Abstract. Background: The M6P/IGF-II receptor belongs to the IGF system which plays a crucial role in tumorigenicity. While the role of the IGF-I receptor in signal transduction is well documented, previous experiments failed to uncover a clear signalling function for the M6P/IGF-II receptor. However, more recent studies have shown the capability of M6P/IGF-II receptor to initiate transmembrane signalling. 

Materials and Methods: Human melanoma cells were used to detect the cell surface expression of the M6P/IGF-II receptor and its modulation by different effectors and monoclonal anti-receptor antibodies. Results: M6P (5 mM) caused an increase of the luminescent receptor signal of about 50%. Pre-incubation of cells with Act-D (5 μg/mL) or CHI (10 μg/mL) following M6P stimulation in the presence of the inhibitors caused a reduction of receptor cell surface expression of 27% or 31%, respectively. The monoclonal antibody (mAb) 2G11 was able to mimic the M6P effect on the receptor up-regulation but the mAb MEM-238 did not. The synergistic effect detected with the combination of M6P and the mAb 2G11 and the failure of 2G11 to compete with the M6P action suggests that both effectors have different binding sites on the receptor.

Conclusion: Up-regulation of the tumour suppressor M6P/IGF-II receptor might represent an approach for anticancer therapy. In addition, results support recent data on the receptor’s capability of signal transduction. Due to their accessibility, receptors and other cell surface proteins of the plasma membrane constitute the main targets for protein-based drugs (1). Therefore, the detection and modulation of M6P/IGF-II receptor on human melanoma cells were investigated. This receptor belongs to the IGF system (2) comprising the growth and survival factors IGF-I and IGF-II, their receptors – the IGF-I and IGF-II receptor (also named M6P/IGF-II receptor) – and the IGF-binding proteins 1-6 (IGFBPs). The IGFBPs and the IGFBP proteases determine the ligand availability and their response to the receptors. IGF-II can not only bind to the IGF-II receptor but also to the IGF-I receptor and to the insulin receptor isoform A (IR_A). Therefore, the IR_A may be added to the IGF system. The IGF system plays an important role in normal growth and development as well as in a multitude of pathological situations, particularly tumorigenesis. Several lines of evidence suggest that the M6P/IGF-II receptor acts as a tumour suppressor. It reduces the IGF-II concentration outside the cell by its internalization and degradation in lysosomes. This prevents the accumulation of high extracellular IGF-II levels and its binding and action via the IGF-I receptor. The M6P/IGF-II receptor recaptures secreted lysosomal enzymes (e.g. cathepsin B and cathepsin L) at the cell surface for their subsequent endocytosis and has the potential to activate pro-TGF-β1 into a potent growth inhibitor. Additionally, the receptor promotes the uptake of granzyme B, a serine protease involved in cytotoxic T-cell induced apoptosis. Thus, loss of M6P/IGF-II receptor function or its down-regulation is associated with progression of tumorigenesis. The receptor expression and tumour development in different tumours was reviewed by Hebert (3). Extensive summaries covering structure, properties and functions of the M6P/IGF-II receptor were given by Gosh et al. (4) and Hawkes and Kar (5).
While the IGF-I receptor is a tetrameric transmembrane tyrosine kinase triggering different signal transduction pathways, the single transmembrane IGF-II receptor with a relatively short cytoplasmic tail has no comparable enzymatic activity. Although recent studies have shown the capability of the M6P/IGF-II receptor to initiate transmembrane signalling (6-8), currently this remains a controversial problem (9).

This contribution describes the detection and modulation of the M6P/IGF-II receptor on the cell surface of human melanoma cells (cell line IGR 1). To modulate the receptor expression M6P, brefeldin A, forskolin and two different monoclonal anti-receptor antibodies were used. The application of immunoluminescence as a highly sensitive method was also suitable to detect moderate changes in cell surface receptor expression produced by different effectors and their specific intracellular reactions. Results obtained on the M6P-stimulated receptor up-regulation and its inhibition by CHI and Act-D suggest that the regulation of the receptor concentration occurs via the activation of transcription and subsequent enhanced protein synthesis. Thus, these data support recent results of the M6P/IGF-II receptor’s capability of signal transduction. The up-regulation of the receptor on the cell surface triggered by a monoclonal anti-receptor antibody or forskolin might represent an approach for anticancer therapy. The limitations to this consideration are discussed.

