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Quaternary Structure of the M_r 46 000 Mannose 6-Phosphate Specific Receptor: Effect of Ligand, pH, and Receptor Concentration on the Equilibrium between Dimeric and Tetrameric Receptor Forms†

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ABSTRACT: The M_r 46 000 mannose 6-phosphate specific receptor exists in solution as a mixture of noncovalently associated dimeric and tetrameric forms. The two quaternary forms were separated by sucrose density centrifugation, and their composition was assessed by cross-linking with bifunctional reagents followed by SDS–polyacrylamide gel electrophoresis. The dependence of equilibrium between the dimeric and tetrameric forms on pH, receptor concentration, and presence of mannose 6-phosphate was studied. The formation of tetrameric forms is favored by pH values around 7, high receptor concentration, and presence of mannose 6-phosphate ligand. Tetrameric forms bind stronger at pH 7 to phosphomannan–Sepharose 4B than dimeric forms. Both quaternary forms dissociate at the same pH from a mannose 6-phosphate affinity matrix. When starting with dimeric or tetrameric forms, the equilibrium between dimeric and tetrameric forms is reached at pH 7.5 and 4 °C after 6–8 days. The presence of 5 mM mannose 6-phosphate shifts the equilibrium toward tetrameric forms. At pH 4.5 and 4 °C, the association of dimeric to tetrameric forms is negligible, while tetrameric forms dissociate to dimeric forms within 12 h. The results demonstrate that oligomerization is an intrinsic property of MPR-46 that is affected by ligand binding, pH, and receptor concentration.

Mannose 6-phosphate specific receptors are involved in the sorting of newly synthesized lysosomal enzymes [for reviews, see von Figura and Hasilik (1986) and Dahms et al. (1989)]. They bind mannose 6-phosphate containing lysosomal enzymes in the trans Golgi and direct their transport to the prelysosome. Due to the acidic pH in the prelysosome, the lysosomal enzymes dissociate from the receptors and are than sorted to lysosomes by an yet unknown mechanism, whereas the receptors recycle back to the trans Golgi.

Two different mannose 6-phosphate recognizing receptors with apparent sizes of 46 kDa (MPR-46)[1] (Hoflack & Kornfeld, 1985a; Stein et al., 1987b; Dahms et al., 1987; Pohlman et al., 1988) and 215 kDa (MPR-215) (Sahagian et al., 1981; Oshima et al., 1988; Lobel et al., 1988) have been characterized. The larger of the two receptors binds insulin-like growth factor II in addition to mannose 6-phosphate and is therefore now referred to as M6P/IGF II receptor (Waheed et al., 1988; Kiess et al., 1988). Both receptors mediate transport of newly synthesized lysosomal enzymes from the Golgi to prelysosomes (Gartung et al., 1985; Stein et al., 1987c). The M6P/IGF II receptor mediates in addition the

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[1] Abbreviations: MPR-46, M_r 46 000 mannose 6-phosphate specific receptor; MPR-215, M_r 215 000 mannose 6-phosphate specific receptor; ECD, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DSS, disuccinimidyl suberate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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endocytosis of lysosomal enzymes (Gartung et al., 1985).

Both receptors are integral membrane glycoproteins (von Figura et al., 1984; Sahagian & Steer, 1985; Stein et al., 1987a). Earlier studies have indicated that purified MPR-46 can be cross-linked at pH 9 by bifunctional reagents to dimeric, trimeric, and tetrameric forms (Stein et al., 1987b) and exists in solubilized membranes of murine BW 5147 cells and oocytes expressing bovine MPR-46 mainly as dimer (Gasa & Kornfeld, 1987; Dahms & Kornfeld, 1989). In membranes of U937 cells, only dimeric forms of the MPR-46 were detectable after cross-linking (Stein et al., 1987a). These studies suggested that MPR-46 exists in different quaternary conformations.

The biological significance of the quaternary structure of MPR-46 is not understood. In the present study, we have used a combination of sucrose density gradient centrifugation and cross-linking to separate and characterize the dimeric and tetrameric forms of MPR-46. We show that the quaternary structure of the receptor depends on the pH, receptor concentration, and the presence of ligand.

