The EMBO Journal vol.4 no.7 pp.1725-1730, 1985

Internalization of blocking antibodies against mannose-6-phosphate specific receptors

Carsten Gartung, Thomas Braulke, Andrej Hasilik and Kurt von Figura

Physiologisch-Chemisches Institut der Westfälischen Wilhelms-Universität, Waldeyerstrasse 15, D-4400 Münster, FRG

Communicated by B.Dobberstein

Antibodies against mannose-6-phosphate specific receptors inhibit the receptor-dependent endocytosis of exogenous lysosomal enzymes as well as the sorting of endogenous lysosomal enzymes. This inhibition was correlated with an apparent loss of the receptors. We report here that treatment of cells with the antibody results in the formation of receptor-antibody complexes that are not extracted by the procedure used for the solubilization of receptors prior to immunoprecipitation and detection of the receptor. The apparent loss of receptors is observed with both native antibody and the $F(ab)_2$ fragments, but not with Fab fragments. In contrast the transport of lysosomal enzymes is inhibited by all three forms of the antibody. The inhibition is ascribed to masking by the antibody of the enzyme-binding site in the receptor. The inhibition of the sorting of endogenous lysosomal enzymes by antibodies added to the medium indicates that the mannose-6-phosphate specific receptors at the sorting site are in dynamic equilibrium with those at the cell surface. The receptor-antibody complexes formed at the cell surface appear to cycle between the cell surface and intracellular membranes. A fraction of the internalized antibodies dissociates from the receptors and is degraded after transfer into lysosomes. Complexing with Fab increases the concentration of the receptor in the lysosomes and decreases 2- to 3-fold the half-life of the receptor.

Key words: antibodies/mannose-6-phosphate receptors

Introduction

In mammalian cells, newly synthesized lysosomal enzymes are endowed with mannose-6-phosphate-containing oligosaccharide side chains. In many cell types, transport to lysosomes depends on binding of the enzymes to mannose-6-phosphate specific receptors in the Golgi complex. Analogously, uptake of exogenous lysosomal enzymes depends on binding of lysosomal enzymes to receptors at the cell surface (for review, see Creek and Sly, 1984). In keeping with their function, mannose-6-phosphate specific receptors are found in membranes of the Golgi complex, of the compartment of uncoupling receptor and ligand (CURL), of coated vesicles, as well as in the plasma membrane (Brown and Farquhar, 1984; Geuze *et al.*, 1984a, 1984b). Exposure of fibroblasts to antibodies recognizing the receptors interferes with endocytosis of exogenous, as well as with the sorting of endogenous, lysosomal enzymes, indicating an exchange of receptors between Golgi complex and plasma membrane (Creek and Sly, 1983; von Figura *et al.*, 1984). Furthermore, a rapid loss of receptors, ascribed to enhanced degradation of antibody-tagged receptors, is observed (von Figura *et al.*, 1984). Here we compare the effects of anti-receptor Ig, and of their bi- and monovalent fragments, $F(ab)_2$ and Fab, on the receptor and its functions. In addition, we describe the binding, uptake and degradation of these antibody forms in fibroblasts.

Results

Effects of the anti-receptor antibody and of its fragments

We showed previously that exposure of fibroblasts to antibodies against mannose-6-phosphate specific receptors inhibited the receptor-dependent endocytosis and sorting of lysosomal enzymes, and induced an apparent loss of the receptor (von Figura *et al.*, 1984). To distinguish between effects resulting from the binding to, and the cross-linking of the receptors, we prepared $F(ab)_2$ and Fab fragments of the antibody. Table I shows that the specificity of the association to fibroblasts of iodinated Fab was comparable with that of iodinated Ig. Treatment with Ig, $F(ab)_2$ or Fab fragments inhibited endocytosis of β-hexosaminidase (Figure 1A), which is known to depend on the receptor. Further, like Ig, Fab interfered with sorting of endogenous lysosomal enzymes, as indicated by enhanced secretion of cathepsin D (Figure 1B).

In a short-term experiment (2 h incubation of cells with antibodies) in the presence of up to 12 μg/ml of Ig or $F(ab)_2$, a marked loss of receptor was noted, whereas the presence of similar amounts of Fab had no effect on the concentration of the receptor measured by analyzing immunoprecipitates obtained from lysates of metabolically-labelled fibroblasts (Figure 2). In the continuous presence of Fab the half-life of the receptor was decreased 2.2- to 3-fold, although only after a lag of 4 h (Figure 3). The results indicated that the increased rate of degradation of the receptor in the presence of Fab was due to a relocation of the receptor to lysosomes. In fact, from fibroblasts that were metabolically labelled with [^{35}S]methionine and subsequently exposed to 6 μg/ml anti-receptor Fab for 2 h, 2.3% of their receptors could be recovered in the high buoyant density lysosomal fraction (Figure 4). In contrast, in controls no receptor ($\leq$0.5%)

Table I. Association of anti-receptor [^{125}I]Ig and [^{125}I]Fab to fibroblasts

Antibody	Addition	Bound [^{125}I]antibody (ng/mg cell protein)
Anti-receptor [^{125}I]Ig	–	37.4
	0.1 ml antiserum	4.0
	0.1 ml pre-immune serum	35.0
Control [^{125}I]Ig	–	3.6
Anti-receptor [^{125}I]Fab	–	185
	0.1 ml antiserum	19
	0.1 ml pre-immune serum	172
Control [^{125}I]Fab	–	26

Fibroblasts in 35 mm dishes were incubated for 2 h at 37°C with 0.5 ml medium containing 0.5 μg [^{125}I]Ig or 3 μg [^{125}I]Fab and the additions indicated. The cell-associated radioactivity was referred to the cellular protein.

