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Regulation of Apoptosis by Histone H2B Modifications

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Abstract.- Histone 2B (H2B) is located along with histone 2A, 3 and 4 in the core of the nucleosome. The N-terminal tails of the histone protrude the chromatin structure and become accessible to various enzymes for post translational modifications (PTMs). Phosphorylation of H2B has been found to be associated with apoptosis in vertebrates. Another equally abundant PTM is the glycosylation at serine/threonine by *O*-GlcNAc (*O*-linked glycosylation) that occurs on the same or neighboring Ser or Thr residues, which also are accessible to kinases (Yin Yang sites). *O*-GlcNAc is removed by *O*-GlcNAcase (OGN), and is found exclusively in the nucleus or cytoplasm of the cell. By using computational methods like Netphos 2.0 and Yinoyang 1.2 we found that OGN, Mammalian 20 sterile kinase and OGN, Protein kinase C- delta, work together during apoptosis and thereby might prevent uncontrolled cellular growth.

Keywords: Human histone H2B, phosphorylation, *O*-linked glycosylation, apoptosis.

INTRODUCTION

The basic unit of chromatin, the nucleosome, contains an octamer of core histones (H2A, H2B, H3 and H4) around which two superhelical turns of DNA are wrapped. Each histone within the octamer consists of a structured domain (the histone fold) and non-structured positively charged N-terminal tails (Luger *et al*., 1997). The N-terminal domain of H2B is reported to lie outside the nucleosomes associated with linker DNA (Csordas, 1990).

The N-terminal tails of histones are subjected to a large number of post-translational modifications (PTMs), which influence chromatin structure to facilitate transcription, DNA replication and DNA repair. Especially phosphorylation of H3 on Ser 10 is widely studied, and has been found to play a role in induction of immediate early genes (Thomson *et al.*, 1999) and in chromatin compaction during mitosis (Kaleem *et al.*, 2006). Phosphorylation of H1, H2A and H3 occur during the cell cycle, while phosphorylation of H2B during the cell cycle is negligible (Ajiro, 2000). Phosphorylation of H2B on Ser 32 has been found to be associated with mammalian apoptotic cells, and is catalyzed by protein kinase C (PKC) (Ajiro, 2000). Furthermore only H2B is essential for chromatin condensation in *Xenopus* cell-free systems (de la Barre et al., 2001). These data are consistent with the idea that H2B phosphorylation may be important for apoptotic chromatin condensation.

Apoptosis or programmed cell death is a normal cell suicide mechanism. This regulated process serves to remove unwanted or deleterious cells such as self-reactive lymphocytes, tumor cells, or virus-infected cells. Formation of condensed apoptotic chromatin bodies, which differ from mitosis specific chromatin (Hendzel *et al.*, 1998) and digestion of DNA into oligonucleosomal fragments are hallmarks for apoptosis. The major executers of apoptosis are proteases called caspases (Cryns and Yang, 1998). Caspase-3 has been found to cleave the mammalian sterile twenty kinase 1 (Mst-1) (Cheung *et al.*, 2003). The cleaved Mst-1 is translocated into the nucleus, where it is able to phosphorylate histone H2B at Ser-14 *in vitro* and *in vivo* (Cheung *et al.*, 2003). Phosphorylation of Ser 14 leads to chromatin condensation and apoptosis (De Souza and Lindsay, 2004). Caspase-3 is also reported to cleave histone deacetylase 4 (HDAC4) and promote the nuclear localization of the HDAC4's N-terminal fragment, where it acts like a

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transcriptional repressor (Yang and Grégoire, 2005). The yeast histone deacetylase hda1 (related to mammalian HDAC4) is found to mediate deacetylation of histones H3 and H2B, but not H4 and H2A *in vivo* (Wu *et al.*, 2001).

Different PTMs have been reported to regulate the function of histone proteins, and a combination of these on one or multiple histone tails serve a specific code, the histone code. The code is executed by histone modifying enzymes of defined specificity and read by non-histone proteins to carry out their function (Turner, 2002). Phosphorylation of histone proteins is documented to play a vital role in relaxed and compact chromatin. *O*-GlcNAc (or *O*-β-GlcNAc) modification like phosphorylation acts as a modulator of the protein function. The addition of *O*-GlcNAc to the protein backbone by O-GlcNAc transferase (OGT) is dynamic, and has been found to act in a reciprocal manner with phosphorylation in the C-terminal of RNA-polymerase (Comer and Hart, 2001) amongst others, suggesting that *O*-GlcNAc and phosphorylation may modulate each other. Love and Hanover (2005) have suggested that OGT and *O*-GlcNAcase (OGN) may participate in chromatin remodeling by associating with HDAC and histone acetyltransferase, respectively. Moreover, *O*-GlcNAc plays a positive role in cellular survival (Zachara *et al.*, 2004), and OGN is rapidly cleaved by caspase-3 after apoptosis and remains active, suggesting that removal of *O*-GlcNAc is part of the apoptotic cascade (Wells *et al.*, 2002).

