Addition of Truncated Oligosaccharides to Influenza Virus Hemagglutinin Results in Its Temperature-conditional Cell-surface Expression

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Abstract. In the preceding paper (Hearing, J., E. Hunter, L. Rodgers, M.-J. Gething, and J. Sambrook. 1989. *J. Cell Biol.* 108:339–353) we described the isolation and initial characterization of seven Chinese hamster ovary cell lines that are temperature conditional for the cell-surface expression of influenza virus hemagglutinin (HA) and other integral membrane glycoproteins. Two of these cell lines appeared to be defective for the synthesis and/or addition of mannose-rich oligosaccharide chains to nascent glycoproteins. In this paper we show that at both 32 and 39°C the two mutant cell lines accumulate a truncated version, $Man_5GlcNAc_2$, of the normal lipid-linked precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$. This is possibly due to a defect in the synthesis of dolichol phosphate because in vitro assays indicate that the mutant cells are not deficient in mannosylphosphoryldolichol synthase at either temperature. A mixture of truncated and complete oligosaccharide chains was transferred to newly synthesized glycoproteins at both the permissive and restrictive temperatures. Both mutant cell lines exhibited altered sensitivity to cytotoxic plant lectins when grown at 32°C, indicating that cellular glycoproteins bearing abnormal oligosaccharide chains were transported to the cell surface at the permissive temperature. Although glycosylation was defective at both 32 and 39°C, the cell lines were temperature conditional for growth, suggesting that cellular glycoproteins were adversely affected by the glycosylation defect at the elevated temperature. The temperature-conditional expression of HA on the cell surface was shown to be due to impairment at 39°C of the folding, trimerization, and stability of HA molecules containing truncated oligosaccharide chains.

We have isolated a series of mutant Chinese hamster ovary (CHO)[1] cell lines that express the hemagglutinin (HA) of influenza virus on the cell surface in a temperature-conditional fashion (see accompanying paper, Hearing et al., 1989). Two of these cell lines, clones 4B and 4J, differed from the others in that they synthesize abnormally modified glycoproteins. Our initial experiments suggested that the apparent defect in the addition of asparagine-linked oligosaccharide chains to nascent glycoproteins was independent of temperature. This observation prompted us to investigate further the defect in glycosylation in these mutant cell lines and to determine how the attachment of aberrant carbohydrate chains influenced the movement of HA through the secretory pathway, resulting in the temperature-conditional expression of this glycoprotein on the cell surface. The results presented in this paper demonstrate that clones 4B and 4J accumulate a truncated oligosaccharide precursor, $Man_5GlcNAc_2$, and transfer both truncated and full length oligosaccharide chains to nascent glycoproteins. At the permissive temperature the folding and trimerization of HA occurred more slowly than in the parental cell line, although HA molecules bearing abnormal oligosaccharide chains could be detected on the surface of the mutant cells. Such molecules were not detected on the plasma membrane at the restrictive temperature. Rather, most HA molecules fail to fold properly or to form trimers at the elevated temperature, events that are required for the movement of this integral membrane protein from the endoplasmic reticulum to the Golgi complex (Gething et al., 1986).

Materials and Methods

Cells and Cell Culture

The origin of the cell lines used in these experiments and the growth conditions have been described (Hearing et al., 1989, accompanying paper).

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[1] *Abbreviations used in this paper*: CHO, Chinese hamster ovary; endo H, Endo-β-*N*-acetylglucosaminidase H; HA, hemagglutinin; LCA, *Lens culinaris* agglutinin; L-PHA, *Phaseolus vulgaris* phytohemagglutinin; RIC, *Ricinus communis* toxin; WGA, wheat germ agglutinins.

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Determination of Lectin Toxicity

A semiquantitative test (Stanley, 1985) was used to measure the cytotoxic effects of plant lectins on parental and mutant cell lines. *Lens culinaris* agglutinin (LCA) and Con A were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Wheat germ agglutinin (WGA), phytohemagglutinin (L-PHA), and *Ricinus communis* lectin (RIC) were purchased from Sigma Chemical Co. (St. Louis, MO). Cells were incubated at 32°C in the absence or presence of increasing concentrations of each lectin until cells receiving no lectin became confluent (7-8 d). The culture medium was removed and the cells stained with 0.2% (wt/vol) methylene blue in 50% methanol.

Radiolabeling of Polypeptides and Immunoprecipitations

Cells were radiolabeled with [^{35}S]methionine and polypeptides were immunoprecipitated and analyzed by SDS-PAGE as described in the preceding paper (Hearing et al., 1989).

Analysis of Lipid- and Protein-linked Oligosaccharides

Cells were seeded at 1.0 × 10^6 cells per 60-mm culture dish in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% FBS and incubated overnight at 32°C. Appropriate cultures were shifted to 39°C for 4 h before radiolabeling. All media used for the in vivo labeling of glycoproteins and glycolipids were warmed to either 32 or 39°C before use. Cells were washed twice with RPMI 1640 containing 5% dialyzed FBS, 5 mM sodium pyruvate, and 100 μg/ml glucose, and then labeled for 40 min with 200 μCi/ml [2-^{3}H]mannose (23.5 Ci/mmol; New England Nuclear, Boston, MA) in the same medium. Radiolabeling was stopped by rapidly washing the cells with ice-cold Dulbecco's PBS (Gibco Laboratories) and scraping the cells in 400 μl cold methanol. The extracts were transferred to microfuge tubes containing 600 μl chloroform and 200 μl water and rapidly mixed. Lipid-linked oligosaccharides were extracted from these samples and subjected to mild acid hydrolysis as described by Huffaker and Robbins (1982). Interfacial material insoluble in chloroform/methanol/water (3:2:1, vol/vol/vol) was dispersed three times by sonication in 1.0 ml of 4 mM $MnCl_2$, extracted three times with 1.0 ml chloroform/methanol/water (10:10:3, vol/vol/vol), and dried under a stream of nitrogen. Glycopeptides were prepared from this material by digestion with pronase (protease type XIV, Sigma Chemical Co.; 5 mg/ml in 100 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and incubated for 60 min at 50°C to inactivate potential glycosidases before use). Digestion was carried out under a toluene atmosphere for 72 h at 37°C with 84 μl additions of pronase at 24 and 48 h. Glycopeptides and oligosaccharides cleaved from lipid by mild acid treatment were digested with endoglycosidase H (endo H; Miles Scientific, Naperville, IL) in 150 mM sodium citrate, pH 5.5, for 16 h at 37°C.

Oligosaccharides and glycopeptides were resolved on a 1 × 115-cm Bio-Gel P-4 column (minus 400 mesh; BioRad Laboratories, Richmond, CA), which was equilibrated and eluted with 50 mM sodium phosphate, pH 6.5, 0.02% sodium azide. The radioactivity in each fraction was determined by liquid scintillation spectrometry using a fivefold volume of Scinti Verse II (Fisher Scientific, Springfield, NJ). Bovine serum albumin and [^{3}H]mannose were included in each sample as markers for the included (V_o) and excluded (V_i) volumes, respectively. The relative elution coefficients (K_d) of peaks were calculated as previously described (Hubbard and Robbins, 1980).

Synthesis of Dolichol-P-Mannose by Crude Membrane Fractions of Parental and Mutant Cells

Crude membrane fractions were prepared from subconfluent cultures of parental and mutant cell lines grown at 32°C. The cultures were washed once with PBS and the cells released from the dishes with PBS supplemented with 10 mM EDTA. The cells were washed once with PBS and crude membranes were prepared (Chapman et al., 1980) and assayed for protein content using the Pierce BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

Reaction mixes contained 1.65 μM GDP-[^{14}C]mannose (302 mCi/mmol; Amersham Corp., Arlington Heights, IL) and 2 mg/ml crude membrane protein in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.2 Triton X-100. Where indicated, dolichol phosphate (Sigma Chemical Co.) in chloroform/methanol (2:1) was added to each reaction tube and the solvent removed under a stream of nitrogen before adding the membrane protein. The final concentration of dolichol phosphate was 0.69 mM. Samples were incubated for 3 min at 32°C before adding GDP-[^{14}C]mannose to initiate the reaction. Aliquots (100 μl) were removed at the times indicated and the radioactivity incorporated into lipid was determined as described (Jensen and Schutzbach, 1985).

