The Emerging Significance of *O*-GlcNAc in Cellular Regulation

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Contents

1. Introduction

O-GlcNAc, the modification of Ser and Thr residues of nuclear and cytoplasmic proteins with *O*-linked β-*N*-acetylglucosamine, is one of a growing number of posttranslational modifications of proteins thought to modulate the function/activity of proteins in cells.[1] Three features suggest that *O*-GlcNAc plays an analogous role to protein phosphorylation in cellular regulation: (1) like phosphorylation, *O*-GlcNAc is highly dynamic with rapid cycling in response to cellular signals or cellular stages;[2−4] (2) *O*-GlcNAc occurs at sites on the protein backbone that are similar to those modified by protein kinases;[5−7] and (3) *O*-GlcNAc is reciprocal with phosphorylation on some well-studied proteins, such as RNA Pol II,[8] estrogen receptor-β,[9,10] SV-40 large T-antigen,[11] and the c-Myc proto-oncogene product.[12] Recently, perturbations in the regulation of *O*-GlcNAc have been implicated in the etiology of type II diabetes, cancer, and neurodegenerative diseases.[1] We will review our current understanding of the importance of the unique carbohydrate modification *O*-GlcNAc, in regulating functions within the cell.

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2. Background

In 1984, a significant body of indirect evidence suggested the presence of nuclear and cytoplasmic glycoproteins.[13] Torres and Hart[14] observed cytoplasmic and nuclear radiolabeling of proteins, when probing living lymphocytes with β-D-1−4-galactosylaminyl transferase (Gal-T). Gal-T recognizes terminal *N*-acetylglucosamine (GlcNAc) residues and modifies them by the addition of a single galactose (Gal) residue via the phosphonucleotide donor uridine diphosphate (UDP)-Gal.[15] Further refinements of this experiment led them to propose that the product *N*-acetyllactosamine (βGal1−4βGlcNAc), was the result of Gal-T recognizing and modifying a single β-GlcNAc residue *O*-linked to Ser and Thr residues (*O*-GlcNAc) of nuclear and cytoplasmic proteins.[16] This contradicted the then current dogma that the addition of carbohydrates was restricted to the ER and Golgi apparatus.

Many proteins modified by *O*-GlcNAc, from a wide range of eukaryotes and tissues, have been detected. These include cytoskeletal proteins,[17] nuclear pore proteins,[16,18] chromatin associated proteins,[19] RNA polymerase II (RNA Pol II)[8] and its transcription factors,[20−23] hnRNPs,[24,25] proto-oncogenes,[12,26] tumor suppressors,[27] hormone receptors,[28−30] phosphatases,[31] and kinases.[32,33] *O*-GlcNAc has now been identified in all eukaryotic organisms studied.[1,6,7,13,34] However, direct evidence of *O*-GlcNAc-modified proteins in simple eukaryotes remains speculative, as many of these organisms also modify proteins with *O*-linked α-GlcNAc. Unfortunately many of the techniques used to detect *O*-GlcNAc do not discriminate between α- and β-GlcNAc residues[35] (Zachara, N. Unpublished observations). As yet, there have been no reports of *O*-GlcNAc in prokaryotes, although several other carbohydrates have been found *O*-linked to Ser, Thr, and Tyr residues.[36]

3. O-GlcNAc: The Enzymes

Consistent with a regulatory role, enzymes for both the addition (UDP-GlcNAc: polypeptide *O*-β-*N*-acetylglucosaminyltransferases; OGT, EC 2.4.1.94, GI:6006036) and removal of *O*-GlcNAc (*O*-β-*N*-acetylglucosaminidase; *O*-GlcNAcase, EC 3.2.1.52, GI: 13646137) have been cloned and characterized. OGT was originally isolated and characterized from rat liver cytoplasm.[37,38] Cloning of the OGT gene indicated that unlike prototypical glycosyltransferases OGT was not a type 2 membrane protein.[39,40] OGT

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is highly conserved, the human gene having 80% homology to that of *Caenorhabditis elegans*.

Although there is evidence for multiple splice variants of OGT, the major species appears to be a 110 kDa protein, which forms a homotrimer.[5,39,40] A 78 kDa protein is sometimes observed in OGT preparations, but it is believed that this is a proteolytic fragment. The OGT protein can be broken into two functional domains, a *C*-terminal catalytic domain and an *N*-terminal domain containing 9−12 tetratricopeptide repeats (TPRs). The TPR domain, commonly involved in protein−protein interactions, is important for trimerization and stability of OGT[41] and is required for enzymatic activity toward protein substrates.[33] Interestingly, the protein is both tyrosine phosphorylated and modified by *O*-GlcNAc.[39,41] OGT is also regulated by UDP-GlcNAc, the protein has multiple K_m values for UDP-GlcNAc, and the glycosylation efficiency toward different substrates changes at different UDP-GlcNAc levels.[41]

While many sites of *O*-GlcNAcylation have been identified, there is no consensus concerning the motif for the addition of *O*-GlcNAc.[5,39,40] This led researchers to propose that there is more than one OGT, but no other eukaryotic genes have thus far been identified. The lack of an agreed upon motif for the addition of *O*-GlcNAc may be explained by the role of the TPR domain in substrate recognition.[33,40−42] While many different peptides are acceptors for GlcNAc[41] with varying K_m values, proteins are more efficient substrates.[33,40] Moreover, Comer and Hart[42] have shown that cooperativity between OGT subunits is required for efficient glycosylation of the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II).

O-GlcNAcase, β-*N*-acetylglucosaminidase, or hexosaminidase C[43,44] was purified from the cytosol of rat spleen[45] but was not successfully cloned until recently.[46,47] This study identified a 130 kDa protein from bovine brain, which had previously been identified as a putative hyaluronidase.[48] However, it is clear from the kinetic data that the protein prefers *O*-GlcNAc as a substrate.[47]

O-GlcNAcase is expressed in all tissues and is predominantly localized to the cytoplasm.[46] *O*-GlcNAcase is expressed as two splice variants, the full-length protein (p130) and as a shorter protein (p75) with a different C-terminal tail.[49] The shorter of the two variants (p75) appears to have a predominantly nuclear distribution,[49] but is *not* an active *O*-GlcNAcase.[47] *O*-GlcNAc has recently been characterized, with a turnover rate of 1.1 s^{-1} and a catalytic efficiency of 1×10^3 M^{-1} s^{-1} toward *p*-nitrophenyl-GlcNAc. The activity of the *O*-GlcNAcase suggests that it is regulated. In support of this, Wells and coworkers have shown that the protein is phosphorylated and purifies as part of a larger complex.[47] Future studies will elucidate the role of these proteins in the regulation of *O*-GlcNAcase. The inhibition of *O*-GlcNAcase by *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc)[50] rapidly increases *O*-GlcNAc levels in cells, further demonstrating the dynamics of this modification.