By using bioinformatics tools, we predict phosphorylations –,*O*-GlcNAc – and Yin Yang sites in H2B, and propose that phosphorylation of H2B on Ser 14 and 32 is associated with apoptosis, whereas *O*-GlcNAc modification of these residues, protect the cell from apoptosis and DNA fragmentation.

MATERIALS AND METHODS

The sequence data used to predict phosphorylation and glycosylation potentials of H2B protein in *Mus musculus* was retrieved from the Swiss Prot database (Boeckmann *et al.*, 2003) with the entry name H2B histone family, member 1B and primary accession no. Q64475. BLAST search was carried out by using NCBI database of non-redundant sequences using all default parameters. The NCBI Blast searches databases and finds regions of local similarity among the sequences of proteins or nucleotides, which can be used to elucidate evolutionary relationships (Altschul *et al.*, 1997). The searches were performed on known species and were divided into vertebrates and invertebrates (Table I).

Table 1: Selected species for prediction of evolutionary conserved residues in H2B

Vertebrates	
Q64475	*Mus musculus*
CAA15668.1	*Homo sapiens*
701196A	*Bos taurus*
NP_001026652	*Gallus gallus*
0506206A	*Rattus norvegicus*
P69069	*Oncorhynchus mykiss*
Q75VN4	*Rhacophorus schlegelii*
Invertebrates	
Q8I1N0	*Drosophila yakuba*
AAK58064	*Rhynchosciara americana*
P17271	*Drosophila hydei*
CAD37816	*Mytilus edulis*
P21897	*Chironomus thummi*
EAT45030	*Aedes aegypti*
Q27442	*Anopheles gambiae*

The sequences were multiply aligned using ClustalW (Thomson *et al.*, 1994) using all default parameters. ClustalW is a multiple sequence alignment program for DNA or proteins, and is used to characterize protein families, to distinguish between new sequences and existing sequences, to help predict the secondary and tertiary structures of new sequences and are essential in evolutionary analysis (Thompson *et al.*, 1994).

Prediction methods

For prediction of Yin Yang sites in *Mus musculus* H2B computational methods like Netphos 2.0 (Blom *et al.,* 1999) and YinOYang 1.2 (unpublished) were used. Yin Yang prediction sites are residues, where both kinase and OGT compete for the same site (Wells *et al.,* 2003). These methods are useful for assessing the modification potential (glycosylation and phosphorylation) of a given

protein. Residues, important for maintaining the 3D structure of a protein and/or its function(s), such as catalytic activity, binding to ligand, DNA or other proteins, have often been found to be highly conserved evolutionarily, and are of biological importance.

Netphos 2.0 is a neural network prediction method. Netphos 2.0 assesses the potential phosphorylation sites on Ser, Thr and Tyr, and is trained to recognize 9-11 amino acids around these phosphorylation sites (Blom *et al.,* 1999). It is developed by training the neural networks with phosphorylation data from the phosphobase 2.0, which comprise 414 phosphoprotein entries covering 1052 phosphorylatable Ser, Thr and Tyr residues (Kreegipuu *et al.,* 1998). Netphos 2.0 (http://wwwcbs.dtu.dk/services/NetPhos/) was used to predict phosphorylation potential in *Mus musculus* H2B. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential site for phosphorylation. All sites that cross the threshold value are considered to have positive potential for phosphorylation.

The YinOYang 1.2 server produces neural network predictions for O-ß-GlcNAc attachment sites in eukaryotic intracellular/nuclear protein sequences. This server uses Netphos to identify possible phosphorylation sites and thus identify "Yin-Yang" sites. YinOYang 1.2 employs the sequence data to train a jury of neural networks on 40 experimentally determined O-GlcNAc acceptor sites for recognizing the sequence context and surface accessibility. YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) was used to predict O-GlcNAc modification potential in *Mus musculus* H2B. Similar to Netphos 2.0 a threshold value is also used by YinOYang 1.2 which varies depending upon surface accessibility of different residues. The residues possessing higher potential than the threshold are determined as possible O-linked (O-ß-GlcNAc) glycosylation sites.

