Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1701–1705, March 1991
Biochemistry

Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins

(O-linked *N*-acetylglucosamine/nucleoplasmic and cytoplasmic glycosylation/Pro-Glu-Ser/Thr regions)

KELLY P. KEARSE AND GERALD W. HART

Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

Communicated by M. Daniel Lane, December 4, 1990

ABSTRACT **A unique form of nucleoplasmic and cytoplasmic protein glycosylation, O-linked GlcNAc, (*O*-GlcNAc) is present on proteins ranging from those of yeast to man, including many chromatin proteins, transcription factors, nuclear pore proteins, and certain types of cytoskeletal proteins. In this report we have studied the effects of cellular activation on *O*-GlcNAc-modified proteins, using T lymphocytes as a model system. Results indicate that the apparent levels of *O*-GlcNAc on many nuclear proteins increases rapidly after lymphocyte activation, returning to control levels after a few hours. In contrast, the apparent levels of *O*-GlcNAc on a distinct population of cytosolic proteins decreases rapidly after cellular activation and also returns to control levels after a few hours. These data are consistent with the hypothesis that *O*-GlcNAc is a regulatory modification and suggest that *O*-GlcNAc modification may play an important role in the early stages of T-lymphocyte activation.**

A unique form of nucleoplasmic and cytoplasmic protein glycosylation in which *N*-acetylglucosamine monosaccharides are O-glycosidically linked to serine or threonine residues (*O*-GlcNAc) has been described in organisms including viruses, yeast, protozoa, insects, frogs, rodents, and man but not bacteria (for reviews, see refs. 1 and 2). *O*-GlcNAc-modified proteins are enriched in nuclei (3) and nuclear pores and are particularly dense in chromatin (4). Subsets of the family of proteins compromising RNA polymerase II transcription factors, but not RNA polymerase I or III transcription factors, appear to be glycosylated by *O*-GlcNAc (5, 6). One such glycosylated transcription factor, c-fos, is important for the activation of the interleukin 2 gene, which encodes a lymphokine critical to T-cell activation and commitment (7).

Aside from the apparent role of *O*-GlcNAc-bearing nuclear pore glycoproteins in nuclear transport (8–11) and the possible involvement of *O*-GlcNAc in enhancing the activities of transcription factors (5), virtually nothing is known concerning the functions of these monosaccharide moieties. It has been suggested that *O*-GlcNAc might be required for the proper assembly of multimeric protein complexes (12) or that it might serve as a nuclear targeting signal (13) analogous to the mannose 6-phosphate-mediated targeting of proteins into lysosomes (14). Based upon the sequences surrounding sites of attachment of *O*-GlcNAc on three proteins (15), it also has been suggested that *O*-GlcNAc might reversibly block potential phosphorylation sites on proteins (1, 12). The sites of attachment of *O*-GlcNAc are similar to the phosphorylation sites of several known serine/threonine kinases (for a review, see ref. 16) and a recently described growth factor-sensitive proline-directed serine/threonine kinase (17, 18). In addition, the sites of attachment of *O*-GlcNAc are strikingly homologous to previously defined proline-acidic residue-(serine or threonine) regions, which are believed to regulate proteolysis of rapidly turning-over regulatory proteins (19).

To further evaluate the hypothesis that *O*-GlcNAc is a regulatory modification, we have examined the effects of cellular activation of murine T lymphocytes on *O*-GlcNAc-modified proteins. We report here that short-term activation of T lymphocytes results in rapidly decreased levels of *O*-GlcNAc in cytosolic proteins and a concomitant increase in the levels of *O*-GlcNAc-modified proteins in the nuclear-enriched fractions of the cell. Kinetic analyses of these changes indicate that they are transient, with individual proteins being affected at different rates. These findings suggest that *O*-GlcNAc addition/removal appears to be highly regulated and may be an important step in the early stages of T-cell activation.

MATERIALS AND METHODS

Mice. Mice (C57BL/6 strain) used in these studies were from The Jackson Laboratory and were 6–12 weeks of age. Breeding colonies were maintained on the premises.

Isolation of Splenic T Cells. Mice were sacrificed by cervical dislocation, and spleens were removed and transferred to sterile tissue culture tubes containing RPMI 1640 medium (GIBCO) with 10% heat-inactivated fetal calf serum (GIBCO) and 50 μM 2-mercaptoethanol. Single-cell suspensions were prepared by homogenization in a Stomacher device (Tekmar, Cincinnati). Cells were centrifuged (1200 rpm for 10 min), and the resulting pellet was depleted of erythrocytes by centrifugation over Histopaque (Sigma) or treatment with isotonic 17 mM Tris/14.4 mM NH_4Cl (pH 7.2). Splenic T lymphocytes were isolated by passage over nylon wool columns as described (20).

