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Biosynthesis and Oligosaccharide Structure of Human CD8 Glycoprotein Expressed in a Rat Epithelial Cell Line*

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The biosynthesis, post-translational modifications, and oligosaccharide structure of human CD8 glycoprotein have been studied in transfected rat epithelial cells. These cells synthesized and expressed on the plasma membrane high amounts of CD8 in a homodimeric form stabilized by a disulfide bridge. Three different CD8 forms were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis after metabolic labeling and immunoprecipitation: a newly synthesized, unglycosylated 27-kDa (CD8u), a palmitylated and initially *O*-glycosylated 29-kDa (CD8i), and the mature, terminally glycosylated 32–34-kDa doublet (CD8m). CD8i is a transient intermediate form between CD8u and CD8m: characterization of carbohydrate moiety of [^{3}H]glucosamine-labeled CD8i showed that it comprises for the vast majority non-elongated *O*-linked GalNAc closely spaced on the peptide backbone. Structural analysis of oligosaccharides released by mild alkaline borohydride treatment from the [^{3}H]glucosamine-labeled CD8 34-kDa form showed that the neutral tetrasaccharide Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcOH, and an homologous monosialylated pentasaccharide, predominate; the disialylated NeuAc2,3Galβ1,3(NeuAcα2,6) GalNAcOH tetrasaccharide appeared to be poorly present. In the CD8 32-kDa form the neutral tetrasaccharide was by far the prominent *O*-linked chain, and no disialyloligosaccharides were identified. These results indicate that the maturation of CD8 glycoprotein in transfected rat epithelial cells results in the formation of branched *O*-linked oligosaccharides and that a higher degree of sialylation is responsible for the production of the heavier 34-kDa form.

Several single or multistep post-translational modifications may occur to plasma membrane proteins, and these maturation events frequently reflect the progression of the protein through consecutive compartments of the intracellular transport pathway. A good example comes from the addition and remodeling of oligosaccharides in the case of *N*-linked glycosylation (1), whereas the other glycosylation process, the biosynthesis of *O*-linked oligosaccharides, has given less information. *O*-glycosylation of plasma membrane proteins is a very heterogeneous process which occurs by a sequence of individual sugar transfer reactions, and the first step is usually the addition of GalNAc to serine or threonine (2). This *O*-linked GalNAc is frequently elongated with Gal and the chain terminated by sialic acid, but different core regions have been found to contain GlcNAc and a long and complex elongation process may occur (3). This process comprises the assembly of additional Gal, GlcNAc, and GalNAc as well as fucose. All of these sugars are probably added late in the Golgi complex, whereas it is still debated where the first GalNAc is attached (see Ref. 4 for a detailed list). To study the formation of *O*-linked oligosaccharides during the intracellular transport, we have analyzed the biosynthesis of human CD8α glycoprotein (henceforth referred to as CD8). Previous reports have suggested that CD8 has only *O*-linked oligosaccharide side chains (5) and that it is expressed as disulphide bond hetero- or homodimer on the surface of thymocytes and peripheral cytotoxic T lymphocytes (6). Several lines of evidence indicate that CD8 is involved in adhesion and signaling function (see Ref. 7). More recently, CD8 cDNA has been cloned, sequenced and successfully expressed in several mammalian cell lines by transfection experiments (8–11). In this paper we present the results obtained studying the biosynthesis of CD8 in a permanently transformed clone derived from an epithelial rat cell line, where it is expressed at much higher level than in lymphocytes. We show that a palmitylated and initially *O*-glycosylated form is an intermediate precursor of the mature glycoprotein, and we report the characterization of the sugar moiety carried by this precursor and by the mature form.

EXPERIMENTAL PROCEDURES

Materials—All culture reagents were supplied by GIBCO. Solid chemicals and liquid reagents were obtained from E. Merck, Darmstadt, Federal Republic of Germany (F. R. G.), Farmitalia Carlo Erba, Milano, Italy; Serva Feinbiochemica, Heidelberg, F. R. G. SDS[1] was purchased from BDH, Poole, United Kingdom. [^{35}S]Cysteine (specific activity 1000 Ci/mmol) was obtained from Du Pont-New England Nuclear; [^{3}H]glucosamine (specific activity 20 Ci/mmol), [^{3}H]galactose (specific activity 30 Ci/mmol), and [^{3}H]palmitic acid (specific activity 30 Ci/mmol) were from Amersham, Buckinghamshire, United Kingdom. Mouse monoclonal OKT$_8$ was supplied by Ortho, Raritan, NJ. Rhodamine-conjugated goat anti-mouse IgG were from Cooper, Malvern, PA. Neuraminidase (*Vibrio cholerae*) was obtained from Boehringer, Mannheim, F. R. G. *Aspergillus niger* and *Escherichia coli* β-galactosidase as well as jack bean β-*N*-acetylglucosaminidase were from Sigma. [^{3}H]GalNAcOH was prepared by reduction of

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ER, endoplasmic reticulum.

GalNAc with KB^3H_4 (specific activity 6.7 mCi/mmol) as described by Crimmin (12). Galβ1,3GalNAcOH was from gG2 of herpes simplex virus (13). The Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcOH tetrasaccharide marker was a generous gift from Dr. M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). The [^{3}H]GalNAcβ1,4Galβ1,4Glc trisaccharide marker was isolated by Bio-Gel P-4 chromatography after desialylation (0.05 M H_2SO_4 for 2 h at 80 °C) of the [^{3}H]GalNAcβ1,4 (NeuAcα2,3)Galβ1,4Glc oligosaccharide prepared as previously described (14). NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAcOH was a gift from Dr. F. Dall'Olio (Department of Experimental Pathology, Bologna, Italy). Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden.

Cell Culture and Transfection—Parental FRT cells (15), and the FRT-U_{10} clone isolated after transfection, were cultured in Coon's modified Ham's F-12 medium containing 10% fetal calf serum and maintained in a 95% air, 5% CO_2 incubator. The human CD8 cDNA was manipulated and co-transfected with a plasmid carrying the bacterial *Neo* gene as described previously (11). Stable transformants were selected in the presence of G_{418} and positive clones screened by immunofluorescence.

Radioactive Labeling—In all experiments the cells were allowed to grow at subconfluency and then manipulated as follows. For [^{35}S]cysteine, 1 h before labeling the normal medium was replaced with Dulbecco's modified Eagle's medium containing 1% fetal calf serum (labeling medium) but lacking cysteine, and the cells were then pulse-labeled for 15–60 min with the labeling medium containing 100 μCi/ml [^{35}S]cysteine; chase was performed with labeling medium containing a 10-fold excess of cold cysteine and 10 μg/ml cycloheximide. For [^{3}H]glucosamine and [^{3}H]galactose, 2 h before labeling the normal medium was replaced with labeling medium containing only 100 mg/liter glucose, and the cells were then labeled for 1–4 h with labeling medium as above containing 100 μCi/ml one of the two radioactive sugars. For [^{3}H]palmitic acid; 24 h before labeling normal medium containing only 1% fetal calf serum was given to the cells; 5–20 min of labeling was then performed with labeling medium not containing fetal calf serum.