To determine the feasible interplay between phosphorylation and glycosylation, Yin Yang sites were determined in H2B. YinOYang 1.2 predicts potential Yin Yang sites in protein. False negative sites (sites, where the residue is positively predicted phosphorylation sites, fully evolutionarily conserved, but not predicted to be O-glycosylated even though the potential is very close to the threshold line) were also identified coupling conservation status and modification potential results of the two methods.

RESULTS AND DISCUSSION

We have predicted the potential phosphorylations-, *O*-glycosylations sites (Figs. 1, 2; Table II), and Yin Yang sites in *Mus musculus* H2B protein (Fig. 2, Table III). All the predicted sites were found to be fully conserved in vertebrates and invertebrates, except Ser 14 and 32, which were found to be conserved in vertebrates. Phosphorylation of Ser 14 and 32 are associated with apoptosis in mammals. Homology of apoptosis in vertebrates (mammals) and invertebrates (like *Drosophila*) is the presence of orthologs apoptotic component like the CAD family (regulators of DNA degradation) (Aravind *et al*., 2001). In unicellular species like yeast (*Saccharomyces cerevisiae*) phosphorylation of H2B occurs on Ser 10 during

Table II.- Predicted phosphorylations – and glycosylations sites of H2B in *Mus musculus*

Predicted phosphorylations sites	Ser 6, 14, 19, 32, 36, 38, 55, 91, 112, 123 Thr 88, 90, 96, 115 Tyr 37, 121
Predicted O-Glycosylations sites	Ser 4, 6, 32, 87, 112, 123, 124 Thr 52, 119, 122

Table III.- Conservation status of the positively predicted and false negative (in italic) Yin Yang sites of H2B in *mus musculus*

Conservation status		
Non-conserved substitution residue	**Conserved substitution residue**	**Conserved residue**
Ser 6		
		Ser 14[1]
	Ser 32[2]	
		Ser 55
		Thr 90
		Ser 112
	Ser 123	

[1]Ser 14 is fully conserved in vertebrates, but not conserved in inverterbrates

[2]Ser 32 is conserved in vertebrates, but not conserved in invertebrates

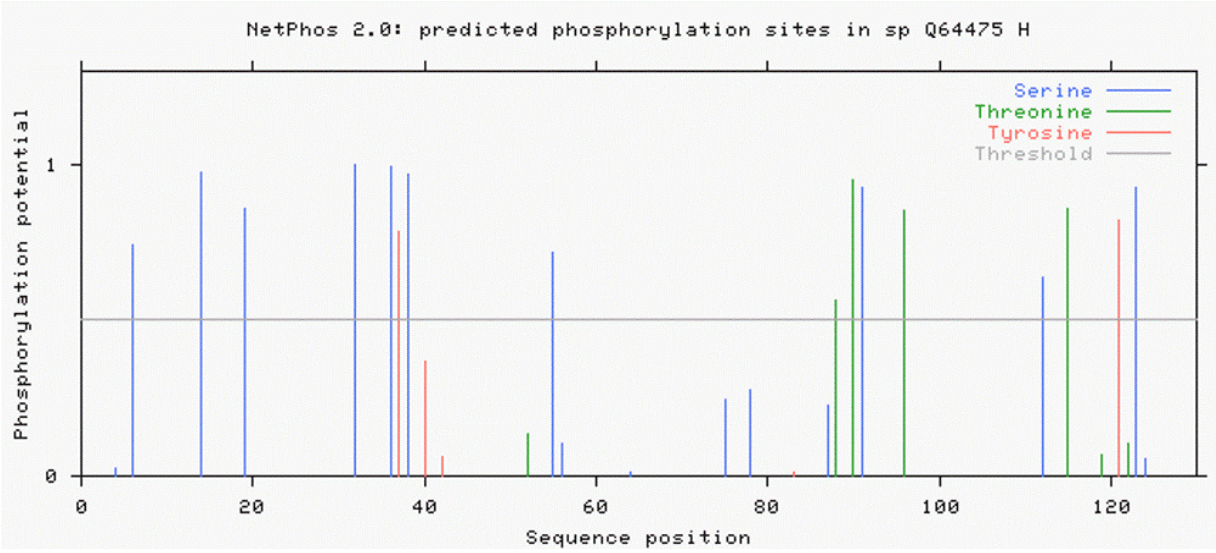

Fig. 1. Graphic representation of the potential phosphate modification on Ser and Thr residues in *Mus musculus* histone 2B. The blue vertical lines show the potential phosphorylated Ser residues; the green lines show the potential phosphorylated Thr residues; the red line show the potential phosphorylated Tyr residues. The light blue horizontal wavy line shows threshold for modification potential.