Materials and Methods

Homogeneous MPR-46 from human liver was prepared as described (Stein et al., 1987). MPR-46 was iodinated with iodogen (Pierce Chemical Co.; Parker & Strominger, 1983) to a specific activity of 3000–4000 cpm/ng of receptor protein. Phosphomannan from *Hansenula holstii* was a gift of Dr. M. Slodki, USDA Northern Regional Research Center, Peoria, IL. AH-Sepharose-4B was purchased from Pharmacia. Mannose 6-phosphate, glucose 6-phosphate, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (ECD) were from Sigma. Disuccinimidyl suberate (DSS) was obtained from Pierce. All other reagents used were of analytical grade.

Protein Determination. Protein concentration was determined according to the procedure of Peterson (1977) using bovine serum albumin as a standard.

Cross-Linking Experiments. Except where indicated otherwise, cross-linking reactions were performed at 37 °C in buffers containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Triton X-100. Cross-linking reactions with DSS were generally performed by incubating 3 nM [^{125}I]MPR-46 in 50 μL of 50 mM sodium phosphate buffers, pH 7–9, or 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffers, pH 6.5–5, for 30 min with 0.6 mM DSS. In some experiments, the receptor incubated in 50 mM sodium acetate, pH 4.5, was titrated to pH 7.5 with 0.5 M Na_2HPO_4 before cross-linking with DSS. Cross-linking reactions with ECD were carried out at room temperature in 50 μL of 50 mM HEPES buffers, pH 4.5–7.0, containing 0.1 M ECD. The reactions were terminated by addition of 50 μL of double-concentrated Laemmli's solubilizer (Laemmli, 1970). The samples were analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) followed by autoradiography.

Sucrose Density Gradient Centrifugation. [^{125}I]MPR-46, 40 000–700 000 cpm, was dissolved in 0.2 mL of 50 mM sodium phosphate containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Triton X-100. In some experiments, $MgCl_2$ was replaced by 10 mM EDTA. The sample was layered on top of a 1.8-mL continuous 5–25% sucrose gradient in the same buffer above a cushion of 0.2 mL of 60% sucrose. The samples were centrifuged for 12 h at 4 °C in Beckman TLS-55 rotor at 55 000 rpm. Gradients were fractionated from the top in 19 fractions and monitored for radioactivity and density. The receptors in each fraction were analyzed by SDS–PAGE before and after cross-linking with DSS. The dimeric and tetrameric forms of the receptor were recovered in fractions 7–11 and 12–15, respectively (see Figure 3). The ratio of dimeric over tetrameric forms was calculated from the radioactivities in these fractions.

Equilibrium between Dimeric and Tetrameric Receptor Forms. [^{125}I]MPR-46, 3 nM, was incubated in 50 mM sodium phosphate (pH 7–9) or HEPES (pH 5–6.5) or sodium acetate (pH 4–5) containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Triton X-100 for 24 h at 4 °C. In some experiments, the incubation was done in the presence of 5 mM mannose 6-phosphate or 5 mM glucose 6-phosphate, or $MgCl_2$ was omitted and 5 mM mannose 6-phosphate was present. The samples were loaded on sucrose gradients prepared in the same buffers and separated by centrifugation as above.

Isolation of Dimeric and Tetrameric Receptor Forms. The sucrose gradient fractions enriched in dimeric and tetrameric receptor forms were desalted on a Sephadex G-10 column (5 mL), equilibrated and eluted with distilled water containing 0.05% Triton X-100. The desalted dimeric and tetrameric forms were characterized by cross-linking with 0.6 mM DSS at pH 7 for 30 min.

Binding of [^{125}I]MPR-46 to Phosphomannan–Sepharose 4B. Phosphomannan–Sepharose 4B was prepared as described (Stein et al., 1987b). The affinity column, 0.2-mL bed volume, was equilibrated with binding buffer (50 mM sodium phosphate, pH 7.0, containing 150 mM NaCl, 5 mM β-glycerophosphate, 10 mM $MgCl_2$ + 0.05% bovine serum albumin, and 0.05% Triton X-100). [^{125}I]MPR-46 [(2–4) × 10^4 cpm] in 100 μL of binding buffer was loaded on the column at 4 °C, and the sample was allowed to incubate for 10 min. Then an additional 100 μL of binding buffer was applied; the flow through was reapplied on the column. The column was washed with 8–10 bed volumes of binding buffer before bound receptor was eluted with 6 bed volumes of binding buffer containing mannose 6-phosphate. For determination of pH-dependent elution of [^{125}I]MPR-46 from phosphomannan–Sepharose 4B, the receptor was loaded in the binding buffer, and the column was washed with 0.8 mL of buffer with the pH decreasing from 7 to 4 (pH 7–5, 50 mM HEPES, pH 4.5 and 4, 50 mM sodium acetate). All buffers contained 150 mM NaCl, 0.05% Triton X-100, and 10 mM $MgCl_2$. The fractions were analyzed for radioactivity, and the radioactive receptor was characterized by SDS–PAGE before and after cross-linking with DSS. Prior to cross-linking, the pH was adjusted to pH 7.5 with 0.5 M Na_2HPO_4.

