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Cytologic assessment of nuclear and cytoplasmic O-linked *N*-acetylglucosamine distribution by using anti-streptococcal monoclonal antibodies

(nucleus/nuclear lamina/IgG3/galactosyltransferase)

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ABSTRACT **Recent studies have demonstrated the existence of single O-linked *N*-acetylglucosamine (*O*-GlcNAc) residues on cytoplasmic and nuclear glycoproteins. Labeled lectin and enzymatic techniques have been used to identify *O*-GlcNAc-bearing proteins, but no antibodies generally reactive with such O-linked GlcNAc moieties have been described. We have previously characterized monoclonal antibodies (mAbs) specific for the GlcNAc residues of streptococcal group A carbohydrate, which is composed of a polyrhamnose backbone with GlcNAc side chains. We now report that these mAbs recognize *O*-GlcNAc-bearing proteins. By immunofluorescence, the mAbs reacted strongly with the nuclear periphery and nucleoplasm of mammalian cells and stained the cytoplasm less intensely. The distribution was not consistent with labeling of the endoplasmic reticulum, Golgi complex, or plasma membrane. Furthermore, the staining pattern of a mutant cell line, which retains terminal GlcNAc residues on many N-linked glycans, was indistinguishable from that of wild-type cells. Nuclear and cytoplasmic staining were inhibited by free GlcNAc and were completely abolished by galactosylation of terminal GlcNAc residues. Indirect ELISA demonstrated GlcNAc- and galactosylation-inhibitable binding of the mAbs to a 65-kDa human erythrocyte cytosolic protein known to contain *O*-GlcNAc. Thus, these mAbs react with *O*-GlcNAc without apparent influence of peptide determinants, do not show detectable binding to *N*- or *O*-glycans, and, therefore, represent a valuable tool for the study of *O*-GlcNAc moieties. In addition, these mAbs provide the first cytologic analysis of the distribution of *O*-GlcNAc residues throughout the nucleus and the cytoplasm of mammalian cells.**

Single O-linked *N*-acetylglucosamine (*O*-GlcNAc) residues are present on many cytoplasmic and nuclear glycoproteins (1, 2). Although these glycoproteins are concentrated in nuclear pores (3) and have been implicated in nucleocytoplasmic transport (e.g., see ref. 4), the broader significance of this carbohydrate modification is unknown. It is striking that transcription factors (5), the erythrocyte cytoskeletal protein band 4.1 (6), and polytene chromosomes (7) also bear *O*-GlcNAc.

O-GlcNAc moieties have generally been detected by using galactosyltransferase to attach labeled galactose to *O*-GlcNAc-bearing proteins or by the binding of labeled wheat germ agglutinin to such proteins (8, 9). Neither method is ideal. For example, the galactosyltransferase procedure is not specific for *O*-GlcNAc monosaccharides, labeling terminal GlcNAc residues of glycans (10), while wheat germ agglutinin binds to terminal GlcNAc and sialic acid residues without absolute linkage specificity (11). Therefore, antibodies that bind single *O*-GlcNAc residues would be of great value in the study of *O*-GlcNAc and the glycoproteins that bear this structure. Although several monoclonal antibodies (mAbs) elicited against nuclear pore proteins recognize epitopes that include *O*-GlcNAc (3), these epitopes also include protein determinants present on subsets of pore complex polypeptides. Furthermore, these mAbs stain only the nucleus of whole cells (12), suggesting that they recognize a subset of the total cellular proteins containing *O*-GlcNAc.

We have previously studied the immunochemical properties of mAbs that recognize GlcNAc residues of streptococcal group A carbohydrate (GAC; ref. 13), a cell wall polysaccharide composed of a polyrhamnose backbone and β-1,3-linked GlcNAc side chains (14). We now report that these anti-GAC mAbs react with *O*-GlcNAc-bearing nuclear and cytoplasmic glycoproteins.

METHODS

Cell Culture. FR 3T3 rat fibroblasts (15) and clone 9 rat hepatocytes (American Type Culture Collection) were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum (GIBCO) at 37°C in 5% CO_2/95% air and subcultured three times weekly. For experiments, cells were plated onto sterile glass coverslips (diameter, 12 mm) (Bellco Glass) and used in subconfluent condition after 3 days of growth.

