AAK69529.1	0.00	1,462	94
6	Sus scrofa	GenBank	AAK69529.1	0.00	1,431	92
7	Mus musculus	SWISSPROT	ITB3_MOUSE	0.00	1,404	90
8	Rattus norvegicus	EMBL	CAD29521.1	0.00	1,402	89
9	Gallus gallus	EMBL	CAA51069.1	0.00	1,269	82
10	Xenopus laevis	GenBank	AAA17427.1	0.00	1,189	75
11	Tetraodon nigroviridis	EMBL	CAG02646	0.00	1,068	67

dicts *O*-β-GlcNAc sites in eukaryotic proteins. The *NetNGlyc* 1.0 [Gupta et al., http://www.cbs.dtu.dk/services/NetNGlyc/] was used for predicting *N*-glycosylation sites. These four methods for predicting the glycosylation sites are neural network based.

Phosphorylation Prediction Method

For prediction of phosphorylation sites in integrins NetPhos2.0 [Blom et al., 1999, http://www.cbs.dtu.dk/services/NetPhos/] was used. The NetPhos2.0 is also a neural network-based program designed by training the neural networks through protein phosphorylation data from phosphobase 2.0.

Prediction methods described above are neural network-based and are designed by memorizing the known sequence environment data of glycosylated/phosphorylated serine/threonine and non-glycosylated/non-phosphorylated serine/threonine. A jury of networks is used to evaluate the performance of neural networks. The results obtained from all the networks are sigmoidally arranged and averaged to obtain a value between zero and one by these prediction methods. Usually a threshold of 0.5 is used for prediction, which means that a site with an output of more than 0.5 is assigned as having a potential to be glycosylated or phosphorylated.

YinOYang 1.2 employs a jury of neural networks on 40 experimentally determined *O*-β-GlcNAc acceptor sites for recognizing the sequence context and surface accessibility. The number of non-acceptor serine/threonine residues was reduced from 1,251 to 626. The method [Gupta et al., http://www.cbs.dtu.dk/services/YinOYang/] is efficient in a cross-validation test as it correctly identifies 72.5% of the glycosylated sites and 79.5% of the non-glycosylated sites in the test set, verifying the Matthews [1975] correlation coefficient of 0.22 on the original data, and 0.84 on the augmented data set. The method has the capability to predict the Yin Yang sites that can be glycosylated and alternatively phosphorylated. NetPhos 2.0 predicts phosphorylation on the OH-function of serine, threonine, or tyrosine residues with a sensitivity range of 69%–96% [Blom et al., 1999].

RESULTS

O-Glycosylation Sites

The results for *O*-glycosylation sites were predicted by three methods including *NetOGlyc* 3.1 (for prediction of *O*-GalNAc sites), *DictyOGlyc* 1.1 (for prediction of *O*-α-GlcNAc sites), and YinOYang 1.2 (for prediction of *O*-β-GlcNAc sites).

NetOGlyc 3.1 predicts protein modification by *O*-GalNAc in mammalian proteins and has been developed by training the artificial neural networks through the sequence context of glycosylated and non-glycosylated serines and threo-nines. The results obtained for the prediction of *O*-GalNAc sites in the three subunits (α_{IIb}, α_v, and β_3) of platelet integrins showed that both α_{IIb} and α_v, as well as the β_3 subunit had no appreciable potential in their cytoplasmic domains for GalNAc addition.

The prediction results obtained by *DictyOGlyc* 1.1 for *O*-α-GlcNAc sites in human platelet integrins showed that there were three potential sites for *O*-α-GlcNAc modification in the α_{IIb} subunit at Ser 77, 876, and 878, whereas no potential site was predicted for threonine of the α_{IIb} subunit. The α_v subunit had been predicted to have the potential for *O*-α-GlcNAc modifica-

tion at Ser1046 in its cytoplasmic domain and β_3 subunit had no potential serine and threonine residue for *O*-GlcNAc modification.