Results

Chemical Cross-Linking of MPR-46. In a previous study, cross-linking of purified MPR-46 with DSS at pH 9.0 yielded a mixture of di-, tri-, and tetrameric forms of the receptor (Stein et al., 1987b). In order to establish the optimal conditions for cross-linking at pH 7.0, the concentration of DSS (0.06–2 mM), the reaction time (2–60 min), and the receptor concentration (3 nM–7.2 μM) were varied. At 3–900 nM [^{125}I]MPR-46, a DSS concentration of 0.6 mM and a reaction time of 30 min were found to be optimal. At lower concentrations of DSS and shorter incubation periods, the yields of cross-linked di- and tetrameric receptors were lower, and trace amounts (≤7% of total) of incompletely cross-linked trimeric forms were obtained. Increasing the concentration of DSS above 0.6 mM or prolonging the reaction time beyond 30 min had no effect on the distribution and yield of cross-linked receptor forms. In Figure 1, the typical result of incubating [^{125}I]MPR-46 with 0.6 mM DSS for 30 min is shown. Before cross-linking, 83% of the receptor is present as monomer and 17% as dimer, which is resistant to reducing agents in the presence of SDS (Figure 1, lane 1) and is considered to be an artifact of receptor solubilization resulting in intermolecular

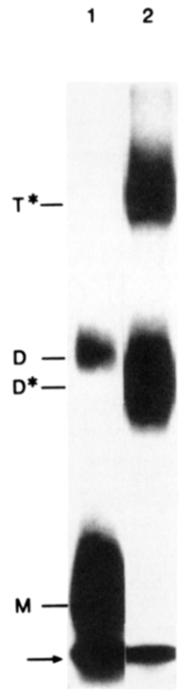

FIGURE 1: Chemical cross-linking of MPR-46. [^{125}I]MPR-46, 3 nM, was incubated without (lane 1) and with (lane 2) 0.6 mM DSS at pH 7 and 37 °C for 30 min. The cross-linked quaternary structures were analyzed by SDS–PAGE followed by autoradiography. Positions of free iodine (→), monomeric (M), dimeric (D), dimeric cross-linked (D*), and tetrameric cross-linked (T*) receptors are marked.

disulfide bonds between the cytoplasmic tails of MPR-46 [see Hille et al. (1989) and Dahms and Kornfeld (1989)]. After cross-linking, 50% of the receptor was recovered as dimeric and 45% as tetrameric cross-linked receptor. Only trace amounts were left as monomeric forms or cross-linked to higher molecular weight forms not entering the gel (Figure 1, lane 2). The cross-linked dimer migrated faster than the dimer present in the receptor preparation, suggesting that intramolecular cross-linking by DSS impairs the unfolding by SDS (Peters & Richards, 1977). MPR-46 shuttles between a neutral or near-neutral compartment, where it binds the ligands (Golgi), and an acidic compartment (prelysosomes), where it releases the ligand. It was therefore of interest to determine the effect of pH on the quaternary structure of the receptor. With lowering the pH from 7 to 6, the ratio of DSS-mediated cross-linked tetrameric over dimeric forms decreased from initially 0.72 to 0.58. At pH 5.0, no cross-linked species were formed (Figure 2). The latter may be ascribed to the slow reaction kinetics of *N*-hydroxysuccinimide esters at pH 5.0 (Cuatrecasas & Parikh, 1972). ECD, which is more reactive at lower pH, mediated at pH 5.0 cross-linking of 20% and 62% of the receptors to tetrameric and dimeric forms, respectively. Increasing the pH to 7 decreased the ECD-mediated cross-linking of the receptor (Figure 2). The pH dependence of the cross-linking reactions severely limits the potential of this approach to study the effect of pH on the quaternary structure of MPR-46. In the following experiments, we therefore tried to separate the different quaternary forms of the receptor prior to analysis.