Cell Lines. The T-cell hybridomas DO-11.0 and SK45.1 were provided by J. Kappler and P. Marrack (National Jewish Center for Immunology and Respiratory Medicine, Denver), and were maintained as described (21).

Reagents. Concanavalin A (Con A) was obtained from Sigma and prepared fresh for each experiment. Phorbol 12-myristate 13-acetate (PMA; Sigma) was maintained as a stock solution (1 mM) and stored at −20°C until use. The calcium ionophore ionomycin (free acid) was purchased from Calbiochem and kept frozen in dimethyl sulfoxide at 2 mg/ml until use. Bovine milk galactosyltransferase (GalTase; Sigma) was autogalactosylated prior to use in cell-labeling experiments as described (22).

Activation Experiments. Splenic T cells were washed once in fresh medium and resuspended at a final concentration of 5×10^6 cells per ml, and tubes were placed in a CO_2 incubator (5% CO_2/95% air) at 37°C for the duration of the experiment. Mitogen (Con A) was added to obtain a final concentration of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: *O*-GlcNAc, O-linked *N*-Acetylglucosamine; GalTase, bovine milk galactosyltransferase; Con A, concanavalin A; CHX, cycloheximide; PMA, phorbol 12-myristate 13-acetate.

4–5 μg/ml. T-cell hybridomas were washed once in fresh medium, and viable cells were separated via centrifugation over Histopaque (Sigma). Cells were then washed twice in fresh medium, resuspended at a final concentration of 5×10^6 cells per ml, and placed in a CO_2 incubator as described above. For cell stimulation, PMA was added with ionomycin directly to cell suspensions to obtain final concentrations of 10 and 250 ng/ml, respectively, whereas Con A was added alone to obtain a final concentration of 15 μg/ml. Experiments were terminated by the addition of 10 vol of cold medium, and cells were immediately centrifuged (1200 rpm for 10 min at 4°C). Cell pellets were washed and then resuspended in RPMI 1640 10% fetal calf serum containing digitonin at 100 μg/ml for splenic T cells and 500 μg/ml for hybridoma T cells, and the suspensions were incubated at room temperature for 10 min to prepare cytosolic material (23). The efficiency of permeabilization was determined by the inability of the cells to exclude trypan blue (GIBCO). After permeabilization, the cellular membrane fractions were removed by centrifugation at 200 × g, and the soluble released fraction was removed. In some experiments, the remaining pellets were resuspended in buffer H (50 mM Hepes/500 mM NaCl/2% Triton X-100/0.1% protease inhibitor cocktail/0.02% NaN_3) and called "digitonin pellet material." Samples were stored at −20°C until labeling.

Enzymatic Labeling Experiments. GalTase was used to label *O*-GlcNAc-modified proteins with [^{3}H]galactose as described (3, 22). Reactions in the presence of GalTase were initiated by the addition of radioactive nucleotide sugar (e.g., UDP-[^{3}H]galactose), allowed to proceed for 90 min at 37°C, and terminated by the addition of 10–20 μl of GalTase stop buffer (10% SDS/10 mM EDTA).

Other Analytical Procedures. Densitometry was performed on a Biomed Instruments (Fullerton, CA) Zeineh soft-laser scanning densitometer. Nonequillibrium pH gradient electrophoresis was performed as described (24), and SDS/PAGE was performed as described by Laemmli (25).

RESULTS

Mitogenic Activation of T Lymphocytes Induces Rapid Changes in the Levels of Cytosolic and Nuclear *O*-GlcNAc-Modified Proteins. Purified murine splenic T lymphocytes were incubated with various doses of the T-cell mitogen Con A for 1 hr at 37°C and washed, and cytosolic fractions were prepared by mild digitonin treatment (23). Cytosolic material was enzymatically labeled with [^{3}H]galactose by using UDP-[^{3}H]galactose and purified bovine milk GalTase under saturating conditions of enzyme and donor substrate (unpublished data and ref. 26). As expected (22), nearly all of the [^{3}H]galactose radioactive probe was located on protein-bound *O*-GlcNAc monosaccharides, based upon resistance to peptide *N*-glycosidase F, and was released as the disaccharide Galβ1-4GlcNAcitol, upon alkali-induced β-elimination (see the legend to Fig. 1). After only 1 hr of Con A activation, the levels of *O*-GlcNAc in the cytosol of splenic T cells decreased by nearly half (Fig. 1). The apparent level of *O*-GlcNAc on most but not all (see asterisks Fig. 1) cytosolic proteins was drastically reduced by short-term cellular activation. Similar but more protein-specific results were seen in the cytosolic fractions of Con A-activated DO-11.10 (27) hybridoma cells (Fig. 2 *Left*). Coomassie blue-detectable protein levels appeared to be unchanged by this short-term activation, but the apparent levels of *O*-GlcNAc associated with several proteins declined markedly, while that on at least two cytosolic proteins (unfilled arrowheads) increased. In contrast to the cytosol, the apparent levels of *O*-GlcNAc on most glycoproteins in the nuclear-enriched fractions (Fig. 2 *Right*) increased in response to short-term activation. These types of studies have been repeated several times with different activating agents and both primary splenic T cells and the T-cell hybridomas,