The prediction results obtained for *O*-β-GlcNAc sites by YinOYang 1.2 showed that the number of potential *O*-β-GlcNAc sites among all three types of *O*-linked modifications was highest (Fig. 1). The α_{IIb} subunit is predicted to have a total of 14 potential sites for *O*-β-GlcNAc modifications at Ser; 130*, 168*, 248, 249, 503, 514*, 876, 964, and at Thr; 73, 327, 444, 607, and 646, with three Yin Yang sites marked by an asterisk. The α_v subunit had been predicted to have six potential sites for *O*-β-GlcNAc modifcation at Ser; 271, 429, 546, 757, 778* and at Thr; 602, with only one Yin Yang site marked by an asterisk. The β_3 subunit was found to have a total of 13 potential sites for *O*-β-GlcNAc modifications at Ser; 46, 85, 96*, 100, 104*, 110*, 123, 188*, 471, 699, and Thr; 33, 107, 276 with 4 Yin Yang sites marked by an asterisk. Apart from all the above predicted sites, there were a number of other Ser and Thr residues that were very close to the threshold and were likely to be modified by *O*-β-GlcNAc possibly resulting in Yin Yang sites which were actually false negative Yin Yang sites. For example, in the β_3 subunit Ser752 and Thr758 were very close to the threshold and likely to be *O*-β-GlcNAc modified. Thus, Ser752 and Thr758 in the cytoplasmic domain of β_3 have potential for phosphorylation and could act as possible Yin Yang sites (Fig. 1). Multiple sequence alignment results also showed that Ser752 and Thr758 were conserved throughout mammals and other vertebrates (Fig. 2).

N-Glycosylation Sites

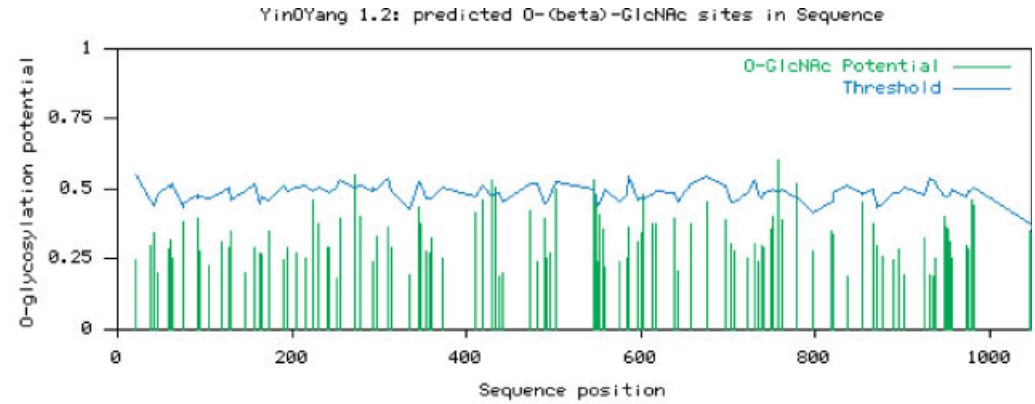
Prediction of N-glycosylation sites by *NetNGlyc 1.0* showed that integrin has more potential for *N*-linked glycosylation as compared to *O*-linked glycosylation. The α subunit has a total of 37 Asn out of 1,039 amino acids. Of these 37 Asn, 5 lie within the sequon Asn-Xaa-Ser/Thr. The 18 other Asn residues, which do not lie within the sequon N-Xaa-S/T could also be modified by *N*-linked sugars. The α_v subunit consists of 1,048 residues comprising 50 Asn. A total of 13 Asn out of 50 were found within the sequon Asn-Xaa-Ser/Thr, and 9 of these were predicted for *N*-glycosylation. The β subunit is composed of 788 amino acids with a total 34 Asn. The Asn residues that are part of the sequon Asn-Xaa-Ser/Thr are only seven and three of them had potential to be glycosylated (Asn 346, 478, and 680), while the other four Asn (125, 397, 585, and 782) showed a negative score for modification.