Determination of the Protease Sensitivity of HA Molecules

The conformation of HA molecules at various times after synthesis was probed by digestion with trypsin. Cells grown at 32°C, or duplicate cultures incubated 4 h at 39°C before the start of the experiment, were harvested with trypsin-EDTA, washed with ice-cold growth medium, and incubated for 30 min at 32 or 39°C in methionine-free RPMI 1640 at 1 × 10^6 cells/ml. All media were warmed to either 32 or 39°C before use. Cells starved for methionine were pelleted and resuspended in 100 μl methionine-free RPMI 1640 containing 1 mCi/ml [^{35}S]methionine (>800 Ci/mmol; New England Nuclear, Boston, MA). After a 5-min incubation at 32 or 39°C, 800 μl of RPMI 1640 supplemented with 2 mM nonradioactive methionine was added to each sample, rapidly mixed, and a 200-μl aliquot was immediately transferred to tubes containing ice-cold PBS. The remaining cells were incubated at the same temperature and 200-μl aliquots were removed at various times. Cells were pelleted and lysed by the addition of 200 μl of 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40. Cell extracts were clarified by centrifugation at 12,800 *g* for 10 min and then divided into two portions. Half of each extract was incubated at 37°C for 10 min with 1.25 μg trypsin (treated with L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone; Sigma Chemical Co.). Each sample was then diluted with 800 μl of NET-Gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.25% gelatin, 0.05% Nonidet P-40) containing 20 μg/ml soybean trypsin inhibitor (Worthington Biochemicals, Malvern, PA) and 5 U/ml Aprotinin (Sigma Chemical Co.). HA molecules were immunoprecipitated as described previously (Gething et al., 1986) and analyzed by electrophoresis through 12.5% SDS-polyacrylamide gels and fluorography (Bonner and Laskey, 1974).

Analysis of HA Molecules by Sedimentation Velocity Centrifugation

The oligomerization of HA polypeptide chains was assayed by sedimentation velocity centrifugation on sucrose gradients (Gething et al., 1986). Cells were seeded in 60-mm culture dishes at 3 × 10^5 cells/dish and incubated for 24 h before radiolabeling. Where indicated, cultures were shifted to 39°C 4 h before the start of the experiment. Cultures were labeled for 5 min with 100 μl of 1 mCi/ml [^{35}S]methionine and either washed with ice-cold PBS and extracted immediately, or incubated for 60 or 120 min in growth medium containing 2 mM nonradioactive methionine before extraction. Cell lysates were prepared in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM *N*-octylglucoside (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and then clarified by centrifugation at 12,800 *g* for 10 min before loading on 5-20% sucrose gradients prepared in the lysis buffer. Gradients were centrifuged at 40,000 rpm for 16 h at 17°C in a SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The gradients were fractionated from the bottom of the tube into 400-μl fractions which were immunoprecipitated with a rabbit anti-HA antiserum and analyzed by SDS gel electrophoresis and fluorography as described above.

Results

Abnormal Carbohydrate Side Chains Are Attached to Cellular Glycoproteins in Clones 4J and 4B

Our previous results indicated that abnormal oligosaccharide chains were added to nascent glycoproteins in the mutant cell lines 4J and 4B at both 32 and 39°C, although HA is expressed on the cell surface in a temperature-dependent fashion (see preceding paper, Hearing et al., 1989). The presence of these abnormal oligosaccharides on cellular glycoproteins at the permissive temperature is further supported by the finding (Table I) that clone 4B and clone 4J cells growing at 32°C are more resistant to certain cytotoxic plant lectins than the parental HA-CHO cells (Stanley, 1983, 1985, 1987*a*,*b*). Both mutant cell lines showed increased resistance to L-PHA, Con A, RIC, and LCA compared to the parental HA-CHO cell line, although clone 4J was more resistant

*Table I. Effects of Cytotoxic Plant Lectins on Cell Growth**

	Lectin resistance (μg/ml)				
Cell line	WGA	L-PHA	ConA	Ric	LCA
HA–CHO	10	15	20	0.5	20
clone 4B	10 (–)	25 (R)	>35 (R)	>1 (>R_2)	25 (R)
clone 4J	10 (–)	>50 (R_3)	>35 (R)	>1 (>R_2)	100 (>R_5)
Lec9	(R)	(R)	(–)	(R_{10})	(R)
Lec15	(–)	(–)	(–)	(R_4)	(–)

* The effects of various lectins on the growth of parental and mutant cells was determined by a semiquantitative test (Stanley, 1985). The values presented in this table represent the concentration of each lectin preparation that inhibited cell growth by 90% or more compared to cells cultured in the absence of lectin. The values in parentheses are fold differences in lectin resistance from parenta cells. (–) Equivalent to parental cells; (*R*) less than twofold more resistant tha parental cells. The data for Lec9 and Lec15 were taken from Stanley (1984).

than clone 4B to L-PHA and LCA. These patterns of resistance differ from those described for lectin-resistant mutants belonging to 22 different complementation groups (Stanley, 1987*b*). This result suggests that clones 4B and 4J may carry mutations in previously unrecognized genes involved in the synthesis of oligosaccharide precursors or in their transfer to newly synthesized polypeptides. However, direct comparison with existing CHO mutants of the patterns of lectin resistance under uniform experimental conditions, combined with precise biochemical and genetic analysis will be necessary to assign clone 4B and 4J cells to currently described or novel complementation groups (Stanley, 1985).

Abnormal Mannose-rich Oligosaccharides Are Added to HA at Both 32 and 39°C in Clone 4J and 4B Cells

The enzyme endo-β-*N*-acetylglucosaminidase H (endo H; Tarentino and Maley, 1974) was used to investigate further the nature of the glycosylation defect(s) in the 4J and 4B cell lines. Sensitivity to endo H is dependent upon the number and configuration of sugar residues in the oligosaccharide chain, requiring the presence of a mannose residue linked α-1,3 to a second mannose residue that is itself linked α-1,6 to a third mannose residue attached to *N*-acetylglucosamine in a β-1,4 linkage (Tai et al., 1977). During the biosynthesis of dolichol-linked $Glc_3Man_9GlcNAc_2$ precursor oligosaccharides, this structure is first present in $Man_6GlcNAc_2$-*P-P*-dolichol (reviewed by Hubbard and Ivatt, 1981; see below for further discussion), which is therefore the first biosynthetic intermediate to be sensitive to endo H. Resistance to endo H is subsequently regained after transfer of the precursor oligosaccharide to protein and processing by glucosidases and mannosidases to a $Man_3GlcNAc_2$ structure.