4. O-GlcNAc: Cellular Regulation

The modification of key cellular proteins, such as transcription factors and tumor suppressors, by *O*-GlcNAc has suggested its potential in cellular regulation. However, the most compelling evidence for the importance of *O*-GlcNAc to cellular regulation is studies where the addition and removal of *O*-GlcNAc have been blocked. Use of a Cre-Lox mutagenesis system to delete the OGT gene from mouse germ cell lines has indicated that OGT, and thus *O*-GlcNAc, is required for embryonic stem cell viability and mouse ontogeny. Interestingly, the gene for OGT maps to Xq13, a locus commonly associated with neurodegenerative diseases.[51]

Researchers have also blocked the ability of cells to add *O*-GlcNAc to proteins by altering the levels of UDP-GlcNAc, the donor substrate for OGT, within the cell by inhibiting components of the hexosamine biosynthesis pathway. In one recent study, a gene for glucosamine-6-phosphate acetyltransferase was

knocked out.[52] The authors showed that while levels of GlcNAc in *N*-linked carbohydrates and GPI-anchors remained unaffected, levels of *O*-GlcNAc were reduced. The affect was lethal in mice, with embryos dying at day 7.5. In cell culture, fibroblasts without this gene exhibited reduced proliferation and adhesiveness and, interestingly, reduced susceptibility to apoptotic stimuli.[52]

Preventing the removal of *O*-GlcNAc from proteins is also toxic to cells. Until recently the gene for *O*-GlcNAcase had not been cloned, and to study the effects of preventing the removal of *O*-GlcNAc researchers have used Gal-T (see above). Two approaches have been taken, Gal-T has either been expressed as a soluble cytosolic/nuclear protein or it has been microinjected into cells.[53,54] Expression of a soluble cytosolic/nuclear Gal-T resulted in cell death.[53] Microinjection into oocytes prevented their exit from mitosis, as the cells were unable to form a viable mitotic spindle.[54] It should be noted that toxicity may be due to the disruption of protein−protein interactions, rather than blockage of the removal of *O*-GlcNAc.

It has long been accepted that phosphorylation is a key posttranslational modification, required for balancing the activity of proteins within the cell. These and other data discussed below argue that *O*-GlcNAc plays a similar, perhaps competitive, role.

A. *O*-GlcNAc: A Modification Analogous to Phosphorylation

Studies that have shown that *O*-GlcNAc was dynamic, with rapid cycling in response to cellular signals or cellular stages,[2−4,55] led researchers to propose that *O*-GlcNAc was a regulatory modification like protein phosphorylation.

To investigate the possible roles of *O*-GlcNAc in cellular regulation, Kearse and Hart[3] investigated changes in *O*-GlcNAcylation in nuclear and cytoplasmic pools of proteins from lymphocytes before and after activation with the T-cell mitogen Con-A. The authors observed a decrease in cytoplasmic glycosylation and a concomitant increase in nuclear glycosylation. The authors suggested several explanations for their observations: (1) *O*-GlcNAc was a transient signal analogous to phosphorylation; (2) *O*-GlcNAcylated proteins were rapidly translocated to the nucleus; and (3) proteins were rapidly degraded. However, careful analysis of the data showed that the changes in glycosylation were transient, returning to basal levels within hours. Moreover, the populations of proteins disappearing from the cytoplasm were largely distinct from those appearing in the nucleus. Last, for a subset of proteins this affect was independent of protein translation, suggesting that *O*-GlcNAc was a signaling event. Supporting this, other researchers have now shown that *O*-GlcNAc turns over faster than the protein backbone on cytokeratins and α-B-crystallin.[2,4] Finally, mutations in a putative OGT from *Aribodopsis*, called Splindly, led to constitutive activation of the gibberellin signaling pathway.[56] This suggests a role for *O*-GlcNAc in the regulating of proteins in this signaling pathway.

B. The Yin–Yang Hypothesis

Careful site-mapping of *O*-GlcNAc-modified proteins showed that *O*-GlcNAc occurs at sites on the protein backbone that are similar to those modified by protein kinases.[3,5−7] Furthermore, *O*-GlcNAc is reciprocal with phosphorylation on some well-studied proteins, such as RNA Pol II,[8] estrogen receptor-β,[9] SV-40 large T-antigen,[11] and the c-Myc proto-oncogene product.[12,26] These data led to the development of the "yin−yang" hypothesis, in which *O*-GlcNAc and phosphorylation compete for the same site or region on the polypeptide backbone. In support of this, only a handful of proteins modified by *O*-GlcNAc are modified by phosphate at the same time.[57] In more compelling studies, it was shown that prior glycosylation prevents[42] or reduces[58] phosphorylation of a peptide substrate in an in vitro kinase assay. The study by Comer and Hart,[42] which examined the reciprocity between *O*-GlcNAc and *O*-phosphate on the CTD of RNA Pol II, went on to show that phosphorylation of a similar substrate prevented glycosylation by OGT.

A global relationship between *O*-GlcNAc and *O*-phosphate has been demonstrated by comparing the levels of *O*-GlcNAc and *O*-phosphate before and after treatment with kinase and phosphatase inhibitors. Treatment of cells with phosphatase inhibitors and kinase activators leads to decreased levels of *O*-GlcNAc within the cell.[59,60] The converse is also true: treatment of cells with kinase inhibitors increases levels of *O*-GlcNAc.[59] These data led to a complex model of protein regulation where many different posttranslationally modified forms of a protein can exist (see Figure 1) and a series of deglycosylation/phosphorylation and/or dephosphorylation/glycosylation events would be required to alter the behavior of a protein. It has recently been observed that OGT copurifies with an unknown Ser/Thr phosphatase,[61] which supports a model for some proteins where phosphate is replaced with GlcNAc via the same enzyme complex. Studies are currently underway to determine if kinase activity associates with *O*-GlcNAcase.

