RUNX1 Transcription Repression and Stability: Interplay Between Phosphorylation and O-GlcNAc Modification

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Abstract:- RUNX1 is a transcription factor that plays major role in hematopoiesis. RUNX1 activation is dependent on phosphorylation in its transactivation domain. Ser 249 and 276 are the potential sites for phosphorylation that lead to transcriptionally active RUNX1 which has transient action and is prone to proteasome mediated degradation. We propose that Ser 249 and 276 have the highest potential for O -GlcNAc modification, and are the Yin Yang sites (potential for both phosphorylation and O-GlcNAc modifications). In contrast to phosphorylation modification, O-glycosylation modification of RUNX1 leads to its transcriptional repression. This novel O-GlcNAc modification on RUNX1 provides a mechanism for regulation of transcription activity and stability of RUNX1. OGT mediated O-GlcNAc modified RUNX1 is more stable and is resistant to proteasome mediated degradation.

Keywords: Transcription repression, post-translational modifications, phosphorylation, O-GlcNAc modification, Yin Yang sites.

INTRODUCTION

RUNX proteins are transcription factors that bind to specific regulatory sequences of DNA, in various promoters and enhancers and are involved in regulation of gene expression (Otto et al., 2003; Licht, 2001). In mammals, the RUNX family consists of RUNX1/AML1, RUNX2 and RUNX3 (van Wijnen et al., 2004). Each of these three RUNX proteins 1, 2 and 3 are known to heterodimerize with non-DNA binding protein CBFβ subunit to control gene regulation. Being transcriptional regulators, these proteins play important roles in normal development and neoplasia (Lund and van Lohuizen, 2002; Coffman, 2003; Ito, 2004). RUNX1 is involved in hematopoiesis and is associated with acute human leukemias due to chromosomal gene translocations (Otto et al., 2003). RUNX2 is involved in osteogenesis and is associated with human autosomal bone disorder cleidocranial dysplasia.
five amino acid motif is present at C-terminus (VWRPY) of every RUNT protein which is known to bind transcriptional repressors like Groucho/TLE/R-esp proteins (Imai et al., 1998; Javed et al., 2000).

Post translational modifications (PTMs) are often involved in regulating the functions of proteins like transcription factors. Nearly all transcription factors are post-translationally modified either by phosphorylation, glycosylation, acetylation, methylation, or a combination of these modifications. These PTMs regulate functions of transcription factors including subcellular localization, stability, interactions with cofactors, and transcriptional activities (Tootle and Rebay, 2005). RUNX1 is also post-translationally modified by phosphorylation, acetylation, methylation, and ubiquitination (Zhang et al., 2004; Tanaka et al., 1996; Zhao et al., 2004; Huang et al., 2001). Extracellular signal regulated kinase (ERK), after its stimulation by interleukin 3 and epidermal growth factor, phosphorylates RUNX1 protein at Ser 249 and 266, located in PST region, leading to its transactivation (Tanaka et al., 1996). Similarly, paramethoxyamphetamines (PMA) also induce activation of ERK and phosphorylates RUNX1 at four serine residues 249, 266, 276, and Thr 273 (Zhang et al., 2004). Mutation of these four serine residues to alanine decreases transcription activity while their substitution to aspartic acid increases transcriptional activity (Ito, 1999; Kanno et al., 1998). The mSin3A, a corepressor of RUNX1 binds to dephosphorylated form of RUNX1 and results in suppression of transcription. Phosphorylation of RUNX1 results in dissociation of mSin3A and hence upregulation of transcription (Imai et al., 2004). Two other important PTMs of RUNX1 are acetylation and methylation of lysine and arginine residues. RUNX1 undergoes acetylation on two lysine residues at positions 24 and 43 that augments the DNA binding activity of RUNX1 (Yamaguchi et al., 2004). Three arginine methylation site are known to be present in RUNX1, one in the Runt domain, and two in the carboxy-terminal region. One of these sites is in the region shown to interact with both the mSin3A and with p300 (Zhao et al., 2004).

