Glycosylation of Recombinant Prorenin in Insect Cells: The Insect Cell Line Sf9 Does Not Express the Mannose 6-Phosphate Recognition Signal

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ABSTRACT: Sf9 cells infected with a recombinant baculovirus containing the gene for human prorenin were cultured in the presence of [^{3}H]mannose. *In vivo* labeled prorenin was isolated by immunoprecipitation from the culture medium and digested with Pronase. The oligosaccharide structures on the resulting glycopeptides were analyzed by a combination of lectin, ion-exchange, paper, and high-pressure liquid chromatography. Of the N-linked oligosaccharides isolated from the Sf9-produced prorenin, 98% were of a truncated (trimannosyl) high-mannose type, approximately two-thirds of which contained a fucose residue linked to the reducing *N*-acetylglucosamine. The remaining 2% constituted a mixture of high-mannose-type structures containing six, seven, or eight mannose residues; none of these structures were core-fucosylated. None of the oligosaccharide structures recovered from recombinant prorenin synthesized by Sf9 cells were phosphorylated or contained any other form of charge. Furthermore, assays for UDP-GlcNAc–lysosomal-enzyme *N*-acetylglucosamine phosphotransferase demonstrated no activity above background in lysates prepared from Sf9 cells. Blotting of Sf9 cell lysates with an ^{125}I-labeled, soluble form of the cation-independent mannose 6-phosphate receptor failed to detect any proteins carrying the mannose 6-phosphate recognition signal. Taken together, the data suggest that Sf9 cells do not synthesize high-mannose-type oligosaccharides containing mannose 6-phosphate, and consequently it appears unlikely that these cells utilize the mannose 6-phosphate receptor mediated pathway for targeting of lysosomal enzymes.

Newly synthesized lysosomal enzymes are in many cell types directed to the lysosomes by a specific targeting signal, a 6-phosphorylated mannose (Man-6-P)[1] residue(s) linked to a high-mannose-type oligosaccharide on the protein. The initial step in the synthesis of this (Man-6-P) structure is the transfer of *N*-acetylglucosamine phosphate to a certain mannose residue(s) on the acceptor molecule, a reaction catalyzed by the enzyme UDP-GlcNAc–lysosomal-enzyme, *N*-acetylglucosamine phosphotransferase. Subsequent to this reaction, the terminal *N*-acetylglucosamine on the product is removed by the enzyme *N*-acetylglucosamine-1-phosphodiester *N*-acetylglucosaminidase, such that the underlying phosphorylated mannose is exposed. Available data suggest that both these reactions are carried out in an intermediate compartment between the endoplasmic reticulum (ER) and the Golgi apparatus [reviewed by Kornfeld and Mellman (1989)]. Two separate receptors capable of recognizing molecules carrying the Man-6-P recognition signal have been described [reviewed by Kornfeld (1992)]. Binding of a newly synthesized lysosomal enzyme to these receptors results in its sequestering from the secretory pathway into a vesicular compartment and subsequent transport to the lysosomes, a reaction believed to be localized primarily in the trans Golgi network (Kornfeld, 1992).

Renin has several characteristics of a lysosomal enzyme. The amino acid sequence of the human molecule is 46% identical with that of cathepsin D (Faust et al., 1985), and it has been suggested that there is an evolutionary relationship between the two molecules (Tang, 1979). Reports have also appeared demonstrating that human renin, in analogy with lysosomal enzymes, carries phosphorylated N-linked oligosaccharides (Faust et al., 1987; Aeed et al., 1992). In a previous study, we characterized the oligosaccharides on human prorenin expressed in Chinese hamster ovary cells (Aeed et al., 1992). Approximately 40% of the oligosaccharide structures on this molecule were found to be of high-mannose and hybrid type, and of these, approximately 6% of the high-mannose-type structures and 10% of the hybrid-type structures contained phosphate residues (primarily as monoesters) which could be removed by treatment with alkaline phosphatase.

Little data have appeared on the terminal substitutions on oligosaccharides synthesized by insect cells. Previous work has demonstrated that in many insect cells the *N*-glycans conjugated to secreted proteins differ from those synthesized by most mammalian cells; typically, the insect cell derived structures have been shown to be exclusively of high-mannose and/or truncated high-mannose type (*e.g.*, Hsieh & Robbins, 1984; Kuroda et al., 1990; Wathen et al., 1991; Hård et al., 1993; Kubelka et al., 1993). However, an exception to this rule, human plasminogen, has been reported (Davidson et al., 1990; Davidson & Castellino, 1991).