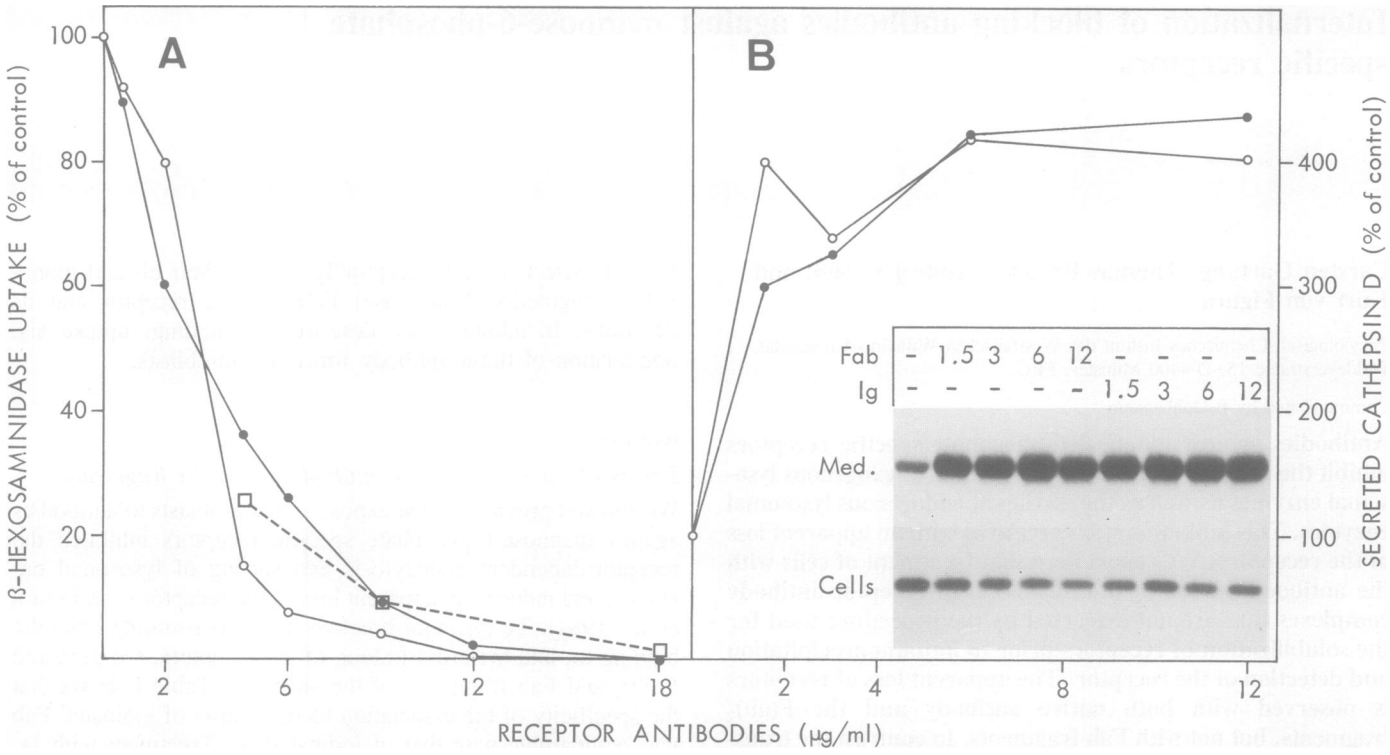

Fig. 1. Effect of receptor antibodies on endocytosis of β-hexosaminidase **(A)** and secretion of cathepsin D **(B)**. **(A)** Sandhoff fibroblasts (deficient in β-hexosaminidase) were incubated for 24 h in 1 ml of medium containing up to 8 μg of Ig (○), $F(ab)_2$ (□) or Fab-fragments (●) against mannose-6-phosphate specific receptors, and 50 mU of β-hexosaminidase prepared from fibroblast secretions. All values were corrected for residual β-hexosaminidase acivity in the recipient fibroblasts. Controls internalized 14% of the added enzyme. **(B)** Fibroblasts were labelled for 24 h with [^{35}S]methionine in the presence of up to 12 μg/ml of Ig (○) or Fab-fragments (●) against mannose-6-phosphate specific receptors. Cathepsin D was isolated from the media and from one-tenth of the cell extracts by immunoprecipitation and gel electrophoresis (see insert). Radioactivity in the cathepsin D polypeptides was quantified by densitometry. Controls contained 8% of the cathepsin D polypeptides in the medium.

was detected in the lysosomal fraction. Thus Fab was as potent as $F(ab)_2$ and Ig in inhibiting receptor function, whereas its effect on the concentration of the receptors was much weaker than those of $F(ab)_2$ or Ig. Apparently, the inhibition of the function correlated with the binding to, rather than with the cross-linking of, the receptors. Binding is likely to involve or alter the mannose-6-phosphate recognition site in the receptor, since incubation of the latter with Ig blocked subsequent binding of β-hexosaminidase by 90% (Table II).

