The Predominant Secreted Protein of Transformed Murine Fibroblasts Carries the Lysosomal Mannose 6-Phosphate Recognition Marker*

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We have found that the major excreted protein (MEP) of transformed mouse fibroblasts, a phosphoglycoprotein of \( M_r = 35,000 \), carries the mannose 6-phosphate recognition marker. MEP secreted by Kirsten virus-transformed NIH 3T3 cells binds to a purified preparation of lysosomal enzyme phosphomannosyl receptor, and this binding is specifically inhibited by mannose 6-phosphate. \(^{32}P\), introduced into MEP by metabolic labeling of intact cells is exclusively associated with asparagine-linked oligosaccharides as indicated by sensitivity to endohexosaminidase H. Labeling studies utilizing \(^{2-3H}\)mannose indicate that approximately one-fifth of the mannose residues of MEP are phosphorylated. Comparative studies of the synthesis, secretion, and uptake of MEP and of the lysosomal enzyme \( \beta \)-galactosidase indicate that MEP made by Kirsten virus-transformed NIH 3T3 cells is not handled in the same manner as are other lysosomal enzymes. MEP may be an unusual lysosomal protein, a mannose 6-phosphate-containing secretory protein, or both.

Proteins secreted by transformed cultured fibroblasts differ significantly from those secreted by nontransformed counterparts (1, 2). The secretion of some proteins such as the procollagens (3–5) is decreased, whereas the secretion of other proteins including plasminogen activator (6), a phosphoglycoprotein of \( M_r = 58,000 \) (2), and the major excreted protein of transformed mouse fibroblasts (1) is increased. In most cases, a role for these proteins in the establishment or maintenance of the transformed phenotype has not been defined.

We have been studying MEP, a phosphoglycoprotein (7) whose secretion is increased 50- to 100-fold in many virally transformed mouse fibroblasts, including KNIH cells (1, 8). A severalfold increase in synthesis and/or secretion of MEP has also been demonstrated with tumor promoters (8–10) and in mouse fibroblasts treated with platelet-derived growth factor (11), fibroblast growth factor (12), or epidermal growth factor (13). When KNIH cells were labeled with \([4,5-^3H]\)leucine, the following procedures were used. Cells are grown in 100-mm culture dishes to approximately 5 x 10^6 cells/dish. Before labeling, the cells were rinsed three times with Waymouth MAB 87-3 medium (22), as formulated in the GIBCO catalogue, with added antibiotics and 5% dialyzed fetal bovine serum, but with leucine reduced to 1.7 g/ml. Cells were labeled in 4 ml of the same medium containing 0.1 mCi/ml of \([4,5-^3H]\)leucine (Amersham Corp., 40 Ci/mmol). For pulse-chase experiments, labeling was terminated by the addition of 0.1 ml of 1-leucine solution (5 mg/ml).

At the indicated times, the growth medium was collected and protein precipitated with 80% (NH_4)_2SO_4. The precipitate was dissolved in 1 ml of 10 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl (Buffer A) and dialyzed for 16 h at 4 \( ^\circ \)C against 1000 volumes of the same buffer. Man-6-P and Nondet P-40 were added to the dialyzed samples to final concentrations of 10 mM and 1%, respectively. The cells were harvested by trypsinization, washed with Eagle's medium to remove trypsin, and extracted for 10 min at 0 \( ^\circ \)C with 1 ml of Buffer A containing 10 mM Man-6-P and 1% Nondet P-40. The cell extracts and medium extracts were adjusted to a final volume of 2 ml and frozen.

