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Cation-Independent Mannose 6-Phosphate Receptor Contains Covalently Bound Fatty Acid

Keith R. Westcott and Leonard H. Rome

Department of Biological Chemistry and Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, California 90024

The cation-independent mannose 6-phosphate receptor (215,000 daltons) was isolated from embryonic bovine tracheal cells and embryonic human skin fibroblasts labelled with [^{3}H]palmitic acid. the tritium label was detected in the protein upon fluorographic analysis of SDS-polyacrylamide gels of the purified receptor. The label was not sensitive to hydroxylamine, methanolic KOH, or β-mercaptoethanol, but labelled fatty acid was recovered from the protein by acidic methanolysis. Labelled receptor protein could not be isolated from cells grown in the presence of [^{3}H]myristic acid. The results suggest the presence of amide-linked palmitic acid in the structure of the cation-independent mannose 6-phosphate receptor.

Key words: lysosomal targeting, acylation, palmitate, proteolipid

The covalent attachment of lipids to proteins of eukaryotic cells and their viruses has recently become recognized as a novel class of posttranslational modification. Three distinct types of lipid attachment have been defined. The first, and most well defined type of linkage, has been shown to be attachment of fatty acids through O-ester or thioester bonds to integral membrane proteins [for review, see 1] Examples of this type of attachment include brain proteolipids [2], vesicular stomatitis virus G glycoprotein [3], Sindbis virus E1 and E2 glycoproteins [4], transferrin receptor [5], human histocompatibility antigens [6] and murine glycophorins [7]. The second type of fatty acid attachment to proteins is by an amide bond. This type of modificaiton usually involves the covalent attachment of myristic acid to cytosolic proteins such as cAMP-dependent protein kinase [8], murine leukemia membrane associated protein, p15 [9], pp60^{src} [10]

Abbreviations used: M6PR, mannose 6-phosphate receptor (nominal Mr = 215,000) cation-independent form, as opposed to the cation-dependent form recently described [36,37]; SDS, soldium dodecyl sulfate; EBTr cells, embryonic bovine tracheal cells; DMEM, Dulbecco's modified Eagle's medium.

Keith R. Westcott's present address is Amgen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.

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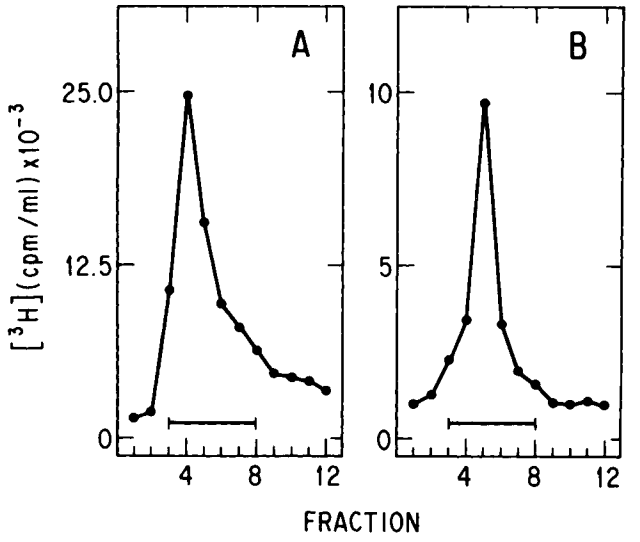

Fig. 1. Chromatogrpahy of labelled M6PR samples. Elution profiles of M6PR from phosphomannan-Sepharose. Receptor solubilized from ^{3}H-labelled EBTr cells was eluted in 2-ml fractions with 10 mM mannose 6-phosphate, as described in Methods. **A:** [^{3}H]Leucine-labelled M6PR. **B:** [^{3}H]Palmitate-labelled M6PR. The bars represent pooled fractions.

and calcineurin b [11]. A modification exhibiting the characteristics of this type of linkage has also been described for the α and β subunits of the nicotinic acetylcholine receptor, both integral membrane proteins of BC_3H1 cells [12]. The third type of modification involves the covalent attachment of fatty acids through a carboxyl terminal glycophospholipid tail. These phospholipase labile linkages have been described in torpedo acetylcholine esterase [13], *Trypanosoma brucei* variant surface glycoprotein [14], and the Thy-1 glycoprotein [15].