Experiments were done to establish whether the decreased concentration of receptors observed after treatment with Ig and $F(ab)_2$ was real, or due to interference of the antibody with the solubilization and/or detection of the receptor. The labelled antibody was bound to the receptor by incubation with the cells at 37°C for 2 h. 90% of the cell-associated radioactivity was recovered in a membrane fraction, from which the receptors are extracted routinely with detergents. More than 80% of the antibody (and probably of the receptors too) was found in the insoluble residue (Table III). We conclude that cross-linked receptors are not extractable by our routine procedure, and that the loss of receptors illustrated in Figure 2 is at least partially apparent.

Binding, internalization, reappearance at the cell surface and degradation of the antibody

The dose dependence of the binding of anti-receptor [^{125}I]Ig and [^{125}I]Fab was determined by incubating the cells for 1 h at 0°C. Saturation of the binding was not achieved at concentrations of up to 30 μg/ml of antibodies (Figure 5). Scatchard plots indicated multiple binding sites for both Ig and Fab fragments. Incubation of cells at 37°C is expected to allow both binding and uptake of iodinated antibodies. To differentiate between cell surface-bound and internalized radioactivity, the cells were treated with 0.1% trypsin for 1 h at 0°C, to release cell surface-associated material. More than 70% of the radioactivity associated with cells after incubation for 1 h at 0°C with iodinated anti-receptor Ig or Fab was trypsin-releasable. In contrast, <20% of the radioactivity associated with cells after incubation for 1 h at 37°C was releasable with trypsin. Thus, the radioactivity bound at 0°C reflected largely cell surface-bound Ig or Fab, whereas that bound at 37°C was mainly internalized Ig or Fab. Within the whole range of concentrations tested, the amount of anti-receptor Ig and Fab associated with the cells during incubation for 2 h at 37°C was ~7–8 times greater than the amount bound at 0°C (Figure 5). At equimolar concentrations (calculated for 0.2 mM antibody) association of Fab at 0°C and 37°C was 2.6 and 3.6 times that of Ig.

A portion of the internalized antibody was subjected to degradation. We followed in time the uptake, binding and degradation of [^{125}I]Ig (Figure 6). Degradation was defined as the amount of trichloroacetic acid-soluble ^{125}I-material released into the medium, and uptake as the sum of cell-associated radioactivity and degraded ^{125}I-material in the medium. Uptake was maximal at the beginning, and continued for at least 24 h. Degraded antibody fragments appeared in the medium after the first hour of incubation and continued thereafter. The amount of the cell-associated radioactivity reached a plateau within about 2 h, that was maintained for nearly 24 h, indicating that an equilibrium between uptake and degradation had been established. At equilibrium ~500 000 antibody molecules were associated with a single fibroblast. Assuming that all Ig molecules are receptor-

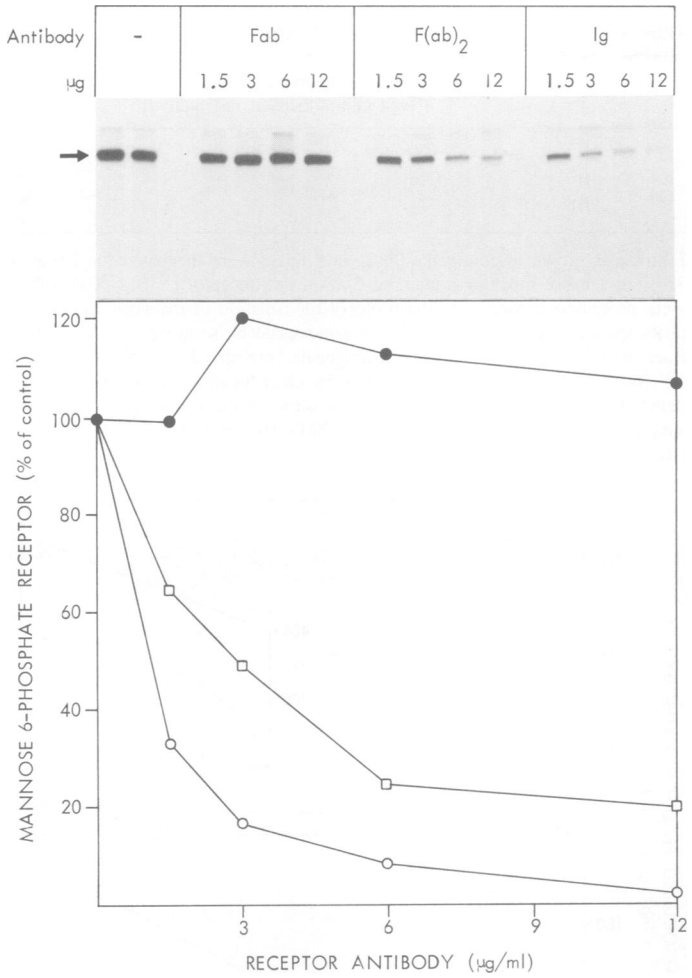

Fig. 2. Loss of extractable receptors in fibroblasts exposed to anti-receptor antibodies. Fibroblasts were subjected to a 6 h pulse−2 h chase labelling with [^{35}S]methionine. The chase was performed with up to 12 μg Ig (○), $F(ab)_2$ (□) or Fab-fragments (●) of the mannose-6-phosphate specific antibody. The receptors (→) were isolated by immunoprecipitation and gel electrophoresis **(upper part)**. Radioactivity in the receptor was quantified by densitometry **(lower part)**.