Prior to immunoprecipitation of labeled proteins, the extracts were
treated with formalin-treated Staphylococcus aureus, Cowan strain, to reduce nonspecific precipitation of labeled materials. S. aureus (100 μl, Bethesda Research Laboratories) was added to each extract and after incubation for 15 min at 0 °C, the extracts were clarified by centrifugation at 18,000 rpm for 30 min in a Sorvall SM 24 rotor. MEP was immunoprecipitated from 200-μl aliquots of the extracts by sequential incubation at 0 °C for 30 min with 2 μl of rabbit anti-MEP (7) and 25 μl of S. aureus β-galactosidase was immunoprecipitated from 0.9-ml aliquots in a similar manner with rabbit anti-bovine β-galactosidase (25). S. aureus pellets were washed four times with 1 ml aliquots of a buffer comprising 10 mM Tris-HCl, pH 8.6, 0.6 M NaCl, 0.05% Nonidet P-40, and 0.1% SDS and then 10 μl Tris-HCl, pH 7.5, containing 0.15 M NaCl. Radioactive proteins were solubilized by heating the S. aureus pellets at 95 °C for 5-10 min in 50 μl of 1% (w/v) sodium dodecyl sulfate (SDS), 10 μl dithiothreitol, and 10% glycerol. After centrifugation in an Eppendorf microfuge for 2 min, supernatants containing the labeled protein were subjected to SDS-PAGE as previously described (18). Radioactive bands were detected by fluorography (24).

Treatment of MEP with Endo-β-N-Acetylhexosaminidase H or Trifluoroacetic Acid—Immunoprecipitated MEP was dissociated from S. aureus pellets by incubation at 95 °C for 10 min in 10 mM sodium acetate, 10 mM sodium phosphate, pH 5.5, containing 0.3% SDS and 10% peptatin. For treatment with endo-β-N-acetylhexosaminidase H, this solution was diluted 3-fold with the same buffer without SDS. Endo-β-N-acetylhexosaminidase H (0.01 unit, Health Rese-arch Inc., Albany, NY) was added and the mixture incubated at 37 °C for 10 h. The incubation mixtures were concentrated by lyophilization.

Acid hydrolysis of MEP was performed in 2 M trifluoroacetic acid as previously described (25). Immunoprecipitated MEP was solubilized with SDS buffer as above, diluted 2-fold with 4 M trifluoroacetic acid (Aldrich), and incubated in a sealed tube at 110 °C for 2 h. The protein was diluted 10-fold with H2O and lyophilized to remove excess trifluoroacetic acid.

Paper Electrophoresis—High voltage paper electrophoresis was performed at 50 V/cm in a Caisson model D electrophoresis on Whatman 3MM paper saturated with 30 mM NH4HCO3 (25). Radioactive bands were detected by scanning electrophoretogram on a Vanguard Systems model 930 Auto-scanner by counting 1-cm pieces of electrophoretograms by liquid scintillation spectrometry.

Binding of Radiolabeled MEP to Purified Phosphomannosyl Receptor—Purified bovine liver phosphomannosyl receptor and rabbit antisera directed against the receptor were prepared as previously described (26, 27). An insoluble receptor-S. aureus complex (27) was prepared to study the binding of MEP to the receptor. S. aureus (0.5 ml of a 10% cell suspension in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 10 mg/ml of bovine serum albumin (Buffer B)) was incubated at 37 °C for 30 min with 75 μl of anti-receptor serum. Excess antibody was removed by centrifugation and the pellet was resuspended in 0.5 ml of Buffer B. Phosphomannosyl receptor (56.3 μg of protein) in 0.5 ml of Buffer B was added and the mixture incubated at 37 °C for 30 min. The resulting receptor-S. aureus complex was collected by centrifugation and washed several times with Buffer B. The final product contained 36 μg of receptor protein and bound approximately 62 pmol of bovine testicular β-galactosidase, a lysosomal enzyme for which the binding to the receptor has been extensively characterized (23, 27).

The following procedure was used to study the binding of 35S-labeled MEP to phosphomannosyl receptor. Culture medium (5 μl) containing metabolically labeled MEP was incubated at 0 °C with insoluble receptor-S. aureus complex in 50 μl of Buffer B. After incubation for 90 min, 1 ml of Buffer B was added and the complex collected by centrifugation. The pellet was then washed three times with cold Buffer B and proteins bound specifically to the receptor were dissociated from the complex by incubation at 0 °C for 30 min in 50 μl of Buffer A containing 10 mM mannose 6-phosphate. Insoluble material was removed by centrifugation and the resulting supernatant was subjected to SDS-PAGE.