Phosphorylation Sites

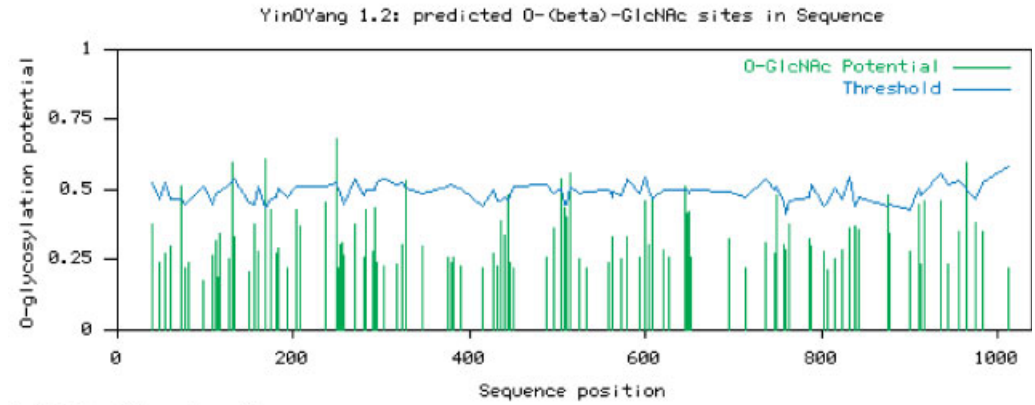
Utilizing *NetPhos* 2.0, *O*-linked phosphorylation at serine and threonine was predicted. In the α_{IIb} subunit, there are 75 (7.21% of total amino acids) serine, 37 (3.56% of total amino acids) threonine, and 28 (2.69%) tyrosine residues (Fig. 2). Out of these 75 serine residues, 21 (26.25%) were found to be potentially phosphorylatable, 4 (10.81%) threonine, and 10 (35.71%) tyrosine residues also showed potential for phosphorylation. The α_v subunit consists of 73 (6.96%) serine, 49 (4.6%) threonine, and 37 (3.53%) tyrosine residues. Of the 73 total serine residues, 25 (34.24%) were predicted to have potential for phosphorylation. Similarly, 6 (12.24%) of the total of 49 threonine residues, and 9 (24.32%) of 37 tyrosine residues had potential for phosphate modification. The β_3 subunit of human platelet integrin possesses 63 (7.99%) serine, 47 (5.96%) threonine, and 27 (3.42%) tyrosine residues. There were 23 (36.50%) serine, 9 (19.10%) threonine, and 11 (40.74%) tyrosine residues with potential to be phosphorylated (Fig. 2). Some other residues were found very close to the threshold level, as shown by the horizontal threshold line in Figure 3 that were likely to be modified by phosphate and appeared to be false negative phosphorylation sites.

DISCUSSION

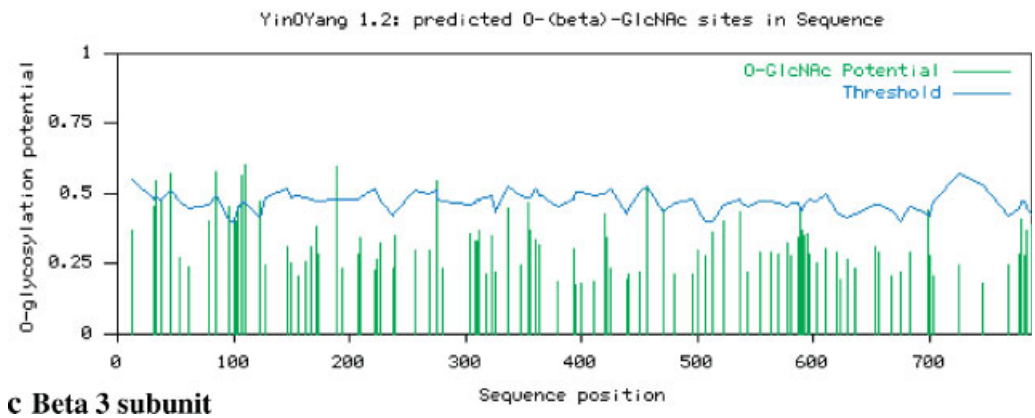
Protein chains are subjected to a variety of modifications, of which some are co-translational (such as *N*-linked glycosylation). Important amongst the post-translational modifications is the folding of the protein chain in its distinctive three-dimensional structure. Proteins are also subjected to other modifications, such as chemical modifications of specific amino acids, including glycosylation and phosphorylation. Such modifications significantly augment the language of proteins. Lastly, the highest level of protein organization occurs in the quaternary structure. The important aspect of protein organization is that each level of organization is dependent on the lower levels, and indeed the most important structural features are dictated by the primary structure



a Alpha V subunit



b Alpha IIb subunit



c Beta 3 subunit

Fig. 1. Graphic presentation of the potential of all Ser and Thr residues for *O*-GlcNAc modification in three subunits of the beta-3 family of integrins (**a**), alphaV; (**b**), alpha IIb and (**c**), beta-3. There are residues other than those crossing the threshold, very close to the threshold in the cytoplasmic domain of beta-3 (Ser752, Thr758) that also show potential for phosphorylation, possible Yin Yang sites.