Many transcription factors as well as other cellular proteins are directly modified by O- β-GlcNAc at Ser/Thr residues and affect their activity (Bowe et al., 2006). Competition of O-β-GlcNAc modification and phosphorylation on the same Ser/Thr residues has been observed in many nuclear proteins. Phosphorylation on serine or threonine residues of a protein can be blocked by the addition of O-β-GlcNAc on these residues (Kearse and Hart, 1991). This mechanism is named as Yin Yang regulation and the sites as Yin Yang sites. Transcription repression activity mediated by corepressors (Sin3A) is mostly because of their interaction with histone deacetylases (HDACs) that reduces DNA availability (Laherty et al., 1998). Addition or removal of O-GlcNAc on proteins has been shown to function in parallel with HDACs to repress transcription. Computer-assisted program, YinOYang 1.2 can predict potential sites in a protein on which phosphorylation and O-glycosylation compete with one another for the same site.

In RUNX1, O-glycosylation has not yet been reported. In this study, we describe potential sites of RUNX1 for O-glycosylation, phosphorylation and their interplay with possible role in RUNX1 transcriptional activity and its stability. These sites have been predicted using different computational methods to describe the PTMs at phylogenetically conserved sites that may regulate key functions of RUNX1 during gene transcription.

MATERIALS AND METHODS

To predict phosphorylation and glycosylation potentials and interplay of these two modifications, in regulation of RUNX1 activity during transcription, sequence of RUNX1 protein in mouse was retrieved from the SWISSPROT database with accession number Q03347. BLAST search was carried out by NCBI database of non-redundant sequences with all default parameters (Altschul et al., 1997). Eight mammalian sequences, including Pan paniscus (ABM54418.1), Macaca nemestrina (ABM88136.1), Takifugu rubripes (AAU14191.1), Gorilla gorilla (ABM46824.1), Danio rerio (571678.1), Xenopus laevis (AAC41269.1), Rattus norvegicus (059021.1), and mouse Mus musculus
sequences chosen from BLAST search are summarized in Table I with their respective E values and Bits score. Multiple sequence alignments were carried out using ClustalW with all default parameters (Thompson et al., 1994).

The potential for phosphorylation and O-β-GlcNAc modification in human RUNX1 was predicted by Netphos 2.0 (Blom et al., 1999) (http://www.cbs.dtu.dk/services/NetPhos/) and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) (unpublished), respectively.

NetPhos 2.0 is a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences. This method predicts phosphorylation sites in independent sequences with sensitivity range from 69% to 96%. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential for phosphorylation. The YinOYang 1.2 server produces neural network predictions for O-β-GlcNAc attachment sites in eukaryotic protein sequences. The YinOYang 1.2 can also predict the Yin Yang sites by predicting the phosphorylation potential utilizing Netphos 2.0. The threshold value used by YinOYang 1.2 is varying depending upon surface accessibility of the different amino acid residues.

RESULTS

In this study potential phosphorylation and O-β-GlcNAc modification sites have been predicted on phylogenetically conserved Ser/Thr of RUNX1.

NetPhos 2.0 prediction results showed that there were 30 Serine, 4 Threonine, and 3 Tyrosine, with a total of 37 possible phosphorylation potential sites (Fig. 1) (Table III). Among these 37 potential sites, Ser 8, 10, 212, 249, 253, 276, 304, 430 and Thr 9, 219, have high phosphorylation potential of 0.925, 0.974, 0.992, 0.978, 0.626, 0.978, 0.934, 0.771, 0.707, and Thr 0.933, respectively.

Table II shows the respective serine, threonine and tyrosine residues in other mammalian sequences that have been predicted for their potential Yin Yang sites. From Tables I and II, it is clear that conservation status of these Yin Yang sites is higher in case of human, and Rattus norvegicus.

DISCUSSION
RUNX1 is involved in regulation of large number of genes like IgA1, BLK, IL-3, Granzyme B, CD3, M-CSF receptor, etc (Otto et al., 2003).

