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Regulation of the mannose 6-phosphate/IGF II receptor expression at the cell surface by mannose 6-phosphate, insulin like growth factors and epidermal growth factor

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Mannose 6-phosphate, insulin like growth factors I and II (IGF I, IGF II), insulin and epidermal growth factor (EGF) induce a 1.5- to 2-fold increase of mannose 6-phosphate binding sites at the cell surface of human skin fibroblasts. The increase is completed within 10-15 min, is dose and temperature dependent, reversible and transient even in the presence of the effectors. It is due to a redistribution of mannose 6-phosphate/IGF II receptors from internal membranes to the cell surface, while the affinity of the receptors is not affected. Combinations of mannose 6-phosphate with IGF I, IGF II or EGF stimulate the redistribution of the receptor to the cell surface in an additive manner, while combinations of the growth factors result in a non-additive stimulation of redistribution. The redistribution is not dependent on extracellular calcium and appears also to be independent of changes of free intracellular calcium. Pre-treatment of fibroblasts with cholera toxin or pertussis toxin increases the number of cell surface receptors 2- and 1.5-fold, respectively. Neither of the toxins affects the redistribution of mannose 6-phosphate/IGF II receptors induced by the growth factors, while both toxins abolish the receptor redistribution induced by mannose 6-phosphate. These results suggest a multiple regulation of the cell surface expression of mannose 6-phosphate/IGF II receptors by G_s- and G_i-like proteins sensitive to cholera toxin and pertussis toxin and by stimulation of mannose 6-phosphate/IGF II, IGF I and EGF receptors. Binding of mannose 6-phosphate/IGF II receptors with mannose 6-phosphate triggers receptor redistribution via a signal transduction pathway sensitive to cholera and pertussis toxin, while binding with IGF II triggers receptor redistribution by a mechanism resistant to these toxins.

Key words: M6P/IGF II receptor/IGF I/IGF II/EGF/mannose 6-phosphate/expression/cell surface

Introduction

The mannose 6-phosphate/insulin like growth factor II (M6P/IGF II) receptor is a multifunctional receptor binding two structurally unrelated families of ligands (Morgan *et al.*, 1987; Roth, 1988). The M6P-containing ligands, such as lysosomal enzymes and IGF II bind to different sites of the receptor and a single receptor can bind the two types of ligands simultaneously (Roth *et al.*, 1987; Kiess *et al.*, 1988; MacDonald *et al.*, 1988; Tong *et al.*, 1988; Waheed *et al.*, 1988). The role of the M6P/IGF II receptor in targeting of newly synthesized lysosomal enzymes to lysosomes and endocytosis of lysosomal enzymes has been established (von Figura and Hasilik, 1986). The lysosomal enzymes bind to the M6P/IGF II receptor via M6P residues in their oligosaccharide chains. IGF II binding to M6P/IGF II receptors has been shown to stimulate DNA-synthesis and cell proliferation, glycogen synthesis and Na^+/H^+ exchange (Mellas *et al.*, 1986; Nishimoto *et al.*, 1987; Sessions *et al.*, 1987; Hari *et al.*, 1987).

About 10-20% of the M6P/IGF II receptors are expressed at the cell surface of fibroblasts (Fischer *et al.*, 1980; Braulke *et al.*, 1987). The surface receptors are in equilibrium with receptors in internal membranes and cycling of the receptors between the cell surface and internal membranes is independent of occupation with M6P-containing ligands (Braulke *et al.*, 1987) or IGF II (Oka and Czech, 1986). The expression of the M6P/IGF II receptors at the cell surface can be stimulated by insulin (Oka *et al.*, 1984; Wardzala *et al.*, 1984) and growth hormone (Bryson and Baxter, 1987). Insulin induces a redistribution of receptors from internal membranes to the cell surface, which is associated with a decreased phosphorylation of the receptor in the plasma membrane (Covera *et al.*, 1988a).

In the present study we report on the modulation of the expression of M6P/IGF II receptors at the cell surface by M6P, growth factors and toxins that modify G-proteins.