Separation of Dimeric and Tetrameric Forms of the Receptor by Sucrose Density Gradient Centrifugation. For several proteins, sucrose density gradient centrifugation has been successfully used to separate the quaternary structures (Biswas et al., 1985; Copeland et al., 1986; Northwood & Davis, 1988; Matlin et al., 1988; Böni-Schnetzler & Pilch, 1987). When [^{125}I]MPR-46 was subjected to a sucrose density

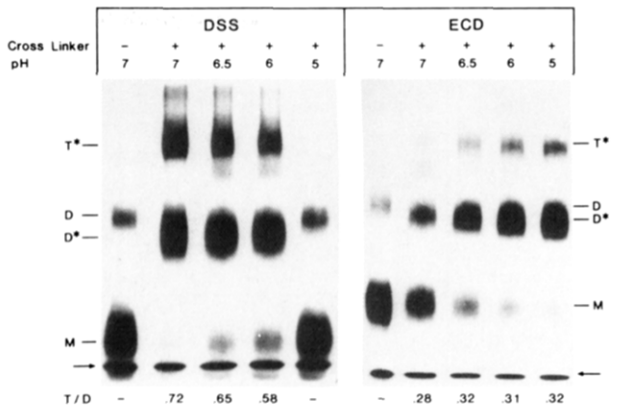

FIGURE 2: Effect of pH on cross-linking of MPR-46 by DSS (left) or by ECD (right). [^{125}I]MPR-46, 3 nM, was incubated for 1 h with 0.6 mM DSS or 0.1 M ECD at the pH indicated. Numbers at the bottom of the gel indicate the ratio of tetrameric/dimeric forms of the receptor (T/D). For symbols, see Figure 1.

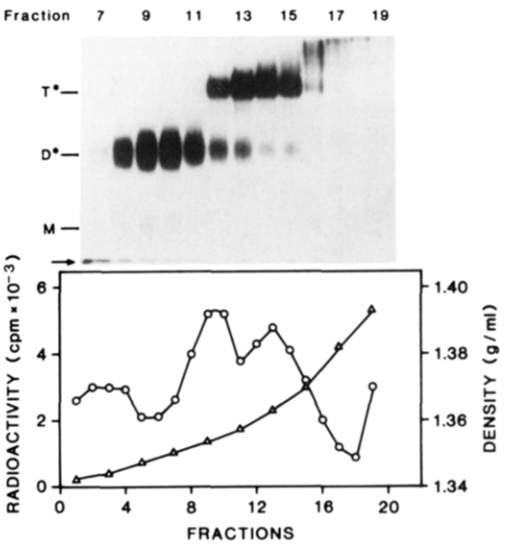

FIGURE 3: Separation of dimeric and tetrameric MPR-46 forms by sucrose density gradient centrifugation. [^{125}I]MPR-46, 3 nM, in 50 mM HEPES, pH 7.5, was subjected to density gradient centrifugation (see Materials and Methods). The fractions were analyzed for radioactivity (○) and density (△) in the lower panel. The receptor was recovered in fractions 7–19. The radioactivity in the top fractions 1–6 represented free ^{125}I (about 25% of total). Fractions 7–19 were cross-linked with 0.6 mM DSS and subjected to SDS–PAGE (upper panel). The positions of free iodine (→), monomeric (M), cross-linked dimeric (D*), and cross-linked tetrameric (T*) receptor are indicated.

gradient at pH 7.5, the receptor radioactivity migrated with two peaks at sucrose densities of 1.355 and 1.363 g/mL, respectively (Figure 3, lower panel). Each fraction was analyzed by SDS–PAGE. The radioactivity in fractions 1–6 comigrated with bromophenol blue and represents the free radioactivity (between 15 and 30% in different receptor preparations). The receptor was recovered in fractions 7–19. Cross-linking with 0.6 mM DSS demonstrated that the receptors in fractions 7–11 (representing 50% of total [^{125}I]MPR-46) are predominantly dimers and receptors in fractions 12–15 (representing 45% of total [^{125}I]MPR-46) tetramers. The radioactivity in the bottom fraction 16–19 represented receptor aggregates of undefined size (Figure 3, upper panel). These results show that the quaternary forms of MPR-46 can be separated by sucrose density gradient centrifugation and that the receptor is at pH 7.5 and 4 °C a 1:0.9 mixture of dimeric and tetrameric forms. In the following, we used this method to study the effect of several parameters on the equilibrium between dimeric and tetrameric forms of the receptor.