The mannose 6-phosphate receptor (M6PR) is a ubiquitous integral membrane protein involved in the transport of lysosomal enzymes to lysosomes in mammaliam cells [for reviews see 16–19]. Purified M6PR is a very large protein, with a monomeric molecular weight of 215,000 daltons [20–22], which binds mannose 6-phosphate moieties present on the oligosaccharide chains of lysosomal enzymes [23,24]. The receptor is a transmembrane protein with a large exocytoplasmic N-terminal domain containing its binding site and a smaller C-terminal cytosolic domain [25,26]. The M6PR has been shown to be posttranslationally modified by the addition of oligosaccharide chains [22,27] and serine-linked phosphate [27]. In this report we describe another posttranslational modification of the mannose 6-phosphate receptor, the covalent attachment of fatty acid.

METHODS

Materials

L-[4,5-^{3}H]leucine (142 Ci/mmol), [9,10 (u)-^{3}H]palmitic acid (50 Ci/mmol) and [9,10 (n)-^{3}H] myristic acid (55 Ci/mmol) were purchased from Amersham. Mannose 6-phosphate, palmitic acid, palmitic acid methyl ester, and cerulenin were obtained from

Sigma. Phosphomannan from *Hansenula holstii* was a gift of Dr. M. Slodki, USDA Northern Regional Research Laboratory, Peoria, Illinois. Dulbecco's modified Eagle's medium was purchased from Grand Island Biological Co. Autofluor was obtained from National Diagnostics, Somerville, NJ. All other reagents were purchased from standard commercial suppliers.

Cell Culture and Cell Labelling Conditions

Embryonic bovine tracheal (EBTr) cells (ATCC No. CCL44) and embryonic human fibroblasts (ATTC No. CCL110) were obtained from the American type Culture Collection. Cells were maintained in Dulbecco's modified Eagel's medium (DMEM) containing 9% fetal calf serum; the concentration of $NaHCO_3$ was reduced to 2.2 g/l and the concentration of NaCl was raised to 10.4 g/l. Each liter of medium was also supplemented with 10^5 units of penicillin, 0.1 g of streptomycin sulfate, and 5×10^4 units of polymyxin B sulfate. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

For labelling studies, cells were grown in 175-cm^2 culture flasks to a highly postconfluent state (approximately 3 weeks growth). Growth medium was changed twice weekly. Two or three flasks of each cell type were labelled in DMEM supplemented with dialyzed fetal calf serum (labelling medium). When cells were labelled with [^{3}H]leucine, leucine-free DMEM was used. Prior to labelling, growth medium was replaced by 30 ml of labelling medium per flask and the cells were incubated for 2 hours. This medium was replaced by 15 ml labelling medium per flask to which had been added either 0.5 mCi [^{3}H]fatty acid in 40 μl 80% ethanol or 0.5 mCi [^{3}H]leucine in 0.5 ml H_2O. After equilibration for 1 hr in 5% CO_2/95% air at 37°C, the flasks were capped and incubated for 15 hr with gentle agitation (50 rpm) on an orbital shaker at 37°C. The radioactive medium was replaced with 30 ml per flask of complete DMEM and the cells were allowed to equilibrate for 1 hr at 37°C in 5% CO_2/95% air. The cells were washed three times with 30 ml of ice-cold phosphate-buffered saline (0.8% NaCl, 0.2% KCl, 0.115% Na_2HPO_4 $7H_20$, 0.2% KH_2PO_4, 0.01% $CaCl_2$ $2H_20$, 0.01% $MgCl_2$ $6H_20$) and harvested (by scraping) in 15 ml cold phospate-buffered saline containing protease inhibitors (2 mM each phenylmethylsulfonyl fluoride, benzamidine, iodoacetamide, and sodium EDTA). The combined material was homogenized by ten strokes in a motor-driven glass-teflon homogenizer and centrifuged for 15 min at 40,000g. The pellet was recovered, homogenized in 2 ml phosphate-buffered saline containing protease inhibitors, frozen in dry ice-acetone, and stored at −20°C.

