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MINI REVIEW

O-GlcNAcylation of key nuclear and cytoskeletal proteins: reciprocity with O-phosphorylation and putative roles in protein multimerization

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Introduction

O-Linked N-acetylglucosamine, O-GlcNAc, was discovered (Torres and Hart, 1984) as part of our studies using highly-purified glycosyltransferases to investigate saccharide structures on lymphocytes and tumor cells (Powell *et al.*, 1987; Whiteheart and Hart, 1987; Passaniti and Hart, 1988; Reichner *et al.*, 1988; Whiteheart *et al.*, 1989). Our glycosyltransferase studies are a direct outgrowth of the pioneering work of Professor Hill and his colleagues, which developed the methods for the purification of glycosyltransferases (Paulson *et al.*, 1977; Beyer *et al.*, 1980, 1981; Sadler *et al.*, 1979, 1981, 1982). In particular, it was the purification (Trayer and Hill, 1971; Barker *et al.*, 1972) and ready availability of bovine milk galactosyltransferase that allowed us to employ the enzyme as a highly specific and sensitive probe for O-GlcNAc on nuclear and cytoplasmic proteins (for methods, see Roquemore *et al.*, 1994b). Using this enzymatic probe and other methods, we and others (for review, Hart *et al.*, 1989, 1995a–c) have now documented that O-GlcNAc is ubiquitous and abundant on nuclear and cytoskeletal proteins of virtually all eukaryotes, including protozoans (Ortega-Barria *et al.*, 1990; Dieckmann-Schuppert *et al.*, 1993, 1994; Stanley *et al.*, 1995) and fungi (Machida and Jigami, 1994). In addition, O-GlcNAc is highly dynamic, with turnover rates much higher than the protein backbones to which it is attached (Kearse and Hart, 1991; Chou *et al.*, 1992; Roquemore *et al.*, 1996). Virtually all O-GlcNAc proteins examined to date are also phosphoproteins, and in some instances Ser(Thr)-O-GlcNAc and Ser(Thr)-O-phosphate appear to reciprocally occupy the same hydroxyl groups (Kelly *et al.*, 1993; Chou *et al.*, 1995a,b). The dynamic characteristics of O-GlcNAc, and the importance of O-GlcNAcylated proteins in cellular regulation and in cytoskeletal structure, have led us to hypothesize that O-GlcNAc is a regulatory modification that not only regulates protein phosphorylation, but also is involved in modulating protein multimerization (Hart *et al.*, 1995b,c).

O-GlcNAcylation is most abundant in the nucleus

Early studies using highly-purified rat liver subcellular fractions (Holt and Hart, 1986), demonstrated that most O-GlcNAc bearing proteins are localized to the nucleus or cytoplasm. The highest concentration of O-GlcNAc was found in the nuclear envelope on proteins that appeared to comigrate with nuclear pore proteins. Using monoclonal antibodies to the nuclear pore proteins (Snow *et al.*, 1987), the nucleoporins were found to be multiply O-GlcNAcylated, and the sugar was shown to be a major epitope recognized by nearly all of the monoclonal antibodies (Holt *et al.*, 1987b). Later work has shown that monoclonal antibodies that are selective for O-GlcNAc and the lectin, wheat germ agglutinin (WGA), which binds clustered GlcNAc moieties, block nuclear transport of macromolecules (Wolff *et al.*, 1988; Forbes, 1992; Hanover, 1992). However, capping of many of the O-GlcNAc residues by the *in vitro* attachment of galactose residues does not appear to block nuclear transport of macromolecules (Miller and Hanover, 1994).