Sample Preparation and Analysis—Preparation of cell extracts, immunoprecipitation, methanolic KOH treatment, *in vitro* transcription and translation, SDS-PAGE and fluorography, and indirect immunofluorescence were performed as detailed previously (16–18). Throughout the work the mouse monoclonal OKT8 antibody has been used. In the case of neuraminidase treatment, the washed protein A-Sepharose beads were treated as described in Ref. 16 except that sodium acetate buffer (50 mM) pH 4.8, and neuraminidase (15 milliunits/ml) were used.

Preparation of [^{3}H]Glucosamine-labeled Glycopeptides by Pronase Digestion—The procedure was as described by Cummings *et al.* (19) with slight modifications. The polyacrylamide gels were fluorographed to locate CD8 bands. The areas of interest in the gel were excised, cut in small portions and incubated at 60 °C with Pronase (10 mg/ml) overnight in 0.1 M Tris-HCl, pH 8. Pronase had been incubated in the same buffer for 1 h at 37 °C to inactivate contaminating enzymes. At the end of the incubation 10 ml of water was added, the mixture boiled for 10 min and then centrifuged. The pellet was suspended in 10 ml of water and retreated as above. In the two supernatants about 90% of the radioactivity present in the gel was recovered.

Column Chromatography—The chromatography of glycopeptides or oligosaccharides on Bio-Gel P-10 or P-4 (400-mesh) was conducted on columns (1 × 75 cm) equilibrated in 0.1 M pyridine-acetate buffer, pH 5; 1-ml fractions were collected. DEAE-Sephacel chromatography was performed in a column (1 × 12 cm) as previously described (13). Recovery of radioactivity after chromatography on either column was 85–95%.

Mild Alkaline Borohydride Treatment of Glycopeptides—Glycopeptides were treated with 1 M $NaBH_4$ in 0.1 M NaOH for 72 h at 37 °C (20). At the end of treatment, samples were acidified with acetic acid and lyophilized. To remove borate as methylborate, samples were evaporated four times with methanol:acetic acid and then chromatographed on the Bio-Gel P-10 or P-4 columns.

Glycosidase Treatments and Non-enzymatic Desialylation—Oligosaccharides were digested with *A. Niger* β-galactosidase (0.1 unit) and β-*N*-acetylglucosaminidase (0.1 unit) in 50 mM sodium citrate buffer, pH 4.5, in a final volume of 25 μl at 37 °C for 24 h. Treatment with *E. coli* β-galactosidase was done as described by Carlson *et al.* (21). Neuraminidase digestion was performed as previously described (22). Oligosaccharides were either desialylated by 0.01 N HCl at 80 °C for 2 h.

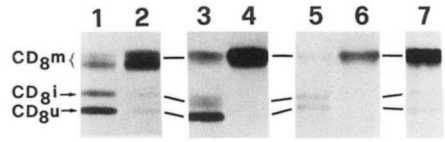

FIG. 1. **Biosynthesis of CD8 in heterologous host cell lines.** SDS-PAGE analysis of the immunoprecipitated products obtained from transfected FRT (*lanes 1* and *2*), MDCK II (*lanes 3* and *4*), CV_1 (*lane 7*), and untransfected HPB-ALL cells (*lanes 5* and *6*). Cells were pulse-labeled with [^{35}S]cysteine for 15 min (*lanes 1, 3,* and *5*) or 60 min (*lane 7*) and subsequently chased for 30 min in the presence of cold cysteine (*lanes 2, 4,* and *6*). Only the relevant portion of the gel is shown.

Analysis of Radioactive Amino Sugars—If glycopeptide or oligosaccharide samples contained salts, they were desalted by a Bio-Gel P-2 column equilibrated and eluted with water. The composition in radioactive amino sugars was performed after acid hydrolysis in 4 N HCl for 4 h at 100 °C in a final volume of 0.5 ml. After removal of acid by evaporation under reduced pressure the hydrolyzed samples were applied to silica gel plates and developed with a solvent containing borate, which allows a good separation among galactosamine, glucosamine and galactosaminitol (23).

Thin Layer Chromatography (TLC) and HPLC—Reduced oligosaccharides were resolved on cellulose TLC plates with ethylacetate:pyridine:acetic acid:water (5:5:1:3) as described by Edge and Spiro (24). HPLC analysis was performed with a Waters apparatus equipped with a Merck Lichrosorb-NH_2 column (12.5 × 0.4 cm) as described by Bergh *et al.* (25).

Periodate Oxidation (Smith Degradation) of Oligosaccharides—Oligosaccharides were oxidized with sodium periodate as described by Carlson *et al.* (21). After reduction with borohydride the samples were lyophilized and desalted on the Bio-Gel P-4 column.

RESULTS[2]

Biosynthesis of CD8 in Heterologous Host Cell Lines—Preliminary transfection experiments were performed to analyze the biosynthesis of recombinant CD8 in different host cell lines. The human lymphoma cell line HPB-ALL was used as a control for the biosynthesis of endogenous CD8. As shown in Fig. 1, CD8 was synthesized in a similar manner in all cell lines; a mature form of 32–34 kDa (CD8m) accumulated with time, whereas two other forms of 27 and 29 kDa (named CD8u and CD8i, respectively; see below) appeared to be transient precursors. Slight differences were detected in the electrophoretic migration of the three CD8 forms made in the various cell lines (Fig. 1); in peripheral blood cells mature CD8 migrates as a broad band of about 34 kDa (6), so it appears likely that these minor differences are host-dependent. Because the endogenous CD8 gene was expressed at low levels in the HPB-ALL cells, we isolated permanently transformed clones from the various cell lines (see "Experimental Procedures") and screened these clones for high CD8 synthesis. The best result was obtained with the FRT-U_{10} clone; as shown in Fig. 2, a strong signal was detected by indirect immunofluorescence analysis of these cells, and large amounts of CD8 were exposed on the plasma membrane. When the cells were metabolically labeled and the cell extracts analyzed by SDS-PAGE before and after immunoprecipitation (Fig. 3, *lanes 1* and *2*), a prominent doublet of the expected apparent molecular size (32–34 kDa) was present in the total lysate and specifically immunoprecipitated. Furthermore, when the same sample was analyzed in the absence of reduction and alkylation a major band of about 55 kDa and minor bands of

[2] Portions of this paper (including part of "Results" and Figs. 8–14) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

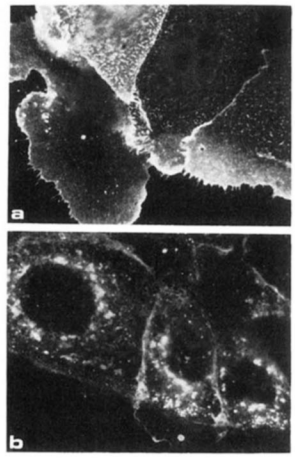

FIG. 2. **CD8 glycoprotein is synthesized and expressed on the surface of FRT-U_{10} cells.** Indirect immunofluorescence analysis. *a*, surface staining; *b*, staining after permeabilization.