Materials and Methods

Antibodies. Anti-mannose-6-phosphate receptor mouse mAb 2G11 (IgG2α) (Calbiochem/Merck Biosciences, Ltd., UK), anti-mannose-6-phosphate receptor mouse mAb MEM-238 (IgG1) (Biozol, Eching, Germany), goat anti human IGF-II receptor (Cat. no. AF2347; R&D Systems, Wiesbaden-Nordenstadt, Germany); isotype controls: mouse IgG2α, mouse IgG1 (Sigma-Aldrich, Taufkirchen, Germany), other controls or blocking reagents: goat IgG (Sigma-Aldrich, Taufkirchen, Germany), goat anti human IGF-II receptor (Cat. no. AF2347; R&D Systems, Wiesbaden-Nordenstadt, Germany), isotype controls, 10 μg/mL; goat anti-human IGF-II R antibody, according to the manufacturer’s instructions for flow cytometry; HRP-conjugates, 1:500.

Reagents. Luminol (3-aminophthalhydrazide) (Boehringer Mannheim, Germany), D-mannose-6-phosphate disodium salt, actinomycin D, cycloheximide, brefeldin A, 3-isobuthyl-1-methylxanthine (IBMX), forskolin (Sigma-Aldrich), 4-iodophenol (Fluka/Sigma-Aldrich).

Cell line. The human melanoma cell line IGR 1 was supplied by Professor J. Wohlrab (Halle, Germany) and originally produced from C. Aubert et al. (10). The cell line was cultured as described previously (11). Briefly, cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with L-glutamine, D-glucose, sodium pyruvate (Gibco, Life Technologies), 10% FCS, and contained penicillin, streptomycin and amphotericin B (Gibco). The cells were maintained at 37°C in a 10% CO2 atmosphere.

Cell labelling (luminescence). Cell harvesting and pre-treatment were carried out as previously described (11, 12) but supplemented by the following steps. Before blocking cells were pre-incubated with the appropriate reagents (30 min: CHI or Act-D; 60 min: M6P or M6P in the presence of CHI or Act-D; 45 min: mAbs 2G11 or MEM-238) at room temperature to modulate the cell surface receptor protein expression. Before incubation with the primary antibody cells were fixed with 4% paraformaldehyde/0.05% glutaraldehyde in PBS for 15 min at room temperature. The following Ab concentrations were used: mAbs 2G11, MEM-238 and isotype controls, 10 μg/mL; goat anti-human IGF-II R antibody, according to the manufacturer’s instructions for flow cytometry; HRP-conjugates, 1:500.

Luminescence. Each 50 μL aliquot of the cell suspension (5×105-106 cells) was mixed with 250 μL of the substrate solution (50 mmol/L phosphate buffer, pH 7.5; luminol/H2O2/4-iodophenol) (13). The light emission was measured as intensity (relative light units, RLU/s) 35 sec after the initiation. Luminescent detection was performed with the single tube luminometer FB12 (Berthold Detection Systems, Pforzheim, Germany).

Results

The incubation of melanoma cells with M6P (5 mM, 60 min) caused an increase of the M6P/IGF-II receptor signal of about 50%. Cycloheximide (CHI) pre-incubation (10 μg/mL, 30 min) of non-stimulated cells inhibited the receptor expression by about 15%. Actinomycin D (Act-D, 5 μg/mL, 30 min) did not decrease the extent of receptor expression. But 30 min pre-incubation of cells with the inhibitors, following M6P stimulation in the presence of Act-D or CHI, caused an inhibition of receptor expression by 27% or 31%, respectively. The mAb 2G11 was used as primary antibody for the receptor detection.

Figure 1. Stimulation of M6P/IGF-II receptor expression by M6P compared to the inhibition by CHI and Act-D with and without M6P. M6P incubation increased the receptor expression on IGR 1 cells to 154%. Cell pre-incubation with CHI (10 μg/mL) in the absence of M6P caused an inhibition of receptor expression of about 15%. Act-D (5 μg/mL) did not decrease the extent of receptor expression. But 30 min pre-incubation of cells with the inhibitors, following M6P stimulation in the presence of Act-D or CHI, caused an inhibition of receptor expression by 27% or 31%, respectively. The mAb 2G11 was used as primary antibody for the receptor detection.
following M6P stimulation in the presence of the inhibitor lowered the receptor expression to 62% without a significant difference between the concentrations used (data not shown).

