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Mannose 6-phosphate potentiates insulin-like growth factor II effects in cultured human neuroblastoma cells

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Insulin-like growth factor II (IGF-II) and mannose 6-phosphate (man-6-P) bind to distinct sites on the same receptor [4,16,20]. In the present study, we examined the effects of man-6-P on the growth promoting effects of IGF-II on SH-SY5Y cultured human neuroblastoma cells. Man-6-P alone increased cell number and neurite outgrowth by approximately 50%; as previously observed [12,18,27,30], IGF-II increased cell number and neurite outgrowth by approximately 110 and 30%, respectively. However, when cells were grown in the presence of both ligands, cell number increased by 330% and neurite outgrowth by 130%. These results suggest that man-6-P can potentiate the known growth promoting effects of IGF-II on human neuroblastoma cells. Furthermore, they indicate that the IGF-II/man-6-P receptor may serve as a means of integrating distinct growth promoting signals in neuronal cells.

INTRODUCTION

Insulin-like growth factor-I and -II (IGF-I and IGF-II) are peptides with both growth-promoting and insulin-like activity[6,9,34]. In vitro, both IGF-I and IGF-II are mitogenic for neurons[10,12,18] and stimulate neuritic outgrowth[19,27,29,30]. The actions of the IGF peptides are mediated by specific cell surface receptors[6,7,9,34]. The type I IGF receptor is structurally and functionally similar to the insulin receptor[34] and receptor binding results in autophosphorylation of tyrosine residues. The type II IGF receptor, unrelated to the IGF-I and insulin receptor, is identical to the mannose 6-phosphate (man-6-P) receptor[1,15,16,20]. The IGF-II/man-6-P receptor, with separate[4] but cooperative binding sites for both IGF-II and man-6-P[33], mediates intracellular and extracellular targeting of lysosomal enzymes via the man-6-P binding site[8]. IGF-II, coupling to its distinct site on the IGF-II/man-6-P receptor, can stimulate DNA synthesis[3], increase amino acid uptake[2,13], enhance Ca^{2+} influx[22] and activate G proteins[23,24]. The biological significance of one receptor capable of binding both IGF-II and man-6-P remains uncertain. Recent work, however, suggests the IGF-II/man-6-P may play a role in integrating multiple growth-promoting signals. Secreted man-6-P containing proteins, including transforming growth factor-β1 precursor[26], proliferin[14] and cathepsin D[17] can bind the man-6-P site of the IGF-II/man-6-P receptor and serve as autocrine growth factors[17].

In the present study, we examined the effects of man-6-P in conjuction with IGF-II on SH-SY5Y human neuroblastoma cell mitogenesis and neuritic outgrowth. This cell line serves as a good model for the growth-promoting effects of the insulin-like growth factors. Both IGF-I and IGF-II receptors are present on SH-SY5Y cells[28], which are stimulated to undergo mitogenesis[12,18] as well as vigorous neuritic outgrowth[19,27,29,30] by both peptides. We report that man-6-P alone can enhance both neuroblastoma mitogenesis and neuritic outgrowth, and in the presence of IGF-II, these effects are potentiated. We speculate that man-6-P-containing proteins may act in an autocrine or paracrine fashion, in concert with IGF-II, to regulate neuronal growth.

MATERIALS AND METHODS

Materials

Human recombinant IGF-II (Bachem, Torrance, CA) was dissolved in 10 mM acetic acid and stored at –20 °C. D-Man-6-P was purchased from Sigma Chemicals, St. Louis, MO.