This study presents a partial characterization of the oligosaccharide structures linked to recombinant human prorenin synthesized by the insect cell line Sf9. In addition, data are presented from experiments aimed at investigating if this cell line expresses the Man-6-P structures used in the mammalian lysosomal enzyme targeting system.

MATERIALS AND METHODS

Materials. Sodium ^{125}I-iodide (14.7 mCi/μg) was from Amersham. AffiGel 10 and BioGel P-6 were from BioRad. Pronase and *Escherichia coli* alkaline phosphatase were from Calbiochem. D-[2-^{3}H]Mannose (22.7 Ci/mmol) was from

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; Man, mannose; pfu, plaque forming units; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* cell line 9; TIU, trypsin inhibitory units.

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Dupont. Peptide:*N*-glycosidase F was from Genzyme. Grace's insect cell culture medium, heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin were from Gibco. Recombinant endoglycosidase H and UDP-[1-^{14}C]-*N*-acetylglucosamine (34 mCi/mmol) were from ICN. McCoy's medium 5A and α-MEM were from Irvine Scientific. Cyanogen bromide-activated Sepharose 4B, concanavalin A–Sepharose, and protein A–Sepharose were from Pharmacia. *Diplococcus pneumoniae* endoglycosidase D, bovine epididymis α-fucosidase, bovine testis β-galactosidase, jack bean α-mannosidase, snail β-mannosidase, *Ricinus communis* agglutinin II (RCA), and QAE-Sephadex were from Sigma. *Pisum sativum* agglutinin (PSA)–agarose was from Vector. Rabbit anti-renin antiserum was provided by Dr. S. Sharma, The Upjohn Co. Soluble cation-independent mannose 6-phosphate receptor (CI-MPR) was a gift from Dr. P. Lobel, The University of Medicine and Dentistry, Piscataway, NJ. Yeast *O*-phosphomannan was provided by M. E. Slodki, Northern Regional Research Center, Agricultural Research Service, USDA, Peoria, IL. All other supplies were from standard sources.

Oligosaccharide Standards. Manα1–3(Manα1–6)Manβ1–4GlcNAc and Manα1–3(Manα1–6)Manβ1–4GlcNAcβ1–4(Fucα1–6)GlcNAc were isolated from [^{3}H]mannose *in vivo* labeled FG glycoprotein (Wathen et al., 1991). $Man_5GlcNAc$, $Man_6GlcNAc$, $Man_7GlcNAc$, $Man_8GlcNAc$, and $Man_9GlcNAc$ were a gift from Dr. R. Cummings, The University of Oklahoma, Oklahoma City, OK.

Preparation of RCA–Agarose. RCA–agarose was prepared as described by Aeed et al. (1992).

Cells and Baculovirus Constructions. Chinese hamster ovary (CHO) cells, provided by Dr. T. Raub, The Upjohn Co. were grown and maintained in McCoy's medium 5A supplemented with 10% FBS, penicillin, and streptomycin. Murine lymphoma BW 5147 cells, kindly provided by Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, MO, were grown and maintained in α-MEM supplemented with 10% FBS, penicillin, and streptomycin. Sf9 cells were cultured, baculovirus constructs were prepared, and recombinant human prorenin was expressed as described previously (Thomsen et al., 1993).

Metabolic Labeling. Approximately 6×10^6 Sf9 cells seeded in a 75 cm^2 tissue culture flask were infected with recombinant baculovirus (5 pfu/cell) containing the prorenin gene. Infected cells were labeled 24–48 h postinfection in 5 mL of Grace's medium containing 10% FBS, 0.4 mM glucose, and 0.1–0.3 mCi/mL [^{3}H]mannose. Following labeling, the culture medium, containing secreted, labeled prorenin, was removed and cooled on ice. Aprotinin (0.1 TIU/mL) and 10 μg/mL each of antipain, chymostatin, leupeptin, and pepstatin were added, and the medium was centrifuged at low speed to remove cells.

Immunoprecipitation and Preparation of Glycopeptides and Oligosaccharides. Prorenin was immunoprecipitated from 5 mL aliquots of conditioned culture medium (see above) as described previously (Aeed et al., 1992). Radiolabeled prorenin glycopeptides were liberated by Pronase treatment as described by Cummings et al. (1989). Oligosaccharides were released from the glycopeptides by hydrazinolysis (Takasaki et al., 1982) or digestion with endoglycosidases (see below).