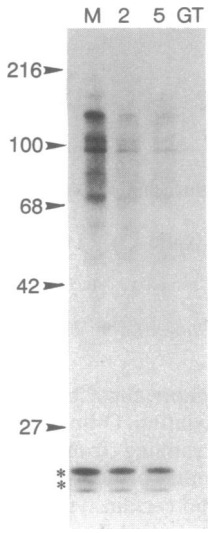

FIG. 1. Effect of short-term cell activation on cytosolic *O*-GlcNAc-modified proteins of splenic T lymphocytes. Splenic T cells (5×10^6) were incubated with various amounts of Con A for 60 min at 37°C in cell culture medium. Digitonin-released cytosolic material was prepared as described and labeled with GalTase. Total [^{3}H]galactose incorporation corresponded to 270,700 cpm in medium with no Con A stimulation, 152,840 cpm with Con A at 2 μg/ml, and 147,330 cpm with Con A at 5 μg/ml. GalTase alone showed 1950 cpm. Material was ≈92% resistant to digestion with peptide *N*-glycosidase F and 94–95% sensitive to alkali-induced β-elimination (unpublished data). Radiolabeled products were electrophoresed through a SDS/10% PAGE gel, and the gel was dried and autofluorographed. Asterisks indicate small molecular mass proteins that do not appear to change after cell activation; mobilities of the standards (shown in kDa) are as marked. Lanes: M, medium; 2, 2 μg of Con A; 5, 5 μg of Con A; GT, GalTase alone.

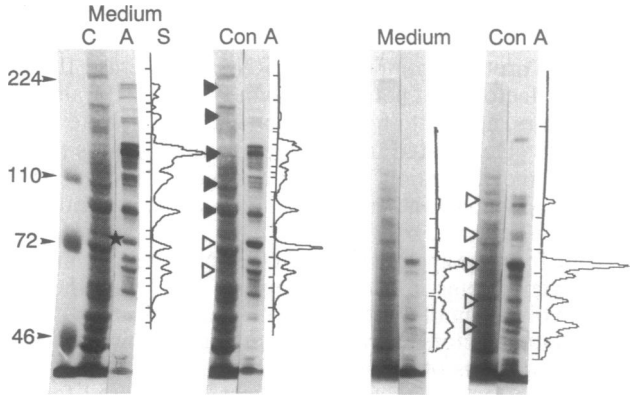

FIG. 2. Effect of short-term cell activation on *O*-GlcNAc-modified proteins of DO-11.10 hybridoma T cells. DO-11.10 cells (5×10^6 cells) were incubated with Con A (15 μg/ml) for 60 min; digitonin-released cytosolic material (*Left*) and digitonin pellet material (*Right*) were prepared as described and labeled with GalTase. Radiolabeled products were electrophoresed through a SDS/10% PAGE gel, and the gel was dried and autofluorographed. Mobilities of molecular mass standards (shown in kDa) are as marked. ▶, Decrease of *O*-GlcNAc-modified proteins following cell activation; ▷, increase of *O*-GlcNAc-modified proteins following cell activation; ★, 75-kDa protein in the cytosolic fraction that appeared to increase following activation. Lanes: C, Coomassie stain; A, autofluorograph; S, densitometry scan of autofluorograph. Radiolabeled material of the cytosolic and pellet fractions was 91% and 85% resistant to digestion with protein *N*-glycosidase F, respectively, and 96% sensitive (cytosolic fraction) and 91% sensitive (pellet) to alkali-induced β-elimination (unpublished data).

DO-11.10 (27) and SK45.1 (28). While the specific effects on each protein appear to be dependent on cell type and the activator used, the overall effect of *O*-GlcNAc in each subcellular fraction is similar in every case to that described here.