Production and maintenance of hybridomas secreting HGAC 85 (16) and HGAC 39 (17) IgG3 mAbs have been described. Tissue culture supernatants of HGAC 85 and HGAC 39 were used for immunofluorescence. In immunofluorescence experiments, culture supernatant from an isotype-matched mAb, 96-G, with specificity for pneumococcal cell wall polysaccharide was used as a negative control (18). For ELISAs, HGAC 39 was affinity purified as described (17) by using GlcNAc-agarose (Sigma). An IgM anti-GAC mAb, HGAC 49 (19), was also used in some experiments. In ELISAs, IgG3 anti-*p*-azophenylarsonate P65J4-1 (20) culture supernatant was used as a negative control for HGAC 85, and FLOPC 21 (IgG3 myeloma protein; Sigma) was the control for affinity-purified HGAC 39.

Immunofluorescence. Cells were fixed for 8 min at room temperature in 2% formaldehyde in phosphate-buffered saline (PBS), washed three times in PBS, quenched for 10 min with 50 mM NH_4Cl in PBS, and permeabilized by a 10-min incubation in PBS containing 0.5% Triton X-100 and 20% normal goat serum (NGS). The mAbs were generally used as culture supernatants to which NGS was added to 15%, and other antibodies were diluted in PBS containing 15% NGS. GlcNAc or GalNAc was added to mAbs from a 220 mM stock to a final concentration of 22 mM. Coverslips were inverted

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Abbreviations: GAC, streptococcal group A carbohydrate; mAb, monoclonal antibody; NGS, normal goat serum.
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on 12-μl drops of antibody and incubated for 45 min at room temperature. The coverslips were washed three to five times with PBS containing 5% NGS between antibody incubations. Binding of the mouse mAbs was detected by incubation with affinity-purified rabbit anti-mouse immunoglobulin (5 μg/ml) (Cappel), followed by affinity-purified rhodamine-conjugated goat anti-rabbit immunoglobulin (5 μg/ml) (Cappel). Texas Red-conjugated wheat germ agglutinin (EY Laboratories) was used at 20 μg/ml. RL12 anti-lamin IgM ascites, kindly provided by Larry Gerace (Scripps Clinic), was used 1:100 with affinity-purified rhodamine-conjugated goat anti-mouse immunoglobulin (20 μg/ml) (Cappel) on cells fixed for 10 min at −20°C in methanol.

Galactosylation of Permeabilized Whole Cells. Cells were fixed for 8 min at room temperature in 2% formaldehyde in PBS, washed three times in PBS, quenched for 10 min with 50 mM NH_4Cl in PBS, and permeabilized by a 10-min incubation in PBS containing 0.5% Triton X-100. Coverslips were then washed in 50 mM Tris·HCl (pH 7.5), inverted on 15-μl drops of 50 mM Tris·HCl/2.5 mM 5′-AMP/10 mM $MnCl_2$/1% aprotinin (Boehringer-Mannheim)/0.4 mM UDP-Gal (sodium salt, Sigma)/bovine galactosyltransferase (4 units/ml) (Sigma), and incubated at 37°C for 40 min (9). For controls, UDP-Gal and/or galactosyltransferase were omitted. After the incubation, coverslips were washed once in PBS at room temperature, incubated with 20% NGS in PBS, and immunostained as described above.

ELISA. A human erythrocyte cytosolic fraction enriched in 65-kDa protein (25% of total protein) known to contain O-linked GlcNAc residues (6), was generously provided by Gerald Hart (Johns Hopkins University). This material in PBS was adsorbed overnight at 4°C to polyvinyl chloride microtiter wells at 1 μg/ml. Binding of GAC-specific and control mAbs was then tested by indirect ELISA as described (21). Binding of test and control mAbs to the solid phase was quantitated with alkaline phosphatase-conjugated goat anti-mouse κ antibody (Fisher) and *p*-nitrophenyl phosphate (Sigma).

Galactosylation of the 65-kDa protein followed adsorption to microtiter plates at 2 μg/ml and was accomplished by incubation for 6.5 hr at 37°C in 50 mM Tris·HCl/2.5 mM 5′-AMP/10 mM $MnCl_2$/2, 0.33, 0.05, 0.009, or 0 mM UDP-Gal (sodium salt)/bovine galactosyltransferase (0.1, 0.05, or 0 unit/ml). Binding of test and control mAbs to the solid phase was quantitated as described above.