Cell culture

Human SH-SY5Y neuroblastoma cells were kindly provided by Dr. Stephen Fisher, University of Michigan Medical Center. Cells

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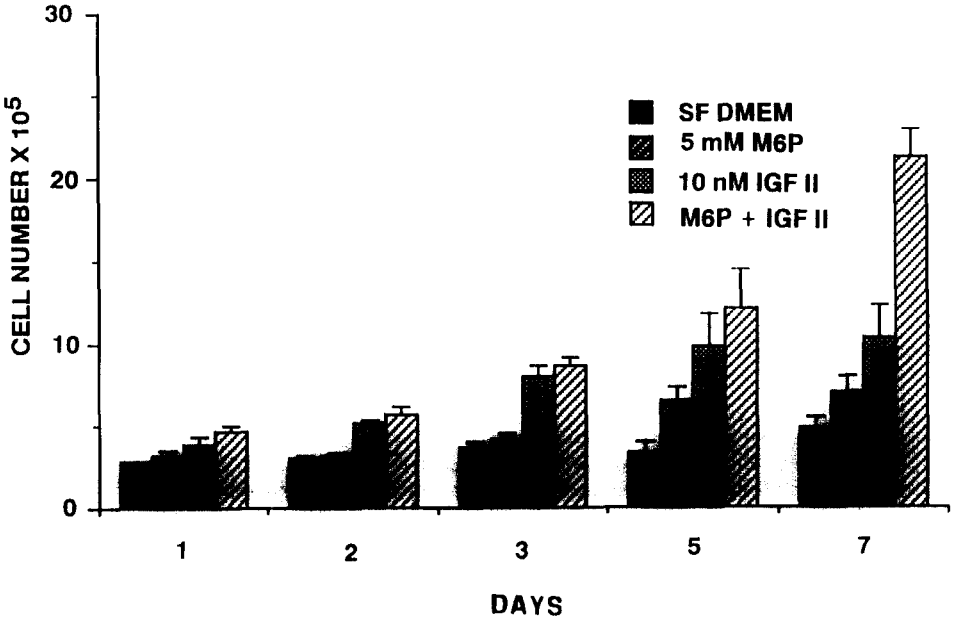
were maintained in plastic tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS). Cells were maintained in an atmosphere of 5% CO_2 and 95% humidified air.

Growth studies[35]

To determine cell doubling, 3×10^5 cells were seeded in 3.5 cm culture wells in DMEM supplemented with 2 mg/ml glucose and 10% CS. After 3 days in culture, cells were rinsed with 1 ml of warm serum-free DMEM before adding 3 ml warm DMEM con-