HA was immunoprecipitated from HA-CHO, clone 4J, and clone 4B cells, treated with endo H, and the products analyzed by electrophoresis on SDS-polyacrylamide gels (Fig. 1). The majority of the HA molecules synthesized in the parental HA-CHO cells during a 30-min radiolabeling period at 32°C migrated with an apparent molecular mass of ~68 kD (Fig. 1, lane 2) and were sensitive to digestion by endo H, which generated a ~61-kD species (Fig. 1, lane *5*). In addition, small amounts of terminally glycosylated HA (~74 kD) and core-glycosylated HA (~66 kD) that have been modified by the removal of glucosyl and mannosyl residues were also observed. Both of these species were resistant to digestion by endo H. Very similar results were obtained after labeling at 39°C (Fig. 1, lanes *8* and *11*). By contrast, the HA synthesized in clone 4J or 4B cells at 32 or 39°C was heterogeneous in size (Fig. 1, lanes *3*, *4*, *9*, and *10*). The majority of the labeled HA molecules migrated as a broad band with significantly increased mobility compared with core-glycosylated HA synthesized in parental HA-CHO cells. However, some of the labeled HA molecules migrated with approximately the same electrophoretic mobility as pulse-labeled HA from the parental cells. Longer exposure of the autoradiogram shown in Fig. 1 indicated that the results obtained with clone 4B were very similar to those seen for clone 4J. Treatment of the immunoprecipitates from the mutant cells with endo H resulted in a broad band of material whose electrophoretic mobility was less than that of endo H-treated HA from the parental cells. Similar results were obtained at both 32 and 39°C (Fig. 1, lanes *6*, *7*, *12*, and *13*). These data are consistent with HA synthesized in clone 4B and 4J cells bearing a mixture of endo H-resistant and endo H-sensitive oligosaccharide side chains. Because the aberrant glycosylation of HA occurred either during or immediately after translation of the molecule in the mutant cells (Hearing et al., 1989), it is likely that endo H-resistant oligosaccharide chains are transferred from a dolichol-lipid precursor to nascent HA molecules in clone 4J and 4B cells. Since the first endo H-sensitive species formed during the assembly of the core oligosaccharide unit is $Man_6GlcNAc_2$ (see above), this result suggests that the endo H-resistant oligosaccharides contain five or less mannose residues.

Both Truncated and Complete Oligosaccharide Units Are Transferred to Nascent Proteins in Clone 4J and 4B Cells

The oligosaccharides transferred to nascent secretory proteins in the parental and mutant cell lines were examined by gel filtration chromatography of glycopeptides prepared from cells labeled for 40 min with [^{3}H]mannose (Fig. 2). The labeled glycopeptides obtained from HA-CHO cells grown at 32 (Fig. 2 *A*) or 39°C (Fig. 2 *C*) eluted from the column as a single peak with a shoulder of more slowly eluting molecules, which was more pronounced at the lower temperature. The major species was sensitive to digestion by endo H (Fig. 2 *B*). Glycopeptides prepared from clone 4J cells labeled at 32°C (Fig. 2 *D*) and clone 4B cells (results not shown) eluted from the column as two peaks whose relative elution coefficients (0.294 and 0.429) corresponded precisely with the coefficients of the peak and shoulder, respectively, observed in the analysis of the HA-CHO glycopeptides. The material in the first peak was sensitive to endo H treatment, while the material in the second peak was resistant to the enzyme (Fig. 2 *E*). These data are consistent with the results shown in Fig. 1, which suggested that both endo H-resistant and endo H-sensitive oligosaccharide chains were transferred to nascent HA molecules in the mutant cells. Qualitatively similar results were obtained with glycopeptides isolated from clone 4J and 4B cells labeled at 39°C (Fig. 2 *F*, and results not shown), further supporting the presence of a temperature-independent glycosylation defect in these mutant cells. However, the total amount of radiolabeled glycopeptides recovered from clone 4J and 4B cells was reduced 5.9-fold and 1.3-fold, respectively, at 39°C

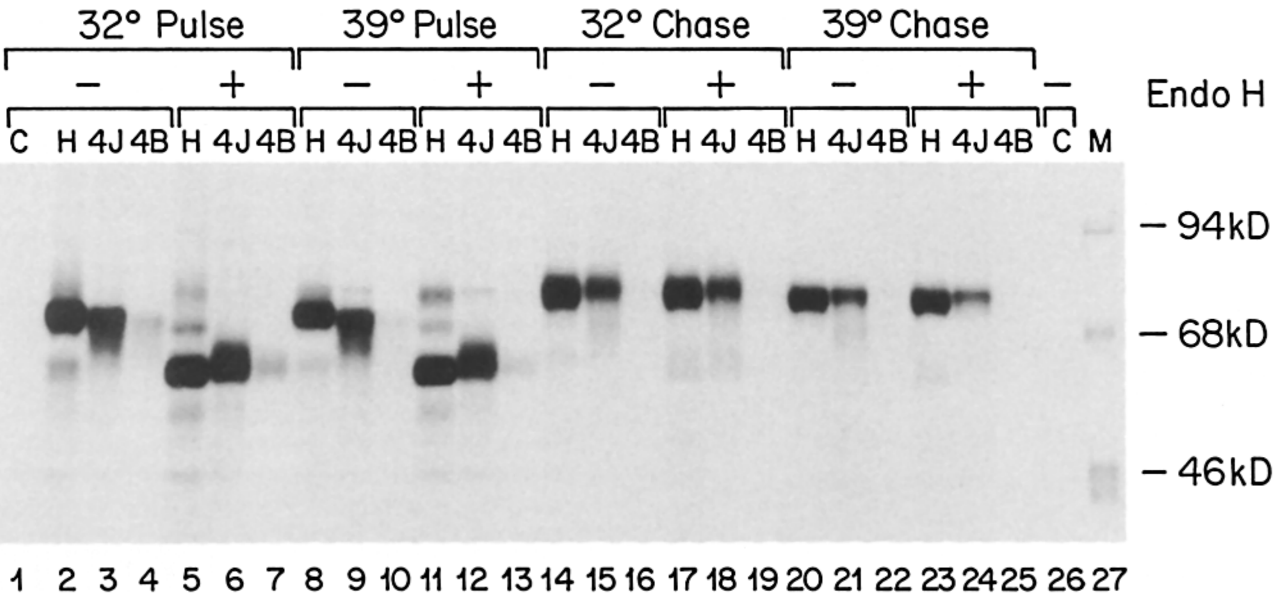

Figure 1. Synthesis and modification of HA in parental HA-CHO cells and the mutant cell lines 4B and 4J. Cells were pulse-labeled for 30 min at either 32 or 39°C with [^{35}S]methionine and then extracted as described in Materials and Methods. Cell extracts were immunoprecipitated with anti-HA serum and the precipitated proteins incubated with (+) or without (−) endo H. The digestion products were analyzed by SDS gel electrophoresis and fluorography (Bonner and Laskey, 1974). CHO cells (*C*); HA-CHO cells (*H*); clone 4J cells (*4J*); clone 4B cells (*4B*); [^{14}C]molecular mass markers (*M*). The size of the markers in kilodaltons is indicated on the right.

compared to 32°C whereas the incorporation of [^{3}H]mannose into glycopeptides in the parental cell line increased slightly (1.2-fold) at 39°C. It is possible that the increased rate of turnover of glycoproteins bearing aberrant oligosaccharide side chains (Hearing et al., 1989) contributes to the decreased yield of labeled glycopeptides at 39°C.