RESULTS

Distribution of Anti-GAC mAb Reactivity in Animal Cells. When either clone 9 rat hepatocytes (data not shown) or FR 3T3 fibroblasts were stained with HGAC 39 (data not shown) or 85 IgG3 anti-GAC mAbs, bright nuclear staining and weaker cytoplasmic staining were detected (Fig. 1). This distribution is consistent with biochemical determinations of *O*-GlcNAc distribution (22). The nuclear membrane (compare to anti-lamin staining; Fig. 1*D*) was intensely labeled, appearing as a bright rim at the nuclear periphery, while the nucleoplasm was diffusely stained. An underlying punctate pattern reminiscent of the distribution of nuclear pores, as seen by immunofluorescence with nuclear pore-specific mAbs (12), was also present. The cytoplasmic staining was also punctate, but it was not confined to any organelle or region of the cytoplasm. The cytoplasmic distribution of mAb staining did not overlap with the pattern of wheat germ agglutinin staining (Fig. 1*C*). Staining of the endoplasmic reticulum, plasma membrane, and Golgi complex, all of

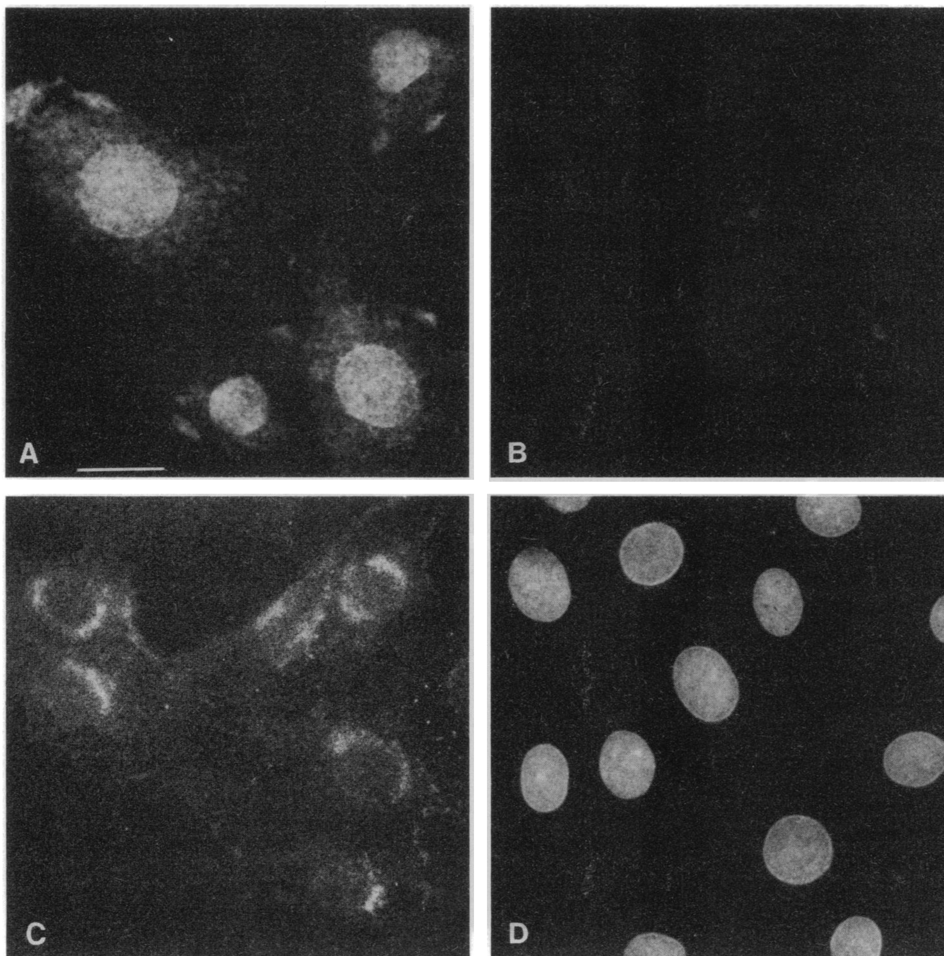

FIG. 1. Immunofluorescent distribution of anti-GAC reactivity. FR 3T3 cells were fixed, permeabilized, and reacted with undiluted HGAC 85 culture supernatant to which 22 mM GalNAc (*A*) or GlcNAc (*B*) had been added. Distributions of wheat germ agglutinin reactivity (*C*) and lamin (*D*) are shown for comparison. (Bar = 20 μm.)

which have been reported to contain terminal GlcNAc residues in other cells (22–24), was not detectable.