of the protein. A similar relation exists between protein structure and function, and it can be argued that function of a particular protein is dictated by its unique three-dimensional structure, which is ultimately dictated by the sequence of the protein. Additionally, certain temporary conformational changes are induced by amino acid modifications, such as GlcNAc or phosphate substitution on the hydoxyl group of Ser, Thr, or Tyr, which often result in signals for regulating specific functions [Blystone et al., 1997; Liu et al., 2004; Ranganathan et al., 2004]. Indeed these amino acid modifications depend on the primary sequence, but they may also

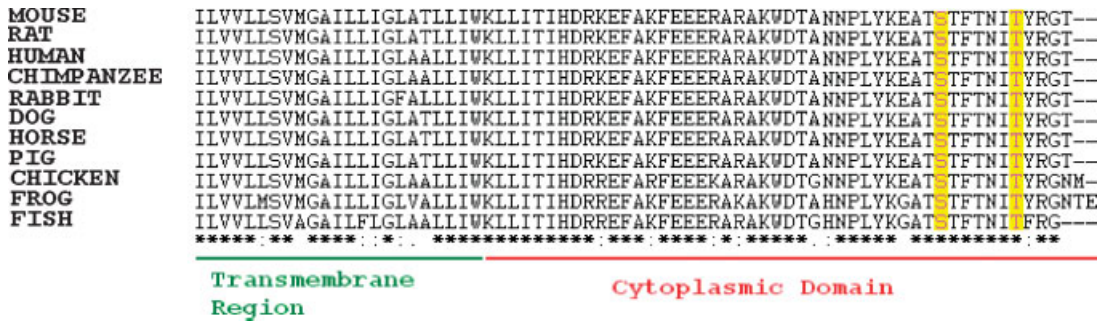


Fig. 2. Multiple sequence alignment by CLUSTALW of 11 sequences of integrin beta-3 chain. The alignment portion shown includes transmembrane region and cytoplasmic region. Ser752 and Thr758 are highlighted by yellow that are conserved throughout all major groups of vertebrates.

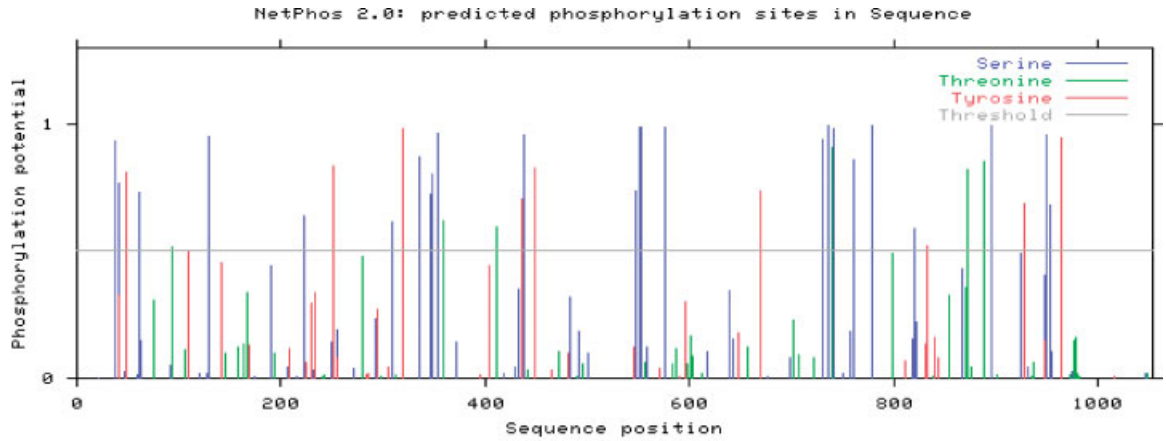
depend on the location of the amino acid in the 3-D structure. For example, a Ser having great potential for phosphate or GlcNAc modification may not be modified because of its location deep inside the 3-D structure.

The anionic group modification in proteins can result in conformational changes [Berlot et al., 2002], leading to the creation of active sites responsible for specific interactions with proteins [Zaia et al., 2001]. Similarly, the negative charge of a phosphate group or groups [Varki and Kornfeld, 1980; Kornfeld and Mellman, 1989] alters the balance of non-covalent interactions that determines secondary, tertiary, or even quaternary structures. In view of its capacity to alter the overall charge, phosphorylation of proteins can induce conformational changes in proteins [Yuan et al., 2003]. The change in conformation of the protein may result in altered biological function, resulting in either association or dissociation of subunits.

