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INSULIN-LIKE GROWTH FACTOR II OVEREXPRESSION DOES NOT AFFECT SORTING OF LYSOSOMAL ENZYMES IN NIH-3T3 CELLS

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The sorting of newly synthesized mannose 6-phosphate (M6P)-containing proteins and of the major excreted protein (MEP), a lysosomal thiol proteinase, was studied in NIH-3T3 cells transfected with the cDNA of human insulin-like growth factor II (IGF II) or with the vector alone. Extracts from media and cells labelled with [^{35}S] methionine were used for chromatography on a M6P/ IGF II receptor affinity matrix or for immunoprecipitation to assess the distribution of newly synthesized M6P-containing proteins and MEP, respectively. The results indicate that the overexpression of IGF II did not affect the synthesis and the sorting of M6P-containing proteins and of MEP. The binding and uptake of the lysosomal enzyme arylsulfatase A were not affected in IGF II overexpressing cells.

Insulin-like growth factor II (IGF II), a polypeptide which is mitogenic for a variety of cell lines, binds to the cation-independent mannose 6-phosphate (M6P) receptor (1). IGF II and M6P bind to different sites of the receptor (2). The receptor mediates the M6P-dependent transport of newly synthesized lysosomal enzymes from the Golgi to prelysosomes and the internalization of exogenous M6P-containing ligands (3). A few data exist on biological effects of IGF II mediated by the M6P/IGF II receptor (4). Addition of IGF II to phospholipid vesicles containing purified receptor and G_{i2} protein activates the GTPase activity of the G

Abbreviations:
IGF, insulin-like growth factor; MEP, major excreted protein; G6P, glucose 6-phosphate; M6P, mannose 6-phosphate; SDS-PAGE, Sodium dodecylsulfate polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor; EGF, epidermal growth factor; BSA, bovine serum albumin.

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protein, while M6P and M6P-containing lysosomal enzymes inhibit the IGF II induced activation (5). In basolateral membranes of kidney tubules binding of IGF II and M6P-containing ligand to the receptor stimulates the formation of inositol trisphosphate (6). While IGF II can sterically inhibit the binding and uptake of some lysosomal enzymes (7) it stimulates binding and uptake of other lysosomal enzymes due to a IGF II induced receptor redistribution to the cell surface (8, 9).

Recently we described the stable expression of biologically active human IGF II in NIH-3T3 cells (10). In this report we studied the relationship between the permanent expression of IGF II and the synthesis and transport of M6P-containing ligands. We showed that overexpression of the IGF II does not affect the synthesis and sorting of endogenous M6P-containing polypeptides as well as the binding and uptake of exogenous lysosomal enzymes.

MATERIALS AND METHODS

Materials: NIH-3T3 cells overexpressing the human IGF II (W15) and cells transfected only with the expression vector pSV2neo (N) were produced as described (10). Polyclonal antibodies to the MEP were a gift of Dr. G. Sahagian (Tufts University, Boston) and Dr. E. Kominami (Jentendo University). Recombinant arylsulfatase A was provided by H. J. Sommerlade of this institute and iodinated with the aid of iodogen (11). [^{35}S]-Methionine (1100 Ci/mmol) and [^{125}I] were from Amersham and [^{14}C]-labelled molecular weight standards were from DuPont, New England Nuclear. M6P (sodium salt) and glucose-6-phosphate (G6P) were purchased from Sigma and Pansorbin from Calbiochem.

M6P/IGF II receptor affinity chromatography: NIH-3T3 cells were labelled with [^{35}S]-methionine (70 μCi/ml) for 3 h at 37°C and chased after the addition of an excess of unlabelled methionine for further 4 h. Extracts of cells and media were prepared and subjected to chromatography on a M6P/IGF II receptor column as described (12). The fractions eluted with 5 mM G6P and 5 mM M6P were precipitated with trichloroacetic acid, analyzed by SDS-PAGE (10% polyacrylamide) and visualized by fluorography.

Immunoprecititation of MEP: MEP was immunoprecipitated from medium or cell lysate containing 60 mM Tris/HCl pH7.4, 1% Triton X-100, 0.15 M NaCl 5 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride and 5 mM iodoacetic acid.