Nuclear and cytoplasmic staining were completely inhibited by inclusion of free GlcNAc with the mAbs, while inclusion of GalNAc had no effect on staining (Fig. 1 *A* and *B*), indicating the specificity of the staining for GlcNAc. Residual staining in the presence of free GlcNAc was indistinguishable from a control isotype-matched mAb specific for pneumococcal cell wall polysaccharide.

Terminal GlcNAc of *N*-Glycans Are Not Recognized by Anti-GAC mAbs. The lack of obvious staining of the secretory path and plasma membrane suggests that the mAbs react with *O*-GlcNAc, but not with terminal GlcNAc of *N*-glycans, in mammalian cells. Therefore, we examined the UDP-Gal transporter-deficient mutant Chinese hamster ovary (CHO) cell line, Lec8, which retains GlcNAc residues at the nonreducing termini of many N-linked glycans (25, 26). Immunofluorescence with the anti-GAC mAbs was similar to that described above for both mutant and wild-type CHO cells (Fig. 2). Thus, either anti-GAC mAbs do not react with terminal GlcNAc of *N*-glycans, or the terminal GlcNAc residues of *N*-glycans are not sufficiently clustered or concentrated to permit detection.

Sites Recognized by Anti-GAC mAbs Are Galactosylatable. To verify that the epitopes recognized by the mAbs were terminal GlcNAc residues, fixed and permeabilized cells were reacted with galactosyltransferase and UDP-Gal prior to immunostaining. This reaction specifically transfers galactose onto terminal GlcNAc residues (10). Enzyme treatment abolished nuclear and cytoplasmic anti-GAC mAb staining (Fig. 3). This confirms that the sites recognized by the anti-GAC mAbs are terminal GlcNAc residues and that the mAbs do not react with terminal galactose residues. If either enzyme (galactosyltransferase) or substrate (UDP-Gal) was omitted, anti-GAC mAb staining of nucleus and cytoplasm was not diminished (Fig. 3), demonstrating the specificity of this inhibition.

Anti-GAC mAbs React with an *O*-GlcNAc Protein by ELISA. The distribution of immunofluorescent staining is consistent with the known distribution of *O*-GlcNAc in the nucleus and cytoplasm (22). As further confirmation of the specificity of the anti-GAC mAbs toward *O*-GlcNAc, we tested binding of these mAbs to a 65-kDa human erythrocyte protein known to contain *O*-GlcNAc, but not *O*- or *N*-glycans (6). The mAbs specifically bound to the 65-kDa protein, as determined by ELISA using a preparation enriched in this protein (Fig. 4). Soluble GlcNAc, but not other monosaccharides, completely inhibited binding (Fig. 4). Isotype-matched control mAbs failed to bind under identical conditions (Fig. 4). Binding was also inhibited by galactosylation (data not shown). A murine IgM anti-GAC mAb, HGAC 49 (19), also bound to the 65-kDa protein in a GlcNAc-inhibitable fashion (data not shown).

DISCUSSION

We have described the cross-reactivity of two mAbs raised against streptococcal group A carbohydrate (GAC) with *O*-GlcNAc on nuclear and cytoplasmic proteins. Reactivity is completely abolished either by addition of free GlcNAc or by galactosylation of terminal GlcNAc residues. In addition, we have shown by ELISA that these mAbs react with a protein preparation known to contain *O*-GlcNAc. Unlike mAbs raised against *O*-GlcNAc-bearing nuclear pore proteins (12), these anti-GAC mAbs apparently recognize *O*-GlcNAc attached to a wide variety of proteins, consistent with a limited influence of peptide determinants on the interactions. The broader reactivity of the anti-GAC mAbs is apparent in the cytoplasmic staining obtained, since, by immunofluorescence, the anti-nuclear pore protein mAbs react only with the nucleus (12). Thus, the immunofluorescent reactivity of anti-GAC mAbs allows cytologic analysis of the subcellular distribution of *O*-GlcNAc.