C. Regulating Proteins

Phosphorylation affects the activity of proteins through a number of different mechanisms, one of which is through the binding of phospho-specific domains, such as SH2, SH3, and WW domains.[62] As of yet, no *O*-GlcNAc binding domain has been identified. Although, there are a number of proteins with lectin-like activity that have been identified in both the cytoplasm and nucleus.[63−67] The role of these proteins in the action of *O*-GlcNAc has not been determined.

Several studies have defined specific roles for *O*-GlcNAc on specific proteins; these include: (1) signaling nuclear transport,[68−73] (2) regulating the involvement of proteins in multimeric complexes,[58,74−76] (3) modulating the half-life and proteolytic processing of proteins,[3,77] and (4) regulating protein activity, by regulating the state of phosphorylation.[8,9,11,12,59,60] More recently, it has been proposed that *O*-GlcNAc

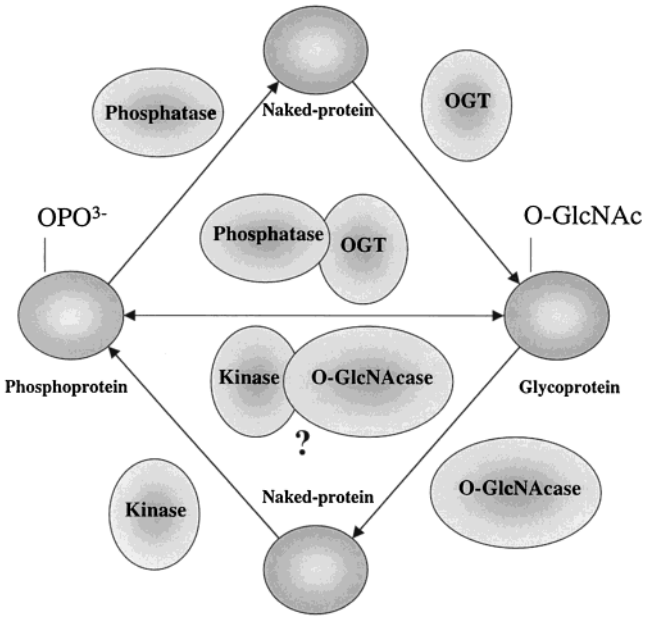

Figure 1. The yin−yang hypothesis models *O*-GlcNAc preventing phosphorylation and vice versa. This is achieved by blocking the site of phosphorylation or modification of an adjacent site. Differently modified proteins may perform different functions or be regulated by swapping between modification states. This can be achieved two ways: the protein must be deglycosylated before phosphorylation can occur, and the protein is dephosphorylated before glycosylation. This could be achieved in a stepwise manner with the enzymes uncomplexed or complexed. Alternatively, *O*-GlcNAc may be important in kinase recognition and phosphorylation important for OGT activity. This latter mechanism would prefer glycosylation and phosphorylation at adjacent sites.

may play a role in sensing glucose (Glc) and is implicated in the etiology of type II diabetes mellitus.

1. Nuclear Transport

Several researchers have proposed that *O*-GlcNAc may play a role in nuclear transport. Much of the focus has been on proteins of the nuclear pore complex, which is highly enriched in *O*-GlcNAc.[16,18,78] Early reports showed that the lectin wheat germ agglutinin (WGA) blocked the energy dependent step of nuclear transport, suggesting that *O*-GlcNAc played a role in the transport process.[79] However, Miller and Hanover[80] showed that capping *O*-GlcNAc on nuclear pore proteins with Gal had NO affect on protein transport. This indicates that the effect of WGA is probably steric.

The nucleus represents one of the greatest concentrations of proteins modified by *O*-GlcNAc,[16] and many *O*-GlcNAcylated proteins shuttle from the cytoplasm to the nucleus.[69,71] This observation led researchers to investigate whether *O*-GlcNAc was an alternative nuclear localization sequence.[25,70−72,81−83] The earliest reports on RNA binding proteins showed that tunicamycin, an inhibitor of *N*-linked glycosylation, could inhibit nuclear uptake of the La-antigen.[25] However, since *N*-glycans have not been structurally documented in the nucleus, the significance of these tunicamycin effects are unclear. The La-antigen, hnRNP G,[24] hnRNP A1, and SR proteins (Zachara, N. E.; Hart, G. W. Unpublished observations) are modified by *O*-GlcNAc. The latter proteins are predominantly glycosylated in the cytoplasm, alluding to the existence of either a glycosylation dependent cytosolic retention mechanism or nuclear transport signal.

This supposition is supported by the data of Monsigny and co-workers, which have shown that the addition of carbohydrates, including GlcNAc, can induce the nuclear transport of heterologous proteins without NLS sequences.[70,72,83] This "carbohydrate dependent" transport is energy dependent but does not require cytosolic factors required by typical nuclear transport mechanisms (Duverger et al.[72]). However, currently there are no direct data with native proteins that support these suppositions.

2. Regulating Protein–Protein Interactions

Many proteins modified by *O*-GlcNAc play key roles in the organization and assembly of the cytoskeleton, including cytokeratins (8, 13, and 18),[2,75] neurofilaments (H, L, and M),[92,93] microtubule associated proteins (MAP 1, 2, 4),[84] adenovirus fiber proteins (types 2 and 5),[94,95] ankyrin$_G$, talin,[85] vinculin, Band 4.1,[86] crystallin,[4,87] synapsin 1,[58,88] and Tau.[17,89] The observation that other *O*-GlcNAcylated proteins are part of multimeric complexes led some researchers to predict that *O*-GlcNAc might play a role in protein−protein interactions. Early studies, which localized *O*-GlcNAc to domains important for protein−protein interaction for Sp1 (see below); keratins 8, 13, and 18;[57,90,91] neurofilament proteins (see below);[92,93] adenovirus fiber proteins 2 and 5;[94,95] and synapsin I[58] promoted this hypothesis. However, for many of these proteins, no clear role for *O*-GlcNAc has been defined.