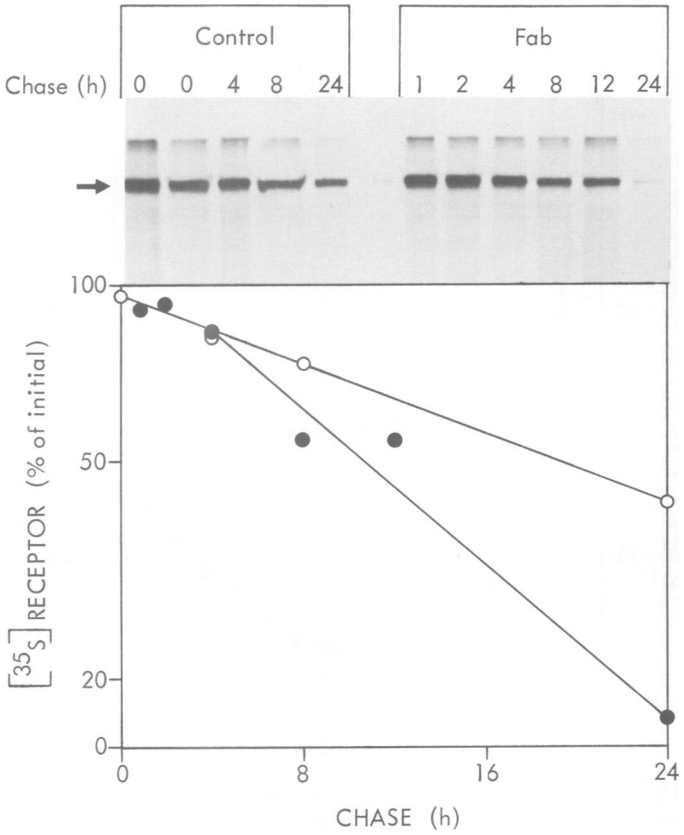

Fig. 3. Mannose-6-phosphate specific receptors in cells exposed to anti-receptor Fab. Fibroblasts were labelled for 16 h with [^{35}S]methionine and then chased up to 24 h in the absence (○) or presence (●) of 6 μg/ml of anti-receptor Fab. The receptors (→) were isolated by immunoprecipitation and gel electrophoresis **(upper part)**. Radioactivity was determined by liquid scintillation counting of excised and solubilized receptor bands **(lower part)**.

bound, and that a single fibroblast contains ~200 000 binding sites (Gonzalez-Noriega *et al.*, 1980; Fischer *et al.*, 1980), the ratio of antibodies to binding sites was therefore 2−3. The continuous uptake of antibody did not depend on the synthesis of new receptors, since fibroblasts treated with cycloheximide showed an unimpaired internalization of antibodies (Figure 6). More than 95% of the cellular receptors are part of cross-linked complexes within 2 h of incubation at the antibody concentration used (see Figure 2). The continued uptake of antibody implies, therefore, that subsequent to internalization, a fraction of the bound antibody is released from the antibody-receptor complexes, and that the complexes are transported back to the cell surface, where vacant antibody-binding sites may become occupied again.

Receptor-antibody complexes reappear at the cell surface. This was demonstrated by measuring the fraction of the [^{125}I]Ig that was released from cells by trypsin treatment at 0°C. About 1/5 of the internalized radioactive antibody reappeared at the cell surface within 2 h of incubation, and this amount did not change much during the subsequent 22 h (Figure 7). After 24 h of incubation, half of the previously internalized [^{125}I]Ig was degraded, and 1/5 was released as acid-insoluble material into the medium. In addition, the fraction of radioactivity associated with lysosomes decreased from 17 to 5%. When cells first labelled with [^{125}I]Ig were subjected to three cyles, each consisting of treatment with trypsin at 0°C followed by incubation for 2 h at 37°C, 20−40% of cell-associated radioactivity was released by trypsin after each cycle.

The binding of [^{125}I]Fab reached a maximum within 4 h of incubation (Figure 6). During the subsequent 20 h, the amount of cell-associated [^{125}I]Fab decreased slightly. Compared with Ig, a smaller proportion of Fab was degraded. The reason for this difference is not understood, although it is tempting to speculate that it reflects differences between Ig and Fab in binding at the cell surface and dissociation in intracellular compartments.

Discussion

Exposure of fibroblasts to anti-receptor antibodies [Ig, $F(ab)_2$ and Fab] results in the inhibition of specific functions of the mannose-6-phosphate receptor, i.e., endocytosis of exogenous and sorting of endogenous lysosomal enzymes. The inhibition of both functions is explained by the inhibitory effect of antibodies on binding of lysosomal enzymes to mannose-6-phosphate specific receptors.

The sorting of newly synthesized lysosomal enzymes depends on their binding to mannose-6-phosphate specific receptors at an intracellular site, presumably within the Golgi complex. The inhibition of sorting indicates that exposure of antibodies is followed by a depletion of functional receptors at the sorting site. The present data suggest that mannose-6-phosphate specific recep-