Purification of the Mannose 6-Phosphate Receptor

M6PR was purified by a modification of the method of Varki et al. [28]. Typically, 120 g bovine liver acetone powder was suspended in 2 liters cold 0.1 M Na acetate, pH 5.0, 0.15 M NaCl in the presence of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 20 mM iodoacetamide) and stirred for 15 min at 4°C. Following centrifugation at 10,000g (30 min, 4°C), the sediment was recovered and resuspended by Polytron homogenization in 2 liters cold water containing the protease inhibitors. Centrifugation was performed as previously described, and the recovered sediment was resuspended in 2 liters cold solubilization buffer (50 mM Na phosphate, pH 7.5, 1% Triton X-100, 0.4 M KCl, 10 mM EDTA) containing the protease inhibitors. This mixture was stirred for 45 min at 4°C and centrifuged for 45 min at 13,800g. The supernatant containing the solubilized protein was retained.

M6PR was isolated by phosphomannan-Sepharose chromatography, as described by Steiner and Rome [22]. The solubilized protein was applied at 4°C to two parallel 60-ml packed bed phosphomanan-Sepharose columns that had been equilibrated in 50 mM Na phosphate, pH 7.5, 0.5% Triton X-100, 10 mM EDTA, 0.9% NaCl, 0.02% NaN_3. The adsorbant was washed with cold equilibration buffer, 1 liter per column, followeed by washing with cold 50 mM Na phosphate, pH 7.5, 0.033% Triton X-100, 0.9% NaCl, 0.02% NaN_3, 3.5 liters per column. Mannose 6-phosphate receptor was eluted at 4°C with the same buffer containing 5 mM mannose 6-phosphate. The eluant was concentrated at 4°C to approximately 5 ml by ultrafiltration with an Amicon XM-50 membrane and stored in small aliquots at −20°C until use. A typical preparation yields 4 to 5 mg of essentially pure M6PR.

Isolation of Labelled M6PR

[^{3}H]Leucine or [^{3}H]fatty-acid-labelled receptor was isolated by a modification of the method just described [28]. All procedures were performed at 4°C. Labelled cell homogenates were thawed and sedimented at 40,000g for 15 min. The pellet was resuspended by ten strokes in a glass-teflon homogenizer in 30 ml 0.1 M sodium acetate, pH 5.0, 0.15 M NaCl containing protease inhibitors (2 mM each phenylmethylsulfonyl fluoride, benzamidine, iodoacetamide, and sodium EDTA). This mixture was incubated for 15 min with end-over-end mixing for 15 min and centrifuged for 15 min at 40,000g. The pellet was resuspended by homogenizing in 30 ml deionized water containing protease inhibitors and sedimented as described earlier. Protein in the pellet was solubilized by homogenization in 30 ml of 50 mM Na phosphate, pH 7.5, 0.4 M KCl, 1% Triton X-100, 1 mg/ml bovine serum albumin containing protease inhibitors. The solubilization mixture was incubated with end-over-end mixing for 45 min, and the insoluble material was removed by centrifugation for 30 min at 40,000g. Purified M6PR (0.15 mg), isolated from bovine liver acetone powder, was added to the solubilized EBTr cell protein as a carrier during subsequent chromatography. No carrier receptor was added before chromatography of the embryonic human skin fibroblast receptor.

The detergent solubilized proteins were recirculated for 15 hr at 10 ml/hr over a 4-ml phosphomannan-Sepharose column. The adsorbant was washed with 100 ml of 50 mM Na phosphate pH 7.5, 0.9% NaCl, 0.1% Triton X-100, 10 mM sodium EDTA, 0.02% NaN_3, followed by 250 ml 50 mM Na phosphate, pH 7.5, 0.9% Nacl, 0.033% Triton X-100, 0.02% NaN_3. M6PR was eluted in 2 ml fractions at 10 ml/hr in the same buffer containing 10 mM mannose 6-phosphate. Receptor containing fractions were identified by liquid scintillation counting, and appropriate fractions were pooled, concentrated by ultrafiltration to 0.4 ml with an Amicon XM-50 membrane, and stored in 50-μl aliquots at 4°C. Protein concentration was determined by the method of Dulley and Grieve [29].