RESULTS

Binding of MEP to Purified Phosphomannosyl Receptor—When KNIH cells were pulse-labeled for 3 h with [35S]methionine, 30-50% of the protein-associated radioactivity in the culture medium was found in MEP (35,000 band, Fig. 1, lane A). When varying amounts of a phosphomannosyl receptor-S. aureus complex were added to aliquots of the culture medium, MEP was specifically bound (lanes B-D). Binding of MEP to the receptor increased with increasing receptor concentration and was nearly quantitative at a receptor concentration of 72 μg/ml. Binding was completely inhibited by 2 mM mannose 6-phosphate (lane E) but not by similar concentrations of mannosamine, fructose 6-P, glucose 6-P, or mannose 1-P (lanes F-I). This pattern of inhibition is similar to that described for binding of β-galactosidase to the receptor (23, 27).

Endo-β-N-Acetylhexosaminidase H Treatment of MEP—Fig. 2 shows the effect of endo H on MEP labeled metabolically with [35S]methionine or [32P]phosphate. SDS-PAGE of total cellular and secreted material (lanes A–D) indicates that MEP (35,000 band) was the major methionine- and phosphate-containing species in the culture medium. Immunoprecipitation of MEP from the media of 35S- and 32P-labeled cells (lanes I and K, respectively) gave a major labeled band of M, = 35,000 (arrow) as well as several minor bands. These minor bands are probably artifacts of sample preparation since they were less prominent when samples were heated for long periods of time (greater than 10 min) in SDS and mercaptoethanol prior to electrophoresis and because peptide maps of these bands from cells were identical with that of the major 35,000 band (7).

When secreted [35S]MEP was treated with endo H (lane J), each labeled MEP band (arrow) shifted to lower molecular weight, indicating that MEP secreted into the culture medium contains asparagine-linked oligosaccharides. Treatment of secreted [32P]labeled MEP (lane K) with endo H (lane L)
resulted in complete loss of the label, suggesting that all of
the phosphate moieties of MEP are associated with endo H-
sensitive oligosaccharides.

Similar results were obtained for cellular MEP (lanes E-H).
After treatment with endo H, the major 32P-labeled MEP
band as well as the minor bands (lane E) was converted to
lower molecular weight bands (lane F) and the correponding
32P-labeled bands (lane G) were lost (lane H). In addition to
the MEP bands found in the culture medium, cells contained
two additional lower molecular weight immunoreactive bands
(lane E and G). Both bands were specifically precipitated
with anti-MEP antiserum. These bands were not seen after
short labeling pulses (≤30 min) but were seen within the cell
after relatively long chase times or after long labeling as in
this experiment. Both lower molecular weight bands contai
phosphorylated endo H-sensitive oligosaccharides as
indicated by a shift in migration of 32P (lanes E and F) and a
loss of 32P (lanes G and H) after treatment with endo H.
These bands may correspond to cellular forms of MEP that
are derived from the 35,000 material by proteolytic processing.

**Partial Characterization of MEP Oligosaccharides**
The oligosaccharides of MEP were examined by metabolically
labeling MEP with [2-3H]mannose. Treatment of secreted
[3H]mannose-labeled MEP with endo H resulted in release
of 94% of the label from the protein, indicating that most of
the mannose residues of MEP are present on asparagine-linked,
endo H-sensitive oligosaccharides. High voltage paper elec
trophoresis of the [3H]mannose-labeled oligosaccharides is shown
in Fig. 3. Most of the label migrated as a single negatively
charged species. When the oligosaccharides were treated ex
tensively with alkaline phosphatase (Fig. 4B), 49% of the label
migrated as less charged material and 31% as neutral material.
These results indicate that most of the oligosaccharides were
phosphorylated. The occurrence of oligosaccharides of inter
mediate charge after treatment with alkaline phosphatase
suggests that the untreated oligosaccharides (Fig. 4A) con
tained two or more negatively charged moieties, some of
which are resistant to the action of alkaline phosphatase.
Examples of such phosphatase-resistant moieties are sialic
acid residues or phosphodiester moieties with the structure

**Man-P-GlcNAc.** Both moieties have previously been dem
onstrated on endo H-sensitive oligosaccharides of lysosomal
enzyme (27-31).