Binding and endocytosis of [^{125}I] arylsulfatase A: For determination of [^{125}I] arylsulfatase A binding cells were chilled to 4°C and endogenous M6P containing ligands removed by incubation with Hank's balanced salt solution containing 2 mM M6P. After washing the cells were incubated for 2 h at 4°C with [^{125}I] arylsulfatase A (3 x 10^5cpm) in MEM containing 0.1% BSA and 20 mM Hepes pH 7.2 (MEM/BSA). After removal of unbound [^{125}I]labelled ligands the specific bound [^{125}I]arylsulfatase A was released by incubation with Hank's buffer containing 2 mM M6P. The cells were

solubilized in 1 N NaOH and the specific cell surface associated radioactivity referred to the cellular protein content (13). The uptake was determined by incubating the cells in MEM/BSA containing [^{125}I]arylsulfatase A (3 x 10^5cpm) for 1 h at 37°C in the absence and presence of 5 mM M6P. Thereafter the cells were chilled to 4°C, washed and the cell surface bound ligand removed by incubation with Hank's buffer containing 2 mM M6P. The uptake is defined as the cell associated radioactivity related to the cellular protein content corrected by the cell associated radioactivity after incubation in the presence of M6P. The presence of 5 mM M6P during the endocytosis period inhibited the uptake of [^{125}I]arylsulfatase A by 96%.

RESULTS

Sorting of endogenous M6P-containing polypeptides

The incorporation of [^{35}S]-methionine into proteins of NIH-3T3 cells overexpressing human IGF II (W15) and NIH-3T3 control cells transfected with the vector only (N1, N2, N3, representing three independent clones) was linear for 2 h and comparable for control and IGF II overexpressing cells. In the following experiments the cells were labelled for 3 h with [^{35}S]-methionine followed by a chase for 4 h. Aliquots of extracts of cells and media were used for immunoprecipitation of MEP (Fig. 1) or applied to M6P/IGF II receptor column (Fig. 2).

The majority of the labelled MEP was recovered in the medium as 39 kD precursor both in the W15 and the three control cell lines

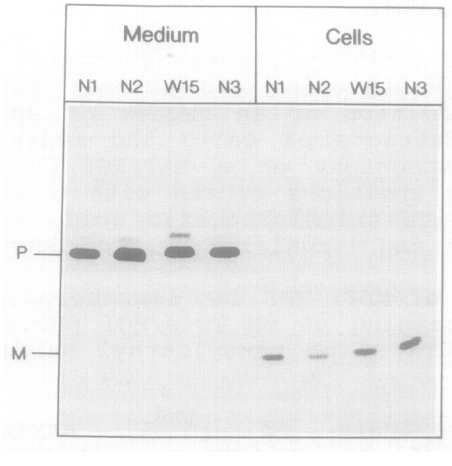

Fig. 1. Immunoprecipitation of MEP.
IGF II overexpressing cells (W15) and three different clones of NIH-3T3 cells transfected only with pSV neo (N1-N3) were labelled for 3 h with [^{35}S]-methionine and chased for further 4 h at 37°C. MEP was immunoprecipitated from extracts of media (7 x 10^5cpm TCA insoluble radioactivity) and cells (1.2 x 10^6cpm) and subjected to electrophoresis and fluorography. The positions of the 39 kD precursor (P) and the 20 kD mature (M) form of MEP are indicated.