Neurofilament proteins are class IV intermediate-filament proteins and are the most abundant structural components of myelinated axons. The proteins are characterized by a 310 amino acid α-helical rod domain, which separates the head domain from the variable length tail domain. The α-helical rod domain is essential for normal filament assembly, while the tail and head domains are required for other protein−protein interactions. Phosphorylation of the head domains induces depolymerization of intermediate filaments and prevents filament assembly. Dong and co-workers were able to show that three neurofilament proteins (H, L, and M) were modified by *O*-GlcNAc.[90] The sites of glycosylation were mapped primarily to the head domain.[93] Deletion analysis of NF-L (AA 31−87) and NF-M (AA 75−126) resulted in disruption of filament formation.[96,97]

3. Regulation of Protein Degradation

The level of many key cellular proteins is controlled both by the rate of synthesis and the rate of degradation. One mechanisms that targets proteins for rapid degradation are PEST sequences, or regions enriched in Pro (P), Glu (E), Ser (S), and Thr (T) residues.[77,98] While some PEST sequences are constitutive, many require activation by mechanisms such as phosphorylation. Many researches have shown that protein sequences modified by *O*-GlcNAc have high PEST scores (http://www.at.embnet.org/embnet/tools/bio/

PESTfind/form.htm). It has been proposed that glycosylation prevents phosphorylation and thus neutralizes the affects of PEST sequences.[3]

When the α-subunit of eukaryotic initiation faction-2 (eIF2) is phosphorylated, eIF2B is sequestered and ternary complex formation is inhibited, preventing protein translation. p67 is a cellular glycoprotein that regulates the activity of the eIF2, by preventing phosphorylation of the α-subunit. Pretreatment of p67 with WGA (a lectin that binds terminal GlcNAc residues) prevented p67 inhibition of eIF2 kinase.[99] In a subsequent study, it was shown that under conditions of serum starvation or heme depletion, p67 was deglycosylated and rapidly degraded.[100] Deglycosylation was controlled by a latent deglycosylase, which was activated upon starvation.[101] This leads to a model where, under normal conditions, p67 is glycosylated and associates with eIF2α, preventing phosphorylation by eIF2 kinase and promoting protein synthesis. When cells are challenged by serum starvation or heme depletion, p67 is deglycosylated and degraded, effectively shutting off protein synthesis.

Studies of the transcription factor Sp1 have also shown glycosylation dependent affects on the rate of protein degradation in a cell type dependent manner.[102] Here the authors modulated the stoichiometry of *O*-GlcNAc on Sp1 by altering the nutritional state of the cells using cAMP or 6-diazo-5-oxonorleucine (DON), an inhibitor of the glucosamine (GlcN) biosynthesis pathway. Under such treatments, Sp1 became hypoglycosylated and was more susceptible to proteosome dependent degradation. The addition of extracellular GlcN abrogated this effect.[102] Only mutagenesis will confirm the role of *O*-GlcNAc in the degradation pathway.

Estrogen receptor α (ER-α) and *β* (ER-*β*) are nuclear hormone receptors, that is, they act as transcription factors upon binding of their ligands. Both are modified by either *O*-phosphate or *O*-GlcNAc, predominantly on sequences with high PEST scores.[9,10,28,30] Initial studies found no difference in the ability of the glycosylated and nonglycosylated proteins to bind DNA or transactivate gene expression.[9,28,30] The role of *O*-GlcNAcylation on ER-*β* protein degradation was also examined. The major site of glycosylation (Ser16)[9] was mutated (Ser15 > Ala, Ser16 > Ala and Thr17 > Val) and the rate of degradation was compared to wild-type ER-*β* and a mutant that would mimic constitutive phosphorylation (Ser15 > Ala, Ser16 > Asp, Thr17 > Val).[10] As expected, the Ser16 > Asp mutant had the fastest turnover rate, while the Ser16 > Ala mutant had the slowest turnover rate. These data suggest that *O*-GlcNAc prevents degradation.[10]

4. Regulating Protein Activity

O-GlcNAc has been detected on every transcription factor examined to date.[23] Sp1, a general transcription factor, is modified by an average of 8 mol of *O*-GlcNAc/mol of protein. To investigate the function of *O*-GlcNAc on Sp1 the authors used protein purified from either HeLa cells or expressed in *Escherichia coli* in an in vitro transcription system. Using HeLa-purified Sp1, the authors were able to show a reduction (~3-fold) in transcriptional activation when the lectin WGA was added to the reaction. Subsequent studies showed that DNA binding was not inhibited. Comparison of the transcriptional activation capabilities of the HeLa and *E. coli* expressed proteins, showed that the HeLa Sp1 was 3−5-fold more efficient that the unglycosylated *E. coli* protein.[20] These studies do not correlate with those of Roos and co-workers (see below), who have shown that glycosylation of the Sp1 transactivation domain inhibits protein−protein interactions key to transactivation.

Roos and co-workers have developed a system for studying the affects of Sp1 *O*-GlcNAcylation on protein−protein interactions.[103,104] In an initial study they determined that the Glu-rich domain (AA 424−542), which was known to be important for self-association and binding to TAF 110, was glycosylated.[103] The authors studied the affect of glycosylation on protein−protein interactions using two different systems. In one, the protein was produced in *E. coli* and shown *not* to be glycosylated; in the other, the major site of glycosylation was mutated from a Ser/Thr residue to an Ala residue. In both cases the unglycosylated protein was able to self-associate and associate with TAF 110.[103,104] In addition, both the mutant and unglycoysylated protein were able to transactivate a reporter system at higher levels than the glycosylated protein.[104] The authors propose that the glycosylation status of Sp1 is required to prevent premature protein−protein interactions of Sp1.

Many of the differences between these two studies could be explained by the different sources of proteins. However, it would be interesting to see if WGA affects the model system developed by Kudlow and others[103,104] in a manner similar to that observed by Jackson and Tjian. It is possible that the different conclusions formed by these two groups could be explained by a model in which glyco-Sp1 binds DNA and is then deglycosylated, phosphorylated, and transactivates gene transcription. In this model, the addition of WGA would not prevent DNA binding and would reduce the efficiency of *O*-GlcNAc removal and thus the transactivation efficiency.