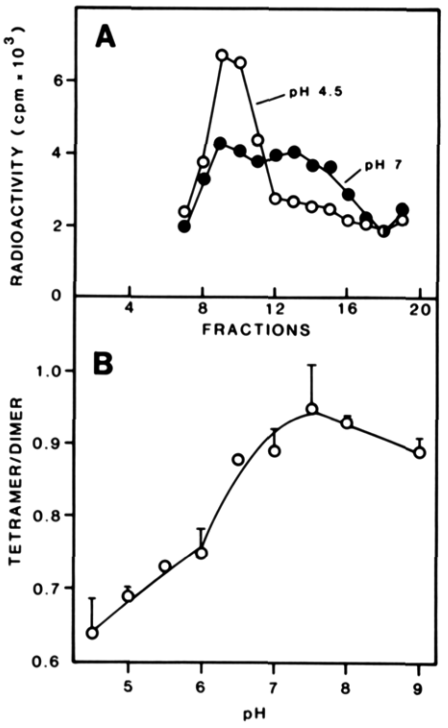

FIGURE 4: Effect of pH on the equilibrium between dimeric and tetrameric MPR-46. (Panel A) [^{125}I]MPR-46, 3 nM, was incubated for 12 h at 4 °C and pH 4.5 or pH 7.0 (see Materials and Methods) and subjected to sucrose density centrifugation. (Panel B) [^{125}I]-MPR-46, 3 nM, was incubated for 12 h at 4 °C and pH 4.5–9.0. After separation by sucrose density centrifugation, the ratio of tetrameric versus dimeric MPR-46 was calculated from the ratio of radioactivity in fractions 7–11 (dimeric MPR-46) and fractions 12–15 (tetrameric MPR-46). The bars indicate the variation in four independent experiments.

pH-Dependent Equilibrium between Dimeric and Tetrameric MPR-46. When [^{125}I]MPR-46, 3 nM, was incubated for 12 h at 4 °C and pH 4.5 before sucrose density gradient centrifugation, the fraction of tetrameric forms decreased on the expense of dimeric forms (Figure 4A). Cross-linking with DSS after adjusting the pH of the gradient fractions to 7 confirmed that radioactivity in fractions 7–11 and 12–15 represented the dimeric and tetrameric forms of the receptor (not shown). To study the effect of pH on the oligomeric state of MPR-46, the receptor was incubated for 12 h at 4 °C in buffers with pH varying from 4.5 to 9 (Figure 4B). Between pH 6.5 and 9, about half of MPR-46 (46–49%) existed as tetramers. A decrease in pH from 6.5 to 4.5 resulted in a continuous increase of the dimeric forms at the expense of the tetrameric forms. At pH 4.5, tetrameric forms represented 38% of MPR-46. These results indicate that near neutral pH, association of the receptor to the tetrameric form is favored and at acidic pH dissociation to the dimeric form.

Effect of Receptor Concentration on the Equilibrium between Dimeric and Tetrameric MPR-46. At 3 nM [^{125}I]-MPR-46, 53% of the receptor was recovered as dimeric and 47% as tetrameric receptor. Raising the receptor concentration to 1.9 and 19 μM by addition of unlabeled receptor and incubation for 12 h at 4 °C increased the fraction of tetrameric receptors to 56% and 68%, respectively. These results indicate that the equilibrium between dimeric and tetrameric forms of the receptor is shifted to tetrameric forms by increasing the receptor concentration.