Enzyme Digestions. Oligosaccharides were released from glycopeptides by digestion with peptide:*N*-glycosidase F (10 units/mL) in 100 mM Tris-HCl, pH 8.6, and 10 mM 1,10-phenanthroline for 24 h or a mixture of endoglycosidase H (300 milliunits/mL) and endoglycosidase D (100 milliunits/mL) in 50 mM citrate–phosphate, pH 6.0, and 20 mM EDTA for 40 h. Digestion with alkaline phosphatase (6 units/mL) was in 50 mM Tris-HCl, pH 8.0, for 2 h. All enzyme digestions were in a final volume of 50 μL, except peptide:*N*-glycosidase F digestions which were in 100 μL, and incubated at 37 °C under a toluene atmosphere.

Column Chromatography. Radiolabeled glycopeptides were fractionated on Con A–Sepharose columns (2.5 mL bed volume) as described (Cummings et al., 1989; Merkle & Cummings, 1987). Bound glycopeptides were either sequentially eluted with 10 mM methyl α-glucoside followed by 100 mM methyl α-mannoside at 55 °C or eluted directly with 100 mM methyl α-mannoside at 55 °C; fraction volumes were 5 mL. Fractionation on PSA–agarose was carried out as outlined by Kornfeld et al. (1981); the bed volume was 2.5 mL (0.5 × 12.5 cm). Bound material was eluted with 10 mM methyl α-glucoside followed by 500 mM methyl α-mannoside; the fraction volume was 1 mL. Chromatography on RCA–agarose was performed on a 3 mL bed volume (0.5 × 15 cm) column as described previously (Cummings et al., 1989). Bound material was eluted with 0.1 M lactose; 1 mL fractions were collected. All lectin chromatography was done at room temperature.

Ion-exchange chromatography of endoglycosidase H and endoglycosidase D released oligosaccharides was carried out on QAE-Sephadex (1 mL bed volume). Bound material was eluted stepwise with increasing concentrations of sodium chloride in 2 mM Tris base; 1.5 mL fractions were collected (Varki & Kornfeld, 1983). Charged species were treated by digestion with either alkaline phosphatase or mild acid hydrolysis in 2 M acetic acid at 100 °C for 1 h followed by digestion with alkaline phosphatase.

High-pressure liquid chromatography of neutral, endoglycosidase H, and endoglycosidase D released oligosaccharides was performed on a Varian Micropak AX-5 column in a linear gradient (65–35%) of acetonitrile and water as described previously (Mellis & Baenziger, 1981).

UDP-GlcNAc–Lysosomal-Enzyme, N-Acetylglucosaminephosphotransferase Assay. N-Acetylglucosamine phosphotransferase activity was assayed essentially as described by Ben-Yoseph et al. (1988) using methyl α-mannoside as an acceptor. Reaction blanks were prepared by replacing methyl α-mannoside with methyl α-galactoside, a nonacceptor.

Detection of Glycoproteins Containing Mannose 6-Phosphate. CI-MPR was iodinated essentially as described by Valenzano et al. (1993). The iodinated CI-MPR was then purified by affinity chromatography on a column of phosphomannan–Sepharose (Ludwig et al., 1991). The phosphomannan–Sepharose column was prepared as described previously (Sahagian et al., 1982).

Cells were lysed by sonication, and microsomal proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with iodinated CI-MPR as described by Valenzano et al. (1993). Bovine testis β-galactosidase was used as a positive control.

Compositional Analysis. To determine the extent of core-fucosylation, aliquots of [^{3}H]mannose-labeled oligosaccharide structures were hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 4 h, and the resulting monosaccharides were separated on descending paper chromatography using ethyl acetate/pyridine/water (8:2:1) as described (Cummings et al., 1983).