Results

Stimulation of M6P-binding by M6P, insulin like growth factors, insulin and epidermal growth factor

For measuring the M6P binding sites pentamannose 6-phosphate substituted [^{125}I]bovine serum albumin (PMP-BSA) was utilized. This neoglycoprotein binds with high affinity ($K_D \cong 5 \times 10^{-10}$ M) to M6P specific receptors (Braulke *et al.*, 1987). Prior to the binding the cells were chilled to 0°C and washed with 2 mM M6P to remove endogenous ligands, which occupy about 20-30% of the cell surface receptors. The cells were then incubated for 4 h at 0°C with the iodinated ligand. [^{125}I]PMP-BSA bound to the cell surface was released by incubation with 2 mM M6P. The radioactivity released by M6P represented 80-90% of the total cell associated radioactivity. In the presence of 2 mM M6P binding of [^{125}I]PMP-BSA was inhibited by more than 85%. For convenience the PMP-BSA binding sites will be referred to as M6P binding sites.

Treatment of the fibroblasts for 10 min with 5 mM M6P or a mixture of 10^{-8} M IGF I and 10^{-8} M IGF II (preparation 1932) increased the M6P binding sites to 140 and 190% of the controls (Table I). The stimulation of PMP-BSA binding in parallel dishes rarely varied by more than 10% while a considerable variability was noted between different experiments carried out over a period of 10 months

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Table I. Effect of M6P and growth factors on binding of [^{125}I]PMP–BSA

Pre-treatment[a]	Bound [^{125}I]PMP–BSA (% of control)
M6P (5 mM)	140 ± 25 (n = 43)
IGF I/IGF II (10^{-8} M) (preparation 1932)	190 ± 48 (n = 22)
IGF I (10^{-8} M)	181 ± 35 (n = 10)
IGF II (10^{-8} M)	143 ± 36 (n = 11)
rIGF I (10^{-8} M)	228 ± 87 (n = 27)
rIGF II (10^{-8} M)	156 ± 47 (n = 25)
EGF (10^{-8} M)	197 ± 43 (n = 30)
Insulin (10^{-7} M)	120
Insulin (10^{-6} M)	197
Fibroblasts growth factor (10^{-8} M)	89
Granulocyte/macrophage colony stimulating factor (10 μg/ml)	92

[a]Fibroblasts were stimulated with M6P or growth factors for 10 min at 37°C except for 10^{-6} M insulin, which was applied for 1 h, controls bound 0.155 ± 0.066 ng/mg cell protein (n = 37).

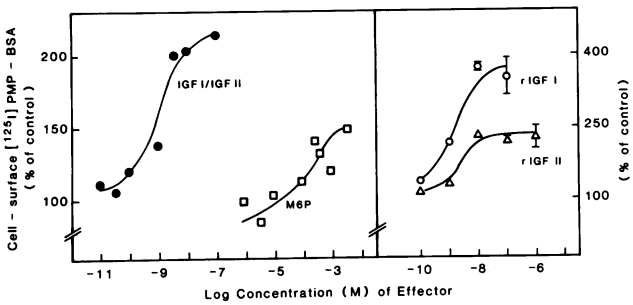

Fig. 1. Concentration dependent stimulation of [^{125}I]PMP–BSA binding by M6P, a mixture of IGF I and IGF II (preparation 1932), rIGF I and rIGF II. For the experiment with rIGF I and rIGF II the range of duplicates is given where it exceeds the size of the symbols.

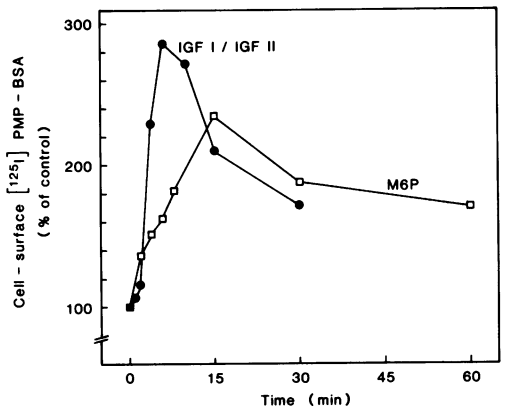

Fig. 2. Time course of the effect of 10^{-8} M IGF I/IGF II (preparation 1932) and 5 mM M6P to increase the cell surface binding of [^{125}I]PMP–BSA.