To check the effect of serum containing growth factors (e.g., EGF, IGF-I and IGF-II), cells were incubated with DMEM (10% FCS) in the presence and absence of M6P (60 min, RT). Cells treated with PBS alone showed the lowest receptor expression and cells incubated with DMEM and M6P the highest (additive effect of growth factors and M6P). DMEM alone was not as effective as in combination with M6P, but led to a higher signal intensity compared to M6P stimulation (data not shown).

Figure 2 shows the stimulation of receptor expression by the monoclonal anti-receptor antibody 2G11 compared with the effect of PBS, M6P and the combination of mAb 2G11 with M6P. As is clearly shown, the stimulating mAb 2G11 in the presence of M6P produced a synergistic effect on the receptor expression. This signal intensity was higher than the sum of each single intensity produced by M6P and 2G11, respectively. The binding epitope of the mAb on the extracytoplasmic part of the receptor is unknown, but obviously both effectors have distinct binding sites. Thus, the mAb 2G11 may be defined as an allosteric activator producing a transmembrane signalling effect. In addition, Figure 2 shows the isotype control (IgG2a, 4.5% non-specific binding) for the mAb 2G11 signal.

Figure 3 shows the indirect detection of the M6P/IGF-II receptor up-regulation by the mAb 2G11. All mAb 2G11 bound in the stimulation step were blocked by a non-detecting Ab (anti-mouse IgG, 45 min, 4˚C). The remaining receptor molecules up-regulated in the stimulation step were detected in the subsequent detection step using the mAb 2G11. See additional discussion in the text.

3. The pre-treatment of cells with the mAb 2G11 was followed by the incubation with goat anti-mouse immunoglobulin G (45 min, 4˚C). This prevented the binding of the secondary Ab to the receptor-bound 2G11 antibodies. Therefore, in the subsequent incubations the HRP-conjugated secondary Ab detected only those receptors that were expressed as a response to the stimulating effect of 2G11. Here, the middle column represents the signal received after quenching of the original receptor moiety, which means this signal constitutes the newly expressed receptor quantity on the cell surface. It is compared with the PBS control and a second control which demonstrates that the non-detecting anti-mouse IgG did not interfere with the receptor detection. Here, the ratio of the signal intensities between the 2G11 stimulation and the PBS control was lower than in Figure 2, probably because of the partial binding of 2G11 antibodies (mAb excess) also on some newly expressed receptor molecules during the stimulation step. This sharing reduced the signal intensity in the detection step. In addition, this experimental result is based on the observation that the pre-treatment of cells at room temperature (22-23˚C) enabled the stimulation of the receptor’s up-regulation by M6P or the mAb 2G11 whereas in the detection step at 4˚C no stimulation occurred. This corresponds with the observation of Braulke et al. (14) that temperatures ≤16˚C did not stimulate an increase in receptor expression on the cell surface of human skin fibroblasts. Figure 4 shows the repression of M6P/IGF-II receptor up-regulation by the mAb MEM-238. Compared to mAb 2G11 the mAb MEM-238 did not induce a receptor up-regulation on melanoma cells. Cell pre-incubation with
M6P in the presence of MEM-238 showed a competition of this Ab with the M6P binding site. This Ab prevented the M6P effect on the receptor up-regulation, confirming the partially overlapping binding epitopes of both molecules on the extracellular receptor domains 1-3. MEM-238 recognizes an epitope between domains 2 and 5. Further experiments have shown a somewhat lower receptor signal after MEM-238 treatment compared to the PBS control. This result suggests that this Ab is subjected to a partial endocytosis because of its simulation as a M6P-binding ligand.