RESULTS

Characterization of the Oligosaccharide Structures on Human Prorenin Synthesized by Sf9 Cells. Separation of glycopeptides prepared by Pronase digestion of *in vivo* [^{3}H]-

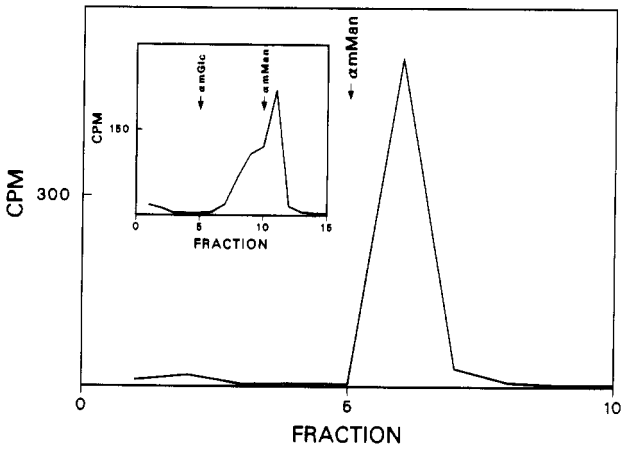

FIGURE 1: Concanavalin A–Sepharose chromatography of prorenin glycopeptides. Recombinant human prorenin was isolated by immunoprecipitation from *in vivo* [^{3}H]mannose-labeled Sf9 cells infected with a baculovirus containing the human prorenin gene. Following purification of the immunoprecipitated prorenin, glycopeptides were generated by Pronase digestion and separated on Con A–Sepharose, as described under Materials and Methods. Bound material was eluted with 100 mM methyl α-mannoside at 55 °C or sequentially with 10 mM methyl α-glucoside followed by 100 mM methyl α-mannoside at 55 °C (inset).

mannose-labeled, Sf9 cell produced prorenin on concanavalin A–Sepharose (Con A) resulted in the profile shown in Figure 1. Essentially all (97%) the radioactivity bound to the column and could subsequently be eluted with methyl α-mannoside. Previous work has demonstrated that this lectin interacts strongly with high-mannose- and hybrid-type structures on glycopeptides and glycoproteins such that elution can only be accomplished at high concentrations of methyl α-mannoside, while biantennary complex type structures are more weakly bound to the lectin and can be eluted with 10 mM methyl α-glucoside. For these experiments, direct elution with methyl α-mannoside was used rather than sequential elution with methyl α-glucoside and methyl α-mannoside, since preliminary experiments showed that elution with methyl α-glucoside results in only a partial release of the trimannosyl structures in the samples from the lectin (Figure 1, inset).

The small amounts of radioactivity which did not bind to the Con A column were insufficient for structural characterization. Specifically, none of this radioactivity bound to PSA or RCA, and size-exclusion separation on BioGel P-6 resulted in several radioactive peaks. Furthermore peptide: *N*-glycosidase F digestion of the structures in this fraction followed by fractionation on QAE-Sephadex failed to detect any charged material (data not shown).

The glycopeptides in the methyl α-mannoside eluate from Con A chromatography were further fractionated on PSA–agarose. This lectin has a high affinity for core-fucosylated N-linked structures (Merkle & Cummings, 1987). The profile from this separation is shown in Figure 2. Approximately 63% of the radioactivity bound to the column and was eluted with methyl α-mannoside; the remaining 37% did not interact with the lectin. The mannose-to-fucose ratio (Figure 2, inset) in the fractions containing material which bound to the column is approximately 3 to 1, suggesting that these structures are relatively small. Consistent with the specificity of PSA, the fractions containing unbound material also contain no detectable fucose.

The glycopeptides recovered from the PSA–agarose chromatography fractions containing bound and unbound material were extensively digested with a mixture of endoglycosidases H and D (>99% of the radioactivity was released), and the released oligosaccharides were separated on HPLC. Figure 3 shows that the material which did not bind to PSA contains predominantly trimannosyl structures (88% of the radioactivity). However, significant amounts of larger oligomannosyl