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [30] on 0.8-mm gels with an Idea Scientific Mini-Slab Apparatus. Protein samples were visualized by Coomassie blue staining in 50% methanol, 10% acetic acid containing 0.25% Coomassie blue, and then destained in the methanol/acetic acid solution. Fluorography was performed on unstained gels fixed in 50% methanol followed by washes in 20% ethanol and deionized water before a 1-hr treatment with Autofluor. Gels were

dried with a Bio-Rad gel-drying apparatus and exposed to Kodak X-Omat XAR-5 film for the indicated times at −70°C.

Delipidation of Fatty-Acid-Labelled M6PR

Labelled M6PR (36μg) was mixed with bovine serum albumin, 500 μg, and precipitated from 0.1 M HCl in 60% acetone overnight at −20°C. The precipitated protein was isolated by centrifugation at 10,000g for 30 min and extracted three times with 1-ml aliquots of petroleum ether [12]. Excess solvent was evaporated with a stream of nitrogen.

Release of Protein-Bound Fatty Acid

The extracted protein was suspended in 1.5 ml 2 N HCl in methanol/water (83:17) containing 50 μg each palmitic acid and palmitic acid methyl ester as internal markers [12]. The mixture was heated for 18 hr at 90°C under vacuum. The hydrolysate was extracted three times with petroleum ether and the extracts were concentrated to dryness in a Speed-Vac concentrator. The residue was dissolved in acetone and analyzed by thin layer chromatography on Silia Gel G plates (EM Science, Cincinnati, OH) developed with petroleum ether/diethyl ether/acetic acid (80:20:1). After separation, the positions of the palmitic acid and palmitic acid methyl ester were determined with iodine vapor. One-centimeter fractions were scraped from the thin layer plate and analyzed for ^3H by liquid scintillation counting.

RESULTS

Isolation of Radioactivity Labelled M6PR

EBTr cells were used as a source of metabolically labelled M6PR. Because these cells were derived from a bovine source, it was assumed that M6PR isolated from them would be similar or identical to receptor isolated in bulk from bovine liver acetone powder [28]. Furthermore, EBTr cells can be grown easily to high density and maintained under standard culture conditions.

A procedure for the isolation of labelled EBTr cell M6PR was developed on the basis of previous work with labelled receptor [22]. Upon washing, homogenization and Triton X-100 extraction of EBTr cells labelled with [^3H]leucine or [^3H]fatty acid, ^3H-labelled M6PR was easily isolated in the presence of cold carrier M6PR (150 μg) by affinity chromatography on a small phosphomannan-Sepharose column (Fig. 1). Using buffer containing mannose 6-phosphate, ^3H-labelled receptor preparations could be eluted in good yield as single protein peaks. The preparations shown in Fig. 1 yielded 176 μg [^3H]leucine-labelled M6PR and 150 μg [^3H]palmitate-labelled M6PR. The ease of these single-step purifications made less reliable isolation techniques such as immunoprecipitation unnecessary.

Electrophoresis and Fluorography of ^3H-Labelled M6PR Preparations

Upon electrophoresis on SDS-polyacrylamide gels both [^3H]palmitate- and [^3H]-leucine-labelled M6PR samples gave charcteristic patterns after staining with Coomassie blue (Fig. 2A). Under nonreducing conditions, both M6PR preparations showed predominant bands at approximately 200 kDa and some aggregated material near the top of the gel. Under reducing conditions with β-mercaptoethanol, only single bands of approximately 215 kDa were observed. Fluorographic analysis revealed a similar pat-