Even though the concentration of O-GlcNAc is the highest at the nuclear pore complex (Holt *et al.*, 1987b; Hanover *et al.*, 1987; Schindler *et al.*, 1987), most of the proteins bearing this modification are within the nucleoplasm. Studies of salivary gland polytene chromosomes from *Drosophila melanogaster* dramatically demonstrated high concentrations of O-GlcNAc along the entire length of the chromosome (Kelly and Hart, 1989). Interestingly, FITC-WGA-staining showed an intense banding pattern similar to commonly used chromatin dyes. The concentration of O-GlcNAc proteins is much reduced at puffs, which are active sites of gene transcription (Kelly and Hart, 1989). Purification and galactosyltransferase probing of various chromatin fractions indicated that a myriad of proteins in chromatin bear O-GlcNAc moieties, and that the majority of the O-GlcNAc chromatin proteins are associated with chromatin at least as tightly as are the histones (Kelly and Hart, 1989).

O-GlcNAc is ubiquitous in all eukaryotes examined

Since 1984, our laboratory and several others have identified many of the multitude of proteins that are modified by O-GlcNAc. Figure 1 represents a list of those that have been identified at the time of this writing. The list includes RNA polymerase II (Kelly *et al.*, 1993) and virtually all of its transcriptional regulatory proteins that have been examined (Jackson and Tjian, 1988, 1989; Hart *et al.*, 1989; Lichtsteiner and Schibler, 1989; Reason *et al.*, 1992). In addition, many key cytoskeletal proteins (Luthi *et al.*, 1991; Holt *et al.*, 1987a; King and Hounsell, 1989; Chou *et al.*, 1992; Chou and Omary, 1993; Dong *et al.*, 1993; Ku and Bishr Omary, 1994; Murphy *et al.*, 1994; Ding and Vandre, 1996; Vostal and Krasnewich, 1996), viral proteins (Benko *et al.*, 1988; Mullis *et al.*, 1990;

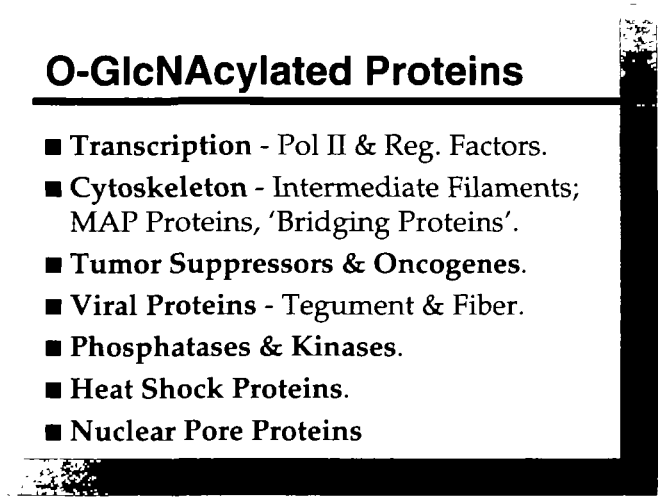

Fig. 1. Identified O-GlcNAcylated proteins. Only a very small percentage of the total O-GlcNAcylated proteins have been identified to date. For references, see text

Gonzalez and Burrone, 1992; Whitford and Faulkner, 1992; Greis *et al.*, 1994), nuclear oncogenes (Privalsky, 1990; Chou *et al.*, 1995a,b), tumor suppressors (Shaw *et al.*, 1996), fungal DNA-binding proteins (Machida and Jigami, 1994), steroid receptors (Jiang *et al.*, unpublished observations), small heat shock proteins (Roquemore *et al.*, 1992; Koga *et al.*, 1996), phosphatases (Meikrantz *et al.*, 1991), kinases (Matsuoka and Hart, unpublished observations), and kinase regulatory proteins (Datta *et al.*, 1989) have been found to be modified by O-GlcNAc. The common feature of these O-GlcNAcylated proteins is that they are all also phosphoproteins and most are known to reversibly form multimeric associations depending upon their phosphorylation states.