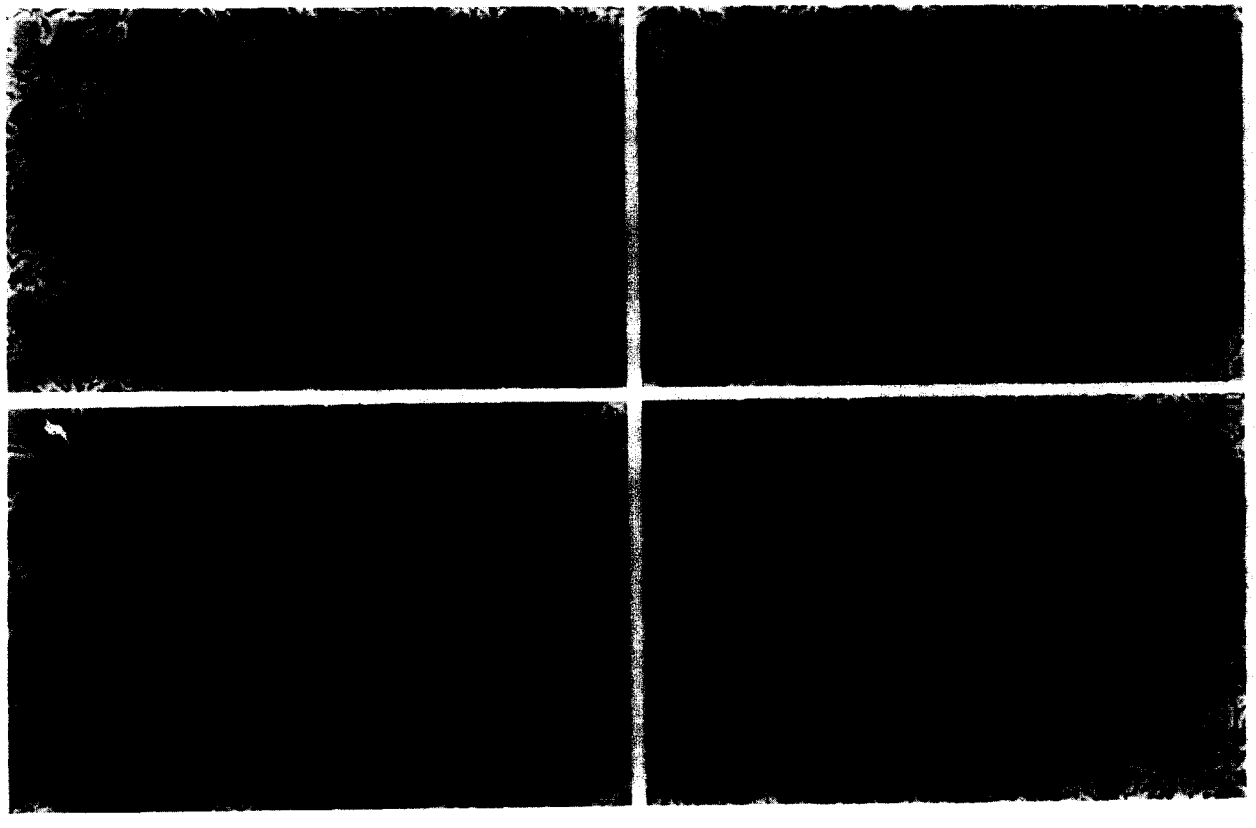


Fig. 1. Effect of man-6-P and IGF-II on SH-SY5Y neuroblastoma doubling. 3×10^5 cells were seeded in DMEM with 10% CS. After 3 days in culture, cells were rinsed with warm serum-free DMEM and placed in DMEM alone (■), 5 mM man-6-P(▨), 10 nM IGF-II (▩)and 5 mM man-6-P plus 10 nM IGF-II (▨) Top: cell doubling was measured as described in Materials and Methods on days 1, 2, 3, 5 and 7, with a media change on day 4. Values are means ± S.E.M. of triplicate replicates, from one of 3 experiments which gave similar results. Bottom: photomicrograph of cells at day 5: A, DMEM alone ; B, 5 mM man-6-P; C, 10 nM IGF-II; and D, 5 mM man-6-P plus 10 nM IGF-II. Bar = 100 μM.

TABLE I

Effect of man-6-P and IGF-II on DNA content

Cells were seeded and cultured as described in Fig. 1. At the indicated days, an aliquot of suspended cells was pelleted and DNA (μg) was determined as described in Materials and Methods. Results are means ± S.E.M. of triplicate replicates, from one of 3 experiments which gave similar results.

	Days				
	1	*2*	*3*	*5*	*7*
Man-6-P	8.8 ± 0.5	10.9 ± 1.7	12.2 ± 1.5	12.0 ± 1.0	13.9 ± 2.5
IGF-II	10.0 ± 0.9	14.6 ± 0.3	17.7 ± 1.2	20.1 ± 2.7	21.2 ± 4.6
Man-6-P + IGF-II	11.4 ± 0.9	14.0 ± 2.9	20.4 ± 1.3	32.2 ± 1.1	50.2 ± 8.9

taining the test agents. Each agent was tested in 3 replicate wells. A baseline cell count was obtained on the day of dosing and cell doubling was measured on days 1,2,3,5 and 7, with a media change on day 4. Floating and attached cells were counted. The media containing floating cells was transferred from each well to tubes, and the attached cells were incubated with 1 ml 0.05% trypsin, 0.53 mM EDTA at 37 °C for 10 min. After terminating trypsinization with 1 ml DMEM with 10% CS, cells were centrifuged for 2 min at 1000 rpm and the cell pellet resuspended in 0.25 ml Hanks' balanced salt solution. A 0.1 ml aliquot was taken and viable cells were counted in a hemocytometer using Trypan blue dye exclusion. The remaining cell suspension was centrifuged at 3000 rpm for 2 min and the cell pellet stored at -20 °C for determination of cellular DNA.