Activation-Induced Changes in Levels of *O*-GlcNAc-Modified Proteins Are Transient. To examine the kinetics of activation-induced changes in *O*-GlcNAc-modified proteins, T-cell hybridoma cells were activated by treating with PMA and a calcium ionophore (29) for various lengths of time up to 6 hr. Cell viability (measured by trypan blue exclusion) was >95% in all groups (unpublished data). Subsequently, both cytosolic and nuclear-enriched fractions were assayed for *O*-GlcNAc-bearing proteins by the GalTase method. Even though the Coomassie blue-detectable proteins did not change over the time course of the experiment, activation induced a rapid decrease in the apparent level of *O*-GlcNAc on most cytosolic proteins and concomitantly caused a rapid increase in the apparent levels of the saccharide on a different set of proteins in the nuclear-enriched fractions (Figs. 3 and 4). Most interestingly, these activation-induced changes were transient, showing a "wave-like" pattern over time.

The changes in *O*-GlcNAc on individual proteins fell into several categories. In activated hybridoma cytosolic fractions, the *O*-GlcNAc levels associated with most proteins (except for a decrease in a major band at 210 kDa) did not change substantially until after about 2 hr (Figs. 3*A* and 4*A*). Also, as was seen in mitogen-activated cells, the levels of *O*-GlcNAc associated with a cytosolic protein of 80 kDa appeared to increase at short times after activation. Overall, the apparent levels of *O*-GlcNAc on the majority of the activated hybridoma cytosolic proteins reached their lowest levels in about 3 hr after activation and appeared to increase gradually thereafter. The activation-induced changes in *O*-GlcNAc of the nuclear-enriched fractions were more striking, and the transient ("wave-like") aspect of the phenomenon was also more evident (Figs. 3*B* and 4*B*). Similar results were obtained with SK45.1 (28) (unpublished data). Unfortunately, we were unable to carry out these activation experiments for longer incubation times (8–10 hr) because of a marked loss in viability (unpublished data). Ashwell *et al.* (30) have shown that the decrease in viability of hybridoma T cells after cellular activation is due to a block in the G_1/S boundary of the cell cycle.

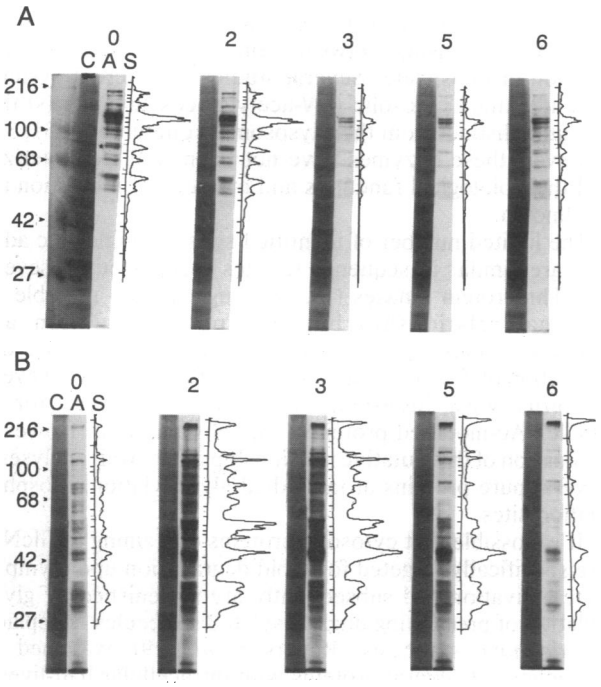

FIG. 3. Activation-induced changes of *O*-GlcNAc-modified proteins of T cells appear to be transient in nature. DO-11.10 hybridoma T cells (5×10^6) were incubated with PMA (10 ng/ml) and ionomycin (250 ng/ml) for various times as indicated in hours above the lanes. All groups were incubated in parallel for a total of 6 hr. Viability was measured by trypan blue uptake at 6 hr and found to be >95% in all groups. Digitonin-released cytosolic material (*A*) and digitonin pellet material (*B*) were prepared and labeled with GalTase as described followed by SDS/PAGE. Lanes: C, Coomassie stain; A, autofluorograph; S, densitometry scan of autofluorograph.

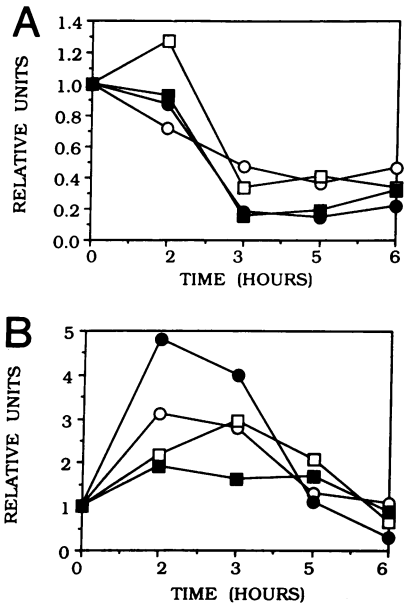

FIG. 4. Specific *O*-GlcNAc-modified proteins in cytosolic and nuclear-enriched fractions change at different rates. (*A*) Peaks corresponding to specific proteins in the cytosolic fractions shown in Fig. 3*A* were cut out, weighed, and integrated by using a digital balance; proteins were of 210 kDa (○), 150 kDa (●), 80 kDa (□), and 60 kDa (■). Values were normalized, and control (no stimulus) groups were assigned a value of 1.0. (*B*) Same as *A* but with nuclear enriched fraction proteins shown in Fig. 3*B* of 115 kDa (○), 55 kDa (●), 45 kDa (□), and 32 kDa (■).