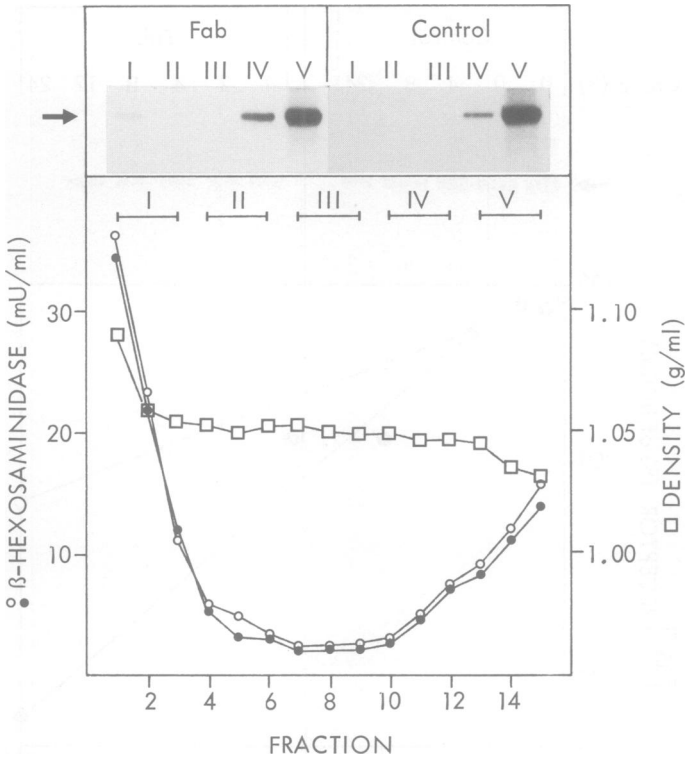

Fig. 4. Partial location of the receptors to lysosomal fractions in cells treated with anti-receptor Fab. Fibroblasts in 75 cm^2 flasks were incubated for 16 h with 0.15 mCi [^{35}S]methionine per flask and then chased for 2 h in the absence (filled symbols) or presence (open symbols) of 6 μg/ml anti-receptor Fab. Postnuclear supernatants of cells were subjected to Percoll density gradient centrifugations. The fractions were analyzed for β-hexosaminidase activity (– ○ –, – ● –) and density (□). Fractions were pooled as indicated (Pool I – V) and analyzed for mannose-6-phosphate specific receptors (→) shown in the fluorograms at the top.

Table II. Binding of β-hexosaminidase to receptor-antibody complexes

	Step I	β-Hexosaminidase (mUnits)	
		Associated with Immuno-Precipitin	Bound to receptors[a]
A	Receptor/antibody/mannose-6-phosphate	0.08	
	Receptor/antibody	0.12	0.04
B	Receptor/mannose-6-phosphate	0.07	
	Receptor	0.47	0.40

In step I, 1 μg of purified human liver mannose-6-phosphate receptor (Geuze *et al.*, 1984b) was incubated overnight at 4°C with 10 μg of receptor-antibody (A) or buffer (B). Then, the receptor-antibody complexes (A) and receptors (B) were incubated for 2 h with 2 mU of affinity-purified β-hexosaminidase from human fibroblast secretions (Geuze *et al.*, 1984a). Subsequently, 10 μg of receptor-antibody (B) or buffer (A) were added. After incubation for 2 h at 4°C, the receptor-antibody complexes were collected with the aid of 3 mg of Immuno-Precipitin. After washing twice, the precipitate was assayed for β-hexosaminidase activity. Where indicated 5 mM mannose-6-phosphate was present throughout all incubations. The incubations were done in 10 mM sodium phosphate, pH 6.5, containing 0.15 M NaCl and 0.1% Triton X-100. The final volumes were 15 μl, 18 μl and 28 μl in steps I, II and III, respectively.
[a]Defined as β-hexosaminidase activity associated with Immuno-Precipitin in a mannose-6-phosphate sensitive manner.

tors of the sorting site are in dynamic equilibrium with those at the cell surface, where binding of antibodies leads to functional inactivation.

Receptor-antibody complexes are subjected to internalization. Within 24 h of incubation the amount of degraded antibodies is four times greater than that bound to the receptor. This finding, and the evidence for continued uptake of the antibody in the absence of synthesis of new receptors, clearly indicate that internalized antibody-receptor complexes partly dissociate, and that receptors are re-utilized for endocytosis. The site of, and the factors triggering the dissociation of, antibodies are unknown. A fraction of the receptors return to the cell surface together with antibodies. A small, additional fraction of the antibody-receptor complex is transported to lysosomes (as shown for Fab-receptor complexes), where both antibodies and receptors are degraded.

Table III. Insolubility of internalized anti-receptor [^{125}I]Ig

Pellet	Radioactivity in pellets (% of cell-associated radioactivity)
I	90
II	76
III	71

Fibroblasts grown to confluency in 25 cm^2 flasks were incubated for 2 h at 37°C in 1 ml of medium containing 6 μg of anti-receptor [^{125}I]Ig. The cells were processed according to the protocol for isolation of mannose-6-phosphate specific receptors. The cells were harvested by scraping and sonicated. Radioactivity associated with cell homogenate corresponded to 577 μg [^{125}I]Ig/mg cell protein. After collecting by centrifugation, the membrane pellet (I) was extracted with detergent. Aliquots of the extract were subjected to centrifugation for 1 h at 50 000 *g* (II) or 6 min at 12 000 *g* (III).