(cf. Table I with Figures 1 and 2). The effect of M6P and IGF I/IGF II was dose-, time- and temperature-dependent. Half maximal stimulation was observed at 10^{-4} M M6P and 10^{-9} M IGF I/IGF II (Figure 1). A bell-shaped curve was observed for the time dependence with maximal stimulation after treatment for 15 and 8 min with M6P and IGF I/IGF II, respectively (Figure 2). After withdrawal of

Table II. Effect of phosphorylated carbohydrates on binding of PMP–BSA

Pre-treatment	Bound [^{125}I]PMP–BSA (% of control)
M6P	159
Fructose 1-phosphate	143
Glucose 1-phosphate	99
Glucose 6-phosphate	116
Pentamannose 6-phosphate (PMP)	139

Table III. Inhibition of [^{125}I]PMP–BSA binding by anti-M6P/IGF II receptor antibodies

Pre-incubation[a]	Antibody	Bound [^{125}I]PMP–BSA (% of control)
–	pre-immune IgG	100
–	anti-M6P/IGF II receptor IgG	10
rIGF I	pre-immune IgG	192
rIGF I	anti-M6P/IGF II receptor IgG	10

[a]Fibroblasts were incubated for 10 min at 37°C in the absence or presence of $^{-8}$ M rIGF I. Cells were then incubated for 90 min at 0°C in MEM/7.5% FCS containing 1.5 mg/ml of pre-immune or anti-M6P/IGF II receptor IgG. After washing with ice-cold Hanks' balanced salt solution the cells were incubated for 4 h at 0°C in MEM/7.5% FCS containing 50 pM [^{125}I]PMP–BSA.

M6P or IGF I/IGF II the number of M6P binding sites at the cell surface returned to control levels with a $t_{½}$ of 29 and 48 min, respectively (not shown). At ≤ 16°C M6P and IGF I/IGF II did not stimulate the binding of PMP–BSA, while at 23°C an intermediate stimulation was observed (not shown).

Fructose 1-phosphate and pentamannose 6-phosphate, but not glucose 1- or glucose 6-phosphate, could mimic the M6P effect (Table II). Fructose 1-phosphate and pentamannose phosphate are known to bind to M6P-specific receptors (Kaplan *et al.*, 1977; Waheed *et al.*, 1988). Thus, M6P is likely to induce the increase in cell surface M6P binding sites through its interaction with M6P receptors.

When IGF I and IGF II purified from human serum were used at 10^{-8} M concentration, IGF I was more effective than IGF II in stimulating the M6P binding (Table I). The similar difference was observed for recombinant (r) IGF I and IGF II. Dose-dependent stimulation with rIGF I and rIGF II showed that rIGF I was 1.6-fold more potent in increasing M6P binding sites, while similar concentrations of rIGF I and rIGF II (~3 × 10^{-9} M) were required to obtain half maximal stimulation of binding (Figure 1). In a separate experiment, in which cells were incubated at 0°C with 10^{-8} M rIGF II and then with [^{125}I]PMP–BSA, we could show that occupation of the IGF II binding site neither increased nor decreased binding of PMP–BSA.

Incubation of fibroblasts for 10 min with 10^{-8} M epidermal growth factor (EGF) increased the M6P binding 2-fold (Table I). Fibroblasts growth factor (10^{-8} M) and granulocyte/macrophage colony stimulating factor (10 μg/ml) had no effect on M6P binding. Treatment with high concentrations of insulin (10^{-6} M) for 1 h stimulated M6P binding about 2-fold (Table I). Lower concentrations of insulin or shorter treatments had little or no effect on M6P binding.

Table IV. K_D values and binding sites for PMP−BSA and binding sites for M6P/IGF II receptor antibodies in fibroblasts stimulated with M6P or growth factors

Pre-treatment[a]	K_D (pM)	M6P binding sites per cell	Bound M6P/IGF II receptor antibodies per cell ($\times 10^{-5}$ M)
− (n = 12)	51 ± 14	3314 ± 1202	1.10 ± 0.37
M6P (n = 3)	52 ± 15	6098 ± 2440	1.57 ± 0.41
IGF I	67	15308	−
IGF II	36	4500	−
rIGF I	32	5113	2.74 ± 0.79
rIGF II	36	4192	2.34 ± 0.47
EGF (n = 3)	63 ± 23	7238 ± 2609	2.10 ± 0.56

[a]Fibroblasts were incubated for 10 min at 37°C with M6P (5 mM) or growth factors (10^{-8} M). Concentration dependent binding of PMP−BSA was determined at 12−200 pM PMP−BSA. For determination of antigenic sites, cells were incubated with pre-immune Ig or anti-M6P/IGF II receptor Ig, followed by incubation with [^{125}I]protein A. For calculation of bound Ig a stoichiometry of 1:1 was assumed for surface bound Ig and [^{125}I]protein A. The values are the mean of two independent experiments and were corrected for [^{125}I]protein A bound to cells incubated with pre-immune Ig (0.68×10^5 per cell).