Binding of Dimeric and Tetrameric MPR-46 to Phosphomannan–Sepharose 4B. The dimeric and tetrameric forms of the receptor were separated by sucrose density centrifugation and desalted. The preparation of dimeric forms was essentially

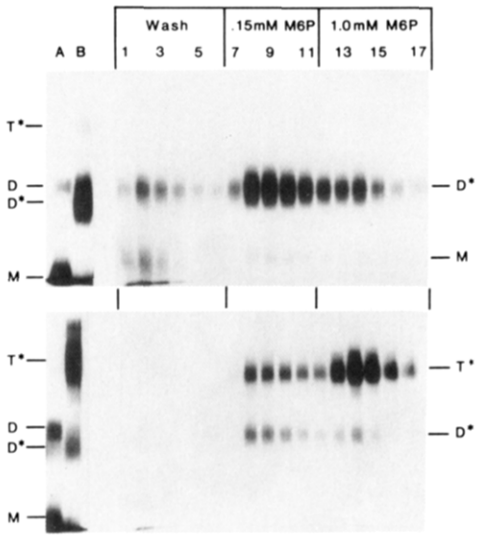

FIGURE 5: Binding of dimeric (upper panel) and tetrameric (lower panel) MPR-46 with phosphomannan–Sepharose. Sucrose density gradient fractions containing dimeric (fractions 7–11 of Figure 3) and tetrameric (fractions 12–15) MPR-46 were pooled, desalted, and analyzed for subunit composition before (A) and after (B) cross-linking with 0.6 mM DSS. The desalted fractions were applied to a phosphomannan–Sepharose column. The column was washed with the binding buffer (lanes 1–6) or with the binding buffer containing 0.15 mM (lanes 7–11) and 1.0 mM (lanes 12–17) mannose 6-phosphate. The fractions were subjected to cross-linking with 0.6 mM DSS and analyzed by SDS–PAGE. For symbols, see Figure 3.

free of tetrameric receptors as judged by cross-linking with DSS (Figure 5, lane B, upper panel), while the preparation of tetrameric forms contained about 15% dimeric forms (Figure 5, lane B, lower panel). The dimeric and tetrameric forms were applied to a phosphomannan–Sepharose 4B column, which was washed and eluted with 0.15 and 1.0 mM mannose 6-phosphate. The fractions were analyzed by SDS–PAGE after cross-linking with DSS. More than 90% of the dimeric receptor bound to the affinity column. The majority, 67% of total, was eluted with 0.15 mM mannose 6-phosphate and the remaining with 1.0 mM mannose 6-phosphate (Figure 5, upper panel). Of the tetrameric receptor, essentially all bound to the affinity column. Only 38% of the radioactivity was eluted with 0.15 mM mannose 6-phosphate (this fraction contained about 70% of the dimeric receptors present in this preparation). The majority of the tetrameric receptors, 62% of the total, was eluted with 1 mM mannose 6-phosphate (Figure 5, lower panel). Thus, tetrameric receptors are bound to phosphomannan–Sepharose 4B more tightly than dimeric receptors.

Effect of pH on the Elution of Dimeric and Tetrameric MPR-46 from Phosphomannan–Sepharose 4B. A preparation of dimeric and tetrameric receptors was bound to phosphomannan–Sepharose 4B and eluted with buffers with pH decreasing from 7 to 4. The amount of radioactivity eluted at the different pHs is shown in Figure 6. Between pH 7 and 5, both forms of the receptor remained bound to the column. When the column was eluted with a buffer of pH 4.5, about 90% of both receptor forms was eluted. These results suggest that the pH dependence of binding is similar for the dimeric and tetrameric forms of the receptor.

Association of Dimeric to Tetrameric MPR-46 as a Function of Time, pH, and Mannose 6-Phosphate. Isolated dimeric receptor forms were incubated for 24 h at 4 °C and pH 7.5 or 4.5 and then subjected to sucrose density centrifugation. The distribution of dimeric and tetrameric forms in the starting material and the gradient fractions was analyzed

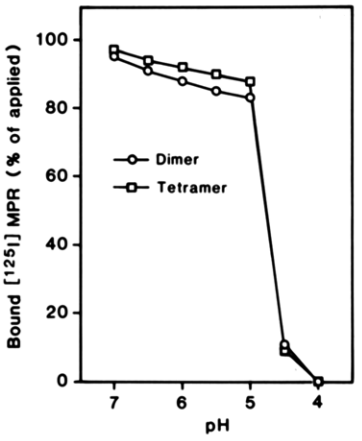

FIGURE 6: pH-dependent elution of dimeric (○) and tetrameric (□) MPR-46 with phosphomannan–Sepharose 4B. The preparations of dimeric and tetrameric MPR-46 (see Figure 5) were applied to the phosphomannan–Sepharose column in the binding buffer with pH decreasing from 7.0 to 4.0. The ordinate gives the percentage of [^{125}I]MPR-46 remaining bound to the affinity matrix.

by cross-linking with DSS. The fraction of tetrameric forms increased from initially 5% to 24% after incubation at pH 7.5 and remained at 5% during incubation at pH 4.5.