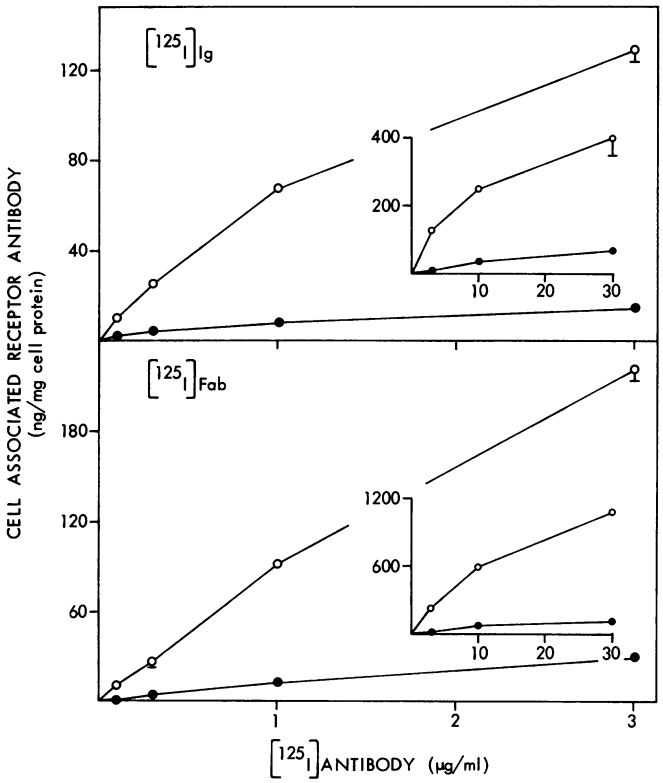

Fig. 5. Dose dependence of binding and uptake of receptor antibodies. Fibroblasts were incubated with 0.5 ml of medium containing up to 15 μg of either anti-receptor [^{125}I]Ig **(upper panel)** or anti-receptor [^{125}I]Fab **(lower panel)**. For determination of binding, cells were incubated for 1 h at 0°C (– ● –). For determination of binding and uptake cells were incubated for 2 h at 37°C (– ○ –). All values were corrected for binding and uptake of control [^{125}I]Ig or control [^{125}I]Fab, respectively. The range of duplicates is indicated where it exceeds the size of symbols. In the inserts, binding (●), and binding and uptake (○) at higher antibody concentrations are shown.

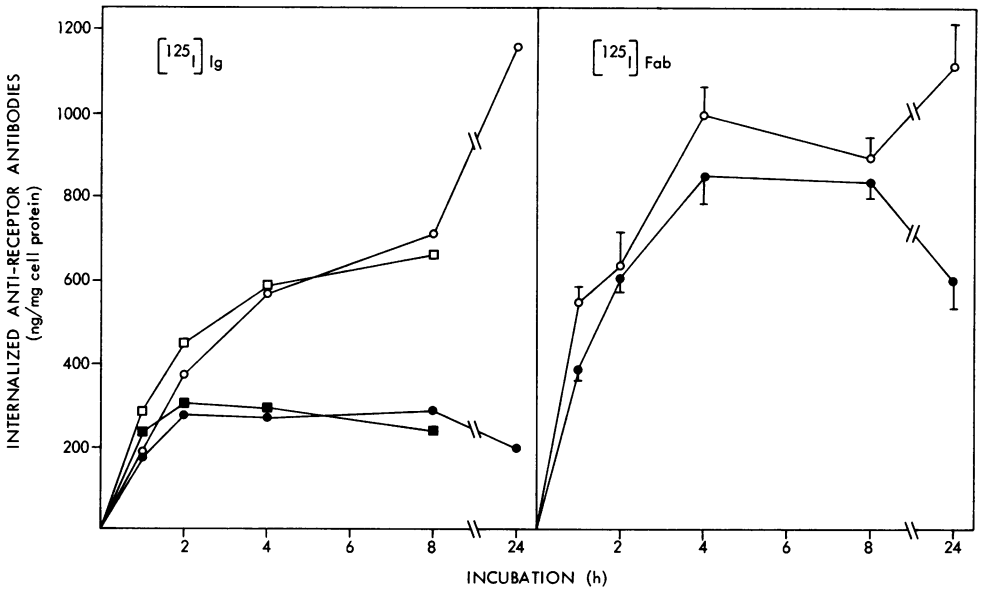

Fig. 6. Time-dependent internalization of anti-receptor antibodies. Fibroblasts were incubated with 0.75 ml medium containing 7.5 μg of anti-receptor [^{125}I]Ig (left part) or [^{125}I]Fab (right part) for up to 24 h. The filled symbols represent the cell-associated radioactivity and the open symbols the internalized radioactivity (sum of cell-associated radioactivity and of TCA-soluble radioactivity in the medium). The rectangular symbols in the left panel give the uptake of anti-receptor [^{125}I]Ig in the presence of 0.5 mM cycloheximide. Bars indicate the range of duplicates.

The loss of receptors in cells treated with anti-receptor Ig has been reported previously, and was attributed to an enhanced degradation of receptor-antibody complexes (von Figura *et al.*, 1984). The present study shows that this loss is not real, but is due, at least in part, to formation of antibody-receptor complexes that are not extracted by the cell-solubilization protocol routinely used prior to immunoprecipitation. The decreased extractability is likely to depend on cross-linking of receptors with anti-receptor Ig and $F(ab)_2$, since in the presence of monovalent anti-receptor Fab the loss of receptors is slow. Cross-linking with antibodies depends on the expression of receptors at the cell surface. The kinetics of the apparent loss of the receptors in cells exposed to anti-receptor Ig, therefore, are related to the kinetics of receptor transport from intracellular sites to the cell surface.

Studies by others had shown that the effects of tagging with antibodies on the movement of receptors involved in endocytosis depends on both the type of receptor and the valency of the antibodies. Macrophage Fc-receptors tagged with polyvalent immune complexes were diverted to lysosomes (Mellman and Plutner, 1984), whereas binding of monovalent anti-receptor Fab did not affect the shuttle of these receptors between cell surface and the endosome compartment (Mellman *et al.*, 1984). Divalent antibodies against the receptors for low density lipoproteins and epidermal growth factor, but not their monovalent Fab-fragments, caused depletion of these receptors at the cell surface, presumably by preventing return of these receptors from the endosome compartment (Anderson *et al.*, 1982; Schreiber *et al.*, 1983). Compared with the receptors mentioned above, the behaviour of mannose-6-phosphate specific receptors appears different in as much as neither polyvalent, nor monovalent antibodies were able to prevent receptor cycling between the cell surface and the intracellular compartment(s).