5. Misregulation of O-GlcNAc

A. *O*-GlcNAc and Diabetes

Recently, researchers have shown that the levels of *O*-GlcNAc change in response to extracellular levels of Glc and GlcN.[41,105−108] This may reflect the change in substrate specificity of OGT at different levels of UDP-GlcNAc.[41] It has been proposed that this is a general response to increased flux through the hexosamine biosynthetic pathway and that the modification of proteins with *O*-GlcNAc is a nutritional sensor.[7,33,41,109] This is an attractive hypothesis, as different mechanisms could result in both positive and negative affects on protein activity and expression. Noteably, changes in the extracellular GlcN levels both increase and decrease protein expression in smooth muscle cells (RASM).[110]

Insulin resistance, the hall mark of type II diabetes, can be induced in animals by prolonged

exposure to elevated levels of Glc or GlcN.[111,112] The role of *O*-GlcNAc in the development of insulin resistance is unclear, though some preliminary studies indicate that the modification of proteins with *O*-GlcNAc may play a key role in signaling Glc uptake by adipose and muscle tissue and in the release of insulin in β-cells. Several studies have shown that elevated levels of GlcN and insulin lead to elevated levels of *O*-GlcNAc in tissues,[113] which has been implicated in the altered expression of proteins within the cell.[110] Hyper-*O*-GlcNAcylation of proteins may alter the balance between *O*-GlcNAc and *O*-phosphate in the insulin signaling cascade, thus preventing insulin-induced phosphorylation signals, triggering increased Glc disposal.

Several studies have indicated that cells of the pancreas have elevated levels of key enzymes required for the addition and removal of *O*-GlcNAc. Treatment of animals with the *O*-GlcNAc analogue streptozotocin (STZ), a weak inhibitor of *O*-GlcNAcase, results in death of pancreatic β-cells.[106,107,114] While several groups were able to show elevated levels of *O*-GlcNAc under these conditions, STZ also causes apoptosis though poly(ADP-ribose) poylmerase-mediated mechanisms.[115] Supporting a role for *O*-GlcNAc in insulin signaling, one recent report has shown that the insulin-promoting factor (PDX-1, IPF-1) is modified by *O*-GlcNAc. In a β-cell line, hyperglycemia concomitantly increases *O*-GlcNAc levels on proteins and increases insulin secretion.[116] In adipocytes, increased levels of GlcN appear to inhibit the transport of Glut4 vesicles from the cytoskeleton to the plasma membrane.[117] Hyperglycemia correlates with decreased phosphorylation and increased glycosylation of the insulin receptor substrates 1 and 2.[118]

B. *O*-GlcNAc and Neurodegenerative Diseases

Many proteins of the neuronal cytoskeleton are modified by *O*-GlcNAc,[17] and of particular interest are the β-amyloid precursor protein[119] and Tau.[89] The role of *O*-GlcNAc in the development of Alzheimer's is unclear. Several reports have shown that Glc metabolism is impaired in Alzheimer's brains. This leads to an attractive model, where decreased Glc metabolism leads to lower levels of UDP-GlcNAc and thus decreased *O*-GlcNAcylation. As a result, increased phosphorylation and misregulation of proteins such as Tau would result from exposure of cryptic phosphorylation sites. Hyperphosphorylation of Tau, in neurofibrillary tangles, is well-documented, supporting this hypothesis. However, the complexity of the relationship between phosphorylation and *O*-GlcNAcylation is highlighted by studies that indicate increased levels of *O*-GlcNAc in some subcellular fractions of Alzheimer brains.[120] Interestingly, both OGT[51] and *O*-GlcNAcase map[46] to chromosomal locations linked to the development of neurodegenerative diseases.[7,121,122]

6. Perspectives

The study of the function of *O*-GlcNAc has been challenging. In part this is due to rapid cycling of the modification, similarity between glycosylation and phosphorylation sites, and a lack of sensitive detection methods. Until recently, the methods for detection of the *O*-GlcNAc modifications have been limited to lectins, antibodies, and Gal-T, which lack specificity and/or sensitivity. Recently, Comer and co-workers have characterized a monoclonal antibody, raised against the C-terminal domain of RNA Pol II, that appears to be specific for *O*-GlcNAc with little requirement for the protein backbone.[123] This antibody will allow the global detection and immunopurification of proteins modified by *O*-GlcNAc. This, in combination with new proteomic tools for the detection of *O*-GlcNAc-modified proteins,[124,125] will expedite studies looking at the global changes in *O*-GlcNAcylation. Study of *O*-GlcNAc-modified proteins in vitro will be aided by an improved glycopeptide synthesis method,[126] which can be combined with nonhydrolyzable substrates[127] to investigate *O*-GlcNAc dependent protein−protein interactions.

It is an exciting time in the field of glycobiology; a combination of tools and knowledge are culminating in a rapid expansion of our understanding of the roles of protein glycosylation,[128] and the modification of proteins with *O*-GlcNAc is no exception to this generalization. A wealth of recent data from many groups is contributing to our understanding of the complex interplay between *O*-GlcNAc and phosphorylation in the regulation of cellular events.