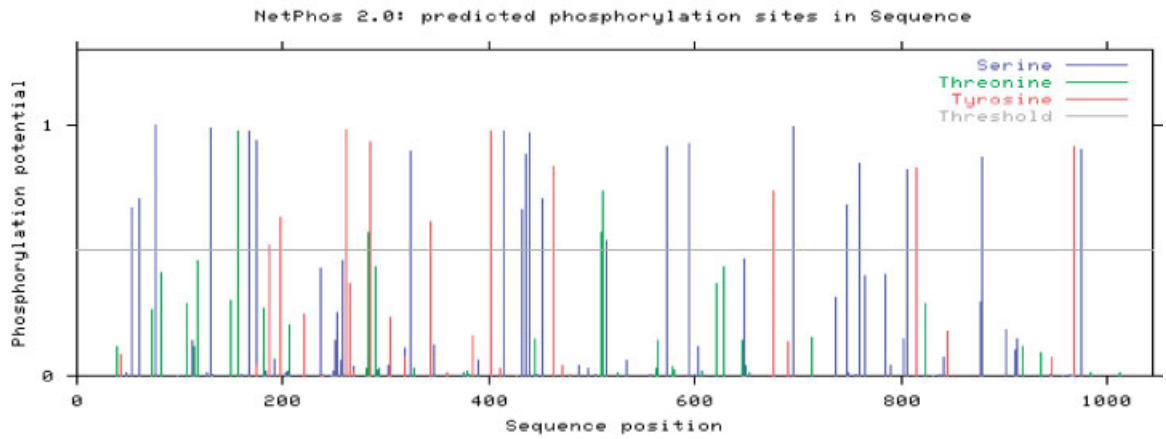
The *O*-GlcNAc modification is known to be dynamic and analogous to phosphorylation [Chou et al., 1992; Roquemore et al., 1996; Shafi et al., 2000] that is, *O*-GlcNAc is a regulatory modification just like protein phosphorylation. Kearse and Hart [1991] established that the changes in glycosylation (and phosphorylation) were transient, returning to their basal level in hours. The *O*-GlcNAc modification sites are similar to the phosphorylation sites [Kearse and Hart, 1991; Kelly et al., 1993; Chou et al., 1995; Medina et al., 1998; Cheng and Hart, 2000], and an interplay between glycosylation and phosphorylation is likely. The occurrence of this phenomenon has been investigated in some proteins, including RNA Pol II [Kelly et al., 1993], estrogen receptor-β_3, SV-40 large T-antigen [Medina et al., 1998], and c-Myc proto-oncogene [Chou et al., 1995]. This led to the formulation of the Yin-Yang hypothesis, according to which *O*-GlcNAc modifications and phosphorylations compete for the same site or region on a protein backbone. Possible roles of *O*-GlcNAc modification have been proposed [O'Donnell, 2002], including prevention of phosphorylation by occupying serine and threonine residues at the same position or in the same region, regulating protein function by blocking phosphorylation-dependent signaling and controlling protein–protein interactions, such as in the formation of functional protein complexes.

Phosphorylations of Tyr, Ser, and Thr in the cytoplasmic domains of integrins are important in the regulation of inside-out signaling [Kirk et al., 2000; Fagerholm et al., 2002; Han et al., 2003]. The highly conserved cytoplasmic domain of the β_3 chain (Fig. 2) in vertebrates stresses its functional importance in β_3 integrin family. Similarly, GlcNAc modification of Ser and Thr in the conserved region of integrin cytoplasmic domains may play equally significant roles. Thus, it becomes mandatory to investigate the possibility of *O*-GlcNAc-phosphate interplay in regulating β_3 integrin function.