Neurite outgrowth[20,23]

For measuring neurite outgrowth, cells were trypsinized as described above, triturated to obtain a single cell suspension and seeded into 12-well plates in 1 ml of DMEM with 10% CS. After 3 days of growth to allow attachment and recovery from trypsinization, the cells were rinsed with 0.5 ml warm serum-free DMEM before dosing with 1 ml warm media containing the test agents. Neurite outgrowth was measured on days 1, 2, 3, 5 or 7. The percentage of neurite-bearing cells was determined by counting 100 cells from randomly selected fields in each of 3 culture wells under low-power phase contrast microscopy. Cells bearing neurites of 40 μm or more were scored positively and cells with multiple neurites were scored only once. Partially obscured neurites caused by cell clustering were not scored. Representative photographs were taken of cells in each test condition on days 1, 2, 3, 5 and 7.

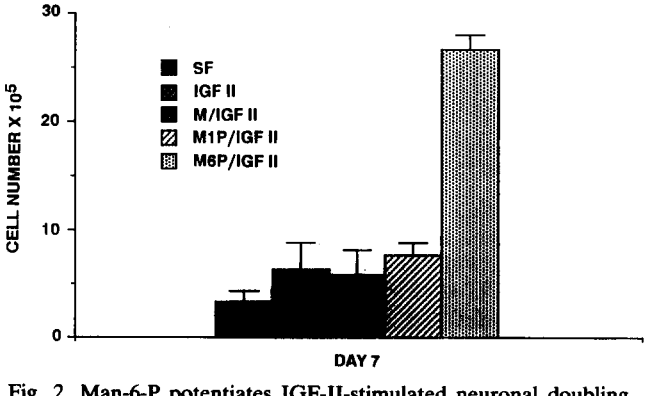


Fig. 2. Man-6-P potentiates IGF-II-stimulated neuronal doubling. 3×10^5 cells were seeded in DMEM with 10% CS. After 3 days in culture, cells were rinsed with warm serum-free DMEM and placed in DMEM alone (■), 10 nM IGF-II (▨), 10 nM IGF-II plus 5 mM mannose(▩), 10 nM IGF-II plus 5 mM man-1-P (▨)and 10 nM IGF-II plus 5 mM (man-6-P(▩). Cell doubling was measured as described in Materials and Methods on day 7, with a media change on day 4. Values are means ± S.E.M. of triplicate replicates.

DNA assay[11]

This method involves hydrolysis of purine nucleotides by heating, allowing the exposed sugars to react with diaminobenzoic acid (DABA). Cell pellets remaining from the cell growth study were dissolved in 0.1 ml of 0.1% sodium dodecyl sulfate (SDS). An equal volume of decolorized, purified 1.8 M DABA was added to each tube. After 30 min at 60 °C, 1.5 ml 1 N HCl was added to each tube and wavelength readings were measured in a Shimadzu spectrofluorophotometer (activation 410, emission 502). Calf thymus DNA (2 mg/ml in 0.1 N NH_4OH) was used to generate a standard curve.

RESULTS

Cell doubling

The effects of man-6-P and IGF-II on neuronal doubling are shown in Fig. 1. In serum-free media, the cells survived for 7 days with no significant change in cell number, as previously reported by Sonnefeld and Ishii[35]. With the addition of 5 mM man-6-P, cell number remained constant for the first 3 days, but after the media change on day 4, there was a 55% increase in cell number observed on day 5 which remained constant at day 7. In the presence of 10 nM IGF-II, cell number increased by 37% by day 2, doubled at day 3 and then remained constant.

When cells were grown in the presence of both man-6-P and IGF-II, neuronal number doubled by day 2, tripled by day 3, increased 4-fold on day 5 and 7-fold on day 7. The effects on neuronal growth of man-6-P and IGF-II together, when compared with each factor separately, were more than additive on day 7. DNA determinations confirmed cell counts (Table I). These effects were specific for man-6-P and did not occur in the presence of mannose or mannose-1-P (man-1-P) (Fig. 2). Concentrations between 5 μM and 5 mM of man-6-P were not toxic to the cells (Fig. 3A) and cell death with corresponding decreases in total DNA (Fig. 3B) were observed only at concentrations equal to or greater than 10 mM man-6-P.