In a separate experiment, the dimeric receptor preparation was incubated for 24 h at 4 °C and pH 7.5 in the absence of a sugar phosphate, in the presence of 5 mM glucose 6-phosphate (nonbinding sugar phosphate), or in the presence of 5 mM mannose 6-phosphate. The fraction of tetrameric receptors increased from 5% to 18% (no sugar phosphate), and 17% (glucose 6-phosphate) and 34% (mannose 6-phosphate), respectively. Replacing the $MgCl_2$ in the buffer by 10 mM EDTA did not affect the mannose 6-phosphate induced tetramer formation.

These results suggest that dimeric forms slowly associate to tetrameric forms at neutral pH and that the rate of association is accelerated by mannose 6-phosphate (independent of Mg^{2+}). In the following experiment, dimeric forms of the receptor were incubated with and without 5 mM mannose 6-phosphate at pH 7.5 and 4 °C for up to 7 days. The quaternary structure of the receptor was characterized by cross-linking with 0.6 mM DSS followed by SDS–PAGE and autoradiography. The results are shown in Figure 7. At zero time, 6% of the receptor was in a tetrameric form. After 6 days, an equilibrium between tetrameric and dimeric forms was reached at a tetramer/dimer ratio of 0.7. The presence of 5 mM mannose 6-phosphate shifted the equilibrium between tetramers and dimers to a tetramer/dimer ratio of 1.4.

Dissociation of Tetrameric MPR-46 as a Function of Time, pH, and Mannose 6-Phosphate. The preparation of tetrameric receptor forms contained dimeric forms, which represented 25% (see Figure 8) of the total receptor. After incubation for 24 h at 4 °C and pH 7.5, no increase of dimeric forms was apparent, while after incubation for 24 h at 4 °C and pH 4.5 dimeric forms represented 80% of the receptor. The rapid dissociation of tetrameric forms at pH 4.5 is illustrated in Figure 8. Within 12 h, the equilibrium between tetramers and dimers at a tetramer/dimer ratio of 0.25 was reached.

When tetrameric forms were incubated at 4 °C and pH 7.5 for a prolonged period, a slow dissociation to dimeric forms was observed, which after 8 days reached the near-equilibrium tetramer/dimer ratio of 0.7 (Figure 9). The presence of 5 mM mannose 6-phosphate stabilized the tetrameric forms. After incubation for 9 days, tetrameric forms still represented more than 60% of the receptor-associated radioactivity (Figure 9).

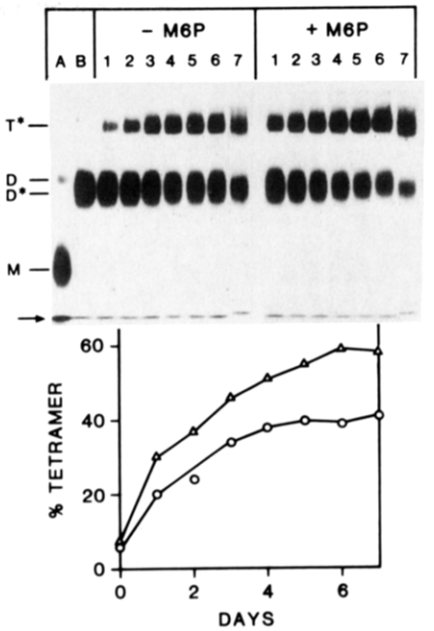

FIGURE 7: Kinetics of association of dimeric MPR-46 to form tetrameric receptor. Dimeric [^{125}I]MPR-46, 3 nM, was incubated at pH 7.5 without and with 5 mM mannose 6-phosphate at 4 °C. At zero time, quaternary structure of the dimeric receptor preparation was characterized before (A) and after (B) cross-linking with 0.6 mM DSS. The conversion of the dimeric to the tetrameric form was determined at different times as indicated by cross-linking the receptor with 0.6 mM DSS. The samples were subjected to SDS–PAGE (upper panel). Gel pieces corresponding to cross-linked dimeric and tetrameric MPR-46 were cut and counted to calculate the percent of tetrameric MPR-46 in the absence (○) or presence (△) of 5 mM mannose 6-phosphate (lower panel).