Two-Dimensional Gel Analyses Show That the Activation-Induced Changes in *O*-GlcNAc-Modified Proteins Are Complex and Protein Specific. To better examine the protein-specific changes in *O*-GlcNAc-modified proteins as a result of cellular activation, DO-11.10 hybridoma cells were activated with PMA and ionomycin for various lengths of time, and the total cellular *O*-GlcNAc-bearing proteins were analyzed by two-dimensional gel electrophoresis. As seen previously, activation rapidly induced pronounced changes in *O*-GlcNAc-modified proteins (Fig. 5). These glycoproteins appeared to fall into two major groups. Most of the *O*-GlcNAc-bearing proteins were large (>80 kDa) and had basic isoelectric points, while another smaller cluster appeared to be of moderate size with acidic isoelectric points. The apparent levels of *O*-GlcNAc on some proteins appeared first to decrease and then to return to normal after activation; others appeared to do the reverse. For example, *O*-GlcNAc associated with protein "a" (see Fig. 5; molecular mass, 100 kDa; pI, ≈4.3) rapidly decreased to its lowest level 4 hr after activation and was restored to slightly above control levels by 5 hr. Similar changes were seen for other proteins (e.g., "g" and "h" in Fig. 5). For some proteins, (e.g., "b" in Fig. 5; molecular mass, 80 kDa; pI, ≈4.7), the apparent *O*-GlcNAc levels increased after 1 hr and rapidly decreased 2–4 hr after activation. Other proteins appeared to show a curious cyclic pattern in their apparent levels of *O*-GlcNAc over the time

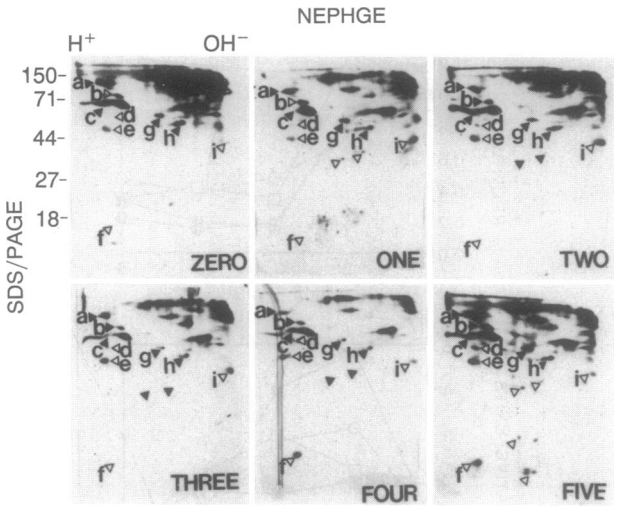

FIG. 5. Two-dimensional gel analysis of activation-induced changes of *O*-GlcNAc-modified DO-11.10 T-cell proteins. DO-11.10 T cells (5×10^6) were cultured for 5 hr in the presence of PMA (10 ng/ml) and ionomycin (250 ng/ml) for the time period indicated. Viability was assayed by trypan blue uptake and found to be >95% in all groups (unpublished data). Digiton-released cytosolic and pellet fractions were prepared as described, labeled separately with GalTase, mixed together, and electrofocused by nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension (horizontal) followed by SDS/PAGE (vertical). Proteins that show marked changes are designated with arrowheads: ▶, decrease relative to zero hour (no stimulus) groups; ▷, increase relative to zero hour.

course of these experiments (e.g., protein "c" in Fig. 5; molecular mass, ≈68 kDa; pI, ≈4.2), whereas for a number of different proteins, the apparent levels increased rapidly after activation (e.g., "d," "e," "f," and "i" in Fig. 5).