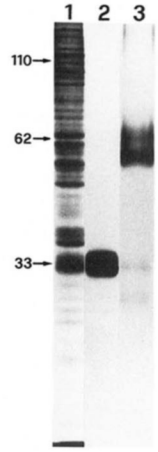

FIG. 3. **Mature forms of CD8 glycoprotein are synthesized by FRT-U_{10} cells.** SDS-PAGE analysis after 15 min of labeling with [^{35}S]cysteine followed by 60-min chase. *Lane 1*, total products; *lanes 2* and *3*, samples immunoprecipitated. Reduction and alkylation were omitted for the sample run in *lane 3*. *Numbers* on the *left* indicate the position of molecular mass standards run on the same gel.

apparently higher molecular mass were detected (Fig. 3); this finding confirms the previous observation that mature CD8 is expressed on the surface of the cell as a dimer and that disulfide bridge(s) stabilizes the structure (7). The parental FRT cells were totally negative by immunofluorescence as well as by metabolic labeling and immunoprecipitation (not shown). These data strongly suggest that the FRT-U_{10} clone synthesizes and expresses on the surface high amount of CD8 glycoprotein.

Identification of Newly Synthesized CD8—CD8 cDNA was cloned under the control of phage Sp6 promoter to perform *in vitro* transcription/translation assays followed by direct SDS-PAGE analysis, omitting the immunoprecipitation step. As shown in Fig. 4, CD8 made *in vitro* migrated as a 28.5-kDa band that microsomal membranes were able to convert to a 27-kDa band comigrating with CD8u pulse-labeled *in vivo*. It has been previously reported that CD8 has an amino-terminal signal sequence of 21 residues (8); thus, most likely, the 28.5-kDa band represents the uncleaved polypeptide chain bearing the amino-terminal signal sequence and the 27-kDa band the processed form inserted in the endoplasmic reticulum membrane. All CD8 forms labeled *in vivo*, as well as the 27 kDa band made *in vitro*, appeared to completely span the membrane leaving a short cytosolic tail accessible to proteolytic degradation.[3]

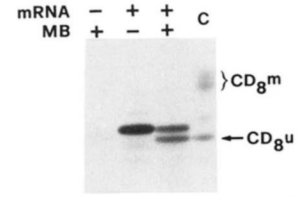

FIG. 4. ***In vitro* synthesis and membrane assembly of newly synthesized CD8.** SDS-PAGE analysis of a coupled transcription-translation assay. [^{35}S]Cysteine labeling and wheat germ-derived protein synthesis system. *MB*, rough microsomal membranes from dog pancreas; *C*, immunoprecipitated products from FRT-U_{10} cells pulse-labeled with [^{35}S]cysteine for 30 min.

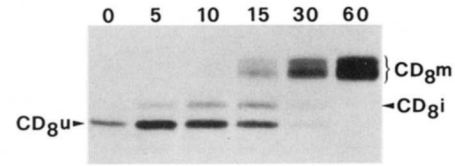

FIG. 5. **Maturation of newly synthesized CD8 protein.** SDS-PAGE analysis of the immunoprecipitated products from parallel cultures of FRT-U_{10} cells pulse-labeled for 5 min with [^{35}S]cysteine and subsequently chased, for the times (in min) indicated on the *top* of the figure, in the presence of 10 μg/ml cycloheximide and a 10-fold excess of cold cysteine. Only the relevant portion of the gel is shown.

Maturation of Newly Synthesized CD8—Parallel cultures of FRT-U_{10} cells were pulse-chase labeled with [^{35}S]cysteine to follow the maturation of the newly synthesized CD8u form. As shown in Fig. 5, CD8u was quickly converted first to the 29-kDa CD8i form and then to the mature 32–34-kDa doublet; CD8i appeared to be a transient precursor form, and even shorter pulse-chase times failed to show complete conversion of CD8u in CD8i before the appearance of the mature CD8m. Furthermore, no maturation of the 32-kDa to the 34-kDa form was detectable by pulse-chase analysis.

Fatty Acylation of CD8—Subconfluent cultures of FRT-U_{10} were pulse-labeled with [^{3}H]palmitic acid, and the immunoprecipitated products analyzed by SDS-PAGE. As shown in Fig. 6 (*lane 1*), only CD8i was labeled with a 5-min pulse, whereas the great majority of the label was in the mature doublet with a 20-min pulse. The labeling appeared to be linked to the protein with a covalent ester bond because it was resistant to chlorophorm-methanol extraction, but was completely removed by incubation in methanolic-KOH (Fig. 6, *lane 3*). However, the removal of the fatty acid did not alter the electrophoretic migration of CD8i and CD8m (Fig. 6, *lane 4*). CD8u was never labeled by [^{3}H]palmitic acid.

Glycosylation of CD8—It has been previously suggested that CD8 has only *O*-linked carbohydrate side chains (5), despite the presence on the polypeptide of an acceptor site for *N*-glycosylation. We first confirmed the absence of *N*-linked chains by several criteria: tunicamicyn did not affect the electrophoretic mobility of the various CD8 forms; labeling with [^{3}H]mannose did not result in any radioactive incorporation; Pronase-digested glycopeptides from various forms of [^{3}H]glucosamine-labeled CD8 did not bind to concanavalin A-Sepharose. Next we performed labeling experiments with [^{3}H]glucosamine, which is intracellularly converted into GlcNAc, GalNAc and sialic acid, and with [^{3}H]galactose. As shown in Fig. 7 (*lanes 1* and *2*), labeling with [^{3}H]glucosamine

[3] M. C. Pascale, M. C. Erra, N. Malagolini, F. Serafini-Cessi, A. Leone, and S. Bonatti, unpublished results.

revealed both CD8i and CD8m, whereas [^{3}H]galactose lighted up only the mature form. This pattern has been fully confirmed by varying the pulse time from 30 min to 4 h, and no labeling of CD8u was ever detected. Neuraminidase treatment converted the 32–34-kDa CD8m doublet to a single broad band of 31 kDa, but was ineffective toward CD8u and CD8i (Fig. 7, *lanes 3* and *4*). Therefore, these results strongly suggested that CD8u is the unglycosylated form and CD8i a neutral glycosylated precursor of CD8m which is more elongated and terminated by sialic acid.

Structural Characterization of Oligosaccharides from [^{3}H] Glucosamine-labeled CD8—The isolation and characterization of *O*-linked oligosaccharides from different forms of [^{3}H] glucosamine-labeled CD8 were performed by a protocol which included: (i) Pronase digestion of each band separately excised from the polyacrylamide gel, (ii) isolation of oligosaccharides generated upon mild alkaline borohydride treatment by Bio-Gel P-10, Bio-Gel P-4, and DEAE-Sephacel chromatographies, (iii) structural elucidation of the oligosaccharides by labeled amino sugar composition, sequential exo-glycosidase digestions, Smith degradation, HPLC, and TLC analysis.