O-GlcNAc is a regulatory modification that can be dynamically reciprocal with phosphorylation

The location of O-GlcNAc at Ser(Thr) sites that are similar or identical to those used by many kinases, and the presence of the modification on many of the cell's most important proteins led us to speculate that O-GlcNAc might be a regulatory modification analogous to phosphorylation. Studies of the activation of murine lymphocytes by mitogens and antigens demonstrated that O-GlcNAc levels on lymphocyte proteins change within minutes after stimulation of the cells (Kearse and Hart, 1991). Pulse–chase studies on cytokeratins (Chou *et al.*, 1992) and on the small heat shock proteins (Roquemore *et al.*, 1996) clearly show that O-GlcNAc turns over many times faster than the polypeptide to which it is attached. These studies, and those that show the reciprocal nature of Ser(Thr)-O-phosphorylation and Ser(Thr)-O-GlcNAcylation (Kelly *et al.*, 1993; Chou *et al.*, 1995a), led us to suggest that the posttranslational modification of many nuclear and cytoplasmic proteins is a ternary process *not* a binary one (Figure 2). Thus, the modulation of some protein's functions may be more complicated than the simple addition and removal of phosphate. Rather, it is clear that some proteins exist in up to three different isoforms, naked polypeptide, phosphorylated polypeptide and O-GlcNAcylated polypeptide, each with distinct physical and biological properties. Thus far, circumstantial evidence suggests the working hypothesis that O-GlcNAcylation of proteins generally favors their associations with other proteins, whereas phosphorylation often appears to favor disassociation. We have evaluated the effect of blocking O-GlcNAc turnover in live cells by transfecting them with cDNAs that encode cytoplasmic or nuclear localizing forms of bovine milk galactosyltransferase, an enzyme that 'caps' terminal GlcNAc residues by transferring galactose from UDP-galactose to form Galβ1–4GlcNAc linkages (Snow *et al.*, 1996). The transfection of Chinese hamster ovary cells with these cDNAs appears to kill the cells within one cell cycle, whereas transfection with the cDNA encoding the normal lumenal Golgi localizing enzyme has no effect on cell viability. These data are consistent with the hypothesis that the dynamic aspects of O-GlcNAcylation are important to the life of the cell. However, the mechanisms by which the nuclear or cytoplasmically localized galactosyltransferase kills the cell remains undetermined.

O-GlcNAcylation sites are similar or identical to kinase sites

As part of our effort to determine the biological functions of O-GlcNAcylation, we and other laboratories have mapped the sites of attachment of the saccharide on many different proteins (Hart *et al.*, 1995b). The overall approach for site mapping of proteins, as illustrated for the c-myc protein, is shown in Figure 3. While about half of the attachment sites for O-GlcNAc occur at or near a 'PVS' type motif, about half of the sites have no obvious 'consensus' sequence (Hart *et al.*, 1995b). Many of these sites are similar to those used by kinases, particularly glycogen synthase kinase 3 (Ishiguro *et al.*, 1993; Skurat and Roach, 1996), and the MAP kinases such as erk2 kinase (Boulton *et al.*, 1991). Site mapping is allowing site-directed mutagenesis approaches to be carried-out in order to evaluate functions. However, if mutation of a specific serine or threonine residue affects a protein functions, it may be difficult to determine if the loss of function is due to inability to attach O-GlcNAc or to attach O-phosphate. Recently, the major site of