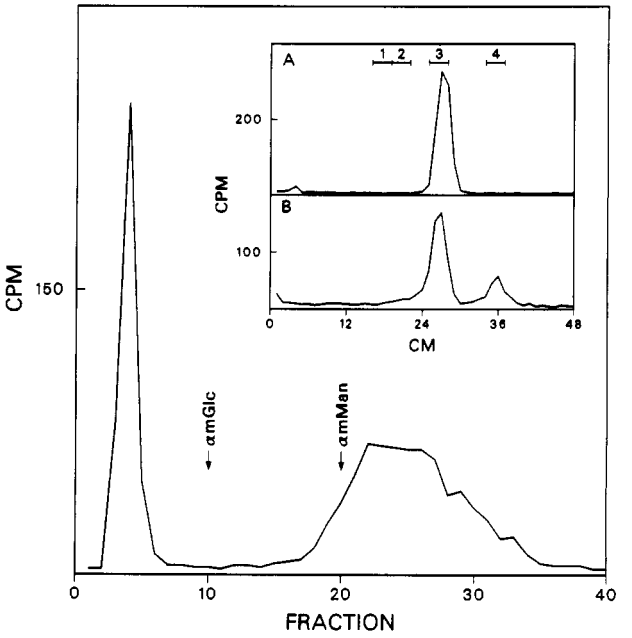

FIGURE 2: PSA–agarose chromatography of prorenin glycopeptides. The methyl α-mannoside eluate from Con A–Sepharose chromatography (see Figure 1) was separated on PSA–agarose. Bound material was eluted with 500 mM methyl α-mannoside. Inset: Aliquots from the PSA fractions containing unbound (A) and bound (B) material were subjected to acid hydrolysis, and the released radioactive monosaccharides were separated on paper chromatography as outlined under Materials and Methods. The migration of standards is indicated: 1, galactose; 2, glucose; 3, mannose; 4, fucose.

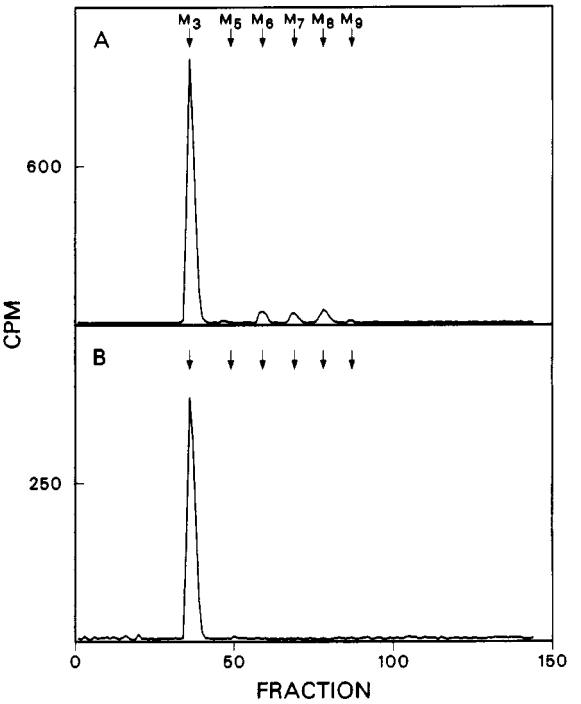

FIGURE 3: Separation of prorenin oligosaccharides on ion-suppression HPLC. [^{3}H]Mannose-labeled oligosaccharides were released from glycopeptides recovered from PSA–agarose chromatography fractions (see Figure 2) by digestion with a mixture of endoglycosidases H and D. Following removal of the peptides by ion-exchange chromatography, the released oligosaccharides were fractionated on a Micropak AX-5 column in a linear gradient (65–35%) of acetonitrile and water. Panels A and B show separations of oligosaccharides recovered from the PSA–agarose fractions containing run-through and bound material, respectively. M_3–M_9 indicate the elution positions of oligosaccharide standards $Man_3GlcNAc$ through $Man_9GlcNAc$.

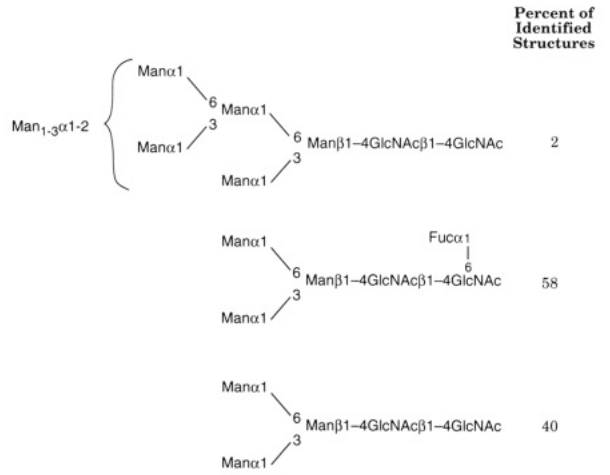

FIGURE 4: Proposed structures of *N*-glycans isolated from human prorenin synthesized by Sf9 cells. Linkage positions proposed in accordance with the common structures on N-linked oligosaccharides and previously published data on glycosylation in Sf9 cells (Kornfeld & Kornfeld, 1985; Kuroda et al., 1990; Wathen et al., 1991).