Table V. Effect of combinations of M6P and growth factors on the binding of [^{125}I]PMP−BSA

Effector 1	Effector 2	Bound [^{125}I]PMP−BSA (% of control)	Effect on binding
M6P (143 ± 22)	+ rIGF I (180 ± 38)	256 ± 92	Additive
	+ rIGF II (140 ± 68)	199 ± 68	Additive
	+ EGF (207 ± 56)	264 ± 78	Additive
rIGF I (180 ± 38)	+ rIGF II (140 ± 33)	172 ± 35	Non-additive
	+ EGF (207 ± 56)	190 ± 55	Non-additive
rIGF II (140 ± 33)	+ EGF (207 ± 56)	212 ± 49	Non-additive

The values for stimulation with M6P (5 mM) or a single growth factor (10^{-8} M) are given in parentheses. The values represent the mean and variation of five independent experiments.

Stimulation of M6P binding is due to a redistribution of M6P/IGF II receptors

At the surface of fibroblasts two M6P-receptors are expressed, the larger M6P/IGF II receptor with an apparent size of 270 000 and the smaller M_r 46 000 receptor. When fibroblasts were incubated with antibodies blocking the M6P binding site of the M6P/IGF II receptor and M_r 46 000 receptors, respectively, only the antibodies against the M6P/IGF II receptor inhibited the binding of PMP−BSA in control and IGF I stimulated fibroblasts (shown for antibodies against the M6P/IGF II receptor in Table III). It had been shown earlier that M6P binding sites at the cell surface of human skin fibroblasts are represented only by the M6P/IGF II receptor, in spite of the expression of the M_r 46 000 receptors at the cell surface (Stein *et al.*, 1987).

The increase in M6P binding could result from an increased affinity and/or an increased number of cell surface M6P/IGF II receptors. To distinguish between these possibilities, fibroblasts stimulated with M6P or growth factors were analysed for dose-dependent binding of PMP−BSA. None of the effectors changed the affinity for PMP−BSA, while the number of M6P binding sites per cell was increased by M6P, IGF I, IGF II and EGF (Table IV). Furthermore, the addition of 0.5 mM cycloheximide (which inhibited protein synthesis by more than 95%) 30 min prior to and during the stimulation did not affect the M6P, IGF I/IGF II and EGF induced increase in M6P binding. These results indicate that the increase in M6P binding site results from a translocation of pre-existing M6P/IGF II receptors from internal membranes to the cell surface. This assumption was supported by the observation that binding of anti-M6P/IGF II receptor antibodies and subsequent binding of [^{125}I]protein A at the cell surface of fibroblasts was increased 1.5- to 2.7-fold after treatment with M6P (5 mM) or growth factors (10^{-8} M) (Table IV).

Combinations of M6P and growth factors have additive effects on receptor redistribution

Combinations of M6P with either rIGF I, rIGF II or EGF stimulated the receptor redistribution in an additive manner, while non-additive effects were observed for combinations of rIGF I with rIGF II or EGF, and of rIGF II with EGF (Table V). These observations indicate that M6P and the growth factors stimulate the redistribution of M6P/IGF II receptors by independent mechanisms. The non-additive stimulation by combinations of growth factors suggests that the mechanisms by which growth factors stimulate the receptor redistribution share common elements.