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8. References

(1) Comer, F. I.; Hart, G. W. *J. Biol. Chem.* **2000**, *275*, 29179.
(2) Chou, C.-F.; Smith, A. J.; Omary, M. B. *J. Biol. Chem.* **1992**, *267*, 3901.
(3) Kearse, K. P.; Hart, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1701.
(4) Roquemore, E. P.; Chevrier, M. R.; Cotter, R. J.; Hart, G. W. *Biochemistry* **1996**, *35*, 2578.
(5) Haltiwanger, R. S.; Kelly, W. G.; Roquemore, E. P.; Blomberg, M. A.; Dong, D. L.-Y.; Kreppel, L.; Chou, T.−Y.; Hart, G. W. *Biochem. Soc. Trans.* **1992**, *20*, 264.
(6) Hart, G. W.; Kreppel, L. K.; Comer, F. I.; Arnold, C. S.; Snow, D. M.; Ye, Z.; Cheng, X.; DellaManna, D.; Caine, D. S.; Earles, B. J.; Akimoto, Y.; Cole, R. N.; Hayes, B. K. *Glycobiology* **1996**, *6*, 711.
(7) Wells, L.; Vosseller, K.; Hart, G. W. *Science* **2001**, *291*, 2376.
(8) Kelly, W. G.; Dahmus, M. E.; Hart, G. W. *J. Biol. Chem.* **1993**, *268*, 10416.
(9) Cheng, X.; Cole, R. N.; Zaia, J.; Hart, G. W. *Biochemistry* **2000**, *39*, 11609.
(10) Cheng, X.; Hart, G. W. *J. Biol. Chem.* **2001**, *276*, 10570.
(11) Medina, L.; Grove, K.; Haltiwanger, R. S. *Glycobiology* **1998**, *8*, 383.
(12) Chou, T.-Y.; Hart, G. W.; Dang, C. V. *J. Biol. Chem.* **1995**, *270*, 18961.
(13) Hart, G. W.; Haltiwanger, R. S.; Holt, G. D.; Kelly, W. G. *Annu. Rev. Biochem.* **1989**, *58*, 841.
(14) Torres, C.-R.; Hart, G. W. *J. Biol. Chem.* **1984**, *259*, 3308.
(15) Brew, K.; Vanaman, T. C.; Hill, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *59*, 491.
(16) Holt, G. D.; Hart, G. W. *J. Biol. Chem.* **1986**, *261*, 8049.
(17) Arnold, C. S.; Hart, G. W. *Trends Glycosci. Glycotech.* **1999**, *11*, 355.
(18) Hanover, J. A.; Cohen, C. K.; Willingham, M. C.; Park, M. K. *J. Biol. Chem.* **1987**, *262*, 9887.