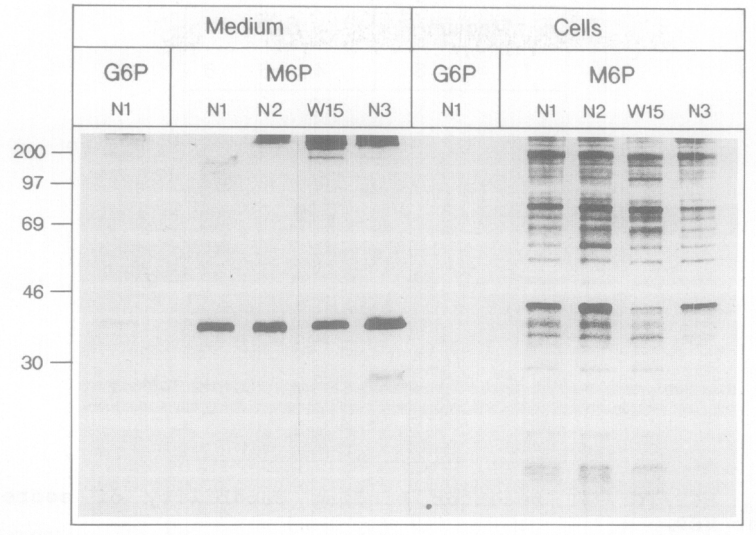

Fig. 2. Synthesis and secretion of M6P-containing ligands. Equal amounts of trichloroacetic acid precipitable radioactivity from media and cellular extracts of IGF II overexpressing cells (W15) and pSV neo transfected NIH-3T3 cells (N1-N3) as described in Fig. 1 were applied on a M6P/IGF II receptor affinity column. Fractions eluted with G6P and M6P were lyophilized, solubilized and subjected to SDS-PAGE. The positions of the molecular mass standards (kD) are indicated at the left margin.

(N1-N3). Between 20 - 30% of the labelled MEP was recovered as the 20 kD mature form in the cells. Neither the synthesis nor the distribution of MEP between cells and media was altered by the overexpression of IGF II (Fig. 1).

Due to the presence of only a single phosphorylated oligosaccharide mouse MEP binds weakly to M6P/IGF II receptor (14,15) and its sorting may not be representative for other M6P-containing polypeptides. We applied therefore the extracts of cells and media to a M6P/IGF II receptor column, which was extensively washed with 10 mM glucose 6-phosphate (G6P) to obtain non-specifically bound polypeptides and then eluted with 10 mM M6P to obtain the M6P-containing polypeptides. The G6P and M6P eluates were separated by SDS-PAGE (Fig. 2).

The radioactivity in the M6P eluates of medium and cell extracts accounts to 2.6 and 1.1% (mean of six independent experiments) of the total radioactivity applied, respectively. The amount of polypeptides in the G6P fraction was $\leq$ 10% of that in the M6P eluates. In control and IGF II overexpressing cells equal amounts and pattern of M6P-containing polypeptides in cellular and medium extracts were observed. The 39 kD MEP precursor was present exclusively in the medium where it accounted for 40-52% of the total secreted M6P-containing polypeptides. In the M6P eluates

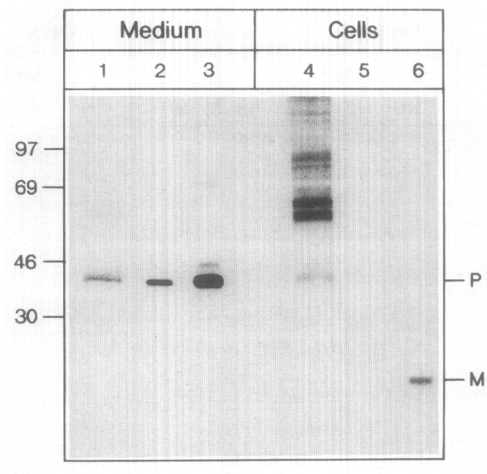

Fig. 3. M6P/IGF II receptor binding capability of secreted and cellular MEP.
Extracts of medium and cells of IGF II overexpressing cells (W15) labelled with [^{35}S]-methionine were applied on a M6P/IGF II receptor affinity column as described in the legend of Fig. 2. Half of the fractions eluted with M6P were directly subjected to SDS-PAGE (lane 1 and 4). The other half of the M6P eluates (lane 2 and 5) and the flow through fractions (lane 3 and 6) were used for immunoprecipitation of MEP and subjected to electrophoresis and fluorography. The identity of the 43 kD protein with the low receptor affinity precipitated from media by the MEP antiserum is unclear.

from cellular extracts the 20 kD mature form was not detectable (see below) suggesting the lost of affinity for the receptor.
We examined therefore the flow through fraction and M6P eluates of the receptor column for the presence of MEP. Similar results were obtained from control and IGF II overexpressing cells. MEP was exclusively recovered in the flow through of cellular extracts as the 20 kD mature form (Fig.3). In the respective fractions of media extracts 65% of the MEP precursor was precipitated from the flow through fraction. This indicates that only part of the MEP precursor binds to the receptor column and that the mature form generated intracellularly has lost the affinity for binding to the receptor. This may result from a dephosphorylation and/or the proteolytic processing.