The stimulation of receptor redistribution does not involve changes in free intracellular calcium

The rapid redistribution of M6P/IGF II receptors suggests that the redistribution and the binding of M6P and growth factors are linked to second messengers. To monitor changes in free intracellular calcium ($[Ca^{2+}]_i$) associated with redistribution of receptors, fibroblasts were loaded with the fluorescent calcium chelator fura-2-AM prior to stimulation with M6P or growth factors. The basal level of $[Ca^{2+}]_i$ in single cells varied between ≤ 0.01 and 0.2 (mean 0.03) μM. Stimulation with 5 mM M6P, 10^{-8} M IGF I or 10^{-8} M IGF II had no effect on $[Ca^{2+}]_i$, while 10^{-8} M EGF in approximately half of the cells studies induced a rise of

$[Ca^{2+}]_i$ of 0.74 (0.4–2.27) μM after a lag period of 35–40 s (not shown).

The redistribution of receptors induced by M6P, a mixture of IGF I and IGF II, or EGF, was not affected by incubating and stimulating the cells in a calcium-free medium supplemented with 10^{-8} M EGTA. Furthermore, addition of 1 mM Co^{2+} to the medium did not inhibit the effector-induced receptor redistribution (Table VI). These observations indicate that the M6P and growth factor induced redistribution of M6P/IGF II receptors is independent of extracellular calcium.

To examine whether the EGF induced receptor redistribution depends on the increase of $[Ca^{2+}]_i$, fibroblasts were loaded with the permeable calcium chelator BABTA. When accumulated intracellularly, BABTA obliterates changes of $[Ca^{2+}]_i$ as a consequence of calcium release from intracellular stores (Gelfand *et al.*, 1987). EGF, 10^{-8} M, led to a similar increase in M6P binding sites in controls and BABTA loaded fibroblasts (not shown). This result suggests that the EGF induced redistribution of M6P/IGF II receptors is independent of the EGF induced rise of $[Ca^{2+}]_i$.

G-protein modulated expression of M6P/IGF II receptors at the cell surface

Fibroblasts were treated for 17 h with cholera toxin (1 μg/ml) or pertussis toxin (0.1 μg/ml). This treatment resulted in an increase of M6P binding to 195 and 144% of controls, respectively (Table VII). The increase in M6P binding was due to an increased number of M6P/IGF II receptors (examined for cholera toxin only, K_D = 50 pM; 7500 PMP–BSA binding sites per cell). These results suggest a control of cell surface expression of M6P/IGF II receptors by G_s- and G_i-like proteins sensitive to cholera toxin and pertussis toxin, respectively (Neer and Clapham, 1988). Pre-incubation of fibroblast with cholera toxin and pertussis toxin did not prevent the redistribution of M6P/IGF II receptors induced by rIGF I, rIGF II and EGF (Table VIII). However, the effect of M6P on receptor redistribution was abolished or greatly reduced by pre-incubation with pertussis toxin or cholera toxin (Table VIII).

Table VI. Effect of M6P and growth factors on the binding of [^{125}I]PMP–BSA in the absence of extracellular Ca^{2+} or presence of Co^{2+}

	Bound [^{125}I]PMP–BSA (% of control)		
	M6P	IGF I/IGF II	EGF
EGTA	168	209	208
Co^{2+}	139	148	175

Fibroblasts were incubated for 1 h at 37°C in Ca^{2+} free medium, supplemented with 10^{-8} M EGTA and then stimulated for 10 min with M6P (5 mM) or growth factors (10^{-8} M). Alternatively, cells were stimulated in the presence of 1 mM Co^{2+}. The values are expressed as percent of controls, which were incubated with the Ca^{2+} free medium or medium supplemented with 1 mM $CoCl_2$.

Table VII Effect of cholera toxin and pertussis toxin on binding of [^{125}I]PMP–BSA

Pre-incubation[a]	Bound [^{125}I]PMP–BSA (% of control)
Cholera toxin (n = 12)	195 ± 44
Pertussis toxin (n = 8)	144 ± 23

[a]Fibroblasts were incubated for 17 h at 37°C with cholera toxin (1 μg/ml) or pertussis toxin (0.1 μg/m) prior to the binding assay. Controls bound 0.101 ± 0.06 ng/mg cell protein (n = 12).

Table VIII. Effects of M6P and growth factors on binding of [^{125}I]PMP–BSA by fibroblasts incubated with cholera toxin or pertussis toxin

Pre-incubation[a]	Cholera toxin (1 μg/ml)	Pertussis toxin (0.1 μg/ml)
Stimulation		
–	260	150
M6P	286	149
rIGF I	528	297
rIGF II	406	221
EGF	455	317

[a]Fibroblasts were incubated for 17 h at 37°C with the toxins and then for 10 min with M6P (5 mM) or growth factors (10^{-8} M). All values represent the mean of duplicates.