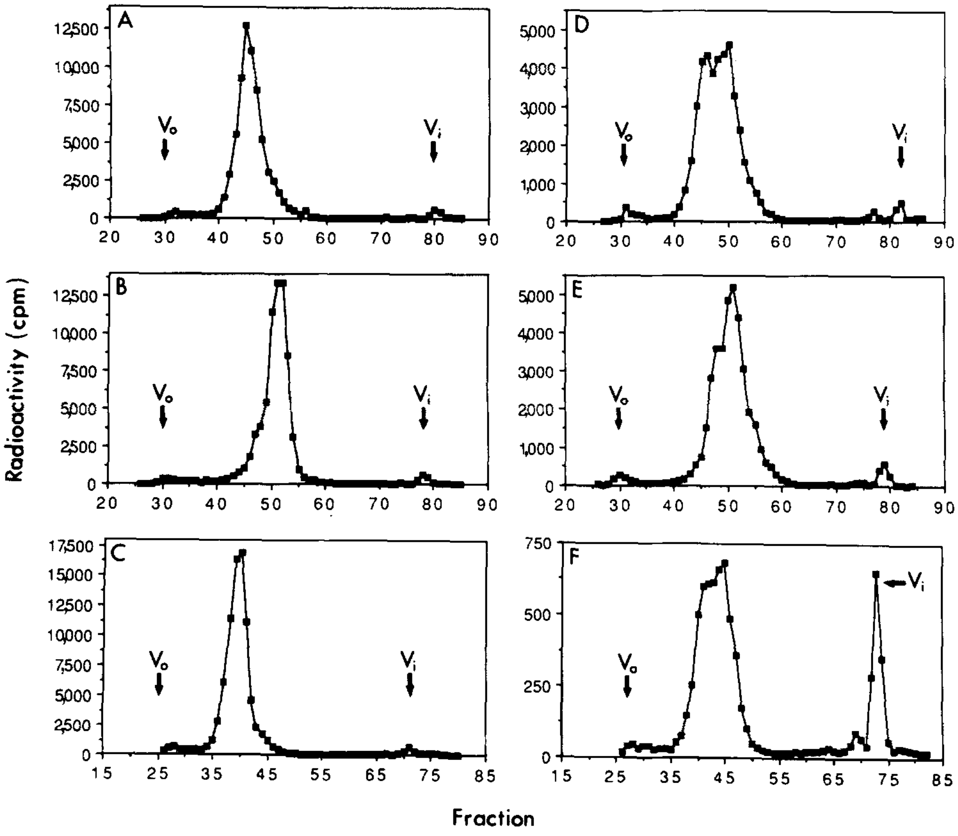

Figure 2. Gel filtration chromatography of [^{3}H]mannose-labeled glycopeptides isolated from HA-CHO and clone 4J cells. HA-CHO cells (*A–C*) and clone 4J cells (*D–F*) were labeled for 40 min with [^{3}H] mannose at 32°C (*A*, *B*, *D*, and *E*) or 39°C (*C* and *F*). Glycopeptides were prepared and analyzed by chromatography on a BioGel P4 column as described in Materials and Methods. The elution positions of BSA (V_o) and mannose (V_i) are indicated. Approximately 1-ml fractions were collected and the radioactivity in each fraction was determined by liquid scintillation counting. The glycopeptides analyzed in *B* and *E* were treated with endo H before analysis.

Clones 4J and 4B Synthesize Reduced Amounts of the Normal Lipid-linked Oligosaccharide Donor Molecule and Accumulate $Man_5GlcNAc_2$-P-P-dolichol

The abnormal glycopeptides detected in clone 4B and 4J cells could have resulted either from a defect in the biosynthesis of the lipid-linked oligosaccharide donor molecule or from a defect in the processing of the oligosaccharide chain after transfer to protein. To distinguish between these possibilities, lipid-linked oligosaccharides were isolated from the parental and mutant cell lines and treated with mild acid to release the oligosaccharides for examination by gel filtration chromatography (Fig. 3). Two major peaks of radiolabeled oligosaccharides were observed in material prepared from HA-CHO cells labeled with [^{3}H]mannose at 32°C (Fig. 3 *A*). The earliest eluting species was sensitive to digestion by endo H (Fig. 3 *B*). This result, together with its observed relative elution coefficient (0.295), suggested that this peak contained $Glc_{2-3}Man_9GlcNAc_2$ (Hubbard and Robbins, 1980). The second peak shown in Fig. 3 *A* was resistant to digestion by endo H (Fig. 3 *B*) and eluted from the column at the position of $Man_5GlcNAc_2$ (K_d = 0.495; Hubbard and Robbins, 1980). The small amount of radiolabeled material that eluted between $Glc_3Man_9GlcNAc_2$ and $Man_5GlcNAc_2$ probably represents biosynthetic intermediates. Similar results have been reported by others (Krag, 1979; Hubbard and Robbins, 1980; Stoll et al., 1982). The same species were detected in samples from parental HA-CHO cells labeled at 39°C (Fig. 3 *C*), although the $Man_5GlcNAc_2$ species was only a minor component at the higher temperature. Samples from confluent cultures of HA-CHO cells labeled under the same conditions at 39°C contained even less $Man_5GlcNAc_2$ (unpublished observations).

The lipid-linked oligosaccharides labeled with [^{3}H]mannose in clone 4J (Fig. 3) and 4B cells (data not shown) differed dramatically from those observed in the parental cells. At both 32 and 39°C (Fig. 3, *D* and *F*) the cells contained a major labeled species that eluted at the position of $Man_5GlcNAc_2$ (K_d = 0.490; Hubbard and Robbins, 1980) and was resistant to digestion by endo H (Fig. 3 *E*). Only minor amounts of larger oligosaccharides were detected after labeling at either temperature. Thus, both clone 4B and 4J cells are defective for the synthesis of the normal, lipid-linked oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$-*P-P*-dolichol) and accumulate $Man_5GlcNAc_2$-*P-P*-dolichol. It is striking that although the full-length precursor constitutes 10% or less of the pool of lipid-linked oligosaccharides (Fig. 3, *D* and *F*), the full-length and truncated oligosaccharides are present in glycopeptides in more equal proportions (Fig. 2, *D* and *F*). This amelioration of the mutant phenotype is probably a consequence of a higher affinity for the full-length precursor of the enzyme that transfers the oligosaccharide units to asparagine residues on nascent polypeptide chains.

Clone 4B and 4J Cells Do Not Lack Mannosylphosphoryldolichol Synthase Activity

The biosynthesis of the mannose-rich oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, for asparagine-linked glycosylation involves the stepwise addition of sugars to dolichol, a polyisoprenoid lipid molecule (reviewed by Hubbard and

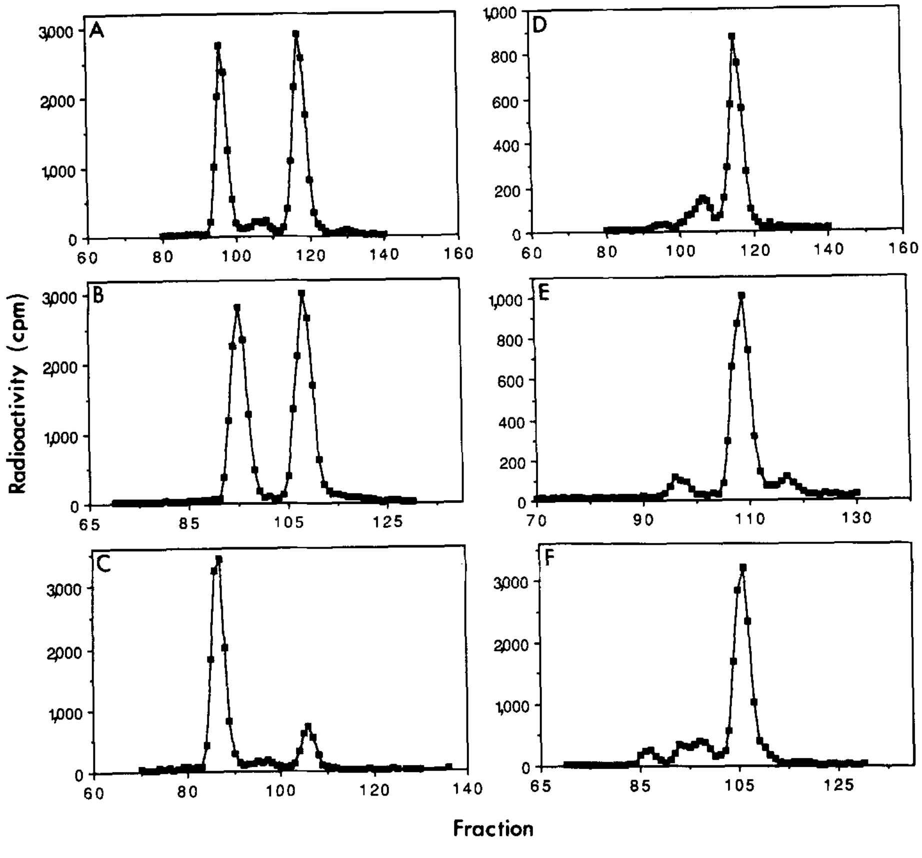

Figure 3. Gel filtration chromatography of [^{3}H]mannose-labeled lipid-linked oligosaccharides synthesized in HA-CHO and clone 4J cells. HA-CHO cells (*A-C*) and clone 4J cells (*D-F*) were labeled with [^{3}H]mannose for 40 min at 32°C (*A*, *B*, *D*, and *E*) or 39°C (*C* and *F*). Lipid-linked oligosaccharides were isolated, the carbohydrate chains released from lipid by mild acid hydrolysis, and the oligosaccharides separated by chromatography on a BioGel P4 column as described in Materials and Methods. Approximately 0.5-ml fractions were collected and the radioactivity in each fraction was determined by scintillation counting. The inclusion and exclusion volumes for the profile shown in *C* were at fractions 156 (V_i) and 58 (V_o) and for *F* were 155 (V_i) and 57 (V_o). The profiles were aligned according to the relative elution coefficients of the observed species. The oligosaccharides analyzed in *B* and *E* were treated with endo H before analysis.