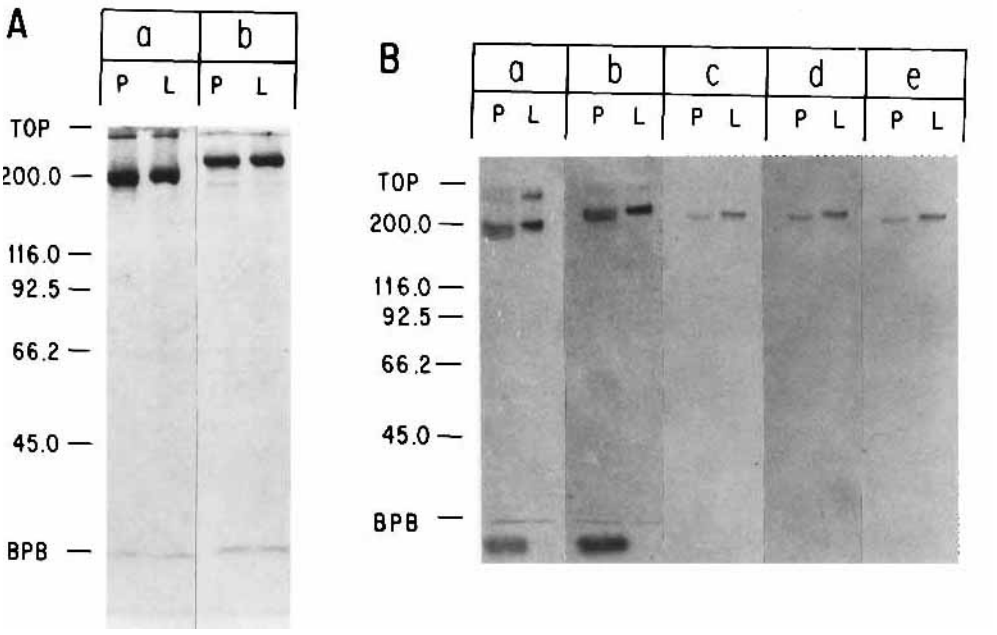

Fig. 2. SDS-polyacrylamide gel electrophoresis and fluorography of [^{3}H]palmitate-and [^{3}H]leucine-labelled M6PR samples. **A:** Coomassie blue staining pattern of 3 M6PR samples on 6% gels. **Lane a:** Nonreducing conditions; **lane b:** Reducing conditions. The letter P designates M6PR derived from [^{3}H]palmitate-labelled EBTr cells; the letter L designates M6PR derived from [^{3}H]leucine-labelled EBTr cells. **B:** Fluorographic analysis, as described in Methods. **Lanes a and b:** Images of samples run as described in part A. Film was exposed for 20 days at −70°C. **Lanes c,d,e:** 1.5μg M6PR samples were run on 6% gels under reducing conditions. After fixing, samples in lanes c, d, and e were pretreated before fluorography by overnight incubation in 1 M Tris HCl, pH 8.5, overnight incubation in 1 M NH_2OH, pH 8.5 or 30 min incubation in 0.2 M KOH in methanol, respectively. Images are derived from film exposed for 35 days at −70°C. The letters P and L indicate the same designations as in part A. Molecular-weight scales were derived with Bio-Rad protein molecular-weight standards. BPB designates the bromphenol blue dye front.

tern of bands for both the [^{3}H]palmitate- and [^{3}H]leucine-labelled M6PR preparation, (Fig. 2B, and lanes a, b). Some of the tritium present in the [^{3}H]palmitate-labelled M6PR preparation, presumably ^{3}H-labelled phospholipid, was found to run between the SDS and bromphenol blue fronts. Boiling in mercaptoethanol was found to have no effect on the amount of label apparent in the [^{3}H]palmitate-labelled receptor protein.

Identification of Covalently Bound Lipid

Covalent attachment of fatty acids to proteins through ester linkages has been demonstrated by their lability to treatment with 1 M hydroxylamine overnight or 0.2 M KOH in methanol for 30 min [31]. M6PR was isolated from cells labelled with [^{3}H]palmitate or [^{3}H]leucine and run on reducing SDS gels. The methanol-fixed polyacrylamide gels (see Methods) were treated with either 1 M hydroxylamine (pH 8.5), 0.2 M KOH or 1 M Tris (pH 8.5) (as a control). Subsequent fluorography showed no loss of label in [^{3}H]palmitate-labelled M6PR from either hydrolytic method, compared with the control gel (See Fig. 2B, lanes c–e).