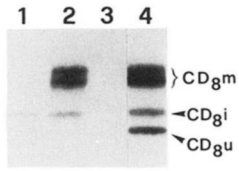

FIG. 6. **Palmitylation of CD8 forms.** SDS-PAGE analysis of the immunoprecipitated products after 5 min (*lane 1*) and 20 min (*lanes 2* and *3*) of labeling with [^{3}H]palmitic acid, or 30 min of labeling with [^{35}S]cysteine (*lane 4*). Before the run, the samples in *lanes 3* and *4* were treated with methanolic KOH.

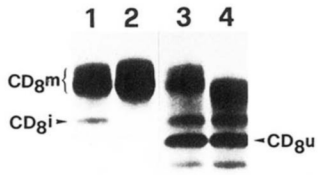

FIG. 7. **Glycosylation of CD8 forms.** SDS-PAGE analysis of the immunoprecipitated products after 60 min of labeling with [^{3}H] glucosamine (*lane 1*), [^{3}H]galactose (*lane 2*), or 30 min of labeling with [^{35}S]cysteine (*lanes 3* and *4*). Before the run, the samples in *lanes 3* and *4* were mock-treated and treated with neuraminidase, respectively.

The major oligosaccharide isolated from CD8i migrated on TLC and HPLC as an authentic sample of free GalNAcOH. Minor amounts of Galβ1,3GalNAcOH and a species probably elongated by GlcNAc were also found (see Miniprint Section). The major neutral oligosaccharides generated by β-elimination of mature forms was elucidated to be Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcOH. Monosialylated chains were isolated from CD8m, in larger amounts from the 34-kDa band. Desialylation of monosialoligosaccharides resulted in the production of two neutral carbohydrate chains to which the structures Galβ1,3GalNAcOH and Galβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcOH have been assigned. At the moment we cannot determine to which sugar the single residue of sialic acid is bound, nor its isomeric linkage. Only 34-kDa CD8 was found to carry the disialylated NeuAcα2,3Galβ1,3-(NeuAcα2,6)GalNacOH oligosaccharide. The detailed experimental procedure for the oligosaccharide isolation and structural elucidation is presented in the Miniprint Section. The results are summarized in Table I.

DISCUSSION

We have assembled a heterologous model system, consisting of FRT cells permanently expressing high level of human CD8 glycoprotein. The FRT cell line, obtained from rat thyroid, displays in culture morphological and functional polarity (15). Several considerations convinced us to assemble these partners in a model system, with the long term aim to study

TABLE I

Proposed structures of O-linked chains from CD8 glycoprotein transfected in FRT rat epithelial cells at different steps of post-translational processing

Oligosaccharides were isolated after mild alkaline borohydride treatment.

Oligosaccharides	CD8i (29 kDa2)	CD8 (32 kDa)	CD8 (34 kDa)
GalNAcOH	+++	−	−
Galβ1→3GalNAcOH	±	−	−
Galβ1→4GlcNAcβ1↘ 6 Galβ1→3GalNAcOH	−	++++	+++
NeuAca2→3/6Galβ1→3GalNAcOH NeuAca2↘ 6 Galβ1→3GalNAcOH	−	+	++
NeuAca2→3/6Galβ1→4GlcNAcβ1↘ 6 Galβ1→3GalNAcOH Galβ1→4GlcNAcβ1↘ 6 NeuAca2→3/6Galβ1→3GalNAcOH	−	+	++
NeuAca2↘ 6 NeuAca2→3Galβ1→3GalNAcOH	−	−	+

the intracellular topology of the O-glycosylation process. First, the difficulty to find another suitable model system, *i.e.* either endogenous or viral O-glycosylated proteins made by mammalian cell in culture in high amount; second, the general interest to acquire informations concerning CD8 structure and function, and the possibility to use this marker for studying the biogenesis and maintenance of cell polarity; third, the opportunity to correlate the post-translational modifications of CD8 with the host-dependent enzymatic pathways.

Two precursors of the mature form of CD8 are detectable by a combination of *in vitro* translation assays and *in vivo* metabolic labeling: first a 27-kDa CD8u form, and then an intermediate 29-kDa CD8i form. CD8u presumably represents the newly synthesized protein inserted in the ER membrane from which the signal sequence has been already removed; CD8i is palmitylated and O-glycosylated, with the initial GalNAc residue linked to serine/threonine and substituted in few cases with one GlcNAc residue. Palmitylation has been shown to be a post-ER modification for G glycoprotein of the ts045 mutant strain of vesicular stomatitis virus and for E1 glycoprotein of Sindbis virus (16); more precisely, it occurred in between the 15 °C block of intracellular transport and the mannose trimming yielding the $Man_5GlcNAc_2$ chains (16). This trimming is due to 1,2-mannosidase, an enzyme which is thought to be localized in the *cis*-Golgi (28). We could not detect palmitylated CD8u even with very short labeling times, and the removal of the palmitic acid did not alter the electrophoretic migration of CD8i. Our carbohydrate characterization of CD8i revealed that it contains mainly nascent O-linked GalNAc. Thus the shift in electrophoretic migration from CD8u to CD8i is due to initial O-glycosylation, and the palmitylation step either follows or happens contemporaneously to the glycosylation. In parallel experiments we found that newly synthesized CD8 was palmitylated and initially O-glycosylated immediately after the 15 °C block.[4] Therefore we believe that CD8i is generated after exit of CD8u from the ER, in between the "15 °C region" and the *cis*-Golgi or directly in the *cis*-Golgi, whereas complete maturation of CD8 takes place in more distal Golgi compartments.

The maturation process of CD8 produces the neutral Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAc tetrasaccharides as major oligosaccharide and a monosialylated chain formed by the addition of a single sialic acid residue to this structure. These results indicate that the full O-glycosylation of CD8 in transfected FRT cells involved the Galβ1,3GalNAc-O-Ser/Thr: β1,6GlcNAc-transferase responsible for the assembly of the core-2 structure (3). Indeed, the FRT cell line does contain a relevant amount of β1,6GlcNAc-transferase.[3] There is evidence that this enzyme is poorly expressed in resting human T lymphocytes (29); thereby one could infer that the prevalence of O-linked chains with the core-2 structure is a peculiar feature of CD8m synthesized by FRT cells. On the other hand, a dramatic stimulation of β1,6GlcNAc-transferase occurs in T lymphocytes activated by interleukin-2 or anti-CD3 antibodies so that O-linked chains of CD43, the major glycoprotein of lymphocytes, shift from the type 1 to type 2 core structure (29, 30). On this basis the carbohydrate sequence we elucidated in CD8m from FRT cells should be that of the glycoprotein expressed by activated T cells rather than by resting lymphocytes. The shift from the type 1 to type 2 core O-linked structure results in a significant increase of the apparent molecular weight of CD43 produced by activated T cells (29). A similar modification of the apparent mass of CD8 has not been described. This could depend on the fact that while CD43 carries 90 O-linked chains, few oligosaccharides occur in CD8 (see below). To prove these considerations other investigations are necessary.

NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc was found in a very low amount in the 34-kDa form of CD8. Different mechanisms may account for the paucity of NeuAcα2,6-linked to GalNAc: i) a low expression of the α2,6-sialyltransferase in FRT cells ii) the previous addition of GlcNAc at the same C-6 position of GalNAc. The first mechanism could be particularly effective because the transfected FRT-U_{10} cells overexpress O-glycosylated CD8. As for the second mechanism, it has been proposed that in leukemic cells the β1,6GlcNAc-transferase responsible for the shift from the core-1 to core-2 structure resides at earlier Golgi cisternae than α2,6-sialyltransferase (31). Current results indicating a low but consistent amount of GlcNAc and the complete absence of sialic acid in the precursor CD8i support this notion for rat thyroid cells as well. Independently of the subcellular site of various glycosyltransferases, other parameters such as K_m, V_{max}, and the post-translational modification of the glycosyltransferases may affect the efficiency of each transferase reaction (31, 32).

In the cell system considered, mature CD8 migrates in SDS-PAGE as a doublet of 32–34 kDa. A similar electrophoretic pattern was seen by Snow *et al.* (33) who purified CD8 from a lymphocyte cell line. Our results are consistent in indicating that this electrophoretic pattern depends mainly on a higher degree of sialylation of the heavier forms.

Our results allow to calculate the approximate number of O-linked chains carried by CD8 because the extent of O-glycosylation is host cell-independent and related to the protein conformation (34). Assuming that at least 1 mol/mol of palmitic acid is linked to CD8i the increment of 2-kDa relative to unglycosylated form accounts for about 8–9 GalNAc residues. Since more than 10% of O-linked GalNAc are elongated with GlcNAc and a small amount of Galβ1,3GalNAc was identified in CD8i, it is more likely that 7–8 Ser/Thr residues are actually O-glycosylated. These figures are much lower than the potential O-glycosylation sites; indeed, 27 residues of Ser/Thr are predicted in the ectodomain of CD8.

Current results evidentiate a large size decrease between the Pronase glycopeptides and oligosaccharides released from all CD8 forms. This supports the notion that the oligosaccharides are closely spaced on the peptide backbone. In fact, this type of spacing hinders the Pronase action, and large glycopeptides are produced carrying more than one oligosaccharide chain. In the predicted amino acid sequence of CD8 three regions of closely spaced Ser/Thr occur at positions 27–34, 117–119, and 126–137, respectively. Although no consensus sequence for O-glycosylation has been found, several workers have suggested that the occurrence of proline residues in vicinity of hydroxy amino acid clusters facilitates the O-glycosylation (34–36). In and around the three CD8 regions, proline is largely present; thereby they are good candidates for O-glycosylation. Analysis of tryptic peptides might help to determine if these regions are actually glycosylated. Parenthetically, threonine at position 30 is part of the unique potential N-glycosylation site of CD8. It is very likely that the lack of N-linked chains in CD8 depends on the fact that proline is at position 29, *i.e.* it is the "X" residue of the Asn-X-Thr consensus sequence for N-glycosylation. There is evidence that the presence of proline in this position interferes with the catalytic site of oligosaccharyl-transferase responsible for the transfer of the high mannose chain to the peptide (37).

In conclusion, this study has established that discrete forms of precursors are produced during the biosynthesis of CD8

[4] M. C. Pascale, M. C. Erra, N. Malagolini, F. Serafini-Cessi, A. Leone, and S. Bonatti, manuscript in preparation.

which mainly diverge for a different degree of *O*-glycosylation. Such a finding has been obtained by combining the isolation of various precursor forms with the carbohydrate analysis of each form. With a similar experimental approach we are planning to ascertain the changes of the oligosaccharide structure and the related modification in the precursor formation due to events or drugs which interfere with the intracellular routing of glycoproteins and with the integrity of the exocytic pathway.

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SUPPLEMENTAL MATERIAL TO

BIOSYNTHESIS AND OLIGOSACCHARIDE STRUCTURE OF HUMAN CD8 GLYCOPROTEIN EXPRESSED IN A RAT EPITHELIAL CELL LINE

By

Maria Carmela Pascale, Nadia Malagolini, Franca Serafini-Cessi, Giovanni Migliaccio, Arturo Leone and Stefano Bonatti

Isolation and characterization of *O*-linked oligosaccharides from the CD8 precursor (CD8i).

Areas of polyacrylamide gel containing [^{3}H]glucosamine-labeled CD8i (4 h of labeling period) were excised, subjected to Pronase digestion and fractionated on a Bio-Gel P-4 column. The [^{3}H]glucosamine-labeled glycopeptides were entirely eluted in the void volume and the analysis of radioactive amino sugars showed a very large prevalence of [^{3}H]GalN on [^{3}H]GlcN (87% versus 13%) (Fig. 8A). It is worth noting that after 4 h of labeling the specific activities of [^{3}H]GalNAc and [^{3}H]GlcNAc were at equilibrium; indeed, the ratio between [^{3}H]GalN and [^{3}H]GlcN of [^{3}H]glucosamine-labeled CD8 did not change after two or four hours of labeling. On DEAE-Sephacel chromatography all of these glycopeptides behaved as neutral components and no radioactive material was released by neuraminidase or mild acid hydrolysis, indicating that CD8i-oligosaccharides were devoid of sialic acid (results not shown). Upon ß-elimination three components were generated, all eluted in included volumes (Fig. 8B). The peak of larger size, which accounted for about 30% of total radioactivity, after strong acid hydrolysis contained mainly [^{3}H]GalN and was interpreted as a glycopeptide resistant to the ß-elimination. It has been reported that mild alkaline borohydride treatment of some glycoproteins does not result in a complete release of *O*-linked chains (13, 26). In our case the high amount of carbohydrate chains resistant to the ß-elimination could be related to the denaturation of the glycoprotein occurring during the fluorography step or to other presently unknown features. In the oligosaccharides of smaller size (peaks B1 and B2 of Fig. 8B) [^{3}H]GalNAc appeared converted in the corresponding alditol, as expected for the innermost residue of *O*-linked chains. The [^{3}H]GalNol was the only radioactive component in the acid-hydrolyzate of the major B2 peak, whereas in B1 oligosaccharides a small amount of [^{3}H]GlcN was found. The latter oligosaccharide peak was not investigated further; on the basis of the amino-sugar composition one can suggest that it contains chains elongated by GlcNAc. The vast majority of B2 oligosaccharides migrated on TLC and HPLC as an authentic sample of free [^{3}H]GalNAcOH and only a small amount of radioactive material with the mobility of Galß1,3GalNAcOH was detected (Fig. 9). These results indicate that CD8i mainly corresponds to a glycoprotein precursor at the initial step of *O*-glycosylation.