**Demonstration of Mannose 6-P as a Constituent of MEP**
The results described above strongly suggest that MEP con
tains mannose phosphate residues. In order to demonstrate
the presence of mannose phosphate directly, [2-3H]mannose-
labeled MEP was hydrolyzed with trifluoroacetic acid under
conditions that give maximum release of monosaccharides
with minimal breakdown of hexosephosphate. Fig. 5 shows
the results of high voltage electrophoresis of acid hydrolysates
of [2-3H]mannose-labeled MEP. Approximately 21% of the
mannose label migrated as mannose 6-phosphate; the remain
der migrated as neutral material (Fig. 5A). The charged
material was converted to neutral material when treated with

**Fig. 2.** Endo H treatment of metabolically labeled MEP.
KNIH cells were labeled for 3 h with [32P]orthophosphate
and [3H]mannose (lanes A, C, E, F, I, and J) or [32P]o
rthophosphate (lanes B, D, G, H, K, and L). MEF was
immunoprecipitated from extracts of the cells (lanes E-H)
and from the media (lanes I-L). Immunoprecipitates were either treated
with endo H (lanes F, H, J, and L) or not treated (lanes E, G, I, and K).
Lane A, [32P]orthophosphate-labeled cell extract used for immuno
precipitation; B, 32P-labeled cell extract; C, [32P]orthophosphate-labeled
medium; D, 32P-labeled medium.

**Fig. 3.** High voltage paper electrophoresis of [2-3H]man
nose-labeled MEP after treatment with endo H. KNIH cells were
labeled for 3 h in complete medium containing 4 mCi of [2-3H]
mannose and [2-3H]mannose was immunoprecipitated from the medium,
treated with endo H, and subjected to high voltage paper electropho
resis for 120 min as described under "Materials and Methods."

**Fig. 4.** High voltage paper electrophoresis of [3-3H]man
nose-labeled oligosaccharides after treatment with alkaline
phosphatase. [2-3H]Mannose-labeled oligosaccharides of MEP were
eluted from the electrophoretogram shown in Fig. 3 and concentrated
by lyophilization. A portion of the labeled oligosaccharides (approximate
ly 2000 cpm) was incubated for 2 h at 37°C with 0.3 unit of
Escherichia coli alkaline phosphatase (Sigma, type III-I in 50 μl of
Tris-HCl buffer, pH 7.5. The alkaline phosphatase-treated oligosacchar
ides (B) and oligosaccharides treated in a similar manner but
without added alkaline phosphatase (A) were subjected to high volt
age paper electrophoresis for 100 min as described under "Materials
and Methods."

Man-P-GlcNAc. Both moieties have previously been dem
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material was converted to neutral material when treated with
Fig. 5. High voltage paper electrophoresis of an acid hydrolysate of [2-3H]mannose-labeled MNP. KNIH cells were labeled with 4 mCi of [2-3H]mannose for 3 h and MEP was immunoprecipitated from the medium. Labeled MEP was solubilized and treated with trifluoroacetic acid as described under "Materials and Methods." The acid hydrolysate was subjected to high voltage paper electrophoresis for 40 min before (A) and after (B) treatment with alkaline phosphatase. Alkaline phosphatase treatment was performed as described in the legend to Fig. 4.

alkaline phosphatase (Fig. 5B). These results suggest that approximately 1 in every 5 mannose residues of MNP is phosphorylated. This number is probably an underestimate since partial dephosphorylation of mannose 6-P may have occurred during the acid treatment.