Table 1: Distribution of Charged Molecules in the Methyl α-Mannoside Eluate from Concanavalin A Chromatography[a]

fraction	radioactivity in fraction (% of total)	recovery of fraction in QAE run-through following alkaline phosphatase treatment (% of total)
QAE 0	95	
QAE 10	2	0
QAE 20	0	
QAE 70	3	<0.03

[a] [^{3}H]Mannose-labeled prorenin oligosaccharides, prepared by digestion of glycopeptides recovered in the methyl α-mannoside eluate from concanavalin A with a mixture of endoglycosidases H and D, were fractionated on QAE-Sephadex columns; bound radioactivity was eluated with stepwise increases in NaCl concentration. Charged oligosaccharides were subjected to digestion with alkaline phosphatase or mild acid hydrolysis followed by digestion with alkaline phosphatase (not shown) and rechromatography on QAE-Sephadex.

structures are also present, ranging in size from 6 to 8 mannose units. In contrast, the methyl α-mannoside eluate contains exclusively trimannosyl structures, *i.e.*, no larger fucosylated structures, suggesting that core-fucosylation, on recombinant prorenin synthesized by Sf9 cells, may be restricted to the more extensively truncated (Man_3) structures on the molecule. A summary of the oligosaccharide types identified on prorenin expressed in Sf9 cells is shown in Figure 4.

In order to investigate if, in analogy to prorenin synthesized by several mammalian cell types, the Sf9-produced molecule contains phosphorylated oligosaccharide structures, the methyl α-mannoside eluate from Con A chromatography was digested extensively with a mixture of endoglycosidases H and D, and the released oligosaccharides were separated on QAE-Sephadex. Table 1 shows that the vast majority (95%) of the radioactivity did not bind to the column, suggesting that this represents radioactivity linked to uncharged structures. Smaller amounts bound to the column and were eluted with 10 mM (2% of the radioactivity) and 70 mM (3% of the radioactivity) NaCl, respectively. However, none of the charge in either of the two fractions containing eluted material could be removed by digestion with alkaline phosphatase or with a combination of mild acid hydrolysis and alkaline phosphatase digestion (Varki & Kornfeld, 1983), suggesting that the charge associated with these structures is not the result of phosphorylation. It appears more likely that the enzymatic release of the oligosaccharides in these fractions (see above) may not have been complete.

Table 2: *N*-Acetylglucosamine–Lysosomal-Enzyme *N*-Acetylglucosaminephosphotransferase Activity in CHO, Murine Lymphoma BW 5147, and Sf9 Cells

cell line	mean activity[a] ± standard error ($N = 3$)
CHO	68.1 ± 6.4
BW 5147	29.2 ± 1.2
Sf9	ND[b]

[a] Transferase activity expressed as picomoles per hour per milligram of protein. [b] ND, none detected.

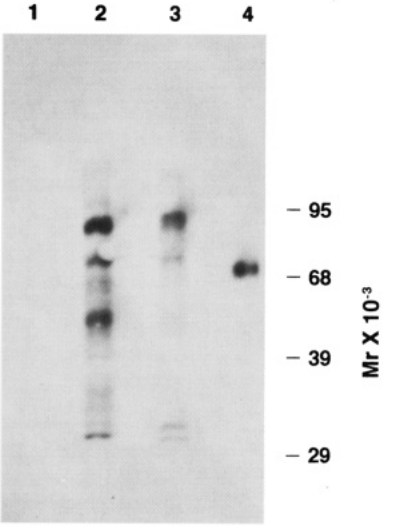

FIGURE 5: Detection of Man-6-P-containing glycoproteins by blotting with the cation-independent Man-6-P receptor. Microsomal fractions isolated from Sf9, CHO, and murine lymphoma BW 5147 cells were separated on SDS–PAGE. Following transfer to a nitrocellulose membrane, the microsomal proteins were blotted with an ^{125}I-labeled soluble form of the cation-independent Man-6-P receptor. Lanes 1, 2, and 3 contain microsomal proteins (100 μg) isolated from Sf9, CHO, and BW 5147 cells, respectively; lane 4 contains 1 μg of bovine testis β-galactosidase. The migration of molecular weight markers is indicated to the right.