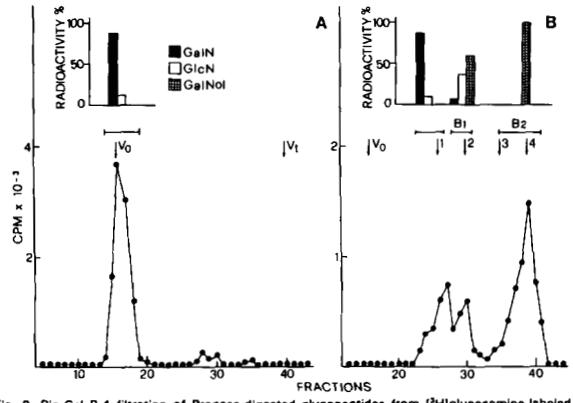


Fig. 8. Bio-Gel P-4 filtration of Pronase-digested glycopeptides from [^{3}H]glucosamine-labeled CD8i before and after mild alkaline borohydride treatment, and their amino-sugar composition.

CD8 was immunoprecipitated from the lysate of FRT-U10 cells labeled for 4 h with [^{3}H]glucosamine and subjected to SDS-PAGE. The band visualized by fluorography at 28 kDa was excised and digested with Pronase as described under the Materials and Methods section.

A, Gel-filtration profile of glycopeptides before mild alkaline borohydride treatment. Fractions under the bar were pooled, an aliquot subjected to strong acid hydrolysis and analyzed for amino sugar composition by TLC. The per cent of radioactivity recovered in the migration position of GlcN, GalN and GalNol is reported in the top of the panel.

B, The remnant glycopeptides were subjected to mild alkaline borohydride treatment and rechromatographed on the same Bio-Gel P-4 column. Fractions were pooled as indicated by horizontal bars and lyophilized. To remove salts, samples were applied to double columns of Dowex 50 and Dowex 1, and the flow-through fractions were freeze dried. An aliquot was used for the amino sugar composition (top of the panel).

Arrows 1, 2, 3, and 4 indicate NeuAcα2,3Galß1,3(NeuAcα2,6)GalNAcOH, GalNAcß1,4Galß1,4Glc, Galß1,3GalNAcOH, and GalNAcOH, respectively.

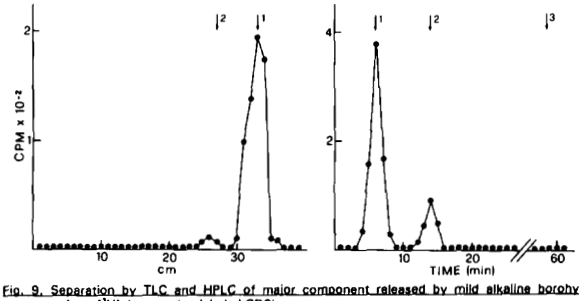


Fig. 9. Separation by TLC and HPLC of major component released by mild alkaline borohydride treatment from [^{3}H]glucosamine-labeled CD8i.

Desalted material from B2 peak of Fig. 8B was divided in two aliquots:

A, One aliquot was subjected to TLC and areas of the cellulose were scraped off of the plates and the radioactivity was determined by liquid-scintillation counting.

B, The other aliquot was injected on a NH_2-Lichrosorb column and subjected to HPLC as indicated in the text. Fractions were collected and counted for radioactivity. Arrows 1, 2 and 3 indicate GalNAcOH, Galß1,3GalNAcOH and NeuAcα2,3Galß1,3(NeuAcα2,6)GalNAcOH.

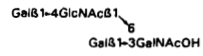
Isolation of *O*-linked oligosaccharides from CD8m.

The two bands of CD8m migrating with an apparent molecular weight of 34 and 32 kDa were separately excised from the gel and digested with Pronase. In both cases the vast majority of labeled glycopeptides were eluted in the void volume of the Bio-Gel P-10 column and their labeled amino sugar analysis showed a ratio between [^{3}H]GlcN and [^{3}H]GalN near 0.62 (Fig. 10A and C). This value is much higher than that found in CD8i-glycopeptides (0.15) indicating that within the maturation of the glycoprotein the oligosaccharides elongated also by the addition of GlcNAc. Upon mild alkaline borohydride treatment two oligosaccharide peaks (C1 and C2) were generated from CD8-34kDa glycopeptides. C2 peak emerged in a more included volume indicating that the oligosaccharides were smaller in size than those of C1 peak. In terms of radioactivity the two peaks were very similar. Conversely, C1 oligosaccharides from the glycopeptides of lighter CD8m contained only one third of the radioactivity of C2 oligosaccharides (Fig. 10B and D).

To ascertain the degree of sialylation the various oligosaccharides were chromatographed on a DEAE-Sephacel column. C1 oligosaccharides from CD8-34kDa were fractionated as neutral, monosialylated and disialylated species, the latter was in a quantity lower than that of the other two chains. Conversely, C2 did not appear to contain disialylated oligosaccharides and the neutral oligosaccharides predominated on the monosialylated species. (Fig. 11A and B). DEAE-Sephacel chromatographic profile of C1 and C2 peaks from CD8-32kDa showed the absence of disialylated oligosaccharides and a large prevalence of neutral chains in both peaks (Fig. 11C and D). From all oligosaccharides bound to the DEAE-Sephacel the neuraminidase treatment cleaved a labeled component which migrated on HPLC as an authentic sample of *N*-acetylneuraminic acid (results not shown). These results indicate that the larger apparent molecular weight of CD8-34kDa depends on a higher sialylation degree of *O*-linked chains.