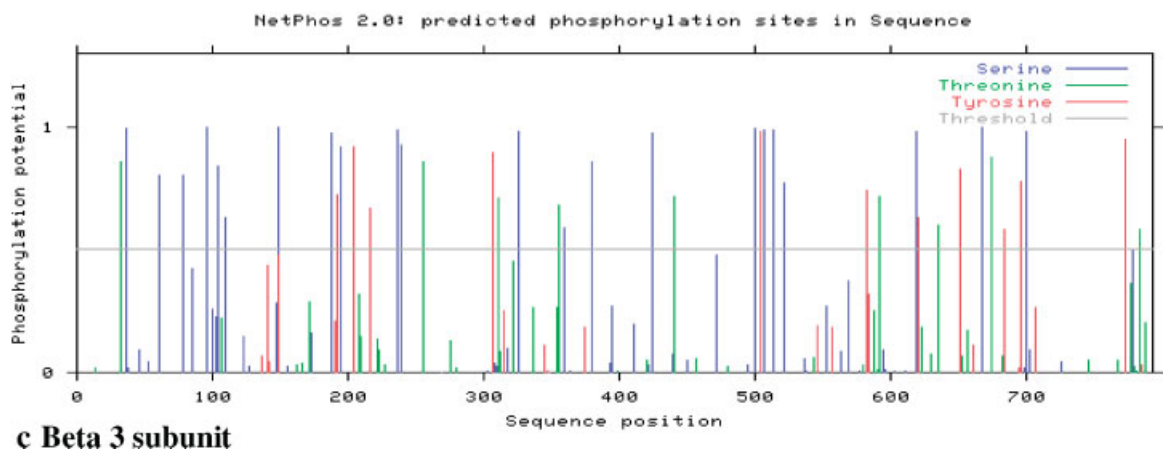
The actual binding of $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ to ligand or to RGD-containing peptides, or the addition of Mn^{2+}, induces a conformational change that increases the affinity for the binding of LIBS (ligand-induced binding site) antibodies to these β_3 integrins [Frelinger et al., 1991]. One consequence of this binding reaction could be the phosphorylation of the cytoplasmic domain of β_3, which is necessary for subsequent steps in the adhesion process [Blystone et al., 1996]. Binding of adhesion receptors to immobilized



a Alpha V subunit



b Alpha IIb subunit



c Beta 3 subunit

Fig. 3. Graphic presentation of the phosphorylation potential of Ser, Thr, and Tyr residues in three subunits of the integrin beta-3 family (**a**), alphaV; (**b**), alpha IIb; and (**c**), beta-3. The cytoplasmic domain region of beta-3 shows potential for Ser, Thr, and Tyr.

ligand is more complex to analyze, but has distinct biological and biochemical consequences that are not revealed by the soluble ligand analyses [Garcia et al., 1999]. The importance of the cytoskeletal connections to β_3 integrin cytoplasmic domains has been reinforced by mutational analysis of the β_3 cytoplasmic domain [Schaffner-Reckinger et al., 1998]. Of particular interest are transgenic knock-in experiments in which the tyrosines in

the cytoplasmic domain of β_3 were substituted with phenylalanines. This mutation did not affect the initial activation of $\alpha_{IIb}\beta_3$ but caused a rebleeding after wounding due to a defect in the clot structure [Law et al., 1999]. Thus, the cytoplasmic domain of β_3 integrin seems to be important for the strength of attachment of cells or platelets through the extracellular domain of β_3 integrin to surface-bound ligands.

Although integrin α and β cytoplasmic tails are devoid of catalytic activity, engagement of integrins by extracellular matrix ligands triggers outside-in signals that collaborate with growth factor-initiated signals to determine cell fate and function [Hynes, 2002]. A prominent biochemical event required for integrin-dependent functional responses is protein tyrosine phosphorylation due to activation of Src and FAK family protein tyrosine kinases [Hynes, 2002]. In the case of the β_3 integrins, $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$, a pool of c-Src co-immunoprecipitates with the integrin in non-adherent osteoclasts and platelets and becomes activated upon cell adhesion.

A familiar characteristic of several integrin β subunits (β_1, β_3, β_6, β_7) is the occurrence of conserved tyrosine residues in sequence contexts resembling that of known phospho-tyrosine binding (PTB) recognition sites, N*XXY* [Van der Geer et al., 1995]. These sequences are necessary for the proper functioning of $\alpha_{IIb}\beta_3$ integrin in platelets [Law et al., 1999] and HEL cells [Liu et al., 1996], and of $\alpha_v\beta_3$ integrin in K562 cells [Blystone et al., 1997]. The phosphorylation of β_3 on tyrosine in activated platelets [Law et al., 1996] and in $\alpha_v\beta_3$-transfected K562 cells [Blystone et al., 1996] correlates with the binding of these cells to specific ligands. Furthermore, it has been demonstrated that phosphorylation of Tyr-747 and Tyr-759 in β_3 integrins generates docking sites for signaling molecules. The presence of Tyr-747 is also necessary for other outside-in signaling events, including focal adhesion kinase and paxillin phosphorylation [Schaffner-Reckinger et al., 1998]. Expression of mutated forms of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in which the β_3 cytoplasmic tyrosine residues are replaced by phenylalanines blocks outside-in signaling during formation of stable platelet aggregates and clot retraction [Blystone et al., 1997].