Ivatt, 1981; Lennarz, 1987; Hirschberg and Snider, 1987). The nucleotide sugars UDP-GlcNAc and GDP-mannose serve as donors for the addition of the two *N*-acetylglucosamine residues and the first five mannose residues, while dolichol-*P*-mannose and dolichol-*P*-glucose are the donors for the elongation of the lipid-linked Man$_5$GlcNAc$_2$ to Man$_9$GlcNAc$_2$ and its subsequent glucosylation.

To investigate the basis for the accumulation of Man$_5$-GlcNAc$_2$-*P*-*P*-dolichol in clone 4B and 4J cells, the ability of crude membrane fractions prepared from the parental and mutant cell lines to catalyze the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to dolichol phosphate was measured. In the absence of exogenous dolichol phosphate, preparations of membranes from the two mutant cell lines incorporated [^{14}C]mannose into lipid to only 60% of the level obtained with membranes prepared from HA-CHO cells (Fig. 4 *A*). As described previously (Krag, 1979; Chapman et al., 1980), the addition of dolichol phosphate to the reaction greatly stimulated the incorporation of radioactivity by membrane fractions prepared from the parental cells (Fig. 4 *B*). Membranes from clones 4B and 4J were also stimulated in transfer activity in the presence of exogenous dolichol phosphate, and in fact catalyzed the transfer of 50–60% more [^{14}C]mannose into lipid than did membranes from the parental HA-CHO cells (Fig. 4 *B*). These results suggest that the clone 4B and 4J mutant cell lines are not lacking in mannosylphosphoryldolichol synthase activity. Whether the apparent deficiency of dolichol phosphate that results in a 30–40% decrease in in vitro activity in the absence of exogenous substrate is sufficient to cause the accumulation of Man$_5$GlcNAc$_2$-*P*-*P*-dolichol in vivo is currently under investigation. It is possible that a compensatory increase in the synthase activity in the mutant cells is responsible for the enhanced incorporation of [^{14}C]mannose into lipid when additional dolichol phosphate is provided.

Addition of Truncated Oligosaccharides to HA in Clones 4B and 4J Alters the Folding and Trimerization of this Integral Membrane Glycoprotein

Previous experiments have demonstrated that newly synthesized, wild-type HA molecules that have been core glycosylated in the endoplasmic reticulum rapidly assemble into trimeric structures that are protease resistant; HA mutants that are not transported to the Golgi apparatus are defective for the formation of native trimers (Gething et al., 1986). We therefore examined the ability of HA molecules synthesized in HA-CHO and clone 4J cells to fold into protease-resistant, trimeric structures. Cells were labeled at either 32 or 39°C for 5 min and then incubated for various periods of time (0–120 min) in growth medium supplemented with nonradioactive methionine before cell extracts were prepared and tested (*a*) by immunoprecipitation and SDS-PAGE for their sensitivity to protease treatment at 37°C (Fig. 5), or (*b*) by sucrose gradient sedimentation for their assembly into trimeric structures (Figs. 6 and 7).

Nascent HA molecules synthesized in parental HA-CHO cells during a 5-min pulse with [^{35}S]methionine were completely degraded during a short incubation with trypsin at 37°C. This result was obtained whether the cells were labeled at 32 or 39°C (Fig. 5). Trypsin-resistant HA subunits (HA1 and HA2) were first observed after a 15-min chase period. After synthesis and incubation at 32°C, the amount of protease-resistant HA species increased with time, although the nascent protein had not become completely resistant to degradation by the last time point (60 min; Fig. 5). By contrast, at 39°C the acquisition of protease resistance by HA was more rapid and reached a maximum by 30 min. The more rapid maturation of HA at 39°C was also indicated by the earlier appearance of terminally glycosylated HA1 and HA2 species (15 min at 39°C compared to 30 min at 32°C; Fig. 5). The mobility difference caused by terminal glycosylation is more pronounced in the HA1 subunit which bears four of the five N-linked oligosaccharide chains that are attached to the A/Japan HA molecule (Gething et al., 1980). The presence of complex oligosaccharide chains on HA1 and HA2 establishes that these molecules have been transported from the endoplasmic reticulum to the Golgi apparatus, a property of trimeric forms of HA (Gething et al., 1986). The half-time for folding of the endogenous HA in the HA-CHO cells into a trypsin-resistant form (∼20 min at 39°C) was much slower than was observed for Japan HA expressed from an SV-40-based viral vector in CV-1 monkey kidney cells ($t_{1/2}$∼7–10 min at 37°C; Gething et al., 1986). We have shown that the rate of trimerization of HA expressed from various recombinant vectors in different cell lines is proportional to the concentration of HA monomers in the rough endoplasmic reticulum, rather than being dependent on host cell factors (Gething, M.-J., K. McCammon, and J. Sambrook, manuscript in preparation).

When the same experiment was performed with clone 4J cells (Fig. 5), no trypsin-resistant HA molecules were detected in extracts of cultures labeled at either 32 or 39°C. In this experiment the protease digestions were performed at 37°C. However, the same result was obtained when the diges-

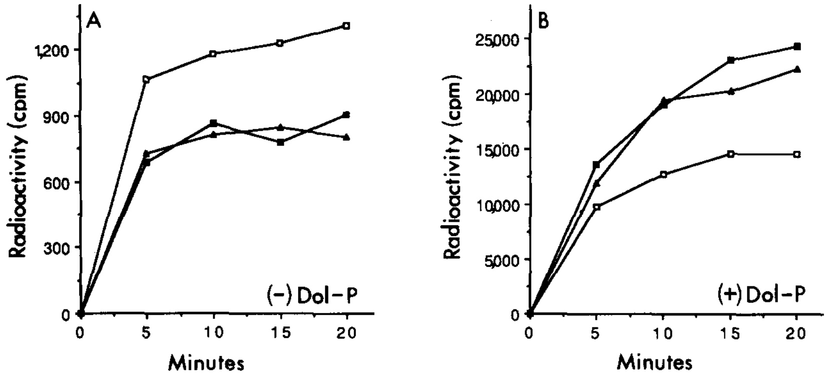

Figure 4. Synthesis of dolichol-*P*-mannose by crude membranes prepared from HA-CHO, clone 4J, and clone 4B cells. Equivalent amounts of membrane protein from HA-CHO, clone 4J, and clone 4B cells grown at 32°C were incubated for the times indicated with GDP-[^{14}C] mannose in the absence (*A*) or presence (*B*) of exogenous dolichol phosphate and the incorporation of ^{14}C into lipid was determined as described in Materials and Methods. (□) HA-CHO cells; (■) clone 4J cells; (▲) clone 4B cells.