Discussion

Control of cell surface receptor expression by cAMP

M6P/IGF II receptors recycle constitutively between intracellular membranes and the plasma membrane. Under steady-state conditions 10–20% of the receptors are expressed at the cell surface of human skin fibroblasts (Fischer *et al.*, 1980; Braulke *et al.*, 1987). Data presented in this study provide evidence that the steady-state distribution of the M6P/IGF II receptor is controlled by G-protein-dependent mechanisms. The cell surface concentration of M6P/IGF II receptors increases after treatment with cholera toxin and pertussis toxin. Cholera toxin is thought to activate permanently G_s-like proteins and pertussis toxin to inactivate G_i-like proteins through toxin catalysed ADP-ribosylation of the respective G-proteins (Gilman, 1987). Since G_s-like proteins stimulate and G_i-like proteins can inhibit adenylyl cyclase, an effect common to both toxins could be the increase of intracellular cAMP. Further evidence for the role of cAMP in the control of M6P/IGF II receptor distribution comes from preliminary observations (T.Braulke, unpublished results) that forskolin and 3-isobutyl-1-methylxanthine increase the cell surface concentration of M6P/IGF II receptors. These drugs elevate intracellular cAMP levels by stimulating adenylyl cyclase (forskolin) or inhibiting cAMP-phosphodiesterase (3-isobutyl-1-methylxanthine).

Stimulation of M6P/IGF II-, IGF I- and EGF-receptors induces redistribution of M6P/IGF II receptors

M6P, IGF I, IGF II, insulin and EGF induced in fibroblasts a redistribution of M6P/IGF II receptors to the cell surface. M6P is likely to induce the receptor redistribution through binding with the M6P/IGF II receptor. The concentration of M6P required to achieve half maximal effect (≅0.1 mM) and the agonistic effect of the structurally related fructose 1-phosphate are characteristic for a signalling via the M6P binding site(s) of M6P/IGF II receptors.

IGF I and IGF II may bind to either IGF I or M6P/IGF II receptors and could therefore induce the receptor distribution by binding with either type of receptor. Two observations suggest that the IGF I effect is mediated by IGF I receptors and that of IGF II by M6P/IGF II receptors. In

general, IGF I receptors have a higher affinity for IGF I than for IGF II and M6P/IGF II receptors a higher affinity for IGF II than for IGF I (Rechler and Nissley, 1985; Maly and Lüthi, 1986). More recent analyses with synthetic and recombinant IGF I suggest that M6P/IGF II receptors may have little or no affinity for IGF I (Tally *et al.*, 1987a; Barenton *et al.*, 1987). Effects of IGF I and IGF II mediated by the same receptor would therefore display a different concentration dependence for the two growth factors. However, similar concentrations of rIGF I and rIGF II ($\cong 3 \times 10^{-9}$ M) induced half maximal receptor redistribution. Furthermore, the extent of receptor redistribution was significantly different for IGF I and IGF II. This suggests that IGF I and IGF II induce the redistribution via different signal transduction pathways, initiated by binding of IGF I and IGF II to the IGF I and M6P/IGF II receptor, respectively.

Insulin has been shown earlier to induce a redistribution of M6P/IGF II receptors from internal membranes to the cell surface by binding with insulin receptors in rat adipocytes (Oka *et al.*, 1984; Wardzala *et al.*, 1984) and rat H35-hepatoma cells (Covera *et al.*, 1988a). The insulin receptor-mediated redistribution is associated with a decrease in the overall phosphorylation of the M6P/IGF II receptor (Covera *et al.*, 1988a). In fibroblasts no significant change in the overall phosphorylation of M6P/IGF II receptors was observed after stimulation with M6P, IGF II, IGF I and EGF (T.Braulke, unpublished results). The high concentrations of insulin that are required to induce redistribution of M6P/IGF II receptors precludes mediation of the insulin effect in fibroblasts by insulin receptors. It is likely to depend on IGF I receptors, to which insulin binds with low affinity (Rechler and Nissley, 1985).