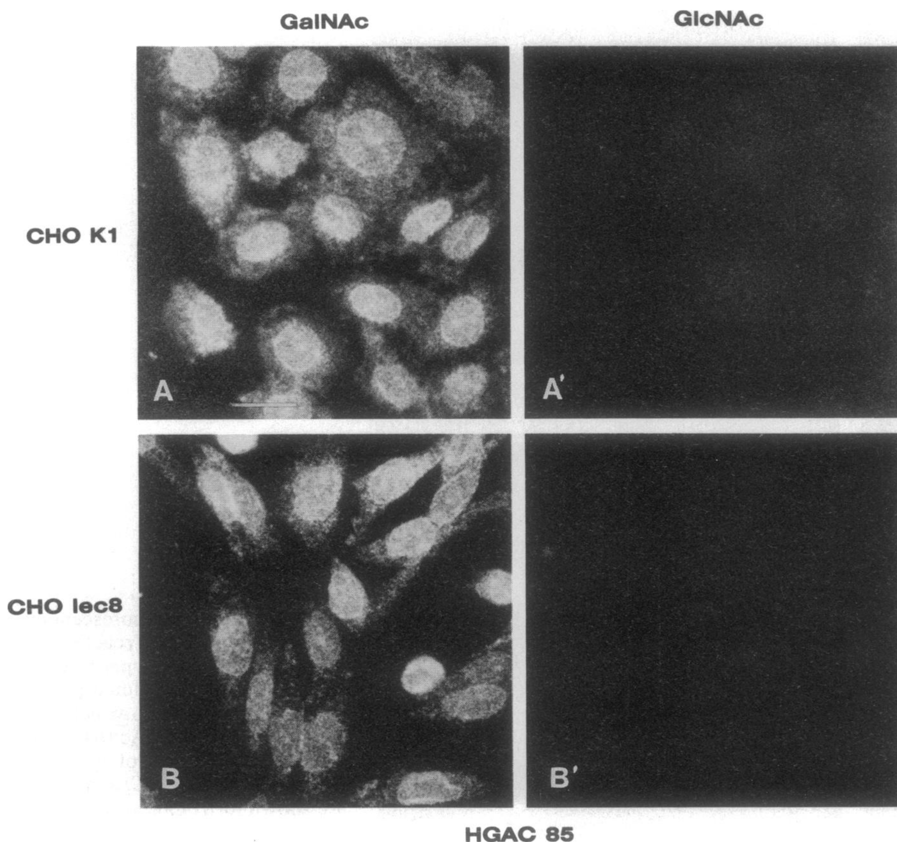

FIG. 2. Immunofluorescent distribution of anti-GAC reactivity in cells with increased terminal GlcNAc on *N*-glycans. The Lec8 CHO cell mutant cell line is UDP-Gal permease-deficient and, as a result, retains GlcNAc residues at the nonreducing termini of N-linked glycans. The immunofluorescent distribution of anti-GAC reactivity in Lec8 cells (*B*) was similar to other cells examined and was indistinguishable from wild-type CHO cells (*A*). There was no accentuated staining of secretory path organelles or plasma membrane in Lec8 cells. Free GalNAc had no effect on the anti-GAC mAb staining (*A* and *B*), but free GlcNAc completely eliminated reactivity (*A'* and *B'*). (Bar = 20 μm.)