(19) Kelly, W. G.; Hart, G. W. *Cell* **1989**, *57*, 243.
(20) Jackson, S. P.; Tjian, R. *Cell* **1988**, *55*, 125.
(21) Jackson, S. P.; Tjian, R.*Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1781.
(22) Reason, A. J.; Morris, H. R.; Panico, M.; Marais, R.; Treisman, R. H.; Haltiwanger, R. S.; Hart, G. W.; Kelly, W. G.; Dell, A. *J. Biol. Chem.* **1992**, *267*, 16911.
(23) Comer, F. I.; Hart, G. W. *Biochim. Biophys. Acta* **1999**, *1473*, 161.
(24) Soulard, M.; Della Valle, V.; Siomi, M. C.; Piñol-Roma, S.; Codogno, P.; Bauvy, C.; Bellini, M.; Lacroix, J.-C.; Monod, G.; Dreyfuss, G.; Larsen, C.-J. *Nuc. Acids Res.* **1993**, *21*, 4210.
(25) Bachmann, M.; Pfeifer, K.; Schröder, H. C.; Müller, W. E. G. *Mol. Cell. Biochem.* **1989**, *85*, 103.
(26) Chou, T.-Y.; Dang, C. V.; Hart, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4417.
(27) Wells, L.; Zhu, L.; Hart, G. W. *Mol. Biol. Cell* **2000**, *Suppl. 2382* (*Abstr.*).
(28) Privalsky, M. L. *J. Virol.* **1990**, *64*, 436.
(29) Jiang, M.-S.; Hart, G. W. *J. Biol. Chem.* **1997**, *272*, 2421.
(30) Cheng, X.; Hart, G. W. *J. Steroid Biochem. Mol. Biol.* **2000**, *75*, 147−158.
(31) Meikrantz, W.; Smith, D. M.; Sladicka, M. M.; Schlegel, R. A. *J. Cell Sci.* **1991**, *98*, 303.
(32) Matsuoka, T.; Dellamanna, D.; Isaacs, J.; Carroll, J. A.; Kreppel, L. K.; Cole, R. N.; Johnson, G. V. W.; Hart, G. W. In preparation.
(33) Lubas, W. A.; Hanover, J. A. *J. Biol. Chem.* **2000**, *275*, 10983.
(34) Hart, G. W. *Annu. Rev. Biochem.* **1997**, *66*, 315.
(35) Elling, L.; Zervosen, A.; Gallego, R. G.; Nieder, V.; Malissard, M.; Berger, E. G.; Vliegenthart, J. F.; Kamerling, J. P. *Glycoconjugate J.* **1999**, *16*, 327.
(36) Messner, P.; Allmaier, G.; Schäffer, C.; Wugeditsch, T.; Lortal, S.; König, H.; Niemetz, R.; Dorner, M. *FEMS Micro. Rev.* **1997**, *20*, 25.
(37) Haltiwanger, R. S.; Holt, G. D.; Hart, G. W. *J. Biol. Chem.* **1990**, *265*, 2563.
(38) Haltiwanger, R. S.; Blomberg, M. A.; Hart, G. W. *J. Biol. Chem.* **1992**, *267*, 9005.
(39) Kreppel, L. K.; Blomberg, M. A.; Hart, G. W. *J. Biol. Chem.* **1997**, *272*, 9308.
(40) Lubas, W. A.; Frank, D. W.; Krause, M.; Hanover, J. A. *J. Biol. Chem.* **1997**, *272*, 9316.
(41) Kreppel, L. K.; Hart, G. W. *J. Biol. Chem.* **1999**, *274*, 32015.
(42) Comer, F. I.; Hart, G. W. *Biochemistry* **2001**, *40*, 7845.
(43) Braidman, I.; Carroll, M.; Dance, N.; Robinson, D.; Poenaru, L.; Weber, A.; Dreyfus, J. C.; Overdijk, B.; Hooghwinkel, G. J. M. *FEBS Lett.* **1974**, *41*, 181.
(44) Overdijk, B.; van der Koef, W. M. J.; van Steijn, G. J.; Lisman, J. W. J. *Biochim. Biophys. Acta* **1981**, *659*, 255.
(45) Dong, D. L.; Hart, G. W. *J. Biol. Chem.* **1994**, *269*, 19321.
(46) Gao, Y.; Wells, L.; Comer, F. I.; Parker, G. J.; Hart, G. W. *J. Biol. Chem.* **2001**, *276*, 9838.
(47) Wells, L.; Gao, Y.; Mahoney, D.; Rosen, A.; Vosseller, K. V.; Hart, G. W. *J. Biol. Chem.*, in press.
(48) Heckel, D.; Comtesse, N.; Brass, N.; Blin, N.; Zang, K. D.; Meese, E. *Hum. Mol. Genet.* **1998**, *7*, 1859.
(49) Comtesse, N.; Maldener, E.; Meese, E. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 634.
(50) Haltiwanger, R. S.; Grove, K.; Philipsberg, G. A. *J. Biol. Chem.* **1998**, *273*, 3611.
(51) Shafi, R.; Iyer, S. P.; Ellies, L. G.; O'Donnell, N.; Marek, K. W.; Chui, D.; Hart, G. W.; Marth, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5735.
(52) Boehmelt, G.; Wakemam, A.; Elia, A.; Sasaki, T.; Plyte, S.; Potter, J.; Yang, Y.; Tsang, E.; Ruland, J.; Iscove, N. N.; Dennis, J. W.; Mak, T. W. *EMBO J.* **2000**, *19*, 5092.
(53) Snow, D. M.; Shaper, J. H.; Shaper, N. L.; Hart, G. W. *Mol. Biol. Cell* **1996**, *6*, 357 *(Abstr.)*.
(54) Fang, B.; Miller, M. W. *Exp. Cell Res.* **2001**, *263*, 243.
(55) Favreau, C.; Worman, H. J.; Wozniak, R. W.; Frappier, T.; Courvalin, J. C. *Biochemistry* **1996**, *35*, 8035.
(56) Thornton, T. M.; Swain, S. M.; Olszewski, N. E. *Trends Plant Sci.* **1999**, *4*, 424.
(57) Ku, N.-O.; Omary, M. B. *Exp. Cell Res.* **1994**, *211*, 24.
(58) Cole, R. N.; Hart, G. W. *J. Neurochem.* **1999**, *73*, 418.
(59) Griffith, L. S.; Schmitz, B. *Eur. J. Biochem.* **1999**, *262*, 824.
(60) Lefebvre, T.; Alonso, C.; Mahboub, S.; Dupire, M. J.; Zanetta, J. P.; Caillet-Boudin, M. L.; Michalski, J. C. *Biochim. Biophys. Acta* **1999**, *1472*, 71.
(61) Wells, L.; Kreppel, L. K.; Hart, G. W. *Mol. Biol. Cell* **1999**, *Suppl. 1112 (Abstr.)*.
(62) Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075.
(63) Felin, M.; Doyennette-Moyne, M. A.; Hadj-Sahraoui, Y.; Aubery, M.; Hubert, J.; Seve, A. P. *J. Cell Biochem.* **1994**, *56*, 527.
(64) Cho, M.; Cummings, R. D. *J. Biol. Chem.* **1995**, *270*, 5207.
(65) Wang, L.; Inohara, H.; Pienta, K. J.; Raz, A. *Biochem. Biophys. Res. Commun.* **1995**, *217*, 292.
(66) Huang. L.; Grammatikakis, N.; Yoneda, M.; Banerjee, S. D.; Toole, B. P. *J. Biol. Chem.* **2000**, *275*, 29829.
(67) Hotta, K.; Funahashi, T.; Matsukawa, Y.; Takahashi, M.; Nishizawa, H.; Kishida, K.; Matsuda, M.; Kuriyama, H.; Kihara, S.; Nakamura, T.; Tochino, Y.; Bodkin, N. L.; Hansen, B. C.; Matsuzawa, Y. *J. Biol. Chem.* **2001**, *276*, 34089.
(68) Finlay, D. R.; Newmeyer, D. D.; Price, T. M.; Forbes, D. J. *J. Cell. Biol.* **1987**, *104*, 189.
(69) Borer, R. A.; Lehner, C. F.; Eppenberger, H. M.; Nigg, E. A. *Cell* **1989**, *56*, 379.
(70) Duverger, E.; Carpentier, V.; Roche, A. C.; Monsigny, M. *Exp. Cell Res.* **1993**, *207*, 197.
(71) Elliot, S. P.; Schmied, R.; Gabel, C. A.; Ambron, R. T. *J. Neurosci.* **1993**, *13*, 2424.
(72) Duverger, E.; Pellerin-Mendes, C.; Mayer, R.; Roche, A.-C.; Monsigny, M. *J. Cell Sci.* **1995**, *108*, 1325−1332.
(73) Duverger, E.; Roche, A. C.; Monsigny, M. *Glycobiology* **1996**, *6*, 381.
(74) Mullis, K. G.; Haltiwanger, R. S.; Hart, G. W.; Marchase, R. B.; Engler, J. A. *J. Virol.* **1990**, *64*, 5317.
(75) Chou, C.-F.; Omary, M. B. *J. Biol. Chem.* **1993**, *268*, 4465.
(76) Hong, J. S.; Engler, J. A. *J. Virol.* **1996**, *70*, 7071.
(77) Rogers, S.; Wells, R.; Rechsteiner. M. *Science* **1986**, *234*, 364.
(78) Davis, L. I.; Blobel, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7552.
(79) Finlay, D. R.; Newmeyer, D. D.; Price, T. M.; Forbes, D. J. *J. Cell. Biol.* **1987**, *104*, 189.
(80) Miller, M. W.; Hanover, J. A. *J. Biol. Chem.* **1994**, *269*, 9289.
(81) Schindler, M.; Hogan, M.; Miller, R.; DeGaetano, D. *J. Biol. Chem.* **1987**, *262*, 1254.
(82) Polet, H.; Molnar, J. *J. Cell Physiol.* **1988**, *135*, 47.
(83) Monsigny, M.; Midoux, M.; Mayer, R.; Roche, A.-C. *Biosci. Rep.* **1999**, *19*, 125.
(84) Ding, M.; Vandré, D. D. *J. Biol. Chem.* **1996**, *271*, 12555.
(85) Hagmann, J.; Grob, M.; Burger, M. M. *J. Biol. Chem.* **1992**, *267*, 14424.
(86) Holt, G.; Haltiwanger, R. S.; Torres, C.-R.; Hart, G. W. *J. Biol. Chem.* **1987**, *262*, 14847.
(87) Roquemore, E. P.; Dell, A.; Morris, H. R.; Panico, M.; Reason, A. J.; Savoy, L.-A.; Wistow, G. J.; Zigler, J. S., Jr.; Earles, B. J.; Hart, G. W. *J. Biol. Chem.* **1992**, *267*, 555.
(88) Luthi, T.; Haltiwanger, R. S.; Greengard, P.; Bahler, M. *J. Neurochem.* **1991**, *56*, 1493.
(89) Arnold, C. S.; Johnson, G. V. W.; Cole, R. N.; Dong, D. L.-Y.; Lee, M.; Hart, G. W. *J. Biol. Chem.* **1996**, *271*, 28741.
(90) King, I. A.; Hounsell, E. F. *J. Biol. Chem.* **1989**, *264*, 14022.
(91) Ku, N.-O.; Omary, M. B. *J. Biol. Chem.* **1995**, *270*, 11820.
(92) Dong, D. L.-Y.; Xu, S.-Z.; Chevrier, M. R.; Cotter, R. J.; Cleveland, D. W.; Hart, G. W. *J. Biol. Chem.* **1993**, *268*, 16679.
(93) Dong, D. L.-Y.; Xu, Z. S.; Hart, G. W.; Cleveland, D. W. *J. Biol. Chem.* **1996**, *271*, 20845.
(94) Caillet-Boudin, M.-L.; Strecker, G.; Michalski, J.-C. *Eur. J. Biochem.* **1989**, *184*, 205.
(95) Hong, J. S.; Engler, J. A. *J. Virol.* **1986**, *70*, 7071.
(96) Gill, S. R.; Wong, P. C.; Monteiro, M. J.; Cleveland, D. W. *J. Cell Biol.* **1990**, *111*, 2005.
(97) Wong, P. C.; Cleveland, D. W. *J. Cell Biol.* **1990**, *111*, 1987.
(98) Rechsteiner, M.; Rogers, S. W. *TIBS* **1996**, *96*, 267.
(99) Datta, B.; Ray, M. K.; Chakrabarti, D.; Wylie, D. E.; Gupta, N. K. *J. Biol. Chem.* **1989**, *264*, 20620.
(100) Ray, M. K.; Datta, B.; Chakraborty, A.; Chattopadhyay, A.; Meza-Keuthen, S.; Gupta, N. K. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 539.
(101) Chakraborty, A.; Saha, D.; Bose, A.; Chatterjee, M.; Gupta, N. K.; *Biochemistry* **1994**, *33*, 6700.
(102) Han, I.; Kudlow, J. E. *Mol. Cell Biol.* **1997**, *17*, 2550.
(103) Roos, M. D.; Su, K.; Baker, J. R.; Kudlow, J. E. *Mol. Cell. Biol.* **1997**, *17*, 6472.
(104) Yang, X.; Su, K.; Roos, M. D.; Chang, Q.; Paterson, A. J.; Kudlow, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6611.
(105) Han, I.; Roos, M. D.; Kudlow, J. E. *J. Cell Biochem.* **1998**, *68*, 50.
(106) Konrad, R. J.; Janowski, K. M.; Kudlow, J. E. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 26.
(107) Liu, K.; Paterson, A. J.; Chin, E.; Kudlow, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2820.
(108) Roos, M. D.; Hanover, J. A. *Biochem. Biophys. Res. Commun.* **2000**, *271*, 275.
(109) McClain, D. A.; Crook, E. D. *Diabetes* **1996**, *45*, 1003.
(110) Akimoto, Y.; Kreppel, L. K.; Hirano, H.; Hart, G. W. *Arch. Biochem. Biophys.* **2001**, *389*, 166.
(111) Robinson, L. J.; Razzack, Z. F.; Lawrence, J. C., Jr.; James, D. E. *J. Biol. Chem.* **1993**, *268*, 26422.
(112) Robinson, K. A.; Sens, D. A.; Buse, M. G. *Diabetes* **1993**, *42*, 1333.
(113) Yki-Jarvinen, H.; Virkamaki, A.; Daniels, M. C.; McClain, D.; Gottschalk, W. K. *Metabolism* **1998**, *47*, 449.
(114) Konrad, R. J.; Mikolaenko, I.; Tolar, J. F.; Liu, K.; Kudlow, J. E. *Biochem. J.* **2001**, *356*, 31.
(115) Gao, Y.; Parker, G. J.; Hart, G. W. *Arch. Biochem. Biophys.* **2000**, *383*, 296.
(116) Gao, Y.; Miyazaki, J.-I.; Hart, G. W. In preparation.