The effects of man-6-P and IGF-I on neuronal doubling are presented in Table II. There was no difference between 1, 3 or 10 nM IGF-I alone, 5 mM man-6-P, or 1 and 3 nM IGF-I plus 5 mM man-6-P on neuronal doubling at day 7. However, cell number more than doubled in the presence of 10 nM IGF-I and 5 mM man-6-P compared to each factor separately.

Neurite outgrowth

The effects of man-6-P and IGF-II on neurite outgrowth are shown in Fig. 4. In serum-free conditions, neurite formation remained stable between days 1 and 3, averaging 35%. After media change, neurite outgrowth decreased by 10% on day 5, but returned to baseline on day 7. Man-6-P enhanced neurite outgrowth to 50% on day 1. This effect was lost by day 2, when neurite outgrowth decreased to 40% where it remained throughout the study. In the presence of IGF-II, neurite outgrowth increased linearly over time, reaching 60% by day 3. After media change on day 4, outgrowth on day 5 remained stable at 57%, but declined to 39% on day 7. In the presence of both man-6-P and IGF-II, neurite outgrowth averaged 50% between days 1 and 3. After media change on day 4, outgrowth increased to 72%, and by day 7, peaked at 80%.

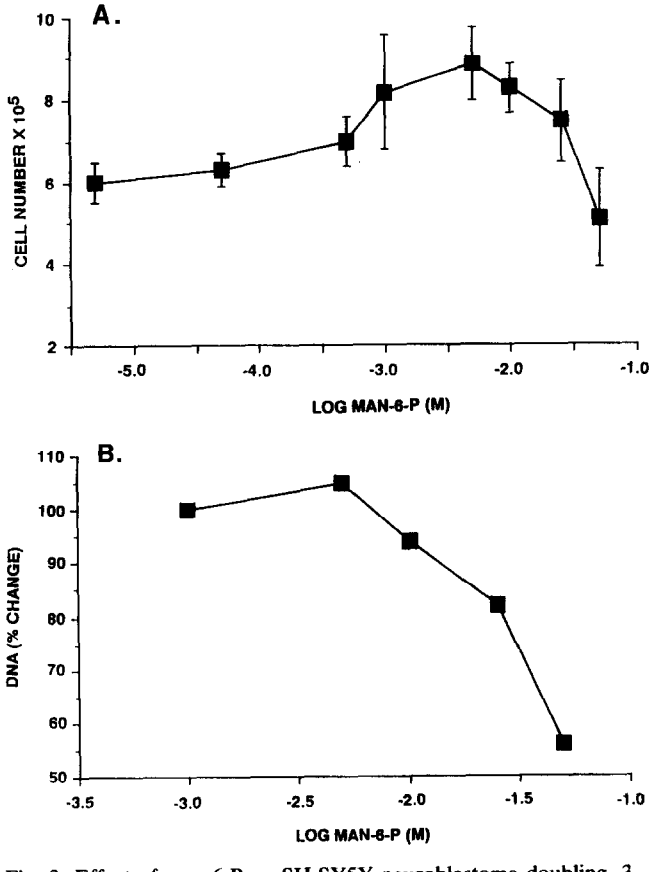


Fig. 3. Effect of man-6-P on SH-SY5Y neuroblastoma doubling. 3 × 10^5 cells were seeded in DMEM with 10% CS. After 3 days in culture, cells were rinsed with warm serum-free DMEM and grown in DMEM plus different concentrations of man-6-P. A: cell doubling was measured as described in Materials and Methods on day 3. Values are means ± S.E.M. of 3 replicate cultures. Cell death was first observed at 10 mM man-6-P. B: DNA determinations were performed after the doubling measurements on each of 3 replicate cultures as described in Materials and Methods. Mean DNA values are expressed as the percent change of the average DNA value at 1 mM man-6-P (13.2 ± 2.4 μg) designated as 100%.