Forskolin and 3-isobutyl-1-methyl-xanthine (IBMX) are substances that enhance the intracellular cAMP concentration in different ways. Forskolin directly activates the adenylate cyclase and IBMX inhibits the cAMP phosphodiesterase activity. Elevation of cAMP facilitates exocytosis or secretion via the reorganisation of the actin filament network. Therefore, forskolin and IBMX were used to enhance the M6P/IGF-II receptor concentration on the cell surface of melanoma cells. Figure 5 demonstrates the influence of forskolin on the receptor expression. Up to a concentration of 10 μg/mL forskolin did not change the receptor signal but 40 μg/mL potentiated the receptor expression by 39%. DMSO as solvent for forskolin did not impair the receptor detection.

In addition, brefeldin A (BFA) was used to investigate the influence of further substances affecting the receptor expression into the opposite direction compared to forskolin. BFA inhibits the transport from the endoplasmic reticulum (ER) to the Golgi apparatus and the protein traffic from the distal Golgi complex to the cell surface. The effect of BFA on the receptor trafficking in melanoma cells is shown in Figure 6. BFA at a concentration of 20 μg/mL was found to inhibit the transport of the receptor to the plasma membrane by 21%. BFA concentrations below 20 μg/mL did not impair the cell surface expression of the receptor. DMSO served as solvent for BFA.

Figure 4. Binding of the mAb MEM-238 to the M6P/IGF-II receptor of melanoma cells did not induce a receptor up-regulation. Further experiments showed a somewhat lower MEM-238 signal compared to the PBS control as shown here. In the presence of M6P, the mAb MEM-238 competes for the second M6P binding site (domains 1-3) preventing the stimulating effect of M6P on the receptor up-regulation.

Figure 5. Influence of forskolin on the M6P/IGF-II receptor expression on the cell surface of melanoma cells. Forskolin directly activates the adenylate cyclase resulting in intracellular cAMP increase. The cellular response to the cAMP-induced processes is an increase in exocytosis. Up to a concentration of 10 μg/mL forskolin did not change the receptor signal but 40 μg/mL potentiated the receptor expression by 39%. DMSO as solvent for forskolin did not impair the receptor detection.

Figure 6. Influence of BFA on the M6P/IGF-II receptor trafficking in melanoma cells. BFA at a concentration of 20 μg/mL (60 min pre-incubation) was found to inhibit the transport of the receptor to the plasma membrane by 21%. BFA concentrations below 20 μg/mL did not impair the cell surface expression of the receptor. DMSO served as solvent for BFA.
Discussion

This investigation was performed to extend our knowledge on the number of functionally important proteins exposed on the cell surface of human melanoma cells. The M6P/IGF-II receptor functions as a tumour suppressor and therefore its up-regulation might represent an approach for anticancer therapy. In addition, the capability of the M6P/IGF-II receptor to initiate transmembrane signalling is currently still a controversial matter. Immunoluminescence, as a highly sensitive method, was suitable to detect changes in cell surface receptor expression produced by different effectors and their specific intracellular reactions.

Obviously, M6P stimulation leads to a transcription activation followed by enhanced receptor protein synthesis in melanoma cells (Figure 1). This is in contrast to the finding of Braulke et al. (14) which define the increase of receptor expression on the cell surface of fibroblasts as a redistribution due to the failure of CHI to inhibit the M6P-stimulated receptor increase. However, the authors do not describe the additional use of Act-D as a transcription inhibitor. If no M6P activation occurs, the antibody detects the normal cell surface receptor level (10-20%) maintained by a basal protein synthesis on low level. The situation in M6P-non-stimulated cells is characterized by a steady state of M6P/IGF-II receptor molecules distributed over several intracellular compartments and the plasma membrane (receptor recycling). CHI was capable of decreasing the cell surface receptor expression in M6P-non-stimulated melanoma cells by 15%. The receptor expression in non-stimulated, PBS-treated cells was not impaired by Act-D indicating that the transcription was not markedly activated or the Act-D concentration of 5 μg/mL was too low to reveal a distinct effect under these conditions. However, in M6P-stimulated cells the same Act-D concentration lowered the receptor expression by 27% (and CHI by 31%). The results presented here clearly show that M6P stimulates not only the redistribution of the M6P/IGF-II receptor from internal membranes to the cell surface, but also to a marked extent the new synthesis of the receptor protein (Figure 1). If M6P stimulates the receptor synthesis, consequently the receptor redistribution to the plasma membrane will be enhanced with a temporary increase of the receptor concentration in the initial phase. Later, receptor recycling may lead to the adjustment of the equilibrium and to a normal receptor concentration on the plasma membrane.