EGF induced a rapid increase in $[Ca^{2+}]_i$, which preceded the EGF induced receptor redistribution. Abolishing the rise in $[Ca^{2+}]_i$ by inhibiting calcium influx from extracellular sources or by chelating calcium released from intracellular storage pools did not impair the EGF induced receptor redistribution. The EGF induced redistribution of M6P/IGF II receptors is therefore likely to be unrelated to the EGF induced rise in $[Ca^{2+}]_i$.

Binding of M6P and IGF II with M6P/IGF II receptors triggers different signal transducing pathways

Pre-incubation of fibroblasts with cholera toxin or pertussis toxin severely decreased or virtually abolished the receptor redistribution induced by M6P. This suggests that binding of M6P to the M6P/IGF II receptor initiates a signal transducing pathway linked to G_s- and G_i-like proteins. IGF II stimulated the M6P/IGF II receptor redistribution via a mechanism insensitive to cholera toxin and pertussis toxin. Moreover, the effects of M6P and IGF II on receptor redistribution were additive. These observations imply that binding of M6P/IGF II receptors with M6P or IGF II initiates the receptor redistribution via different signal transducing pathways. The M6P/IGF II receptor appears therefore to be a multifunctional protein which binds different classes of ligands at its external domain and is linked to different signal transducing systems at its cytoplasmic domain.

Little is known about the coupling of M6P/IGF II receptors to signal transducing pathways. In basolateral membranes of the renal proximal tubular cell, IGF II stimulates the formation of phosphatidylinositol 4,5-bisphosphate and diacylglycerol (Rogers and Hammerman, 1988). In competent BALB/c 3T3 cells primed with EGF, rIGF II stimulates calcium influx via a mechanism sensitive to pertussis toxin (Nishimoto *et al.*, 1987). Since phospholipase C mediated formation of phosphatidylinositol 4,5-bisphosphate results in increased intracellular calcium levels, both effects may result from an IGF II induced activation of phospholipase C. We were unable to demonstrate an increase of $[Ca^{2+}]_i$ in fibroblasts stimulated with M6P or IGF II, suggestging that the M6P and/or IGF II receptor induced redistribution are not linked to a (phospholipase C induced) rise in intracellular calcium.

Biological function of cell surface M6P/IGF II receptors

Fairly little is known about the biological function of M6P/IGF II receptors at the cell surface. These receptors bind and mediate internalization of M6P-containing compounds (lysosomal enzymes, uteroferrin, proliferin) and of IGF II. The biological role of these functions remains to be established. IGF II binding to its receptor has been shown to stimulate glycogen synthesis in rat hepatoma cells (Hari *et al.*, 1987), to stimulate proliferation of K-562 cells (Tally *et al.*, 1987b), to alkalinize cells of the proximal tubular segment from canine kidney by stimulating Na^+/H^+ exchange across the brush border membrane (Mellas *et al.*, 1986) and to stimulate Ca^{2+} influx and DNA synthesis in competent BALB/c 3T3 cells primed with EGF (Nishimoto *et al.*, 1987). Preliminary experiments have shown that IGF II (as well as IGF I and EGF) stimulates in fibroblasts the uptake of PMP−BSA to a similar extent as the binding of PMP−BSA (S.Tippmer and T.Braulke, unpublished results). Growth factors may therefore stimulate both binding and internalization of the ligands of the M6P/IGF II receptor and thereby modulate M6P/IGF II receptor mediated functions. In addition to an increase of the overall concentration of M6P/IGF II receptors at the cell surface, growth factors may distinctly affect subpopulations of receptors within the plasma membrane. For rat adipocytes it was recently shown that phosphorylation of M6P/IGF II receptors in coated pits is ~20−40 times higher than outside of coated pits. Insulin increases the number of M6P/IGF II receptors in the plasma membrane outside of coated pits and decreases the phosphorylation of receptors associated with coated pits (Covera *et al.*, 1988b). Furthermore, it remains to be determined which changes in receptor distribution in internal membranes are induced by growth factors. It may be that some biological responses to the M6P and growth factor induced redistribution of M6P/IGF II receptors depend on changes of the M6P/IGF II receptors in the internal membranes rather than at the cell surface. For example, changes of the M6P/IGF II receptor concentrations in the Golgi/*trans* Golgi network area may affect the secretion or retention of endogenous receptor ligands (lysosomal enzymes or IGF II).