Structural analysis of neutral oligosaccharides from mature CD8

The final fractionation of neutral chains from CD8-32kDa and -34kDa were performed by Bio-Gel P-4 chromatography. A portion of these chains eluted in and near the V_o of the Bio-Gel P-4 column and, after strong acid hydrolysis, appeared to contain [^{3}H]GlcN and unreduced [^{3}H]GalN (Fig. 12A). As is the case of CD8i, this portion was interpreted as a glycopeptide resistant to the ß-elimination. The major peak of neutral chains, emerging in the included volume, contained [^{3}H]GalNol and [^{3}H]GlcN in a similar quantity and only a small amount of unreduced [^{3}H]GalN (Fig. 12A). When this peak was analysed on TLC the major component migrated as an authentic sample of Galß1,4GlcNAcß1,6(Galß1,3)GalNAcOH (Fig. 13A). The same result was obtained on HPLC performed as described by Saitoh *et al.* (27). Preliminary experiments indicated that sequential digestions with ß-galactosidase and ß-*N*-acetylglucosaminidase result in a drastic demolition of the oligosaccharide sequence. Fig. 12B shows that after ß-galactosidase treatment (from *Aspergillus niger*) a large portion of neutral oligosaccharides was eluted by the Bio-Gel P-4 column as a smaller component which contained an identical amount of [^{3}H]GalNol and [^{3}H]GlcN. On TLC this component migrated faster than the untreated chain and with the mobility of a trisaccharide marker (Fig. 13A and B). Since the ß-galactosidase from *Aspergillus niger* specifically cleaves ß1,4-linked galactose, this result indicates that a ß1,4-linked galactose residue is in the terminal non-reducing position of CD8m-neutral chains. The trisaccharide isolated by gel-filtration was then treated with ß-*N*-acetylglucosaminidase. As the Bio-Gel P-4 profile shows (Fig. 12C) this treatment generated two radioactive peaks, the larger one was eluted in the position of Galß1,3GalNAcOH and contained as radioactive sugar only [^{3}H]GalNol. Even on TLC the disaccharide migrated as the Galß1,3GalNAcOH marker (Fig. 13C). The second radioactive component, eluted as monosaccharide on Bio-Gel P-4, migrated on TLC as free [^{3}H]GlcNAc (result not shown). When the disaccharide was digested with ß-galactosidase from *Escherichia coli*, an enzyme which specifically acts towards Galß1,3GalNAcOH (21), free [^{3}H]GalNAcOH was produced (Fig. 12D and 13D). This protocol did not allow to elucidate whether GlcNAc was ß1,6-linked to the innermost GalNAc (as it occurs in *O*-linked oligosaccharides with the core-2 structure) or to the galactose residue of Galß1,3GalNAcOH core-1 unit. To elucidate this point the tetrasaccharide was subjected to Smith degradation. The product obtained after this treatment migrated on TLC faster than a disaccharide (Fig. 13E). This result excluded the structure Galß1,4GlcNAcß-Galß1,3GalNAcOH. Indeed, in this sequence, beside the degradation of GalNAcOH, only one terminal residue of galactose should be sensitive to the degradative oxidation and a component larger than a disaccharide should be produced. Conversely, in the structure Galß1,4GlcNAcß1,6(Galß1,3)GalNAcOH two galactose residues are exposed to the Smith degradation with a consequent larger shortening of the chain. For these reasons we propose that the major neutral chain released from CD8m has the following structure:

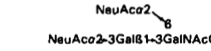


Structural analysis of monosialoligosaccharides from CD8m.

When monosialoligosaccharides from [^{3}H]glucosamine-labeled CD8m were gel-filtrated on the Bio-Gel P-4 column they eluted in two different peaks (Fig. 14A). The composition in radioactive amino sugar showed a very similar content of [^{3}H]GalNol and [^{3}H]GlcN in the oligosaccharide peak of larger size (Mo1), whereas the oligosaccharides of smaller sizes (Mo2) contained only [^{3}H]GalNol. After mild acid hydrolysis the desialylated Mo1 oligosaccharides migrated on TLC with the same mobility of neutral tetrasaccharide (Fig. 13F). By sequential digestions with ß-galactosidase (from *Aspergillus niger*), ß-*N*-acetylglucosaminidase and ß-galactosidase (from *Escherichia coli*) the tetrasaccharide was converted to [^{3}H]GalNAcOH (results not shown). When the Mo2 oligosaccharides were subjected to desialylation, the residual chains for the vast majority behaved on TLC as the Galß1,3GalNAcOH disaccharide (Fig. 13G). Based on these results we propose that the desialylated structure of Mo1 is Galß1,4GlcNAcß1,6(Galß1,3)GalNAcOH whereas that of Mo2 is Galß1,3GalNAcOH.

Structural analysis of disialylated oligosaccharides

Disialoligosaccharides from [^{3}H]glucosamine-labeled CD8-34kDa were eluted from the Bio-Gel P-4 column as a single peak after the void volume and the labeled amino sugar composition showed that apart [^{3}H]GalNAcOH no other amino sugars were present (Fig. 14B). On HPLC these oligosaccharides migrated as the NeuAcα2,3Galß1,3(NeuAcα2,6)GalNAcOH tetrasaccharide isolated from fetuin (results not shown). After desialylation, performed by mild acid hydrolysis, the residual oligosaccharide migrated on TLC as an authentic sample of Galß1,3GalNAcOH (Fig. 13H). Based on these results we assigned to the disialoligosaccharide the following structure:



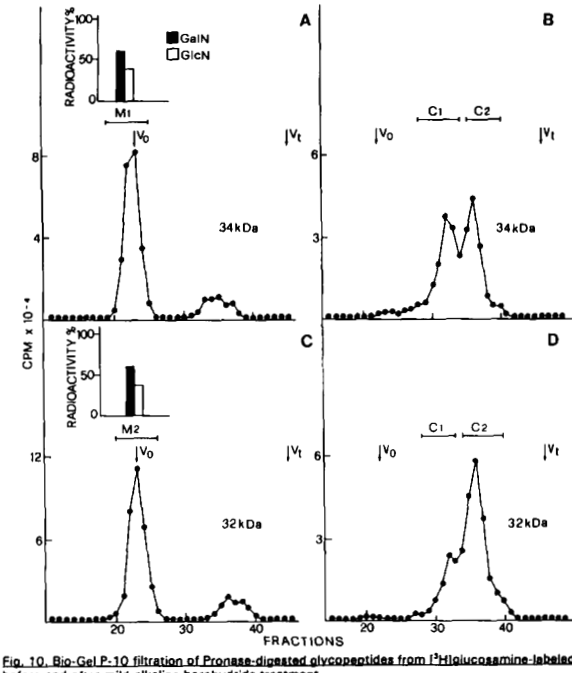


Fig. 10. Bio-Gel P-10 filtration of Pronase-digested glycopeptides from [^{3}H]glucosamine-labeled CD8m before and after mild alkaline borohydride treatment.

Bands of the polyacrylamide gel with the apparent molecular weight of 34kDa and 32kDa of [^{3}H]glucosamine-labeled CD8 obtained as described in the legend of Fig. 8 were separately excised and Pronase-digested.

A, Profile of glycopeptides from 34kDa band before mild alkaline borohydride treatment. The horizontal bar indicates the fractions pooled which were subjected to mild alkaline borohydride treatment. An aliquot was analyzed for the labeled amino sugar composition (top of the panel).

B, Profile of M1 glycopeptides from A after mild alkaline borohydride treatment. Oligosaccharides were pooled and designated as indicate by the horizontal bars.

C, Profile of glycopeptides from 32KDa band before mild alkaline borohydride treatment. The other indications are as in A.

D. Profile of M2 glycopeptides from C after mild alkaline borohydride treatment. Oligosaccharides were pooled and designated as indicate by the horizontal bars.

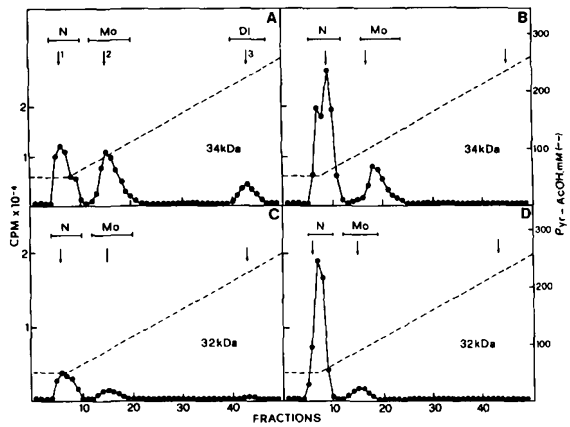


Fig. 11. DEAE-Sephacel chromatography of [³H]glucosamine-labeled oligosaccharides from CD8m.