Table II.- Prediction results of RUNX1 of mouse aligned with other species.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Accession number</th>
<th>Predicted sites for phosphorylation</th>
<th>Predicted sites for O-GlcNAc modification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serine</td>
<td>Threonine</td>
</tr>
<tr>
<td><em>Danio rerio</em></td>
<td>NP_571678.1</td>
<td>25, 55, 72, 145, 150, 200, 211, 246, 250, 273, 303, 434, 436, 438</td>
<td>18</td>
</tr>
<tr>
<td><em>Macaca nemestrina</em></td>
<td>ABM88136.1</td>
<td>8, 10, 21, 46, 50, 67, 140, 145</td>
<td>9, 14</td>
</tr>
<tr>
<td><em>Gorilla gorilla</em></td>
<td>ABM46824.1</td>
<td>2, 48, 50, 52, 56, 69, 82, 86, 106, 110, 125, 133, 138, 144, 152, 171, 203, 205, 288, 290, 292</td>
<td>76</td>
</tr>
</tbody>
</table>
Table III.- Potential Yin Yang Sites.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Position</th>
<th>Context</th>
<th>Phosphorylation score</th>
<th>O-GlcNAc score</th>
<th>Yin Yang Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>8</td>
<td>PVDA*TTSRR</td>
<td>0.925</td>
<td>0.4926</td>
<td>Yes</td>
</tr>
<tr>
<td>T</td>
<td>9</td>
<td>VDAS*TSRRF</td>
<td>0.933</td>
<td>0.5219</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>10</td>
<td>DAS*TSRFT</td>
<td>0.974</td>
<td>0.4883</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>212</td>
<td>AMRV*SEPHEP</td>
<td>0.992</td>
<td>0.5167</td>
<td>Yes</td>
</tr>
<tr>
<td>T</td>
<td>219</td>
<td>HPAP*FEFNR</td>
<td>0.881</td>
<td>0.5030</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>249</td>
<td>QIQP*SEPWS</td>
<td>0.978</td>
<td>0.5886</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>253</td>
<td>SPP*SYDQS</td>
<td>0.626</td>
<td>0.5911</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>276</td>
<td>ATP*SGRA</td>
<td>0.978</td>
<td>0.5974</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>304</td>
<td>LTAF*DFRQ</td>
<td>0.771</td>
<td>0.4473</td>
<td>Yes</td>
</tr>
<tr>
<td>S</td>
<td>431</td>
<td>EAEG*HSNS</td>
<td>0.707</td>
<td>0.4288</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Fig. 1. Phosphorylation potential at different serine (Ser), threonine (Thr) and tyrosine (Tyr) residues of RUNX1. Blue vertical lines show the potential for Ser, green lines show the potential for Thr and red line shows potential for Tyr. Horizontal grey line represent the threshold value of 0.5 bits for phosphorylation.

tightly regulated because of its role in differentiation status and on the ontogenic stage of cell (Liu et al., 2006). Mitogen-activated protein kinase (MAPK) pathway is involved in transcriptional activity of RUNX1 by mediating its phosphorylation. Previously known experimental data has shown that Ser 249, 266, 276 and Thr 273 present in PST regions (AD) of RUNX1 are known to be phosphorylated by ERK pathway (Tanaka et al., 1996; Zhang et al., 2004). Another study showed that AML1 protein levels (and activity) are regulated during cell cycle progression. Phosphorylated AML1 at positions Ser 276, 293, 303 and Thr 300 is targeted for degradation by the APC-Cdc20 complex at early M phase, during the late M and G1 phases, APC-Cdh1 complex degrades AML1 independent of phosphorylation status and during re-entry of the cells into S phase, the SCF-
Skp2 complex slightly degrades phosphorylated AML1 (Biggs et al., 2006). Similarly mutations of these four phosphorylation sites necessary for transcriptional regulation can lead to increased ubiquitinated, matrix bound AML1c (a spliced form of RUNX1), increasing half-life of the matrix-

![Graph showing potential sites where interplay between phosphorylation and O-GlcNac modification can occurs.](image)

Fig. 2. The graph showing potential sites where interplay between phosphorylation and O-GlcNac modification can occurs. Horizontal blue wavy line shows threshold value with change in surface accessibility of the different amino acid residues.