Biogenesis and Secretion of MEP and β-Galactosidase—We conducted several experiments to determine the extent to which MEP and β-galactosidase, a well characterized lysosomal enzyme, are secreted or targeted to lysosomes in KNIH cells. Fig. 6 shows the biogenesis and secretion of MEP and β-galactosidase by KNIH cells and the effect of NH4Cl on these processes. When cells were labeled for 1 h with [5,6-3H]leucine in the presence or absence of 10 mM NH4Cl, most of the labeled MEP (35,000 band, lanes A-D) and β-galactosidase (82,000 band, lanes I-L) was associated with the cells. After a 6-h chase with unlabeled leucine, greater than 85% of the labeled MEP had been secreted into the culture medium (lanes E-H); NH4Cl had no observable effect on secretion of MEP. In contrast to MEP, most of the β-galactosidase remained cell-associated after the 6-h chase (lanes M and N). The enzyme was apparently converted from a precursor form of 82,000 (lane I) to a processed form of 63,000 (lane M). The small amount of β-galactosidase secreted into the culture medium (lane N) had a molecular weight of 84,000, slightly higher than that of the precursor form. When labeling was performed in the presence of NH4Cl, the majority of labeled β-galactosidase was secreted into the culture medium (lanes O and P).

The results presented in Fig. 6 indicate that in KNIH cells, MEP is handled differently than β-galactosidase. MEP is secreted into the culture medium, whereas β-galactosidase is retained by the cells, presumably in lysosomes. The synthesis and packaging of lysosomal enzymes by KNIH cells appear to be normal in that β-galactosidase is synthesized in precursor form and is processed to a 63,000 form that has a molecular weight similar to β-galactosidase isolated from mouse liver (32). Processing of β-galactosidase from an 82,000 form to a 63,000 form has also been observed in mouse macrophages (53). Furthermore, NH4Cl, a lysosomotropic amine that stimulates secretion of lysosomal enzymes in human fibroblasts (17, 18) and CHO cells (34), stimulates secretion of β-galactosidase by KNIH cells. The rise in intralysosomal pH caused by NH4Cl is thought to prevent delivery of lysosomal enzymes to lysosomes by interfering with normal functioning of the phosphomannosyl receptor (17).

Fig. 6. Biogenesis and secretion of MEP and β-galactosidase. KNIH cells were labeled for 1 h with [4,5-3H]leucine in the presence or absence of 10 mM NH4Cl as indicated. Chases (6 h) were carried out by addition of L-leucine to the labeling medium. MEP and β-galactosidase were immunoprecipitated from cell (C) and medium (M) extracts. Fluorograms for MEP and β-galactosidase were exposed for 3 days and 20 days, respectively.

Fig. 7. Uptake of MEP and β-galactosidase by CHO cells. KNIH cells were labeled in the presence or absence of NH4Cl with [4,5-3H]leucine for 1 h and chased with L-leucine for 6 h. Proteins secreted by the cells were concentrated from the media by (NH4)2SO4 precipitation (90% saturation). Precipitates were redissolved in 200 μl of 10 mM NaPO4, pH 6.8, containing 0.15 mM NaCl and dialyzed extensively at 4°C against the same buffer. Normal secretions or secretions from NH4Cl-treated cells were added to 4 ml of Eagle's medium and incubated with CHO cells (2 x 106 cells/100-mm dish) at 35°C for 16 h. Cell (C) and medium (M) extracts were prepared as described for KNIH cells and MEP and β-galactosidase were immunoprecipitated. Fluorograms for MEP and β-galactosidase were exposed for 3 and 30 days, respectively.
Uptake of MEP and β-Galactosidase by CHO cells—The finding that MEP binds specifically to purified preparations of phosphomannosyl receptor prompted us to study the uptake of MEP by CHO cells. CHO cells have been used to study the uptake of lysosomal enzymes present in secretions of human skin fibroblasts and CHO mutants (34). Uptake of lysosomal enzymes by CHO cells is mediated exclusively by the phosphomannosyl receptor.