Endocytosis of exogenous arylsulfatase A: Treatment of human fibroblasts with IGF II increase the binding and endocytosis of M6P-containing ligands due to a redistribution of M6P/IGF II receptors to the plasma membrane (8,9). To determine whether the overexpression of IGF II affects the cell surface expression of M6P/IGF II receptors the binding and uptake of arylsulfatase A were studied. Both W15 and control cells (N1-N3) bind and internalized similar amounts of [^{125}I] arylsulfatase A (Table I).

Table I. Binding and uptake of [^{125}I]arylsulfatase A

Cell line		Binding [a] (ng/mg protein ± SD)	Uptake [b] (ng/mg/h ± SD)
N1 - N3	mean	0.52 ± 0.36	4.80 ± 1.23
	n	13	11
W 15	mean	0.66 ± 0.08	4.83 ± 1.66
	n	5	3

a) Cell surface binding of [^{125}I]arylsulfatase A (60 ng) was determined at 4°C. The values are corrected by binding of [^{125}I]arylsulfatase A in the presence of M6P.

b) Cells were incubated with [^{125}I]arylsulfatase A (90 ng) for 60 min at 37°C. The cells were chilled to 4°C and the cell surface bound [^{125}I]arylsulfatase A removed by M6P. Uptake was defined as the cell associated radioactivity corrected by the values obtained from incubations in the presence of M6P.

DISCUSSION

In order to define the possible regulatory role of IGF II by binding to the M6P/IGF II receptor on the transport of M6P-containing lysosomal enzymes we have examined the synthesis and sorting of the major excreted protein (MEP) and non-defined M6P-containing polypeptides in NIH-3T3 fibroblasts overexpressing IGF II. The synthesis and secretion of IGF II is more than 100-fold increased in the overexpressing cells (10). Previous studies have shown that large quantities of MEP, which is identical with cathepsin L (16) are synthesized and secreted by mouse fibroblasts in response to exogenously added PDGF, EGF or viral transformation (17, 18). The endogenously made IGF II could affect the transport of newly synthesized M6P-containing lysosomal enzymes in different ways. First, newly synthesized IGF II may bind in the Golgi to the M6P/IGF II receptor and induce a redistribution of receptors resulting in either decreased or increased number of receptors. This could impair the binding of M6P-containing lysosomal enzymes,which then would be secreted into the medium or retained in the cells, respectively. Secondly, binding of IGF II to the M6P/IGF II receptor within the secretory route accompanied by sterical inhibition of the M6P-binding site or by a decrease of receptor affinity results in secretion of M6P-containig ligands.

Our results clearly show that sorting and proteolytic processing of M6P-containing proteins and MEP is not affected by overexpression of IGF II. Endogenously produced IGF II appears therefore not to interfere with the targeting of M6P-containing proteins mediated by the M6P/IGF II receptor. In addition, treatment of IGF II overexpressing or control NIH-3T3 cells for various times and concentrations with IGF II failed to stimulate the synthesis or secretion of MEP or other M6P-containing ligands (unpublished results) which support findings in human fibroblasts (8).

In human fibroblasts exogenous IGF II induce a redistribution of M6P/IGF II receptors from intracellular membranes to the cell surface resulting in an increased endocytosis of lysosomal enzymes (8,9). The overexpression of IGF II in NIH-3T3 cells showed no effect on the endocytosis of exogenous lysosomal enzymes compared with the control cells. Additionally, treatment of IGF II overexpressing cells with IGF II did not change the number of M6P/IGF II receptors at the cell surface whereas in control NIH-3T3 cells IGF II induced a receptor redistribution (unpublished results).

These data indicate that IGF II overexpressing cells have lost the capability for regulated M6P/IGF II receptor distribution in response to IGF II. This may result from binding of endogenous IGF II to the receptor which does not interfere with the sorting and endocytosis of lysosomal enzymes.

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