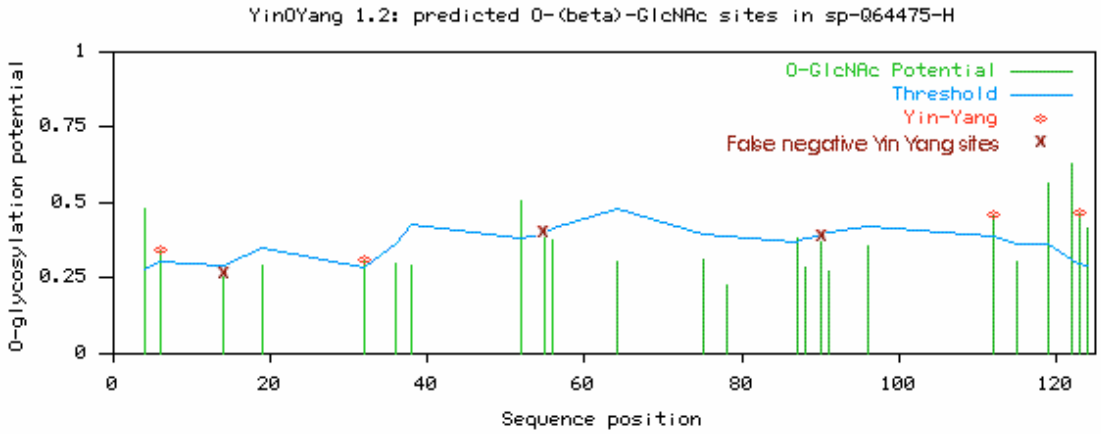

Fig. 2. Graphic representation of potential for *O*-GlcNAc modification of Ser and Thr residues in *Mus musculus* histone 2B. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and light blue horizontal wavy line shows threshold for modification potential. The red ○ shows potential yin-yang sites, where phosphorylation and glycosylation is predicted to occur in *Mus musculus* histone 2B, and the violet X shows the predicted false negative Yin Yang sites.

hydrogen peroxide induced apoptosis (Ahn *et al*., 2005). But the yeast Sterile 20 kinase (Ste20, mammalian homolog Mst-1) that phosphorylates Ser 10 of H2B is not dependent on caspase cleavage, indicating a difference between apoptosis in mammalian and yeast (Ahn *et al*., 2005).

The biochemical hallmark of apoptosis or programmed cell death is the cleavage of chromatin into nucleosomal fragments (Liu *et al*., 1997), but not all apoptotic pathways lead to DNA

fragmentation (Yuste *et al*., 2001). Activated Caspase-3 interacts with other cytosolic proteins to generate DNA fragmentation (Liu *et al.*, 1997). Caspases are activated by either DNA damaging agents, or via binding of death ligands like tumor necrosis factor (TNF) or FAS to their respectively death receptor, which eventually triggers the release of cytochrome c from the mitochondria into the cytosol, and thereby activates initiator caspases such as Caspases-9 (Cryns and Yuan, 1998). This caspase proteolytically activates downstream effector caspases like Caspase-3, which in turn kill cells by cleaving intracellular proteins (Cryns and Yuan, 1998). Mst-1 (De Souza and Lindsay, 2004) and PKC-δ (Anantharam *et al*., 2002) are amongst the several key cellular proteins, which are cleaved by Caspase-3 in the cytosol. Mst-1 is cleaved into a 36 kDa active fragment (Graves *et al.*, 1998), and is translocated into the nucleus before nuclear fragmentation is initiated (De Souza and Lindsay, 2004), where it phosphorylates nuclear proteins like H2B on Ser 14 (Cheung *et al*., 2003). Phosphorylation of H2B at Ser 14 during apoptosis occur immediate prior to DNA laddering, and might operate as an activator for DNA fragmentation during apoptosis (Cheung *et al*., 2003). Furthermore phosphorylation of H2B on Ser 14 has been documented to occur at radiation-induced DNA damage, but is dependent on the phosphorylated isoform of H2AX (Fernandez-Capetillo *et al*., 2004). Caspase-3 also cleaves OGN into a 65 kDa fragment representing the C-terminal portion of OGN (Wells *et al*., 2002). This cleavage does not impair OGN's activity *in vitro*, and the truncated OGN is able to catalyse the hydrolysis of *O*-GlcNAc from endogenous *O*-GlcNAc-modified proteins (Wells *et al*., 2002).