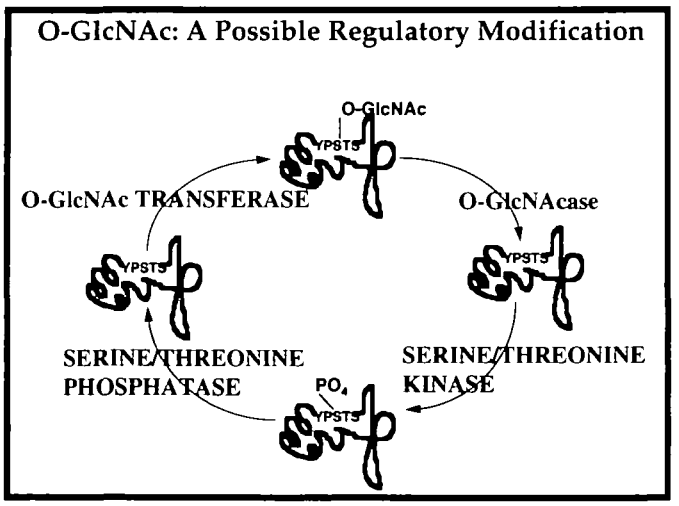

Fig. 2. Dynamic model of the relationship of Ser(Thr)-O-GlcNAc and Ser(Thr)-O-phosphate. In this model, there is often a reciprocal relationship between phosphorylation of a hydroxyl moiety and its O-GlcNAcylation. However, it is anticipated that attachment of O-GlcNAc to nearby or adjacent serine or threonine residues may also influence their phosphorylation.

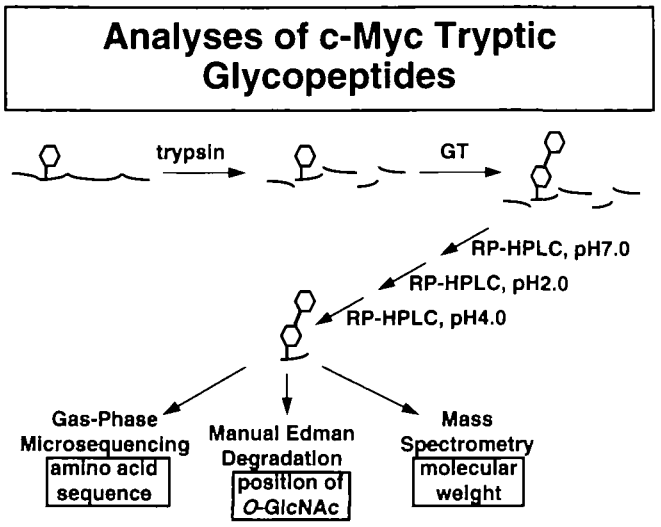

Fig. 3. General scheme for locating the attachment sites of O-GlcNAc. Trypsin has proven to be most useful for fragmentation It is helpful to 'tag' the glycopeptides using UDP-[^{3}H]galactose and galactosyltransferase (GT). Generally, several runs of reverse-phase high performance chromatography (RP-HPLC) under different conditions are required. Site location requires a combination of techniques including, gas phase microsequencing, solid-phase manual Edman degradation, and mass spectrometry.

O-GlcNAcylation of the c-myc oncogene protein was mapped to the transactivation domain (Chou *et al.*, 1995b) at Thr-58 (Chou *et al.*, 1995a), which is also a major phosphorylation site and the most important 'hotspot' in mutations in c-myc associated with lymphomas. Mutation of Thr-58 in c-myc to alanine or to methionine is associated with increased tumorigenicity, suggesting that posttranslational modification at Thr-58 leads to negative regulation of growth and neoplastic phenotype. Clearly, given the reciprocal occupancy of O-GlcNAc and phosphate at this hydroxyl, it is now unclear which of these modifications are beneficial in preventing the oncogenic properties of this protein. However, based upon the literature discussing the role of phosphorylation in the binding of tumor suppressor proteins to oncogenes (Henriksson *et al.*, 1993; Pulverer *et al.*, 1994), it appears likely that the form of c-myc that interacts with tumor suppressors like the retinoblastoma (Rb) protein (Lees *et al.*, 1991; Picksley and Lane, 1994; Weinberg, 1995) or p53 protein (White, 1994; Kastan *et al.*, 1995), is the O-GlcNAcylated form. Recently, the p53 tumor suppressor was shown to bear O-GlcNAc (Shaw *et al.*, 1996). These studies suggested that the O-GlcNAcylated form of p53 has higher biological activity and higher DNA binding activity than the non-O-GlcNAcylated species of p53. Recent studies have also shown that SV40 Large T antigen is O-GlcNAcylated (Medina-Vera and Haltiwanger, 1994). Several O-GlcNAcylated DNA-binding proteins have also been described in the filamentous fungus, *Aspergillus orzae*, suggesting that O-GlcNAcylation may be important in the functions of transcription factors from even the most primitive eukaryotes (Machida and Jigami, 1994).