It has also been reported that β_3 integrin is stoichiometrically phosphorylated on Thr-753 following treatment of platelets with calyculin A, a membrane-permeable inhibitor of protein serine/threonine phosphatases [Blystone et al., 1996]. Interestingly, this treatment also inhibits outside-in signaling events. The phosphorylation of β_3 on Thr-753 may inhibit outside-in signaling events by preventing the tyrosine kinases from phosphorylating β_3 or, alternately, by interfering with the binding of signaling molecules to tyrosyl-phosphorylated β_3. Similarly, Thr phosphorylation in leukocyte integrin (β_2) also plays an important role in its adhesive functions [Fagerholm et al., 2002].

The *O*-linked oligosaccharide chain in the cytoplasmic domain of α_{IIb} subunit of platelet integrin $\alpha_{IIb}\beta_3$ at Ser847 was shown to be involved in the expression of this protein. Similarly, *O*-GlcNAc modification of the cytoplasmic part of integral membrane proteins, such as ankyrin G was reported to regulate the signaling process. According to the prediction results, Thr758 and Thr762 are both very close to the threshold value and are likely to be modified by GlcNAc (Fig. 1). Similarly, Thr758 also shows potential for phosphorylation, whereas Thr762 shows very low potential for phosphorylation; so Thr758 adjacent to Tyr759 can block outside-in signaling events as described earlier for Thr753, which blocks phosphorylation of Tyr747, when itself phosphorylated. Furthermore, Thr758 adjacent to Tyr759 can act as a Yin Yang site for possible interplay of phosphate with GlcNAc (Fig. 4). We propose that interplay of phosphate with GlcNAc at Thr758 (a Yin Yang site) may regulate two-way signaling events during

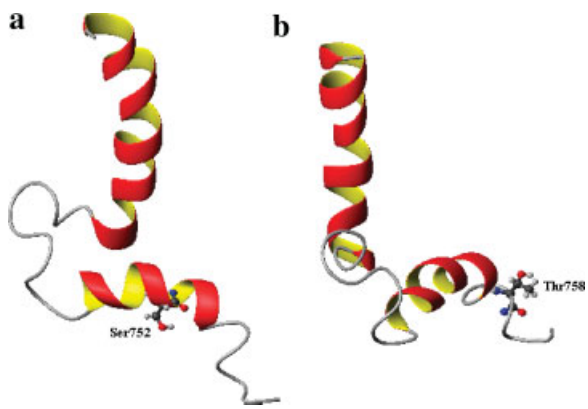


Fig. 4. Three dimensional structure of the cytoplasmic domain of beta-3 viewed and annotated on MolMol (molecular analysis and molecular display) software, representing Ser752 in (**a**) and Thr758 in (**b**) in ball and stick style. The hydroxyl groups of both the residues are easily surface accessible by kinases and OGT for acting as possible interplay sites in regulating the signaling function of integrins.

platelet aggregation and clot retraction. It has already been described that threonine phosphorylation in the cytoplasmic domain of β_3 in $\alpha_{IIb}\beta_3$ integrin results in different functional controls: while it enhances inside-out signaling following the exposure of fibrinogen/von Willebrand factor binding sites [van Willigen et al., 1996], it also inhibits outside-in integrin signaling linked to cell spreading and cytoskeletal rearrangements [Lerea et al., 1999]. Like Tyr759, Thr758 also lies in the NXXY motif, necessary for proper functioning of $\alpha_{IIb}\beta_3$. Thr758 may therefore regulate two-way signaling events through the interplay of GlcNAc and phosphate on this residue. When cytoplasmic Thr758 is GlcNAc-modified, then outside-in signaling in response to ligand binding is favored, leading to phosphorylation of Tyr759 or Tyr747 and clot retraction. But when Thr758 is phosphorylated, outside-in signaling and phosphorylation of Tyr759 or Tyr747 are inhibited.