During apoptosis other sites of H2B becomes phosphorylated as well. Ser 32 has been identified being phosphorylated by protein kinase C (PKC) (Ajiro, 2000). Ser 32 is found in the inner globular region near the N-terminal border of H2B, where it is not easily accessible to kinases. During apoptosis the nucleosome opens and the histone proteins are released (Wu *et al*., 2002). When H2B is released it may become accessible to PKC in the cytoplasm, but no evidence was found to support whether Ser 32 of H2B is phosphorylated in the nuclei or in the cytoplasm. PKC-δ, a PKC isoform, has been documented to be cleaved by Caspase-3 during apoptosis (Anantharam *et al*., 2002). Proteolytic cleavage of PKC-δ by caspase-3 results in persistent activation of PKC- δ in the cytosol (Anantharam *et al*., 2002), and might be the possible kinase that phosphorylates H2B on Ser 32 (Ajiro, 2000). After release the histone proteins then migrate to the cell surface, where they may assist in the recognition and clearance of apoptotic remains (Radic *et al*., 2004).

The two apoptosis related residues (Ser 14 and 32) of H2B, are substituted by Gly and a basic amino acid in invertebrates, respectively (Fig. 3). Furthermore the two predicted Yin Yang sites are in vicinity with basic residues (Lys and Arg) (Fig. 3), whereas the other predicted Yin Yang sites did not show this feature (data not shown). This might be a requirement for recognition by apoptosis related kinases like Mst-1 and PKC-δ. This is in agreement with PKC's consensus sequence, which is rich in basic amino acids (Medler and Bruch, 1999).

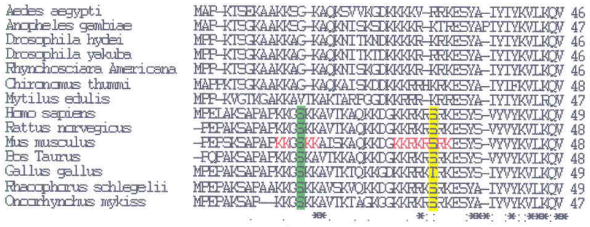

Fig. 3. Multiple alignments of selected sequences (Amino acid 1-48) of H2B in *vertebrates* and *invertebrates.* The consensus sequence is highlighted by asterisk, conserved substitution by double dot and semiconserved substitution by single dot. Different sequences are ordered as in aligned results from CLUSTALW. The positively predicted Yin yang sites are highlighted in yellow, and the negatively predicted Yin yang site is highlighted in green.

Expression of Mst-1, which is cleaved by Caspase-3 on its regulatory C-terminal thereby increasing its kinase activity, also acts as an activator of caspases, emphasizing its role in apoptosis (Ura *et al*., 2001). Furthermore Mst-1 is capable of activating the p38 Mitogen protein kinase and c-Jun N-terminal protein kinase (JNK) pathways as well and thereby induce apoptosis (Ura

et al., 2001). Guo and colleges (1998) reported that a prolonged activation of JNK induces apoptosis in Rat Mesangial Cells. The second kinase, which also is capable of phosphorylating H2B during apoptosis, is PKC-δ (Ajiro, 2000). PKC-δ's kinase activity is also amplified by its cleavage by Caspase-3 (Anantharam *et al*., 2002) and has also been found to activate the JNK pathway (Reyland *et al*., 1999). This suggest, that when apoptosis needs to take place (*e.g.* when a cell is damaged beyond repair, or infected with a virus), Caspase-3 proteolytically starts cleaving endogenous proteins. These protein kinases (Mst-1 and PKC-δ), which activity is increased after cleavage, are translocated into the nucleus, where they are able to phosphorylate H2B, to promote DNA fragmentation (Fig. 4).