O-GlcNAc is involved in transcriptional initiation

The 'mucin-like' sequence of the C-terminal domain (CTD) of RNA polymerase II (Pol II) prompted us to investigate if this protein is O-GlcNAcylated (Kelly *et al.*, 1993). All eukaryotic Pol II proteins have multiple repeats of the consensus sequence, -Tyr-Ser-Pro-Thr-Ser-Pro-Ser- , at their C-terminus (Corden, 1990). Available data indicate that the CTD is unphosphorylated in the transcriptional preinitiation complex (Lu *et al.*, 1991), but becomes heavily phosphorylated with the addition of as many as fifty phosphates preceding transcriptional elongation. We found that a subset of Pol II CTDs are heavily O-GlcNAcylated but contain no phosphates (Kelly *et al.*, 1993). These data suggest a model in which the O-GlcNAcylated isoform of Pol II assembles in the preinitiation complex. The O-GlcNAcs then are a rapidly removed prior to the phosphorylation of these same sites. Phosphorylation of the CTD may be important for breaking apart the initiation complex and allows elongation to take place (Zawel and Reinberg, 1995). This model of the role of O-GlcNAc in transcriptional initiation is consistent with the proposed role of O-GlcNAc in mediating subunit interactions and with its observed reciprocal relationship with phosphorylation. We further postulate that site-specific occupancy of transcription factors and Pol II by bulky O-GlcNAc residues might play an important role in regulating cell specific assembly of the transcriptional initiation complex and thus directly affect cell specific gene expression. Very recently, strong support for a role of O-GlcNAc in transcriptional initiation has been obtained by the ability of specific O-GlcNAcase inhibitors and O-GlcNAc-modified CTD peptides, but not O-GalNAc modified CTD peptides to block mRNA transcription mediated by the major late promoter of adenovirus (Comer and Hart, 1996). Elucidation of the nature of O-GlcNAc's role in transcription will require careful kinetic and biochemical analyses of the relationships of O-GlcNAcylation and phosphorylation on each of the many O-GlcNAc bearing proteins comprising the transcriptional machinery.

O-GlcNAc may mediate cytoskeletal assembly and organization

Site mapping studies on neurofilament proteins indicated that the different types of neurofilaments, H, M, or L, are O-GlcNAcylated (Dong *et al.*, 1993). Based upon prior site-directed mutagenesis studies of these neurofilaments (Gill *et al.*, 1990), the locations of the O-GlcNAc moieties suggest that the saccharide is directly involved in mediating neurofilament assembly. In fact, most of the O-GlcNAc residues on neurofilaments are buried and are inaccessible to galactosyltransferase or hexosaminidase treatments, unless the protein is first denatured or fragmented with protease treatment. The O-GlcNAc moieties on α-crystallins (Roquemore *et al.*, 1992), and those on the adenovirus fiber proteins (Mullis *et al.*, 1990) are also buried on the native molecules. During the analyses of partially purified neurofilaments from rat spinal cord (Dong *et al.*, 1993), we observed an apparently heavily O-GlcNAcylated protein that appeared to be the microtubule-associated protein, tau (Mandelkow and Mandelkow, 1994; Goedert *et al.*, 1995; Ingram, 1995). In normal neurons, tau plays a key role in organizing microtubules in the axons. However, in neurons from Alzheimer disease patients, tau is abnormally hyperphosphorylated causing it to form abnormal filaments (PHF-tau), which may be involved in neuronal death (Mandelkow and Mandelkow, 1993; Mandelkow *et al.*, 1993). We have shown that normal tau from bovine brain is heavily O-GlcNAcylated, with apparently over 12 O-GlcNAcylation sites and an average stoichiometry of ~4 O-GlcNAc residues per mole of protein