![Multiple alignment of sequences selected for finding conservation status of predicted Ser 249 and 276 in different mammalian sequences. These two Yin Yang sites (in red color) are conserved in all mammals.](image)

Fig. 3. Multiple alignment of sequences selected for finding conservation status of predicted Ser 249 and 276 in different mammalian sequences. These two Yin Yang sites (in red color) are conserved in all mammals.

associated AML1c, showing that phosphorylation of AML1c on specific serine/threonine residues not only controls transcriptional activity but also regulate rate of degradation (Biggs et al., 2005).

Among these predicted sites, there were 11 such residues that were predicted to come into Yin Yang sites, that is, they were having potential for both phosphorylation and glycosylation (Tables II and III). Ser 249, and 276, falling above threshold value were chosen to be important residues for phosphorylation modification with the background of experimental data available. In our results Ser
266 has been predicted false negatively with a potential of 0.063 and in fact it is an experimentally proved site for phosphorylation (Tanaka et al., 1996).

For $\textit{O-}\beta\textit{-GlcNAc}$ modification and phosphorylation interplay sites as in Table III and Figure 3 Ser 249 and 276, (glycosylation potential of 0.5610 and 0.6058, and by Netphos potential of 0.978 and 0.978, respectively), were selected as important residues where modification could play role in transactivation and stability of RUNX1.

Ser 249 and Ser 276 both are phylogenetically conserved in mammals with the exception of \textit{Macaca nemestrina} (Fig. 4) and are present in transactivation domain (PST rich region) of RUNX1. Previous experimental data showed that phosphorylation in PST region by ERK leads to activation of RUNX1, as co-repressor mSin3A dissociates (Imai et al., 2004). The mSin3A-interaction domain of RUNX1 has been mapped to the region just C-terminal to the Runt domain. Since deletion of this region (aa 181–210) impaired the association between the two proteins (Lutterbach et al., 2000). Deletion of region 248–287, containing ERK phosphorylation sites Ser 249, 266, 276 and Thr 273 increases the interaction of RUNX1 with
mSin3A (Imai et al., 2004; Lutterbach et al., 2000). We propose that phosphorylation of Ser 249 and 276, the sites among other experimentally known phosphorylation sites having highest potential, may result in inducing temporary conformational changes that dissociate Sin3A leading to RUNX1 transcriptional activity. Phosphorylated RUNX1 has transient action that is less stable and is subjected to proteasome mediated degradation (Bae and Lee, 2006), suggesting that unphosphorylated RUNX1 with bound mSin3A is more stable and is resistant to proteasomal degradation.

The O-GlcNAc transferase (OGT) is ubiquitous regulator of transcription and is known to be involved both in repressing as well as inducing the transcription of genes by modifying transcription factors with O-GlcNAc. In gene repression the Ser/Thr phosphorylation sites in transactivation domains are blocked by O-β-GlcNAc by inhibiting their interactions with general transcription factors or coactivators (Yang et al., 2001). Many transcription factors that are post-translationally modified by O-β-GlcNAc glycosylation are known (Chou et al., 1995; Jackson and Tjian, 1988; Kelly et al., 1993; Reason et al., 1992). The first transcription factor found to contain this modification was Sp1 (Yang et al., 2001). Phosphorylated Sp1 is known to bind DNA (Jackson et al., 1990), and O-GlcNAcylation of Sp1 decreases its transcriptional activity in living cells, as it blocks its interactions with other Sp1 molecules and TATA-binding protein-associated factor II 110 (Yang et al., 2001). O-GlcNAc modification for RUNX1 is not known. Prediction results show that Ser 249 and 276 not only have the highest potential for phosphorylation as compared to other sites but are also important residues for glycosylation with potential of 0.5886 and 0.5974, respectively (Table III). This modification can lead to prediction of a novel mechanism for transcription repression activity of RUNX1.