In stress induced environment a rapid increase of *O*-GlcNAc modified nucleocytoplasmic proteins occur (Zachara *et al*., 2004). This modification protects the cell from apoptosis, whereas a reduction of *O*-GlcNAc modified proteins may lead to a reduced cellular survival (Zachara *et al*., 2004). This suggests that H2B might be *O*-GlcNAc modified on Ser 14 and 32 under normal or stress induced conditions. When apoptosis occurs, OGN is cleaved by Caspase-3, and catalyzes the removal of *O*-GlcNAc from H2B (and other nucleocytoplasmic proteins), thereby making H2B ready to become phosphorylated by Mst-1 and PKC-δ on Ser 14 and 32 respectively. The majority of OGN is present in the cytosol, but is also found in the nucleus at smaller levels (Wells *et al*., 2001). This means that OGN might be able to remove *O*-GlcNAc from H2B and other proteins in the nucleus. Another possibility is, when H2B becomes phosphorylated on Ser 14, DNA fragmentation begins, and H2B is released into the cytoplasm, where OGN removes *O*-GlcNAc from Ser 32, and subsequently becomes phosphorylated by truncated PKC-δ (Fig. 4).

Cells in apoptosis release nucleosomes into the cytoplasm and attach them to the outside of nuclear fragments (Radic *et al*., 2004). These nuclear fragments migrate to the cell surface, break through the plasma membrane. Nucleosomes become accessible for interactions with receptors, including B cell receptors (Mecheri *et al*., 1993), and binding to proteoglycans like Heparan sulfate

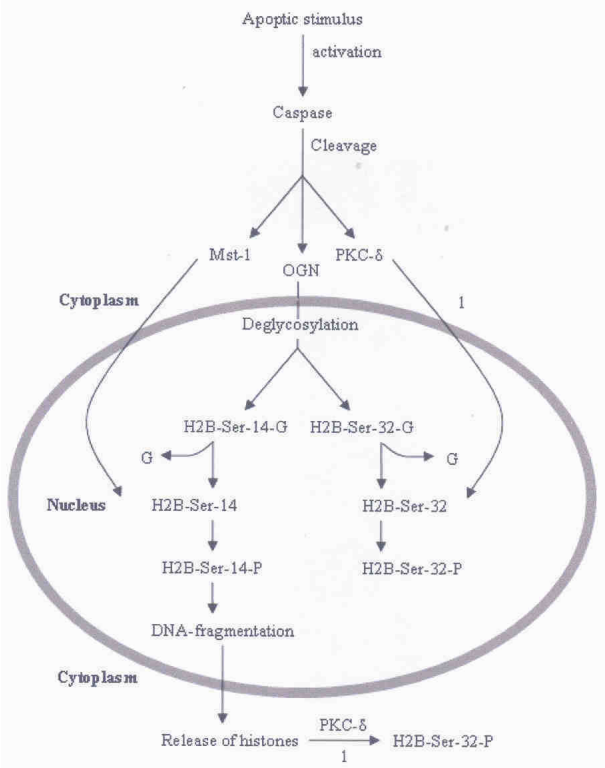

Fig. 4. When apoptosis occurs Caspase-3 is activated, and is able to cleave nucleocytoplasmic proteins like OGN, Mst-1 and PKC-δ. After cleavage OGN is able to deglycosylate H2B on Ser 14 and 32 (and other predicted residues as well). When H2B is deglycosylated, Mst-1 and PKC-δ phosphorylates H2B on Ser 14 and 32 respectively, leading to DNA fragmentation. [1]Ser 32 is present in the inner globular region of H2B, and is not accessible to kinases, as long as the nucleosome structure is intact. PKC-δ phosphorylates Ser 32 either in the nucleus or in the cytosol after release of the histone proteins, but no evidence was found concerning this.

proteoglycans, which are implicated in various biological roles such as presentation and localization of growth factors (Watson *et al*., 1999). The function of nucleosomes present on the cell surface is unknown, but has been proposed to have a role in the synthesis of immunoglobulins in normal lymphocytes (Bell *et al*., 1990). Phosphorylation of H2B, which occur during apoptosis, might be associated with DNA fragmentation (Ajiro, 2000), and might act as recognition for attachment of

cytoplasmic nucleosomes to the nuclear envelope. Furthermore phosphorylation of H2B has been proposed to be part of the apoptotic histone code (Cheung *et al.*, 2003). We propose that O-GlcNAc also play a crucial role by protecting the nucleoprotein H2B from being phosphorylated under normal and stress induced conditions, and when apoptosis occurs, the truncated OGN is able to deglycosylate H2B and kinases are able to phosphorylate H2B.

Other Yin Yang sites in human H2B were predicted (Table III). No specific data was found concerning these sites, which are of equal importance, and the potential for phosphorylation and *O*-glycosylation cannot be ignored.

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