The clean profiles on the HPLC separations discussed above are also consistent with the absence of charged oligosaccharide structures on the molecule; >97% of the radioactivity loaded on the column was recovered in oligomannosyl structures (data not shown).

Assays for UDP-GlcNAc–Lysosomal-Enzyme N-Acetylglucosaminephosphotransferase. The partial analysis discussed above suggests that Sf9 cells do not phosphorylate the oligosaccharide structures on prorenin. To further investigate this phenomenon, we assayed for the enzyme catalyzing the initial reaction in the synthesis of the Man-6-P recognition signal, UDP-GlcNAc–lysosomal-enzyme, *N*-acetylglucosamine phosphotransferase, in lysates from Sf9 cells and two other cell types which reportedly are capable of this substitution (Reitman & Kornfeld, 1981; Gabel & Kornfeld, 1982). Table 2 shows that while both CHO and murine lymphoma BW 5147 cells contain considerable amounts of this activity, none could be detected in Sf9 cells.

Blotting Assay for Molecules Carrying Oligosaccharide Structures Containing Man-6-P. The possible presence of molecules carrying phosphorylated oligosaccharide structures in Sf9 cells was investigated by blotting microsomal proteins separated on SDS–PAGE with ^{125}I-labeled cation-independent mannose 6-phosphate receptor. Figure 5 shows that while CHO and murine lymphoma BW 5147 cells both contain several reactive protein species, no microsomal proteins were recognized by the probe in Sf9 cells.

DISCUSSION

The results from the oligosaccharide characterization experiments in this investigation suggest that Sf9-produced recombinant prorenin contains high-mannose-type and truncated high-mannose-type *N*-glycans similar to those isolated from other insect-produced glycoproteins (*e.g.*, Hsieh & Robbins, 1984; Kuroda et al., 1990; Wathen et al., 1991; Hård et al., 1993; Kubelka et al., 1993). The core-fucosylated, trimannosyl structure, which constitutes the majority of the oligosaccharides on the Sf9-produced prorenin molecule, has also been described previously (*e.g.*, Kuroda et al., 1990; Wathen et al., 1991; Staudacher et al., 1992). It is noteworthy that this type of structure, which has been suggested to be the "insect equivalent" of a mammalian complex-type oligosaccharide (Hsieh & Robbins, 1984), constitutes more than 90% of the oligosaccharide structures on the insect cell produced molecule, while the same protein expressed in a mammalian (CHO) cell contains only 60% complex-type oligosaccharides. However, several glycoforms of renin have been reported in both humans and rats, suggesting that the distribution of oligosaccharide types on this molecule can vary considerably (*e.g.*, Fritz et al., 1986; Hori et al., 1988; Hosoi et al., 1991; Kim et al., 1988, 1991).

The complete absence of charged oligosaccharides on the insect-produced prorenin molecule strongly suggests that Sf9 cells do not phosphorylate the *N*-glycans on recombinant human prorenin. This raises the question whether Sf9 cells synthesize this kind of substitution at all and, additionally, if they utilize the Man-6-P-mediated targeting pathway for lysosomal enzymes. We were unable to detect any UDP-GlcNAc–lysosomal-enzyme *N*-acetylglucosamine phosphotransferase activity in lysates from Sf9 cells. Furthermore, using the cation-independent Man-6-P receptor as a probe, we were also unable to detect any microsomal proteins in Sf9 cells carrying Man-6-P. Taken together, these results suggest that Sf9 cells do not synthesize molecules carrying oligosaccharide structures with 6-phosphorylated mannose residues. Consequently, it appears unlikely that the Man-6-P-mediated targeting pathway is operative in these cells.

Although many mammalian cell types utilize Man-6-P for the targeting of acid hydrolases to the lysosomes, several cell types have also been identified which apparently do not use this pathway. In these cells, an alternative, but as yet uncharacterized, targeting mechanism appears to be used (Kornfeld & Mellman, 1989). Oligosaccharides containing 6-phosphorylated mannose residues have also been demonstrated in several lower eucaryotes (*e.g.*, Freeze et al., 1983; Kornfeld & Mellman, 1989; Lang et al., 1986; Kuan & Ming, 1989). In these organisms, however, none of these structures have been shown to be involved in lysosomal targeting. *Dictyostelium discoideum* and *Saccharomyces cerevisiae*, for example, synthesize oligosaccharides containing Man-6-P but appear to target their lysosomal enzymes independently of these structures (Kornfeld & Mellman, 1989).

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