When secretions from [3H]leucine-labeled KNIH cells were added to CHO cells, β-galactosidase, present in the secretions as the 84,000 form (Fig. 6, lane N), was efficiently taken up by the cells and processed to a 60,000 form (Fig. 7, lane G). β-Galactosidase present in NH4Cl-induced secretions of KNIH cells was also taken up by CHO cells, but to a lesser extent (lanes E and F). In contrast, MEP, in normal secretions or secretions from cells treated with NH4Cl, was not taken up as well by CHO cells (lanes A–D). The small amount of MEP found in the cells (lanes A and C, not clearly seen in this photograph) appeared as a lower molecular weight form of approximately 19,000–20,000 and represents 5–6% of the total labeled MEP recovered from the cells and medium.

**DISCUSSION**

The manifolds increase in MEP synthesis and secretion which correlates with transformation and tumor promotion in cultured mouse cells has allowed us to use this phenomenon as a molecular marker of the transformed phenotype (1, 7, 8). Until now, we have had no hint as to the possible function of this secreted phosphoglycoprotein. In this work, we show that MEP contains mannose phosphate residues and binds quantitatively and specifically to the phosphomannosyl receptor. This membrane-associated glycoprotein receptor has been implicated in the intracellular translocation of lysosomal enzymes (19–21).

The data presented here indicate that MEP has some similarities to other well studied lysosomal proteins. In previous studies, the mannose phosphate receptor has been demonstrated to occur on lysosomal enzymes or glycoprotein fractions enriched in lysosomal enzymes. The recognition marker is thought to serve as a signal to direct these proteins to lysosomes. As for lysosomal enzymes, the mannose phosphate moieties of MEP occur on asparagine-linked oligosaccharides that can be released from the glycoprotein by treatment with endohexosaminidase H. These moieties are presumably synthesized in a manner similar to that for lysosomal enzymes, by transfer of GlcNAc-1-P from UDP-GlcNAc to the oligosaccharides to give the phosphodiester moieties on lysosomal enzymes or glycoprotein (28-31). Most of the oligosaccharides of secreted MEP were sensitive to treatment with alkaline phosphatase, indicating that they contain mannose phosphate in the phosphomannosyl form. A portion of the oligosaccharides also contained negatively charged moieties that were resistant to alkaline phosphatase. While not further characterized, such moieties may correspond to phosphodiester moieties found on lysosomal enzymes.

In contrast to the situation seen with a well characterized lysosomal protein, β-galactosidase (Fig. 6), in KNIH cells, MEP is almost quantitatively secreted into the culture medium. Thus, the presence of the phosphomannosyl recognition marker on MEP is apparently not sufficient for its efficient delivery and retention in lysosomes as is found for true lysosomal proteins. In addition, MEP is not taken up efficiently by CHO cells which are known to have surface phosphomannosyl receptors and which efficiently take up β-galactosidase synthesized by KNIH cells (Fig. 7). It is not clear why MEP binds to the phosphomannosyl receptor but is not efficiently internalized by CHO cells or targeted to lysosomes in KNIH cells, processes thought to be mediated by this same receptor. Perhaps MEP possesses the primary signal (Man-6-P residues) for binding to the receptor but is missing secondary structural features that are required for translocation of the receptor-bound ligand. Secondary structural features may include the number and/or orientation of Man-6-P residues on the oligosaccharides (35), other carbohydrate moieties on the oligosaccharides (27, 36), or structural determinants contained within the polypeptide portion of the glycoprotein (37). Alternatively, MEP may carry another signal specifying secretion which is not found on usual lysosomal proteins. Perhaps, the differently charged species of MEP which have been described (7) may each have different structural features which affect their subcellular distribution.

We have recently obtained evidence that in nontransformed mouse fibroblasts the portion of MEP retained within the cell is greater than in transformed cells and that this MEP has a predominantly lysosomal localization as determined by indirect immunofluorescence and electron microscopic localization. Whether MEP is a novel mannose phosphate-containing secretory protein or a lysosomal protein, the localization of which has been altered by transformation, or both, is currently under investigation.

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