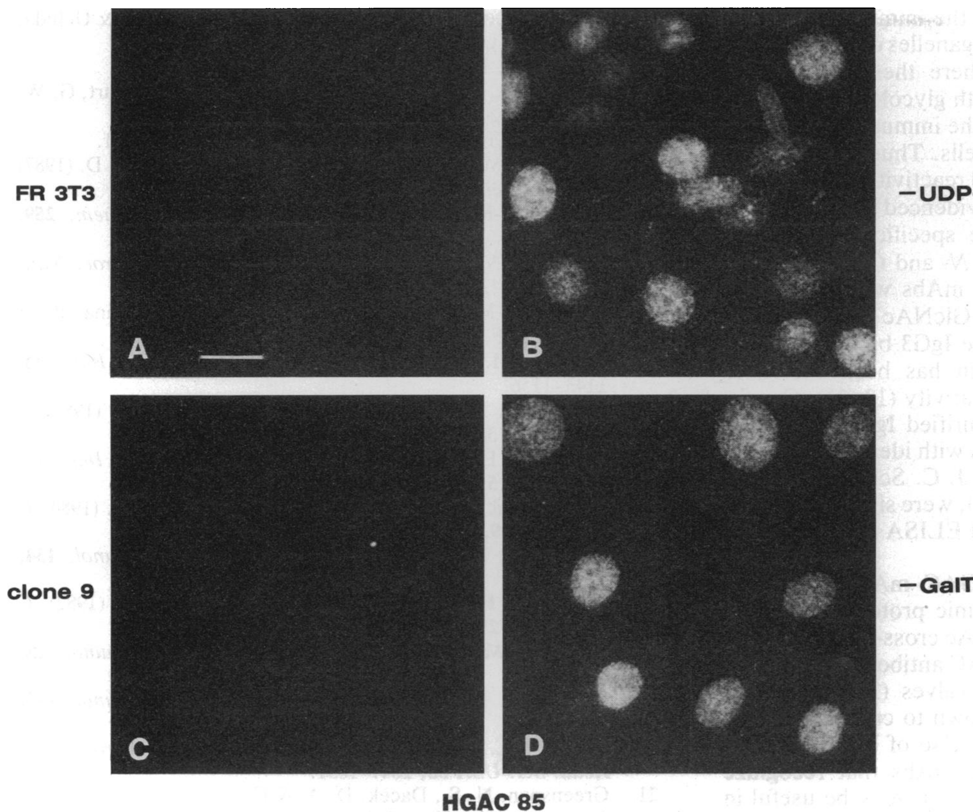

FIG. 3. Galactosylation abolishes anti-GAC reactivity. FR 3T3 (*A* and *B*) or clone 9 (*C* and *D*) cells were fixed, permeabilized, and incubated with galactosyltransferase and UDP-Gal (*A* and *C*), galactosyltransferase (GalT) alone (*B*), or UDP-Gal alone (*D*). The cells were then stained with undiluted HGAC 85 culture supernatant. (Bar = 20 μm.)

The relatively weak cytoplasmic staining is consistent with biochemical determinations of intracellular *O*-GlcNAc distribution (22), but the punctate pattern is unexpected. The punctate distribution of cytoplasmic reactivity might represent "spare" nuclear pore complexes in annulate lamellae (27), nuclear transport factors, extranuclear nucleolar proteins (28) or transcription factors (e.g., NFkB; refs. 5 and 29), or endoplasmic reticulum-associated O-linked GlcNAc residues (23).

Although the Golgi complex contains a significant number of terminal GlcNAc residues (24), no obvious immunofluorescent labeling of the Golgi, plasma membrane, or lysosomes with anti-GAC mAbs was observed. In addition to the direct comparison of anti-GAC reactivity to staining by wheat germ agglutinin (Fig. 1), we have compared anti-GAC reactivity to labeling by a mAb against a Golgi-localized protein (15) and mAbs against endoplasmic reticulum-localized protein disulfide isomerase (30). The distributions are not at all similar in these cells (data not shown). These data and the observation that distribution of anti-GAC reactivity in Lec8 cells was indistinguishable from wild-type CHO cells suggest that the anti-GAC mAbs do not react with terminal GlcNAc residues of *N*-glycans. Alternatively, terminal GlcNAc residues of *N*-glycans may not be sufficiently clustered or concentrated to permit detection.

Since HGAC 85 and 39 anti-GAC mAbs bind to GlcNAc β-1,3-linked to rhamnose (GAC), GlcNAc 1-phenyl-linked to lysine (as in GlcNAc–bovine serum albumin; ref. 21), and *O*-GlcNAc monosaccharides linked to serine and/or threonine (the 65-kDa erythrocyte protein), the mAbs recognize GlcNAc in a variety of linkages to both peptide and saccharide backbones. This does not rule out possible lack of reactivity with terminal GlcNAc in a subset of thus far untested linkages. The data do not address reactivity with