(117) Baron, A. D.; Zhu, J. S.; Zhu, J. H.; Weldon, H.; Maianu, L.; Garvey, W. T. *J. Clin. Invest.* **1995**, *96*, 2792.
(118) Patti, M. E.; Virkamaki, A.; Landaker, E. J.; Kahn, C. R.; Yki-Jarvinen, H. *Diabetes* **1999**, *48*, 1562.
(119) Griffith, L. S.; Mathes, M.; Schmitz, B. *J. Neurosci. Res.* **1995**, *41*, 270.
(120) Griffith, L. S.; Schmitz, B. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 424.
(121) Muller, U.; Steinberger, D.; Nemeth, A. H. *Neurogenetics* **1998**, *1*, 165.
(122) Bertram, L.; Blacker, D.; Mullin, K.; Keeney, D.; Jones, J.; Basu, S.; Yhu, S.; McInnis, M. G.; Go, R. C.; Vekrellis, K.; Selkoe, D. J.; Saunders, A. J.; Tanzi, R. E. *Science* **2000**, *290*, 2302.
(123) Comer, F. I.; Vosseller, K.; Wells, L.; Accavitti, M. A.; Hart, G. W. *Anal. Biochem.* **2001**, *293*, 169.
(124) Greis, K. D.; Hayes, B. K.; Comer, F. I.; Kirk, M.; Barnes, S.; Lowary, T. L.; Hart, G. W. *Anal. Biochem.* **1996**, *234*, 38.
(125) Haynes, P. A.: Aebersold, R. *Anal. Chem.* **2000**, *72*, 5402.
(126) Meinjohns, E.; Vargas-Berenguel, A.; Meldal, M.; Paulsen, H.; Bock, K. *Chem. Soc. Perkin Trans.* **1995**, *1*, 2165.
(127) Ohnishi, Y.; Ichikawa, M.; Ichikawa, Y. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1289.
(128) Hurtley, S.; Service, R.; Szuromi, P. *Science* **2001**, *291*, 2337.

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