In order to confirm that [^{3}H]palmitate-labelled M6PR did indeed contain covalently bound fatty acid, labelled receptor was subjected to extraction with organic solvent to

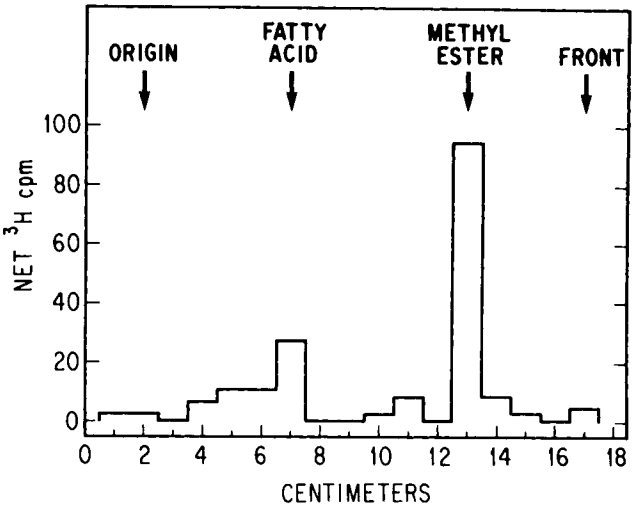

Fig. 3 Thin-layer chromatography of petroleum ether extract of methanolized [^{3}H]palmitate labelled M6PR. Distribution of ^{3}H on the chromatogram compared with the position of palmitic acid and palmitic acid methylester standards. Receptor-bound fatty acid was converted to the methylester, as described in Methods. Chromatogram was developed in petroleum ether/diethyl ether/acetic acid (80:20:1). One-centimeter areas were scraped from the plate and counted for ^{3}H. Background (40 cpm) has been subtracted from all values.

remove noncovalently associated [^{3}H]fatty acid, followed by acidic methanolysis [12]. Extraction of the hydrolysate with petroleum ether and subsequent thin-layer chromatography revealed a major peak of fatty-acid methyl ester and a minor peak of fatty acid (Fig. 3). Unfortunately, this solvent system [12,32] does not separate fatty-acid methyl esters of various chain lengths, and the exact identity of the bound fatty acid has not yet been identified. In this experiment, 40% of the [^{3}H] label in the hydrolyzed sample remained in the aqueous phase after extraction with petroleum ether. The nature of this material is unknown but may represent incomplete hydrolysis, degraded fatty acid produced during the methanolysis procedure or other labelled material resulting from entry of label from [^{3}H]palmitate into metabolic pools during the overnight labelling procedure. Hydroxylamine treatment of organic solvent-extracted receptor and subsequent thin-layer chromatography of $CHCl_3$/methanol soluble material [5] produced no evidence for the presence of hydroxylamine-labile fatty acyl linkages in the receptor protein (data not shown), confirming the data obtained through electrophoresis and fluorography. Taken together, these observations indicate the presence of a very stable, presumably amide-linked fatty-acid moiety attached to the mannose 6-phosphate receptor.

M6PR From EBTr Cells Labelled With [^{3}H]Myristic Acid

Most proteins with amide-linked fatty acids have been shown to contain myristic acid [8–11]. Studies therefore were undertaken to establish whether M6PR could be metabolically labelled with [^{3}H]myristic acid. Although the extent of incorporation of tritium from [^{3}H]myristic acid into the particulate fraction of EBTR cells appeared to be similar to that seen with [^{3}H]palmitic acid, no labelling of M6PR by [^{3}H]myristic