Protein Synthesis Requirements of the Activation-Induced Changes in *O*-GlcNAc-Modified Proteins Are Protein Specific. To determine the involvement of *de novo* protein synthesis in the above changes, identical analyses as described in Fig. 5 were carried out, but cells were pretreated with cycloheximide (CHX) prior to activation. Controls labeled with [^{35}S]methionine showed that protein synthesis was blocked >99% (unpublished data). CHX did not effect the activation-induced changes seen for some proteins (e.g., "c," "g," and "h" in Fig. 6), while it appeared to abrogate the changes in others (e.g., "a," "d," "f," and "i"; compare Figs. 5 and 6). As seen previously, a protein migrating at 75–80 kDa increased slightly after activation and then decreased 2–4 hr after stimulation (protein "b"; pI, ≈4.4). In CHX-treated cells, this protein decreased dramatically at 1 hr and then "reappeared" 2–5 hr after stimulation, presumably because of (re)addition of *O*-GlcNAc (see *Discussion*). Similar results were observed in CHX-treated splenic T cells (unpublished data). Thus, inhibition of *de novo* protein synthesis affects activation-induced changes in *O*-GlcNAc-bearing proteins; however, the extent of this effect is highly protein specific, suggesting that several cellular mechanisms are involved in the regulation of *O*-GlcNAc after cell stimulation (see below).

DISCUSSION

The data presented in this study show that the apparent levels of *O*-GlcNAc on specific nuclear or cytoplasmic proteins change both rapidly and transiently as the result of the activation of lymphocytes by various stimuli. The apparent levels of *O*-GlcNAc on many cytosolic proteins rapidly and transiently decline as a result of lymphocyte activation by mitogens and by phorbol esters. Possible explanations for these findings are (*i*) *O*-GlcNAc is specifically removed by

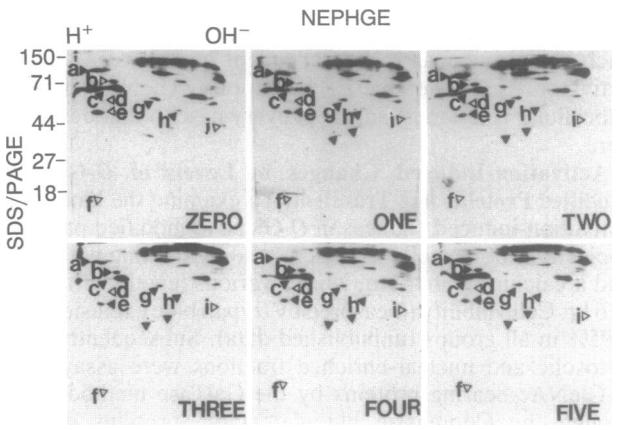

FIG. 6. Effect of CHX on activation-induced changes of *O*-GlcNAc-modified DO-11.10 T-cell proteins. DO-11.10 T cells (5×10^6) were incubated with CHX (50 μM) or medium (control groups) for 45 min at 37°C. At the end of this incubation period, cells were cultured for an additional 5 hr in the presence of PMA (10 ng/ml) and ionomycin (250 ng/ml) for the time period indicated. CHX was present throughout the experiment. Viability was assayed by trypan blue uptake and found to be >95% in all groups (unpublished data). Digitonin-released cytosolic and pellet fractions were analyzed as described in Fig. 5. Changes in individual proteins are designated by arrowheads: ▶, decrease relative to zero hour (no stimulus) groups; ▷, increase relative to zero hour.

β-*N*-acetylglucosaminidase(s) stimulated by activation; (*ii*) *O*-GlcNAc-bearing proteins are specifically proteolyzed, possibly by Pro-Glu-(Ser or Thr)-mediated proteolytic enzymes (see below); (*iii*) *O*-GlcNAc is masked, perhaps by the addition of another sugar by a regulated *O*-GlcNAc-specific glycosyltransferase; or (*iv*) *O*-GlcNAc proteins are rapidly translocated from the cytoplasm to the nucleus upon activation.

The first hypothesis is exciting because it suggests a possible relationship between removal of *O*-GlcNAc and addition of phosphate. Several studies in different systems have identified cytosolic β-*N*-acetylglucosaminidase(s) that are quite distinct from their lysosomal counterparts (31, 32); however, these enzymes have not been well characterized and their biological functions and methods of regulation are not known.

The limited number of identified sites of *O*-GlcNAc addition are similar in sequence to sites recognized by several Ser/Thr protein kinases (13, 16), suggesting a possible reciprocal relationship between phosphorylation and *O*-GlcNAc addition (3). Preliminary evidence indicates that activation of T cells results in the phosphorylation of several proteins with identical electrophoretic behavior as *O*-GlcNAc-modified proteins (unpublished data). However, evaluation of this putative relationship must await analyses of several pure proteins at individual glycosylation/phosphorylation sites.