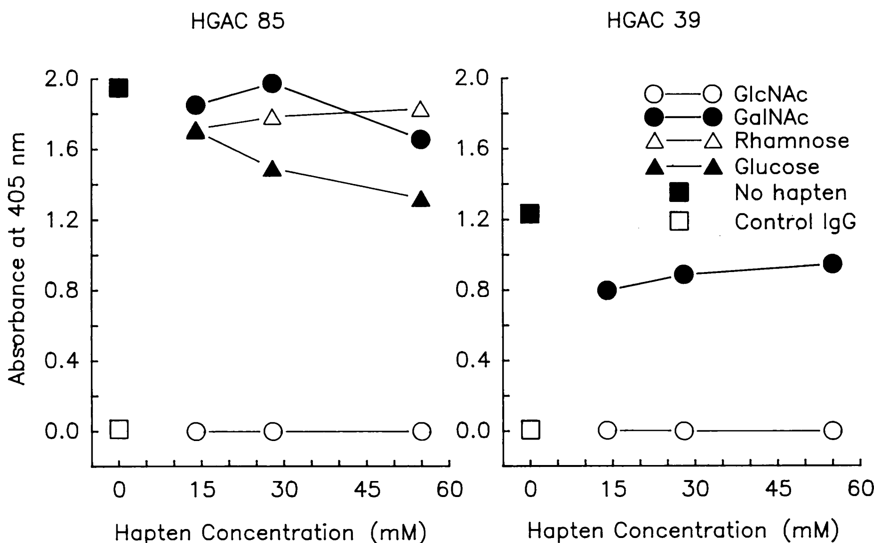

FIG. 4. Anti-GAC reactivity with protein bearing *O*-GlcNAc. Both HGAC 85 tissue culture supernatant diluted 1:2 ($\approx$10 μg/ml) (*Left*) and affinity-purified HGAC 39 (20 μg/ml) (*Right*) show significant reactivity, in the absence (■) of competing hapten, with a preparation enriched in a 65-kDa erythrocyte cytosolic protein known to contain *O*-GlcNAc. Isotype-matched control mAbs were P65J4-1 mAb anti-*p*-azophenylarsonate tissue culture supernatant diluted 1:2 for HGAC 85 (*Left*, □), and FLOPC 21 myeloma protein (20 μg/ml) for HGAC 39 (*Right*, □). Inclusion of free GlcNAc (○) completely eliminated anti-GAC reactivity with the protein, but free GalNAc (●), rhamnose (△), or glucose (▲) did not.

terminal GlcNAc of *O*-glycans, but the immunofluorescent staining pattern did not accentuate organelles of the secretory path or the plasma membrane, where these glycans are found. Anti-GAC mAb reactivity with glycolipids cannot be evaluated from these studies, since the immunofluorescence was on fixed, detergent-extracted cells. Thus, although the anti-GAC mAbs demonstrate a broad reactivity with terminal GlcNAc and even free GlcNAc (as evidenced by competition assays), there appears to be some specificity that limits reactivity with terminal GlcNAc on *N*- and *O*-glycans.

The reactivity of these anti-GAC mAbs with *O*-GlcNAc may depend on the clustering of *O*-GlcNAc along proteins that possess these residues (3), since IgG3 binding to GAC and GlcNAc–bovine serum albumin has been shown to involve Fc region-dependent cooperativity (13, 21). Consistent with this hypothesis, affinity-purified IgG1 and IgG2b isotype switch variants of HGAC 39, with identical variable-region sequences (L. J. N. Cooper, J. C. Schimenti, D. D. Glass, and N.S.G., unpublished data), were significantly less reactive by immunofluorescence and ELISA in experiments similar to those described above.

The data demonstrate that anti-GAC mAbs react with *O*-GlcNAc on nuclear and cytoplasmic proteins. Although the biological significance of *O*-GlcNAc cross-reactivity with anti-GAC mAbs is unknown, anti-GAC antibodies have been shown to cross-react with cardiac valves (31), and other anti-streptococcal antibodies are known to cross-react with myosin and nervous tissue (32, 33). Use of bacterial polysaccharide immunogens to generate mAbs that recognize related mammalian carbohydrate antigens may be useful in other systems. The potential of these anti-GAC mAbs in purification of *O*-GlcNAc-bearing proteins remains to be explored. Finally, the extensive *O*-GlcNAc distribution described suggests that cytoplasmic functions of this posttranslational modification must be considered.

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