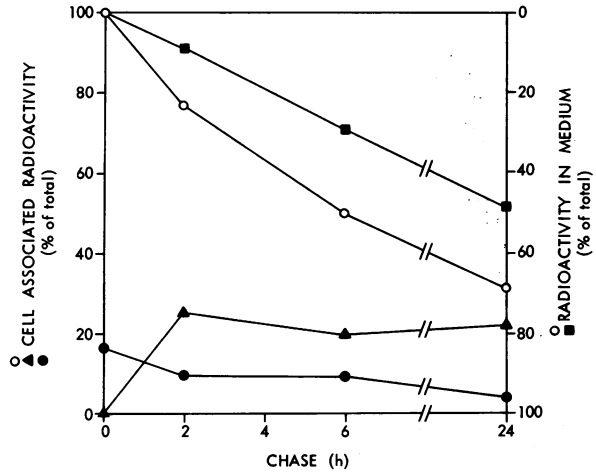

Fig. 7. Metabolism of the internalized anti-receptor [^{125}I]Ig. Fibroblasts were incubated for 1 h at 37°C in the presence of 6 μg/ml of [^{125}I]Ig. Cell surface-associated material was removed by incubation with 0.1% trypsin for 1 h at 0°C. After incubation in fresh medium for up to 24 h at 37°C, the cells were incubated with 0.1% trypsin for 1 h at 0°C and subjected to subcellular fractionation (for details see Materials and methods). Total radioactivity in cells, and in medium (○), TCA-soluble radioactivity in the medium (■), radioactivity associated with cell surface (▲) and dense lysosomes (●) was determined. The sum of radioactivity in cells and medium (ranging from 47 200 to 54 400 c.p.m.) was taken as 100%. It should be noted that the scales on the left-hand side for cell-associated radioactivity and on the right-hand side for medium-associated radioactivity run in opposite directions. The open circle symbol represents total cell-associated radioactivity when referring to the left-hand scale, and total-medium radioactivity when referring to the right-hand side scale.

Materials and methods

Antibodies

Affinity-purified rabbit antibodies against human liver mannose-6-phosphate specific receptor, and human placental cathepsin D were those described (von Figura *et al.*, 1984; Hasilik *et al.*, 1983). $F(ab)_2$ and Fab fragments from anti-receptor and control rabbit Ig were prepared by digestion with pepsin or papain (Davis, 1974; Mage, 1980) and purified on a protein A-agarose (Sigma) column. Iodination with Bolton-Hunter reagent (2000 Ci/mmol, NEN) yielded Ig and Fab preparations with specific activities ranging from 1600 to 3800 c.p.m./ng protein.

Metabolic labelling

Fibroblasts grown to confluence were labelled with [^{35}S]methionine as described (von Figura *et al.*, 1983). For chase, medium containing 0.25 mg/ml methionine

was added. Stock solutions of the antibodies and cycloheximide in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl were added in up to 5% of the final volume.

Subcellular fractionation

Postnuclear supernatants were fractionated by Percoll (Pharmacia) density gradient centrifugation and analyzed for density, radioactivity, β-hexosaminidase, and mannose-6-phosphate specific receptors as described (Lemansky *et al.*, 1984; von Figura *et al.*, 1984). Cells that had been incubated in the presence of iodinated antibody were treated for 1 h at 0°C with 0.1% trypsin to release cell surface-associated material, and washed twice with medium containing 20% fetal calf serum prior to harvesting by scraping.

Immunoprecipitation

The mannose-6-phosphate receptors were extracted and immunoprecipitated as described (von Figura *et al.*, 1984). Briefly, cells were harvested by scraping and suspended in 0.1 M sodium acetate, pH 6.0, containing 0.2 M NaCl, 10 mM EDTA, 1 mM PMSF and 5 mM iodoacetamide. After ultrasonication and centrifugation a membrane pellet was obtained, from which the receptors were solubilized by ultrasonication in 50 mM imidazole-HCl, pH 7.0, containing 0.4 M KCl, 1% Triton X-100, 0.05% sodium deoxycholate, 0.1% bovine serum albumin, 1 mM PMSF and 5 mM iodoacetamide. The extracts were cleared by centrifugation for 1 h at 50 000 *g*, absorbed twice with 10 mg of Immuno-Precipitin and mixed with anti-receptor antibody. The receptor-antibody complexes were collected with the aid of Immuno-Precipitin that had been pretreated as described (von Figura *et al.*, 1985). Cathepsin D was immunoprecipitated as previously (Gieselmann *et al.*, 1983). The immunoprecipitates were solubilized in the absence (receptor) or presence (cathepsin D) of 10 mM dithiothreitol, separated by polyacrylamide gel electrophoresis (12.5% polyacrylamide) and visualized by fluorography (Laemmli, 1970; Laskey and Mills, 1975). Radioactivity in polypeptides was quantitated by densitometry or by liquid scintillation counting (Waheed *et al.*, 1982).