Recently, a model for Src kinase activation by direct interaction with the β_3 cytoplasmic domain was proposed by Arias-Salgado et al. [2003]. These authors further demonstrated that conversion of Ser752 to phenylalanine, a mutation that prevents β_3-mediated outside-in signaling in platelets and osteoclasts [Chen et al., 1994; Feng et al., 2001], completely abolishes Src tyrosine interaction with the integrin β_3-tail [Arias-Salgado et al., 2003], whereas substitution of tyrosines 747 and 759 with phenylalanines had no effect on the interaction of $\alpha_{IIb}\beta_3$ with c-Src [Arias-Salgado et al., 2003]. These findings suggest an important role of Ser752 in Src-kinase activation in $\alpha_{IIb}\beta_3$-mediated adhesion processes. According to our prediction results, Ser752 shows more potential for phosphorylation than the threshold and is very likely to be modified by phosphate (Fig. 3). Similarly, Ser752 also shows potential for GlcNAc modification very close to the threshold (Fig. 1). This residue can thus act as a possible interplay site for phosphate and GlcNAc in controlling Src kinase activation and inactivation in the β_3 cytoplasmic tails of $\alpha_{IIb}\beta_3$ (Fig. 4).

The $\alpha_v\beta_3$ integrin was previously considered a promiscuous receptor involved in the adhesion of many cell types to the extracellular matrix and to other cells. But $\alpha_v\beta_3$ signaling may be different for different ligands resulting in unique cellular processes [Boettiger et al., 2001]. For example, $\alpha_v\beta_3$-mediated adhesion to vitronectin requires tyrosine phosphorylation at Tyr747, Tyr759 within the β_3 cytoplasmic tail and is dependent upon protein kinase C (PKC, also known as Ser/Thr kinase) activation, whereas $\alpha_v\beta_3$-mediated adhesion to fibronectin is constitutive, requiring neither of these events [Boettiger et al., 2001].

During normal flow, hematopoietic cells are non-adhesive but when they encounter pro-inflammatory or thrombotic signals, they exit from the circulation by means of firm adhesion to the vascular walls and migrate to the site of inflammation. The capacity of hematopoietic cells to control their adhesion-dependent extravasation is mediated partially by the integrin $\alpha_v\beta_3$ [Weerasinghe et al., 1998]. Firm adhesion of vitronectin to integrin $\alpha_v\beta_3$ involves phosphorylation at Tyr747 and Tyr759, and activation of PKC, which may be followed by phosphorylation of Ser752, Thr753, or Thr758 and regulation of inside-out signaling events. Prediction results show that Ser752 and Thr758 can act as possible false negative Yin Yang sites. Interplay of GlcNAc and phosphate at these sites may also regulate signaling events antagonistically (Fig. 4). Surface contact and solvent surface area calculations by MolMol [Koradi et al., 1996] also showed that the hydroxyl groups of Ser752 and Thr758 (Fig. 4) are surface accessible. Kinases and GlcNAc transferase (OGT) can access and modify Ser752 and Thr758, which are conserved in mammals and other vertebrates (Fig. 2). Although other Ser and Thr residues in the cytoplasmic domain of β_3 chain are conserved, they show very low potential for both modifications. On the other hand, Ser752 and Thr758 conserved residues in mammals and other vertebrates (Fig. 2), appeared to be false negative Yin Yang sites. Both these residues show a positive phosphorylation and *O*-GlcNAc modification potential very close to the threshold and are therefore unlikely to be false negative sites.

We, therefore, propose that GlcNAc and phosphate modifications at Ser and Thr in the cytoplasmic domain of β_3 subunits of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins control two-way signal transduction events. The Ser752 and Thr758 of β_3 are possible interplay sites for GlcNAc and phosphate modification, which we propose to control both outside-in and inside-out signaling events (Fig. 4). These two modifications may, therefore, control signaling events, antagonistically blocking outside-in signaling events, and promoting

inside-out signaling events and vice versa, as in platelet aggregation during clot formation and clot retraction. For instance, phosphorylation of Thr758 may block the outside-in signals triggered by ligand binding in the extracellular part of $\alpha_{IIb}\beta_3$ integrin that activate Src and FAK protein tyrosine kinase to phosphorylate Tyr747, and/or Tyr759 and prevent platelet aggregation. Reciprocally, *O*-GlcNAc modification of Thr758 would prevent its phosphorylation, leaving outside-in signals unperturbed and promoting platelet aggregation. Similarly, interplay of GlcNAc and phosphate at Ser752 could regulate Src kinase activation and inactivation antagonistically. We expect these prediction studies to accelerate our understanding of the roles of co- and post-translational modifications in integrins as well as in proteins in general, and hopefully lead to novel therapeutic approaches for treatment of human diseases.

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