It is possible that cytosolic proteins containing *O*-GlcNAc are specifically targeted for rapid degradation upon lymphocyte activation and subsequently are replenished by glycosylation of preexisting unglycosylated molecules or replaced by *de novo* synthesis. Rogers *et al.* (19) examined the sequences of several proteins with intracellular half-lives of less than 2 hr and noted that each contained one or more regions rich in proline (P), glutamic acid (E) (and to a lesser extent apartic acid), and serine (S) or Thr (T), referred to as "PEST" domains in single-letter code. This motif is not only similar to known sites of *O*-GlcNAc attachment, but synthetic PEST peptides are good *in vitro* substrates for an *O*-GlcNAc:protein-glycosyltransferase (33). Two of the molecules originally identified as rapidly degraded PEST proteins have been shown subsequently to contain also

O-GlcNAc *in vivo*—i.e., c-fos and c-jun (5). Attachment of *O*-GlcNAc to PEST domains has the potential to either block or target specific protease recognition. However, the observation that the *O*-GlcNAc on cytosolic proteins appears to rapidly decrease and is restored to approximately control levels over a few hours suggests that the majority of cytosolic *O*-GlcNAc proteins are not simply being destroyed as a result of activation but rather may be undergoing a cycle of *O*-GlcNAc removal and addition. Interestingly, CHX has no effect on this apparent "cycle" for several proteins, while affecting both disappearance and reappearance for others.

Several lines of evidence indicate that cytosolic *O*-GlcNAc residues are not masked by the covalent attachment of other moieties during lymphocyte activation. First, when the activation studies are carried out using metabolically incorporated [^{3}H]glucosamine, the apparent disappearance and reappearance of *O*-GlcNAc was found to be similar to that described with the galactosyltransferase probe (unpublished data). Furthermore, analyses of the metabolically labeled *O*-GlcNAc by alkali-induced β-elimination followed by high-resolution sizing of the released saccharides have repeatedly shown that the *O*-GlcNAc in lymphocytes (unpublished data) and other cell-types (R. S. Haltiwanger, G. D. Holt, and G.W.H., unpublished data) exists almost entirely in the form of monosaccharide residues. Recent careful analyses of the topological localization of an endogenous galactosyltransferase that acts on *O*-GlcNAc *in vitro* have shown that all of this enzyme is either lumenal or on the cell surface (34), and thus it likely does not act on nucleoplasmic or cytosolic *O*-GlcNAc *in vivo*.

It is also unlikely that the disappearance of cytosolic *O*-GlcNAc could be explained by transport into the nuclear compartment. A comparison of the *O*-GlcNAc-modified proteins disappearing in the cytosol to those concomitantly increasing in the nuclear fractions (Figs. 3 and 4) clearly shows that the populations of *O*-GlcNAc-modified proteins are largely distinct in each fraction. Also, two-dimensional gel analyses of total cellular *O*-GlcNAc (Figs. 5 and 6) showed increases and decreases in many *O*-GlcNAc-containing proteins; if translocation from one intracellular compartment to another were occurring, these changes would not have been observed. An alternative explanation for these findings is that *O*-GlcNAc-modified proteins are present in precursor forms in the cytosol and attain their final molecular mass in the nucleus after transport, thus accounting for the changes seen electrophoretically. The identification and characterization of individual *O*-GlcNAc-containing proteins will allow us to address these questions more specifically.

Unlike what is observed in the cytosol, lymphocyte activation induces a rapid and transient increase in the apparent levels of *O*-GlcNAc on proteins in the nuclear-enriched fraction. This suggests that either the total glycoproteins are rapidly increasing in response to activation or that activation stimulates transport of *O*-GlcNAc:protein glycosyltransferase (33) to the nucleus. Alternatively, nucleoplasmic forms of this enzyme may be activated following stimulation. As previously observed for cytosolic *O*-GlcNAc proteins, CHX pretreatment does not block the observed changes in several proteins but does abrogate the transient increases seen in others.

Most *O*-GlcNAc-bearing proteins appear to be present in small amounts, and those that have been identified appear to be transcriptional regulatory factors, components of transport systems, or mediators of cytoskeletal interactions. It appears likely that if our hypothesis with respect to *O*-GlcNAc's biological function is correct, then changes similar to that described here must also occur in other biological systems undergoing phenotypic changes. Preliminary studies suggest that variations in apparent levels of *O*-GlcNAc on nuclear and cytoplasmic proteins occur during progression of several different cell types through the cell cycle (W. G. Kelly, K.P.K., and G.W.H., unpublished data). Ultimately, careful analyses of individual proteins, measuring site-specific stoichiometry in conjunction with quantitative bioassays, will be required to evaluate critically the functional significance of *O*-GlcNAc addition/removal on particular proteins. The known importance of many identified *O*-GlcNAc-bearing proteins in cellular activation and the data described in this report encourage further investigation into the role(s) of this form of intracellular glycosylation in fundamental cellular processes.