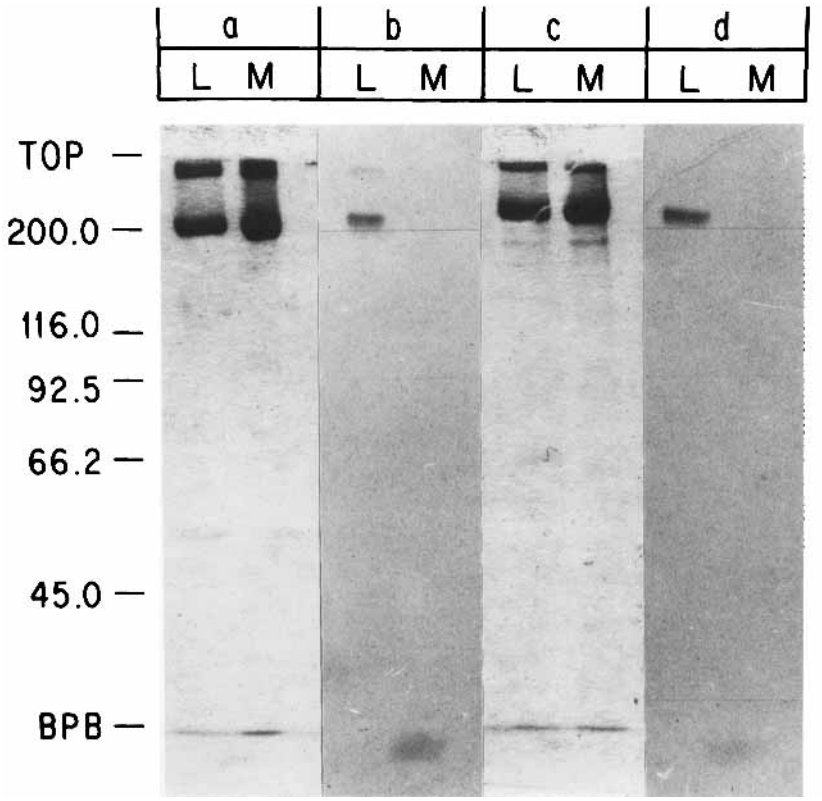

Fig. 4. SDS-Polyacrylamide gel electrophoresis and fluorography of M6PR purified from [^{3}H]leucine- and [^{3}H]myristate-labelled EBTR cells. M6PR samples, 2 μg, were analyzed on 6% gels. The letter L designates M6PR derived from [^{3}H]leucine-labelled EBTr cells; the letter M, M6PR derived [^{3}H]myristate-labelled EBTr cells. **Lanes a, c:** Coomassie blue staining patterns under nonreducing and reducing conditions, respectively. **Lanes b, d:** Fluorographic analysis as described in "Methods" from samples run as described for lanes a, c. Images are derived from film exposed for 20 days at −70°C. Molecular-weight scale was determined with Bio-Rad protein molecular-weight standards. BPB designates the bromphenol blue dye front.

acid could be detected (Fig. 4). This result also supports the idea that the labelling of M6PR by palmitic acid is a specific process and not a result of nonspecific labelling of receptor through degradative entry of tritium into other metabolic pools.

[^{3}H]Palmitate Labelling of Embryonic Human Skin Fibroblast M6PR

To confirm that fatty acylation of the mannose 6-phosphate receptor is a general phenomenon not restricted to receptor in bovine cells, labelling experiments were conducted with cells of human origin. Following labelling of the fibroblasts with [^{3}H]palmitic acid, M6PR was solubilized from the particulate fraction of the cells and isolated by phosphomannan-Sepharose affinity chromatography. Figure 5 shows fluorographic images of SDS-polyacrylamide gels of nonreduced and reduced preparations of the fibroblast receptor. As shown for M6PR from EBTr cells the tritium label in the fibroblast receptor is not sensitive to treatment with β-mercaptoethanol. Further characterization was not

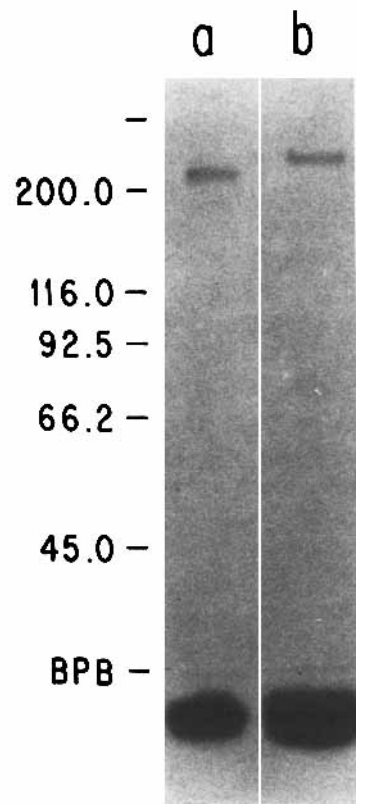

Fig. 5. Fluorographic analysis of [^{3}H]palmitiate-labelled fetal human skin fibroblast M6PR. M6PR samples, 0.3 μg, were run on a 6% SDS-polyacrylamide gel. Fluorographic analysis was performed as described in Methods. Images are derived from preflashed x-ray film exposed for 45 days at −70°C. **Lanes a, b:** Migration under nonreducing and reducing conditons, respectively. Molecular-weight scale was determined with Bio-Rad protein molecular-weight standards. BPB designates the bromphenol blue dye front; the uppermost bar indicates the top of the resolving gel.

attempted because of the limited amounts of labelled fibroblast receptor that were obtained.