RUNX1 repression is mediated by its interaction with mSin3A (Imai et al., 1998). It is known that mSin3A acts as a complex with HDAC to mediate transcription repression (Glass and Rosenfeld, 2000; Kouzarides, 1999). It is now experimentally evident that mSin3A can also repress transcription independently of deacetylation (Glass and Rosenfeld, 2000). This is because of its ability to interact with OGT. Since it has been proved that overexpression of OGT inhibits basal transcription from a minimal promoter (Yang et al., 2002). The OGT interacts with mSin3A via hydrophobic interactions between TPR motifs 1–6 of OGT and PAH4 domain of mSin3A (Yang et al., 2002). Moreover presence of hyperglycosylated proteins that are known to interact with mSin3A at the silenced promoter regions suggests that mSin3A recruits OGT to mediate transcriptional repression (Yang et al., 2002).

In cellular system, OGT and O-GlcNAcase act in combination with kinases and phosphatases generating different isoforms of physiological substrates. As a result of these modifications, the isoforms will differ in properties like protein–protein interactions, protein stability, and enzymatic activity (Hanover, 2001). Here we propose that mSin3A interaction with OGT provides an alternative way for transcription repression by RUNX1. The OGT catalyzes O-GlcNAc modification at Ser 249 and 276 leading to transcription repression.

Phosphorylated RUNX1 has transient action and is degraded by proteasome mediated degradation (Bae and Lee, 2006). Proteasomes are present in both cytoplasm and nucleus (Reits et al., 1997; Enenkel et al., 1998). Bigg et al. (2005) has proposed that AML1c, a spliced form of AML1, is degraded in the matrix and that phosphorylation plays important role in both proteasome mediated degradation and transcriptional activation. In another study, on AML1b, it was proposed that phosphorylation at serine 249 and 266 of AML1b leads to its dissociation from mSin3A making soluble AML1b to be available for both transcriptional activation and degradation (Imai et al., 2004). Here we propose that O-β-GlcNAc modification of RUNX1 on Ser 249 and 276 leads to its resistance to proteasome mediated degradation. It could be explained in context that O-β-GlcNAc modification blocks phosphorylation and thus proteasome degradation and RUNX1 may remains attached to mSin3A in glycosylated form. Thus the sites recognized by proteasome are occupied by O-β-GlcNAc making it resistant to degradation. It has also been well-established in
case of sp1 where hypoglycosylated Sp1 is more susceptible to degradation by proteasomes because of the change in conformation or the exposure of recognition or modification sites that are otherwise occupied by O-GlcNAc (Han and Kudlow, 1997). Another possibility for increased stability of RUNX1 by O-β-GlcNAc modification may be exemplified by the fact that mSin3A recruited OGT can mediate reversible O-β-GlcNAc modification of proteasomes, which in turn leads to the inhibition of its ATPase activity and consequently the proteasome cannot degrade Sp1 (Han and Kudlow, 1997; Zhang et al., 2003). Thus it is quite obvious that OGT attached to mSin3A/RUNX1 complex can also glycosylate proteasome to increase half life of RUNX1 making it resistant to degradation.

In this study, we have predicted that Ser 249 and 276 are the most important potential sites for dynamic modification in transactivation domain of RUNX1. Competition between glycosylation and phosphorylation on these residues may provide a novel mechanism for regulation of transcriptional activity and stability of RUNX1 within nucleus. The O-β-GlcNAc modification at Ser 249 and 276 of RUNX1 may result in blocking phosphorylation, hence inhibiting trans-activation of RUNX1. This blocking of phosphorylation is proposed to be related with the transcriptional repression and increased stability of protein, as mSin3A recruited OGT is also known to glycosylate proteasomes, thus inhibiting their ATPase activity.

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