Materials and methods

Growth factors and chemicals

IGF I and IGF II purified from human serum and a mixture of IGF I and IGF II (preparation 1932) were a kind gift of Dr R.Humbel, University of Zürich. Human IGF I and IGF II produced by recombinant DNA technology were obtained from Dr K.Scheibli, Ciba-Geigy, Basel. EGF, fibroblast growth factor and granulocyte/macrophage colony stimulating

factor were obtained from Boehringer-Mannheim. M6P (sodium salt), cholera toxin and pertussis toxin were from Sigma. Fura-2-AM and BABTA−AM were obtained from Molecular Probes (Junction City, OR, USA), insulin and recombinant protein A were from Serva. Pentamannose 6-phosphate was prepared according to Bretthauer *et al.* (1973), from *Hansenula holstii* phosphomannan, which was kindly provided by Dr M.Slodki (United States Department of Agriculture, Northern Regional Research Center, Peoria, IL, USA). PMP−BSA (bovine serum albumin) was prepared and iodinated with Na[^{125}I] to a specific activity of 350 mCi/mg with the aid of iodogen (Pierce Chemical Co., Rockford, IL, USA) as described (Braulke *et al.*, 1987). Protein A was iodinated with iodogen to a specific activity of 1 mCi/mg.

Cell culture and stimulation protocol

Human skin fibroblasts were grown in 35-mm dishes in minimal essential medium (MEM) with 7.5% fetal calf serum to confluency. The cells were washed, incubated at 37°C for 1 h in MEM containing 0.1% BSA and then stimulated with sugar phosphates or growth factors in the albumin-substituted medium. In experiments with cholera toxin and pertussis toxin, the cells were incubated for 16 h with MEM containing 7.5% fetal calf serum and the toxins and then for 1 h with albumin-substituted MEM containing the toxins.

Binding and uptake of [^{125}I]PMP−BSA

After stimulation at 37°C with the growth factors or M6P, the cells were washed twice for 10 min with 1 ml of ice-cold Hank's buffered salt solution containing 2 mM M6P. The cells were then incubated for 4 h at 0°C with 0.6 ml of MEM containing 7.5% fetal calf serum, 20 mM Hepes, pH 7.2, and 2 ng of [^{125}I]PMP−BSA. Bound [^{125}I]PMP−BSA was displaced by two washes with M6P-supplemented salt solution as above. The cells were solubilized in 1 N NaOH. Radioactivity was determined in the M6P washes and the solubilized cells and referred to cell protein (Lowry *et al.*, 1951). Concentration dependent binding of PMP−BSA was analysed at 12−200 pM PMP−BSA.

Binding of anti-M6P/IGF II receptor antibodies, [^{125}I]protein A

Fibroblasts grown in 24-well plates were incubated for 2 h at 0°C with pre-immune IgG or affinity purified anti-M6P/IGF II receptor antibodies (5 μg/well) in 0.2 ml of MEM, containing 7.5% fetal calf serum, 20 mM Hepes, pH 7.2. After washing the cells were incubated with [^{125}I]protein A (45 ng/well) for 16 h at 0°C. The cells were washed and solubilized in 1 N NaOH for determination of radioactivity and cell protein.

Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by fluorescence measurements on single cells loaded with fura-2-AM (Grynkiewicz *et al.*, 1985; Neher, 1988). Cells were incubated for 30 min at room temperature in saline containing 2 μM fura-2-AM, followed by incubation at 37°C in standard culture medium. Average autofluorescence was measured on non-loaded cells and corrected for. Autofluorescence varied considerably between cells such that absolute values of $[Ca^{2+}]_i$ cannot be considered to be accurate. M6P (5 mM) and growth factors (10^{-8} M) in MEM, 20 mM Hepes, pH 7.2, containing 0.1% BSA were applied locally for 20−60 s through a close-by pipette with an opening of 2−3 μm.

For loading with BABTA, cells were incubated for 30 min at 37°C in MEM containing 20 mM Hepes, pH 7.2, 0.1% BSA and 50 μM BABTA−AM, chased for 30 min in the same medium without BABTA−AM and then stimulated with EGF (10^{-8} M).

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