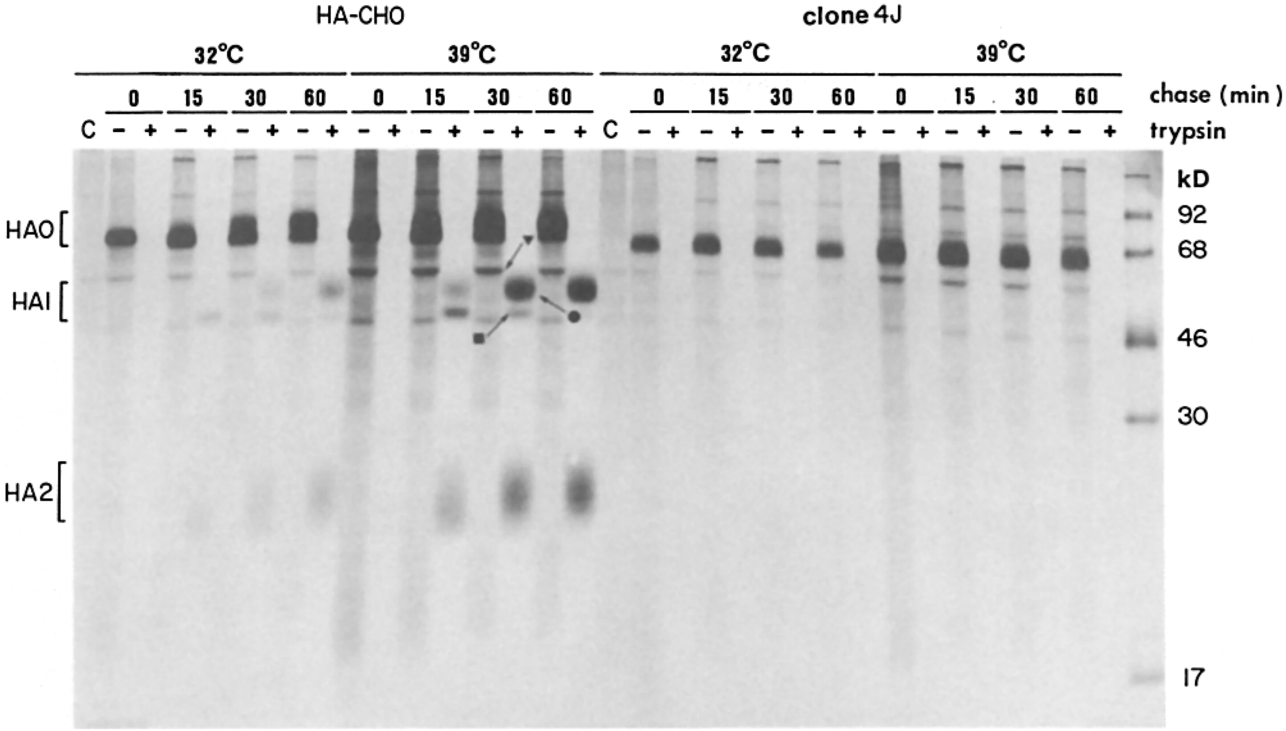

Figure 5. Acquisition of resistance to trypsin by HA molecules synthesized in HA–CHO and clone 4J cells. Cell extracts were prepared from cultures pulse-labeled for 5 min with [^{35}S]methionine and chased for the times shown with nonradioactive methionine. The pulse-chase was performed at both 32 and 39°C. The ability of HA molecules to fold into a trypsin-resistant structure was determined by incubating half of each extract with (+) or without (−) trypsin at 37°C, immunoprecipitating the surviving HA molecules, and analyzing the immunoprecipitates by SDS gel electrophoresis and fluorography as described in Materials and Methods. The positions of uncleaved HA (*HAO*) and the trypsin cleavage products (*HA1* and *HA2*) are indicated on the left. The square indicates the core-glycosylated form of HA1 while the circle indicates the terminally glycosylated form of HA1. The triangle indicates a protease-sensitive, ∼55-kD species that is also present in control cell extracts from labeled CHO cells. The sizes of the molecular mass markers, in kD, are indicated at the right.

tions were carried out at 32°C (data not shown). Thus HA bearing truncated carbohydrate side chains does not attain a trypsin-resistant conformation during the 60-min chase period.

Evidence that the assembly of HA molecules into trimers was impaired by aberrant glycosylation was also obtained by sedimentation velocity centrifugation of nascent HA synthesized in HA-CHO or clone 4J cells. Monomeric and trimeric forms of HA were separated on sucrose gradients, immunoprecipitated from the gradient fractions using a polyclonal anti-HA serum, and visualized by SDS-PAGE and fluorography (Gething et al., 1986). The majority of nascent HA molecules isolated from parental HA-CHO cells pulse-labeled for 5 min at either 32 or 39°C sedimented as a single peak on a 5–20% sucrose gradient (Fig. 6). After a 60-min chase an additional, more rapidly sedimenting peak was observed that contained both core and terminally glycosylated HA molecules. Cross-linking experiments have previously demonstrated that the more rapidly sedimenting peak is composed exclusively of trimeric HA molecules (Gething et al., 1986). A greater proportion of the nascent HA molecules synthesized at 39°C were assembled into the trimeric form during the chase period at the elevated temperature.

The formation of HA trimers was much slower in clone 4J cells than in HA-CHO cells (Fig. 7). Only a small amount of HA synthesized at 32°C during a 5-min pulse sedimented at the position of trimeric HA after a 60-min chase period. However, the amount of trimeric HA continued to increase between 60 and 120 min as did the proportion of HA trimers that contained complex oligosaccharides. By contrast, only a very small proportion of HA molecules synthesized at 39°C were assembled into trimers during the 60-min chase period at 39°C and that proportion did not appear to increase during the next 60-min chase period at the elevated temperature (Fig. 7).

The Secretion of a Nonglycosylated Cellular Protein, β_2-Microglobulin, Is Not Affected in Clone 4J and 4B Cells

Although the glycosylation defect in clone 4J and 4B cells was temperature independent, the cell surface expression of HA and other cellular integral membrane glycoproteins was temperature conditional, apparently as the result of impairment of the intracellular transport and stability of the aberrantly glycosylated molecules at the elevated temperature. However, there was no difference between the ability of the parental or mutant cell lines to synthesize and secrete a nonglycosylated cellular protein, β_2-microglobulin (Fig. 8); in fact, all three lines secreted more of the protein at the higher temperature. β_2-microglobulin is normally complexed with class I histocompatibility antigens on the cell surface but in