DISCUSSION

Studies presented in this report indicate that the mannose 6-phosphate receptor from cultured embryonic bovine tracheal cells and embryonic human skin fibroblasts contains covalently attached fatty acids. Tritium can be incorporated into M6PR in cells incubated with [^{3}H]palmitic acid. The linkage of the fatty acid is resistant to 1 M hydroxylamine (pH 8.5) and methanolic KOH, treatments that would hydrolyse O-linked fatty acyl esters. An S-linked fatty-acid linkage is unlikely because boiling in β-mercaptoethanol did not affect the label. In addition, the linkage was sensitive to methanolysis, and it therefore exhibits characteristics of an amide bond rather than

of any type of ester bond. In this respect, M6PR is similar to α and β subunits of the nicotinic acetylcholine receptor from BC_3H1 cells [12]. The identity of the bound fatty acid in M6PR has not been conclusively determined. Amide-linked fatty-acid moieties in proteins are generally found to be composed of myristic acid bound to the α-amino group of the N-terminal amino acid. Indeed, the N-terminal amino acid of M6PR appears to be blocked[1] and while this location is a possibility for the site of attachment of the fatty acid, it is unlikely, given the exocytoplasmic orientation of the N-terminal portion of the protein [24,25]. Furthermore, it is not likely that the attached fatty acid is myristic acid because labelling of EBTr cells with [^{3}H] myristic acid did not result in incorporation of tritium into the receptor. Recent evidence [33] suggest that amide-linked palmitic acid is not an uncommon modification; the detection of the incorporation of [^{3}H]palmitic acid in an amide linkage may depend on the relative availability of exogenously and endogenously derived palmitate.

The functional significance of fatty acid covalently bound to the mannose 6-phosphate receptor is not clear. It has been suggested that covalently attached lipid may aid in membrane anchoring [1,5]. Indeed, it has been demonstrated that the majority of fatty acylated proteins are membrane-bound [34]. The polypeptide chain of M6PR has been shown to span to the lipid bilayer, but the exact nature of the membrane-anchoring domain has not been elucidated [25,26]. A role for acylation in the control of protein-membrane and protein-protein associations has also been proposed [12,35]. The antibiotic cerulenin has been shown to inhibit fatty acylation of proteins [35]. Furthermore, cerulenin treatment has been shown to block budding of vesicular stomatitis virus virions [35] and the expression of nicotinic acetylcholine receptor on the cell surface of BC_3H1 cells [12]. We have examined whether cerulenin treatment of human fibroblasts impairs uptake of lysosomal enzymes. Although cerulenin treatment was observed to inhibit [^{125}I]β-galactosidase uptake by human fibroblasts, this inhibition was found to be merely a result of the general cytotoxic effects of the antibiotic. Inhibition of [^{125}I]β-galactosidase uptake by added cereulenin occurred to exactly the same extent as inhibition of cellular utilization of added [^{3}H]leucine[2] and therefore could not be conclusively attributed to a decreased acylation of the M6PR.

An understanding of the nature and signficance of the fatty acyl linkage to the mannose 6-phosphate receptor will certainly require further study. The development of methods resulting in increased incorporation of [^{3}H]fatty acid, perhaps involving [^{3}H]acetate labelling [33], will undoubtedly be required to elucidate the full extent of fatty acylation of M6PR and the identity of the bound fatty acid. Identification of the precise structure of the fatty acid will provide important corroborative evidence for the conclusions we have made. A detailed knowledge of the sequence and structure of the receptor as well as models of orientation in the membrane should place constraints on the location of the fatty acyl linkage. Furthermore, future studies on the mechanism of M6PR function in packaging of lysosomal enzymes may also indicate a role for fatty acylation in this process.

[1] Searles RP, Fowler A, Rome LH, unpublished observations.

[2] Westcott KR, Hill DF, Rome LH, unpublished observations.

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