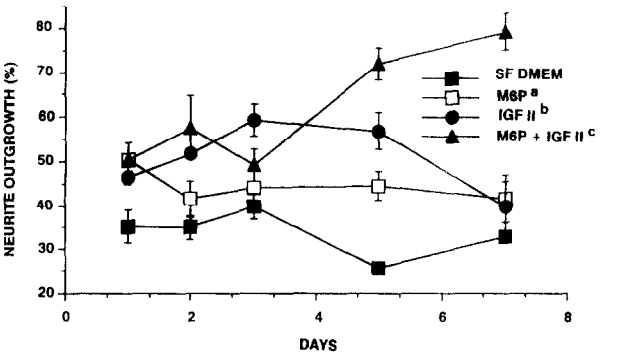


Fig. 4. Effect of man-6-P and IGF-II on SH-SY5Y neuroblastoma neurite outgrowth. 1 × 10^4 cells were seeded in DMEM with 10% CS. After 3 days in culture, cells were rinsed with warm serum-free DMEM and placed in DMEM alone (■), 5 mM man-6-P (□), 10 nM IGF-II (●) and 5 mM man-6-P plus 10 nM IGF-II (▲). Neurite outgrowth was measured as described in Materials and Methods. Values are means ± S.E.M. of triplicate results, from one of 3 experiments which gave similar results. Statistical significance was determined with the Student's *t*-test. [a]$P < 0.01$ compared to SF DMEM cultures on days 1 and 5; [b]$P < 0.01$ compared to SF DMEM cultures on days 1–5; [c]$P < 0.01$ compared to SF DMEM cultures on days 1–7.

DISCUSSION

The man-6-P receptor is structurally and functionally identical to the receptor for IGF-II[16,20], a polypeptide with mitogenic activity[6,9,10,12,18,34]. This multifunctional receptor, with distinct but cooperative binding sites for both man-6-P and IGF-II[4,33], mediates intracellular and extracellular targeting of man-6-P-containing proteins via

TABLE II

Effect of man-6-P and IGF-I on SH-SY5Y neuroblastoma doubling

3 × 10^5 cells were seeded in DMEM with 10% CS. After 3 days in culture, cells were rinsed with warm serum-free DMEM and placed in DMEM alone; 5 mM man-6-P; 1, 3 or 10 nM IGF-I; and 5 mM man-6-P plus 1, 3 or 10 nM IGF-I. Values are means × 10^5 ± S.E.M. of triplicate replicates, from one of two experiments which gave similar results.

	Concentration of IGF-I		
	1 nM	*3 nM*	*10 nM*
Man-6-P	8.7 ± 0.9	9.8 ± 1.0	8.0 ± 0.6
IGF-I	8.5 ± 0.8	12.3 ± 1.0	8.9 ± 2.4
Man-6-P + IGF-I	10.6 ± 1.8	12.9 ± 0.6	20.3 ± 1.4

the man-6-P binding site[8]. Transmembrane signalling of IGF-II's growth promoting effects can occur via the IGF-II binding site[2,3,7,13,17,23,24]. Thus, the man-6-P/IGF-II receptor can serve as a way to integrate the dynamic function of glycoproteins with growth factor signaling and may play a key role in the life cycle of a neuron. We postulated that man-6-P might have a direct effect on neuroblastoma cells and modulate the known mitogenic[12,18] and neurite promoting[19,27,29,30] effects of IGF-II on these cells.

Man-6-P alone stimulated SH-SY5Y neuroblastoma doubling in a dose-dependent fashion. At concentrations less than 5 μM, man-6-P had no effect on neuroblastoma doubling while concentrations equal to or greater than 10 mM were toxic to the cells. Between 5 μM and 5 mM, man-6-P enhanced neuronal doubling, while 5 mM mannose and man-1-P had no effect. Nielsen and Gammeltoft[21] reported a similar stimulation by man-6-P of rat brain neuronal precursor cells. This stimulation was inhibited by antiserum specific for the man-6-P/IGF-II receptor. The mitogenic effect of man-6-P on neuroblastoma cells (our data) and neuronal precursor cells (ref. 21) suggests that man-6-P-containing proteins could modulate neuronal growth via the IGF-II/man-6-P receptor. Mitogenic man-6-P-containing proteins have been identified, including transforming growth factor-β1 precursor[26] and proliferin[14], which bind to the IGF II/man-6-P receptor and activate phospholipase C[32]. A third man-6-P-containing protein is a lysosomal enzyme known as cathepsin D. Cathepsin D binds the IGF-II/man-6-P receptor, stimulates cellular growth and alters IGF-II mediated cell doubling[17,36], supporting our tenet that distinct ligands can effect growth via binding to the multifunctional IGF-II/man-6-P receptor.