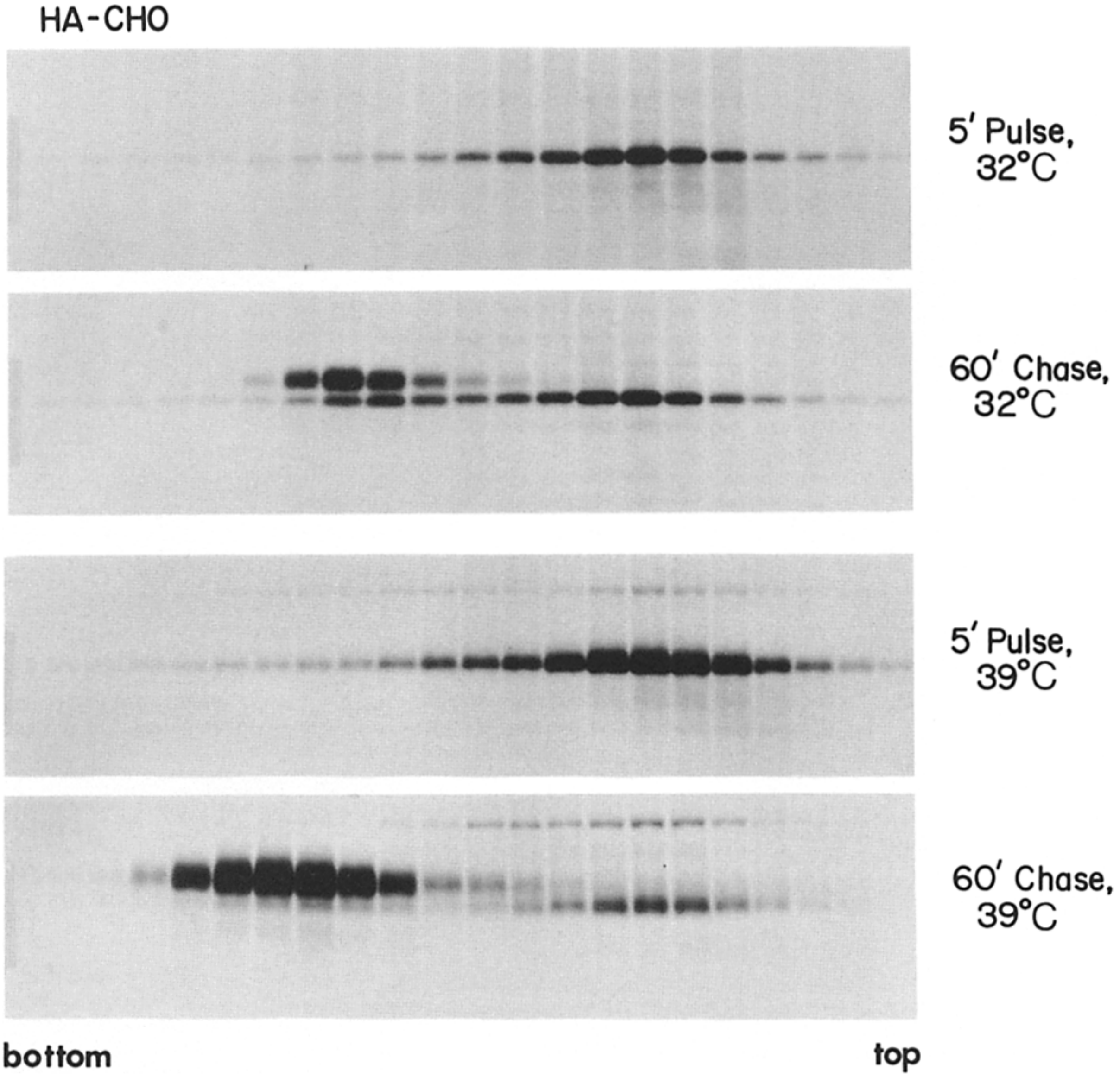

Figure 6. Velocity sedimentation analysis of HA trimer formation in HA-CHO cells. Extracts were prepared from cells pulse-labeled for 5 min with [^{35}S]methionine and then incubated for 0 or 60 min with nonradioactive methionine. The extracts were subjected to velocity sedimentation on 5–20% sucrose gradients, the gradients fractionated, and HA molecules were immunoprecipitated from each gradient fraction. The immunoprecipitates were analyzed by SDS gel electrophoresis and fluorography. Details of the techniques used are given in Materials and Methods. The portion of each gel containing HA molecules is shown and the direction (*top* and *bottom*) of the sucrose gradients is indicated at the bottom of the figure.

a number of cell lines it is synthesized in excess of these antigens and is secreted into the culture medium (M. Krangel, personal communication). The result shown in Fig. 8 suggests that clone 4B and 4J cells are not defective in the transport to the cell surface of nonglycosylated secretory proteins.

Discussion

Glycosylation mutants of animal cells have served as important tools for elucidating the complex biochemical pathways for the synthesis, addition, and subsequent modification of N-linked oligosaccharide chains on secretory proteins (reviewed by Stanley, 1984, 1987*a*,*b*). The majority of such mutant cell lines were obtained using cytotoxic plant lectins to select from populations of mutagenized cells those bearing altered carbohydrate moieties on cell-surface molecules. Glycosylation mutants have also been found among survivors of suicide protocols using [^{3}H]sugars (Hirschberg et al., 1981, 1982) and in cells resulting from selections aimed at individual glycoprotein membrane receptors such as Thy-1 antigen (Trowbridge and Hyman, 1975, 1979; Trowbridge et al., 1978), the mannose-6-phosphate receptor (Robbins et al., 1981; Stoll et al., 1982), and the low density lipoprotein receptor (Krieger et al., 1981; Kingsley et al., 1986). We

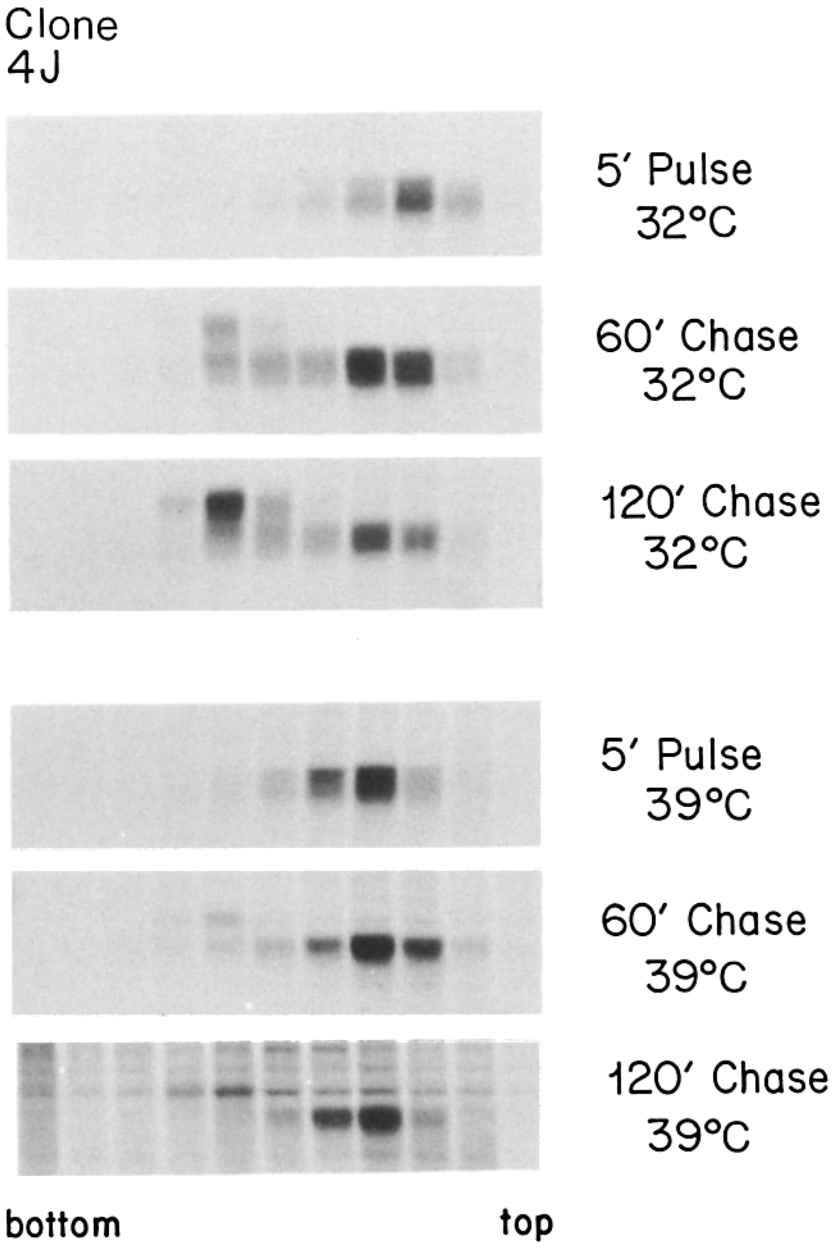

Figure 7. Velocity sedimentation analysis of HA trimer formation in clone 4J cells. HA trimer formation in clone 4J cells was analyzed as described in Fig. 6. An additional chase period of 120 min was included in this experiment.

have developed a protocol for the isolation of secretory pathway mutants of animal cells that uses a cell sorter and a cell line that constitutively expresses large amounts of a well-characterized membrane protein, the HA of influenza virus (Hearing et al., 1989, accompanying paper). During the course of screening cell lines for the temperature-conditional cell surface expression of HA, we identified two independently isolated clones that synthesized glycoproteins which displayed abnormal mobilities on SDS-polyacrylamide gels. The data presented in this paper demonstrate that these cell lines, clones 4B and 4J, are defective for the synthesis of the normal lipid-linked oligosaccharide donor molecule for N-linked glycosylation and illustrate how abnormal modification of an integral membrane glycoprotein may result in its temperature-conditional expression on the cell surface.