Oligosaccharides C1 and C2 fractionated as shown in Fig. 10B and D were chromatographed as detailed in the text. Fractions were designated and pooled as shown.

A and B, Oligosaccharides from C1 and C2 peaks of Fig. 10B, respectively;

C and D, Oligosaccharides from C1 and C2 peaks of Fig. 10D, respectively;

Arrows 1, 2 and 3 indicate the elution position of a neutral oligosaccharide, NeuAcα2,3Galß1,4Glc and NeuAcα2,3Galß1,3(NeuAcα2,6)GalNAcOH.

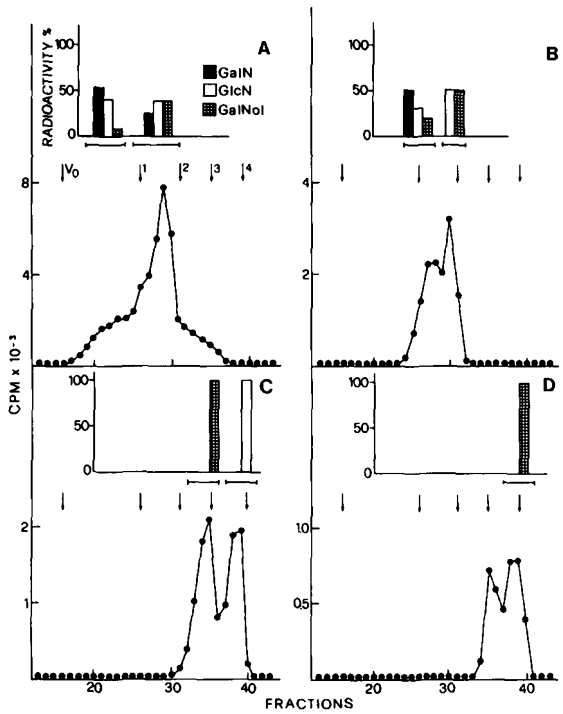


Fig. 12. Bio-Gel P-4 filtration of neutral oligosaccharides from [³H]glucosamine-labeled CD8m after various exo-glycosidase treatments

A, Neutral oligosaccharides from [³H]glucosamine-labeled CD8-34kDa and 32kDa (peaks N of Fig. 11) were mixed, lyophilized and applied to the Bio-Gel P-4 column. Fractions under the bars were pooled and an aliquot was subjected to strong acid hydrolysis for the labeled amino sugar composition.

B, Filtration profile of pooled fractions 25-31 of A digested with *Aspergillus niger* ß-galactosidase.

C, Filtration profile of pooled fractions 29-32 of B digested with jack beans ß-*N*-acetylglucosaminidase.

D, Filtration profile of pooled fractions 32-36 of C digested with *Escherichia coli* ß-galactosidase. On the top of panels the labeled amino-sugar composition of each peak is reported. Arrows as in Fig. 8.

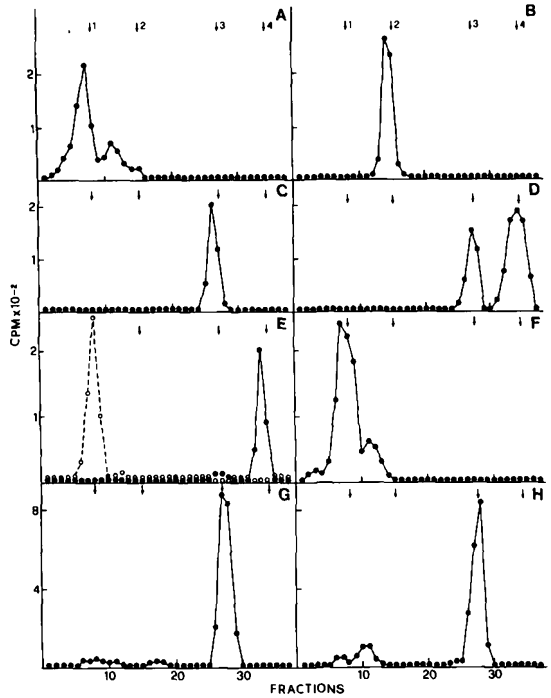


Fig. 13. TLC chromatography of oligosaccharides from mature [³H]glucosamine-labeled CD8 before and after sequential exoglycosidase digestions, Smith degradation and desialylation.

A, Migration of neutral oligosaccharides from pooled fractions 26-31 of Fig. 12A

B, Migration of fractions 29-32 of Fig. 12B corresponding to the oligosaccharides digested with *Aspergillus niger* ß-galactosidase.

C, Migration of fractions 32-36 of Fig. 12C corresponding to oligosaccharides digested with ß-*N*-acetylglucosaminidase.

D, Migration of fractions 38-40 of Fig. 12D corresponding to oligosaccharides digested with *Escherichia coli* ß-galactosidase.

E, Migration of neutral tetrasaccharide (fractions 26-31 of Fig. 12A) before (----) and after Smith degradation ( ).

F, Migration of Mo1 glycans of Fig. 14A after desialylation (0.01 N HCl for 2 h at 80° C).

G, Migration of Mo2 glycans of Fig. 14A after desialylation performed as indicated above.

H, Migration of Di oligosaccharides of Fig. 14B after desialylation performed as indicated.

Arrows 1, 2, 3 and 4, Galß1,4GlcNAcß1,6(Galß1,3)GalNAcOH, GalNAcß1,4Galß1,4Glc, Galß1,3GalNAcOH and GalNAcOH.

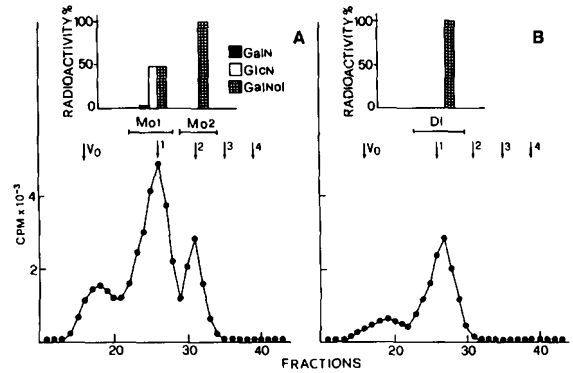


Fig. 14. Bio-Gel P-4 filtration of sialylated oligosaccharides from [³H]glucosamine-labeled CD8m.

A. Monosialooligosaccharides from [³H]glucosamine-labeled CD8-34kDa and -32kDa (peaks Mo of Fig. 11) were mixed lyophilized and applied to the Bio-Gel P-4 column.

B. Profile of disialooligosaccharides from [³H]glucosamine-labeled CD8-34kDa (peak Di of Fig. 11A).

Fractions under the horizontal bars were designated and pooled as shown. An aliquot was used for the composition in radioactive amino sugars (top of panels). Arrows as in Fig. 8.