We thank Drs. J. Kappler and P. Marrack for their generosity in providing the T cell hybridoma lines used in this study, members of the Hart lab for their help in reviewing this manuscript, and Dr. Ann Hubbard for the use of the densitometry scanner. The expert technical assistance of Irene Wood is greatly appreciated. This work was supported by National Institutes of Health Grant HD13563 and Postdoctoral Fellowship GM 13771-01 awarded to K.P.K.

1. Hart, G. W., Holt, G. D. & Haltiwanger, R. S. (1988) *Trends Biochem. Sci.* **13**, 380–384.
2. Hart, G. W., Haltiwanger, R. S., Holt, G. D. & Kelly, W. G. (1989) *Annu. Rev. Biochem.* **58**, 841–874.
3. Holt, G. D. & Hart, G. W. (1986) *J. Biol. Chem.* **261**, 8049–8057.
4. Kelly, W. G. & Hart, G. W. (1989) *Cell* **57**, 243–251.
5. Jackson, S. P. & Tjian, R. (1988) *Cell* **55**, 125–133.
6. Jackson, S. P. & Tjian, R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1781–1785.
7. Crabtree, G. R. (1989) *Science* **243**, 355–361.
8. Finlay, D. R., Newmayer, D. D., Price, T. M. & Forbes, D. J. (1987) *J. Cell Biol.* **104**, 189–200.
9. Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L. & Hart, G. W. (1987) *J. Cell Biol.* **104**, 1157–1164.
10. Hanover, J. A., Cohen, C. K., Willingham, M. C. & Park, M. K. (1987) *J. Biol. Chem.* **262**, 9887–9894.
11. Davis, L. I. & Blobel, G. (1986) *Cell* **45**, 699–709.
12. Holt, G. D., Haltiwanger, R. S., Torres, C. R. & Hart, G. W. (1987) *J. Biol. Chem.* **262**, 14847–14850.
13. Schindler, M., Hogan, M., Miller, R. & De Gaetano, D. (1987) *J. Cell Biol.* **104**, 1143–1156.
14. Kornfield, S. (1990) *Biochem. Soc. Trans.* **18**, 367–374.
15. Hart, G. W., Haltiwanger, R. S., Holt, G. D. & Kelly, W. G. (1989) *Ciba Found. Symp.* **145**, 102–118.
16. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613.
17. Vulliet, P. R., Hall, F. L., Mitchell, J. P. & Hardie, D. G. (1989) *J. Biol. Chem.* **264**, 16292–16298.
18. Hall, F. J., Mitchell, J. P. & Vulliet, P. R. (1990) *J. Biol. Chem.* **265**, 6944–6948.
19. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234**, 364–368.
20. Henry, C., Chen, Y. U., Stout, R. & Swain, S. L. (1980) in *Selected Methods in Cellular Immunology*, eds. Mishell, B. B. & Shiigi, S. M. (Freeman, San Francisco), Vol. 2, pp. 182–185.
21. White, J., Haskins, K. M., Marrack, P. & Kappler, J. W. (1983) *J. Immunol.* **130**, 1033–1042.
22. Torres, C. R. & Hart, G. W. (1984) *J. Biol. Chem.* **259**, 3308–3317.
23. Whiteheart, S. W., Shenbagamurthi, P., Chen, L., Cotter, R. J. & Hart, G. W. (1989) *J. Biol. Chem.* **264**, 14334–14341.
24. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
25. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
26. Whiteheart, S. W., Passaniti, A., Reichner, J. S., Holt, G. D., Haltiwanger, R. S. & Hart, G. W. (1989) *Methods Enzymol.* **179**, 83–95.
27. Kappler, J. W., Skidmore, B., White, J. & Marrack, P. (1981) *J. Exp. Med.* **153**, 1198–1214.
28. Haskins, K., Hannum, C., White, J., Roehm, N., Kubo, R., Kappler, J. & Marrack, P. (1984) *J. Exp. Med.* **160**, 452–470.
29. Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R. & Sigal, N. H. (1990) *J. Immunol.* **144**, 251–258.
30. Ashwell, J. D., Cunningham, R. E., Noguchi, P. D. & Hernandez, D. (1987) *J. Exp. Med.* **165**, 173–194.
31. Overdijk, B., Van Der Kroef, W. M. J., Van Steijn, G. J. & Lisman, J. J. W. (1981) *Biochim. Biophys. Acta* **659**, 255–266.
32. Izumi, T. & Suzuki, K. (1983) *J. Biol. Chem.* **258**, 6991–6999.
33. Haltiwanger, R. S., Holt, G. D. & Hart, G. W. (1990) *J. Biol. Chem.* **265**, 2563–2568.
34. Russo, R. N., Shaper, N. L. & Shaper, J. H. (1990) *J. Biol. Chem.* **265**, 3324–3331.