Binding and uptake of [^{125}I]antibodies

Fibroblasts in 35 cm^2 dishes were incubated for either 1 h at 0°C (placed on ice-water), or up to 24 h at 37°C in 0.5–0.75 ml of Eagle's minimum essential medium containing 7.5% fetal calf serum and the iodinated antibodies (diluted 5- to 50-fold with the respective unlabelled antibodies). After incubation, the cells were washed six times with Hank's balanced salt solution, and solubilized in 1 ml of 1% NP-40 and 1% sodium deoxycholate. Alternatively, prior to solubilization, the labelling was followed by a chase, or the cell surface-associated radioactivity was released by incubation for 1 h at 0°C with 0.1% trypsin. For the determination of the degradation of the endocytosed labelled antibodies (i.e., the radioactive material released by cells at 37°C), 0.1 ml of medium was mixed with 2.5 mg bovine serum albumin and 1 ml 10% (w/w) trichloroacetic acid. The mixtures were kept overnight on ice, and then centrifuged for 6 min at 12 000 *g*. The radioactivity values were corrected for tricholoroacetic acid-soluble radioactivity in media that had been incubated in parallel without cells.

Inhibition of ligand binding by anti-receptor Ig

Purified human liver mannose-6-phosphate receptors, 1 μg, were incubated in the absence or presence of 10 μg anti-receptor Ig for 2 h at 4°C in 15 μl of binding buffer (Sahagian and Neufeld, 1983) containing 0.1% Triton X-100. 18 mU of β-hexosaminidase affinity purified from NH_4Cl-induced fibroblast sections (Ullrich *et al.*, 1979) were added. After incubation for 2 h at 4°C the amount of anti-receptor Ig was adjusted in all assays to 10 μg. After incubation for 2 h at 4°C the immune complexes were collected with the aid of 30 μg Immuno-Precipitin. The Immuno-Precipitin was washed twice with 0.15 M NaCl and assayed for β-hexosaminidase activity. All values were corrected for β-hexosaminidase bound in parallel assays containing 10 mM mannose-6-phosphate.

Other methods

Endocytosis of β-hexosaminidase (Ullrich *et al.*, 1978) was determined as described.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 310) and the Fonds der Chemischen Industrie.

References

Anderson,R.G.W., Brown,M.S., Beisiegel,U. and Goldstein,J.L. (1982) *J. Cell Biol.*, **93**, 523-531.

Brown,W.J. and Farquhar,M.G. (1984) *Cell*, **36**, 295-307.

Creek,K.E. and Sly,W.S. (1984) in Dingle,J.T., Dean,R.T. and Sly,W.S. (eds.), *Lysosomes in Biology and Pathology*, Elsevier, Amsterdam, pp. 63-82.

Creek,K.E., Grubb,J.H. and Sly,W.S. (1983) *J. Cell Biol.*, **97**, p. 253a (abstract).

Davis,W.C. (1974) *Methods Enzymol.*, **32**, 60-70.

Fischer,H.D., Gonzalez-Noriega,A. and Sly,W.S. (1980) *J. Biol. Chem.*, **255**, 5069-5074.

Geuze,H.J., Slot,W.J., Strous,G.J.A.M., Hasilik,A. and von Figura,K. (1984a) *J. Cell Biol.*, **98**, 2047-2054.

Geuze,H.J., Slot,W.J., Strous,G.J.A.M., Peppard,J., von Figura,K., Hasilik,A. and Schwartz,A.L. (1984b) *Cell*, **37**, 195-204.

Gieselmann,V., Pohlmann,R., Hasilik,A. and von Figura,K. (1983) *J. Cell Biol.*, **97**, 1-5.

Gonzalez-Noriega,A., Grubb,J.H., Talkad,V. and Sly,W.S. (1980) *J. Cell Biol.*, **85**, 839-852.

Hasilik,A., Pohlmann,R. and von Figura,K. (1983) *Biochem. J.*, **210**, 795-802.

Laemmli,U.K. (1970) *Nature*, **227**, 680-685.

Laskey,R.A. and Mills,A.D. (1975) *Eur. J. Biochem.*, **56**, 335-341.

Lemansky,P., Gieselmann,V., Hasilik,A. and von Figura,K. (1984) *J. Biol. Chem.*, **259**, 10129-10135.

Mage,M.G. (1980) *Methods Enzymol.*, **70**, 142-150.

Mellman,J. and Plutner,H. (1984) *J. Cell Biol.*, **98**, 1170-1177.

Mellmann,J., Plutner,H. and Ukkonen,P. (1984) *J. Cell Biol.*, **98**, 1163-1169.

Sahagian,G.G. and Neufeld,E.F. (1983) *J. Biol. Chem.*, **258**, 7121-7128.

Schreiber,A.B., Liberman,T.A., Lax,I., Yarden,Y. and Schlesinger,J. (1983) *J. Biol. Chem.*, **258**, 846-854.

Ullrich,K., Mersmann,G., Weber,E. and von Figura,K. (1978) *Biochem. J.*, **170**, 643-650.

Ullrich,K., Gieselmann,V., Mersmann,G. and von Figura,K. (1979) *Biochem. J.*, **182**, 329-335.

von Figura,K., Steckel,F. and Hasilik,A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6060-6066.

von Figura,K., Gieselmann,V. and Hasilik,A. (1984) *EMBO J.*, **3**, 1281-1286.

von Figura,K., Gieselmann,V. and Hasilik,A. (1985) *Biochem. J.*, **225**, 543-547.

Waheed,A., Hasilik,A. and von Figura,K. (1982) *Eur. J. Biochem.*, **123**, 317-321.

Received on 3 April 1985; revised on 5 May 1985