In agreement with Ishii and associates[12,18,19,27,29,30], we found that the IGFs stimulated SH-SY5Y neuroblastoma doubling and neurite outgrowth. Our data suggests that man-6-P potentiates these IGF induced cellular responses via the IGF II/man-6-P receptor. In SH-SY5Y neuroblastoma cells, the half-maximal displacement of ^{125}I-IGF-I by its unlabeled ligand is 2–5 nM while 10 nM IGF-I occupies over 50% of the IGF-II receptor sites[28]. Man-6-P did not potentiate IGF-I stimulated neuronal doubling when the growth factor was present at 1 or 3 nM, concentrations at which IGF-I preferentially binds IGF-I receptors in neuroblastoma cells[28]. In contrast, at 10 nM IGF-I, where there is significant cross-occupancy of IGF-I with the IGF-II receptor[28], we observed man-6-P potentiation of growth factor induced doubling. Similarly, after 7 days in culture, addition of either man-6-P or IGF-II stimulated neuronal doubling by 42% and 110%, respectively. However, when added together, man-6-P and IGF-II increased cell number by 335%, a response much larger than can be accounted for by a simple additive effect. In parallel, by day 7, addition of either man-6-P or IGF-II-stimulated neurite outgrowth by approximately 30%, while together, man-6-P and IGF-II enhanced outgrowth by 132%.

There are other biological examples of man-6-P potentiation of signal transduction by the IGF-II/man-6-P receptor, including phospholipase C activation by IGF-II[31], transforming growth factor-β1 precursor and proliferin[32]. In these studies and in our own work, the mechanism which underlies man-6-P potentiation is not determined, but several possibilities exist. Potentiation could result from a man-6-P enhancement of available man-6-P/IGF-II cell surface receptors. In our studies, the effects of man-6-P were most pronounced in cells which had been in culture for 5 or more days, suggesting increased receptor availability over time. This phenomenon is reported in fibroblasts, where binding of man-6-P to the IGF-II/man-6-P receptor triggers redistribution of the receptor to the cell surface, increasing available ligand binding sites[5]. Alternatively, man-6-P may directly enhance IGF-II binding by displacing inhibitory ligands[25] or increasing the affinity of IGF-II for its receptor[33]. While these explanations remain plausible for our studies, they cannot explain the mechanism of man-6-P-potentiated IGF-II phospholipase C activation in the kidney, reported by Rogers and Hammerman[31]. In this model, man-6-P-potentiated IGF-II induced phospholipase C activation by 215% while specific IGF-II binding was increased by only 26%. Furthermore, man-6-P decreased transforming growth factor-β1 precursor and proliferin binding to the IGF-II/man-6-P receptor, but potentiated peptide stimulated renal phospholipase C activation[32]. It is possible that potentiation of phospholipase C activity (Hammerman's studies) and of neuroblastoma doubling and neuritic outgrowth (our studies) reflects an action of man-6-P to directly enhance IGF-II signal transduction. Further studies are needed to address these issues.

In summary, the IGF-II/man-6-P receptor is a multifunctional protein capable of binding both IGF-II and man-6-P[16,20]. In human cultured neuroblastoma cells, man-6-P stimulated mitogenesis and neurite outgrowth and potentiated the known growth promoting effects of IGF-II. We speculate that the IGF-II/man-6-P receptor can serve as a way to integrate signaling from glycoproteins, including man-6-P containing growth factors, and IGF-II in neuronal cells.

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