Analysis of the lipid-linked oligosaccharides and glycopeptides in the mutant cell lines revealed that clones 4B and 4J accumulate the biosynthetic intermediate $Man_5GlcNAc_2$-*P*-*P*-dolichol and transfer both truncated and full-length oligosaccharide chains to nascent glycoproteins in the endoplasmic reticulum. It initially appeared paradoxical that the 4B and 4J mutants, which had been selected on the basis of temperature-conditional expression of HA on the plasma membrane, should display this defect at both the permissive and restrictive temperatures. However, the experiments described in this and the accompanying paper demonstrate that the basis for the temperature-sensitive phenotype is the defective assembly and instability of HA and other glycoproteins bearing abnormal side chains. Interestingly, members of three complementation groups of CHO cell mutants (lec5,

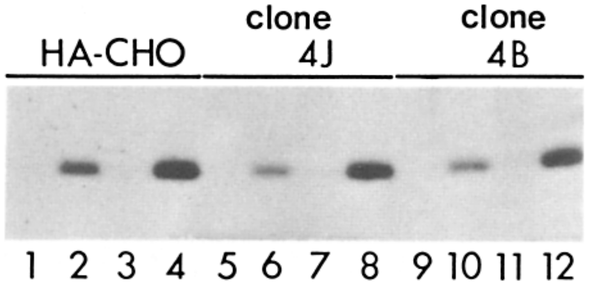

Figure 8. Secretion of β_2-microglobulin by HA-CHO, clone 4J, and clone 4B cells. Cultures growing at either 32°C (lanes *1*, *2*, *5*, *6*, *9*, and *10*) or 39°C (lanes *3*, *4*, *7*, *8*, *11*, and *12*) were pulse-labeled with [^{35}S]methionine for 10 min and then chased for 4 h in the presence of nonradioactive methionine. The culture media were divided into two equal portions and immunoprecipitated with either an anti-HA antiserum (lanes *1*, *3*, *5*, *7*, *9*, and *11*) or an anti-β_2-microglobulin antiserum (lanes *2*, *4*, *6*, *8*, *10*, and *12*). The immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

lec9, and tsK/34C) that have nonconditional defects in various steps in the synthesis and transfer of lipid-linked oligosaccharides also display a temperature-sensitive growth phenotype (Stanley, 1984). No temperature-sensitive phenotypes have been observed for mutants that map in the later steps of oligosaccharide processing. The addition of abnormal oligosaccharides to newly synthesized polypeptides therefore seems to have a more detrimental effect on protein stability and transport, probably by inhibiting the correct folding and assembly of the nascent molecules (Gething et al., 1986), than does abnormal processing of already correctly folded glycoproteins. This possibility is supported by previous observations that drugs that block N-linked glycosylation or inhibit the processing of core oligosaccharides in the endoplasmic reticulum have more deleterious effects on protein transport than do drugs which interfere with the later processing steps located in the Golgi apparatus (reviewed by Elbein, 1987).

The biosynthesis of the mannose-rich oligosaccharide precursor for asparagine-linked glycosylation has been intensely investigated (reviewed by Hubbard and Ivatt, 1981; Lennarz, 1987; Hirschberg and Snider, 1987). This precursor, Glc$_3$Man$_9$GlcNAc$_2$, is synthesized by the stepwise addition of sugars to dolichol, a polyisoprenoid lipid molecule. In exponentially growing cells only the full-length precursor is transferred to protein, although under conditions such as glucose deprivation or treatment with carbonyl cyanide *m*-chlorophenyl hydrazone, an uncoupler of oxidative phosphorylation, the truncated oligosaccharide Man$_5$GlcNAc$_2$ is accumulated and can be glucosylated and transferred to protein (reviewed by Elbein, 1987). Strikingly, cell mutants isolated using diverse selection protocols, including clones 4B and 4J described in this paper, also accumulate and transfer the same truncated oligosaccharides to nascent polypeptides (reviewed by Stanley, 1984, 1987*a*,*b*). Two cell lines in the Lec15 complementation group, a murine class E Thy-1$^-$ lymphoma line that failed to display detectable amounts of Thy-1 glycoprotein on the cell surface, and the CHO B4-2-1 cell mutant that synthesized an altered mannose-6-phosphate receptor, accumulated Man$_5$GlcNAc$_2$-*P*-*P*-dolichol and only transferred endo H–resistant oligosaccharides to nascent glycoproteins (Stoll et al., 1982). A different CHO cell line, Lec9, also accumulated Man$_5$GlcNAc$_2$-*P*-*P*-dolichol (Stanley, 1984), as did a CHO cell variant, PIR, which has been selected for resistance to the processing inhibitors castanospermine and swainsonine (Lehrman, M. A., and Y. Zeng, personal communication). Since the Lec9 and Lec15 mutants belong to different complementation groups (Stanley, 1984), this phenotype can result from the loss of (at least) two enzyme activities.

Three possible sites exist for a defect leading to accumulation of the truncated Man$_5$GlcNAc$_2$ oligosaccharide (reviewed by Chapman and Calhoun, 1988). The first involves inhibition of the synthesis of dolichol-*P*-mannose which could occur as a result of conditions such as drug treatment, energy depletion, insufficient substrate levels, or inactive enzymes. Alternatively, the transport of dolichol-*P*-mannose to the luminal side of the endoplasmic reticulum membrane may be blocked even though the molecule is synthesized normally on the cytoplasmic side of the membrane. Finally, the transferase responsible for synthesis of Man$_6$GlcNAc$_2$ could be inactivated. In only one case is the specific defect leading to the accumulation of the truncated oligosaccharide understood. The mutant cell lines in the Lec15 complementation group have been shown to lack the enzyme mannosylphosphoryldolichol synthase (Chapman et al., 1980; Stoll et al., 1982) and thus are unable to synthesize dolichol-*P*-mannose, the sugar donor molecule for the elongation of Man$_5$GlcNAc$_2$-*P*-*P*-dolichol. The specific defect in Lec9 cells, which have normal levels of mannosylphosphoryldolichol synthetase, has not yet been reported.

Clones 4B and 4J clearly differ from mutants from the Lec15 complementation group since in vitro assays indicated that the cells contained levels of mannosylphosphoryldolichol synthase somewhat greater than that in the parental cells when exogenous dolichol phosphate was added. Complementation analysis will be necessary to determine whether the same cellular gene is defective in 4B, 4J and Lec9 cells. However, clones 4B and 4J displayed some differences in their degree of resistance to various cytotoxic plant lectins (Table I). Furthermore, their patterns of resistance also differed in some respects from that reported for Lec9 cells (Table I; Stanley, 1984), although we have not been able to make a direct comparison under uniform experimental conditions. It is therefore possible that the three mutants are either affected in different reactions necessary for elongation of the Man$_5$GlcNAc$_2$-*P*-*P*-dolichol intermediate, or that there is variability in the severity of the defect common to all three cell lines. Complementation analysis combined with precise biochemical characterization of the levels of activity of the various enzymes involved in the lipid-linked oligosaccharide precursors in these cells should discriminate between these possibilities.

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