As shown in Figure 2, the receptor expression on the cell surface was up-regulated by the monoclonal anti-receptor antibody 2G11 and in the presence of M6P this antibody produced a synergistic effect. However, the sole effect of the mAb 2G11 on the receptor up-regulation was not inhibited by CHI. Thus, this mAb obviously triggers the receptor up-regulation on the cell surface by a mechanism which differs from that of M6P. This consideration is supported by the finding that M6P and the mAb 2G11 do not interfere with each other because of different binding sites on the extracytoplasmic part of the receptor.

Frequently, Bordetella pertussis toxin (PTX, which inhibits Gt proteins) and cholera toxin from Vibrio cholerae (CTX, which affects Gt proteins) were used to test whether the receptor is coupled with a G protein. An overview describing PTX and CTX effects on the M6P/IGF-II receptor-G protein interaction is given by Hawkes et al. (9). For example, Braulke et al. concluded from their data that the binding of M6P/IGF-II receptors with M6P 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 (14).

It was found that the M6P/IGF-II receptor is capable of forming oligomeric structures that are most likely dimers, and the binding of a lysosomal enzyme appears to stabilize the oligomeric state (17, 18). The capability of forming dimers supports previous results on the G protein-coupled ligand-receptor interaction and the putative signal transduction mechanism. In addition, studies on receptor mutants incapable of dimerization have confirmed that receptor dimerization of single chain receptors is essential for signalling and the biological response in vivo.

In contrast to the mAb 2G11, the mAb MEM-238 did not induce a receptor up-regulation. Due to its binding epitope covering the extracellular receptor domains 2 and 5, this Ab is bound at one of the two M6P binding sites (receptor domains 1-3). Therefore, the mAb MEM-238 prevents the stimulating effect of M6P on the receptor up-regulation. Either the M6P binding site between domains 1-3 is essential for its stimulating effect or both M6P binding sites (domains 1-3 and 7-9) have to be occupied by M6P for the receptor up-regulation.

Forskolin is a cell permeable diterpenoid, and therefore bypasses the putative G protein by its direct activation of the adenylate cyclase. cAMP increased by this reaction promotes the exocytosis of receptor-containing vesicles and their facilitated transport to the plasma membrane due to disassembly or reorganization of the actin cytoskeleton. Another possible mechanism triggered by forskolin might be the activation of protein kinase A by cAMP and the subsequent phosphorylation of the transcription factor CREB. CREB has the potential to activate or repress the transcription of different genes (19). If CREB activates the receptor gene transcription it might result in an increase of receptor protein synthesis.

Finally, BFA was shown to inhibit the transport of the receptor to the plasma membrane by reversible disassembly of the Golgi apparatus. In contrast to BFA it was found that M6P, forskolin and the mAb 2G11 cause the up-regulation
of M6P/IGF-II receptor in melanoma cells. Therefore, an activating mAb – preferentially a fully human mAb – or forskolin might constitute an approach for their evaluation as anticancer drugs. The up-regulation of the M6P/IGF-II receptor as tumor suppressor seems to be clear. On the other hand, the IGF-I receptor is overexpressed in many diverse tumour types and is a critical signalling molecule for tumour cell proliferation and survival. Therefore, currently the opposite approach is targeted on the IGF-I receptor and its signal transduction pathway by blocking or neutralizing mAbs and IGF-I R-specific small inhibitory molecules (20-22). In addition, the up-regulation of the M6P/IGF-II receptor in tumour cells is effective only if the tumour repressor function is not impaired by gene mutations. A second problem could be the reversibility of receptor up-regulation by receptor recycling and the adjustment of the equilibrium concentration on the cell surface.

Acknowledgements

I want to thank cordially Professor Dr. med. Johannes Wohlrab and Mrs. Karin Hölsken (Department of Experimental Dermatology, Clinical Centre, University Halle-Wittenberg) for their generous help in cultivation of the melanoma cell line IGR 1.

References