(Arnold *et al.*, 1994). These studies suggest that the abnormal hyperphosphoryation of tau in Alzheimer disease neurons may result from a defect in the normal O-GlcNAcylation of serine or threonine residues that allows abnormal phosphorylation to take place. A putative role for O-GlcNAcylation in Alzheimer disease is even more intriguing with the recent finding of O-GlcNAc on the β-amyloid precursor protein (Griffith *et al.*, 1995), and the direct demonstration of altered O-GlcNAc levels in Alzheimer patients (Griffith and Schmitz, 1995). Likewise, a possible role for O-GlcNAc in generally mediating the associations of microtubule binding proteins must be considered in the light of recent reports describing the O-GlcNAcylation of the high molecular weight MAP proteins, including MAP2 (Ding and Vandre, 1996). It is noteworthy that several of the O-GlcNAcylated cytoskeletal proteins, such as band 4.1 (Holt *et al.*, 1987a), vinculin (Vostal and Krasnewich, 1996), talin (Hagmann *et al.*, 1992), synapsin (Luthi *et al.*, 1991), and others are involved in the phosphorylation-dependent reversible bridging of the cytoskeleton to the membrane or other structures. Thus, while direct data are still lacking, it is our working hypothesis that O-GlcNAcylation plays an important role in mediating protein–protein interactions involved in a wide variety of cellular functions, including the organization of the cytoskeleton.

Nuclear and cytoplasmic enzymes that remove or attach O-GlcNAc to proteins are abundant

Analogous to phosphatases, we have identified and purified a cytoplasmic O-GlcNAcase from rat liver that has selectivity for O-GlcNAc peptides (Dong and Hart, 1994). O-GlcNAcase activities and nonspecific hexosaminidases are widely abundant in all cell types we have examined, making the quantification of O-GlcNAc stoichiometry problematic (see Roquemore *et al.*, 1992, 1994a, for discussion). The O-GlcNAcase we have described and purified is similar to a partially purified class of enzymes (Minami *et al.*, 1981; Overdijk *et al.*, 1981; Ueno and Yuan, 1991; Koga *et al.*, 1996) referred to as hexosaminidase C. As yet we know almost nothing about the regulation of O-GlcNAcases, but the cloning of the cDNA encoding this rat cytosolic enzyme is in process.

Using synthetic peptide substrates we have also purified a UDP-GlcNAc:polypeptide O-N-acetylglucosaminyltransferase (*O-GlcNAc transferase*) from rat liver (Haltiwanger *et al.*, 1990, 1992). The rat liver enzyme appears to have two immunologically related subunits, of Mr = 110,000, and Mr = 78,000 with a molar ratio of 2:1, respectively, in the native enzyme. Photolabeling studies indicate that the 110K subunit contains the active site of the enzyme. The enzyme also appears to be phosphorylated on tyrosine residues and is itself O-GlcNAcylated (Kreppel and Hart, 1996). Using sequence information from 11 tryptic peptides of the catalytic subunit, we have recently cloned the O-GlcNAc transferase from rat liver. The protein is unlike any known glycosyltransferase previously described (Paulson and Colley, 1989; Joziasse, 1992) and is very highly conserved at the primary sequence level based upon a putative open reading frame in the GenBank database obtained from *C.elegans*. Identification of clones encoding the O-GlcNAc transferase has been confirmed by the comparison of the sequences of numerous peptides, overexpression of the protein and corresponding enzyme activity in heterologous systems, and by direct immunoprecipitation and/or immunodepletion of O-GlcNAc transferase activity using a polyclonal antibody made against the recombinant protein overexpressed in *E.coli*. The availability of the cloned DNA encoding an O-GlcNAc transferase should allow rapid progress on the evaluation of both the regulation of O-GlcNAcylation and the determinations of the saccharide's functions in a wide variety of biological systems.

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