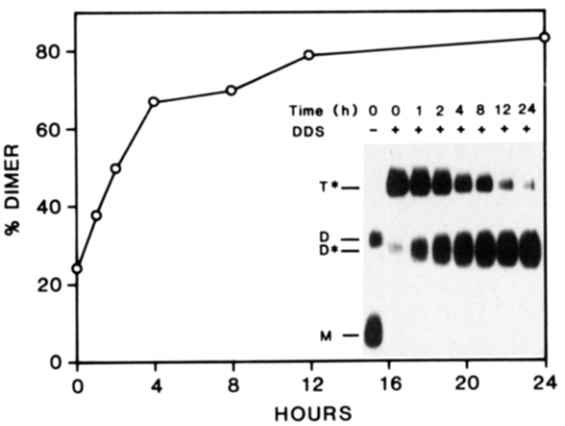

FIGURE 8: Kinetics of dissociation of tetrameric MPR-46 at pH 4.5. Tetrameric [^{125}I]MPR-46, 3 nM (see Figure 5), was incubated at 4 °C in 50 mM sodium acetate, pH 4.5, containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Triton X-100. Aliquots of the reaction mixture were removed at the indicated times, titrated to pH 7.5 with 0.5 M Na_2HPO_4, and cross-linked with 0.6 mM DSS followed by SDS–PAGE. The inset shows the autoradiogram of the gel. Gel pieces corresponding to the cross-linked dimeric and tetrameric MPR-46 were cut and counted to calculate the percent of dimeric MPR-46.

Discussion

An important property of many endocytic receptors is the pH dependence of their binding activity. They bind their ligands near neutral pH and release the ligands at acidic pH. Typically, pH values below 5.5 are sufficient to cause dissociation of the receptor–ligand complexes (Mellman et al., 1986). This pH dependence of binding is one of the means that allow the utilization of receptors for many rounds of transport. Endocytic receptors bind their ligands at the cell surface, become internalized via clathrin-coated vesicles, and

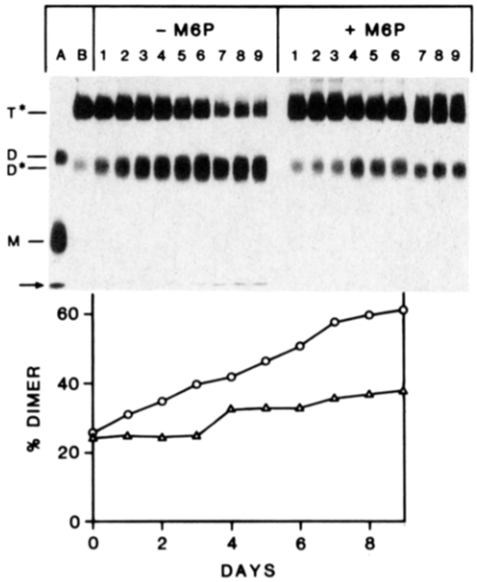

FIGURE 9: Kinetics of dissociation of tetrameric MPR-46 at pH 7.5. Tetrameric [^{125}I]MPR-46, 3 nM, was incubated at 4 °C in 50 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Triton X-100 in the absence (−) or presence (+) of 5 mM mannose 6-phosphate (M6P). The conversion of tetrameric to dimeric form was analyzed at the indicated times by cross-linking with 0.6 mM DSS followed by SDS–PAGE (upper panel). The gel pieces corresponding to cross-linked dimeric and tetrameric MPR-46 were cut and counted to calculate the percent of dimeric MPR-46 formed in the absence (○) or presence (△) of 5 mM mannose 6-phosphate (lower panel).

release their ligands within the acidic environment of endosomes (Goldstein et al., 1985). After being recycled to the cell surface, the receptors can be used for another round of transport. Recycling, however, does not depend on the dissociation of the ligands. For several receptors, it has been shown that not all ligands are released in the endosomal compartment and that occupied receptors can return to the cell surface. Mannose 6-phosphate specific receptors resemble to endocytic receptors with respect to the pH dependence of binding and the recycling behavior. A difference is that the majority of mannose 6-phosphate specific receptors bind their ligands within the Golgi and transport the ligands to the endosomal compartment via an intracellular route (von Figura & Hasilik, 1986; Dahms et al., 1989). Only a small fraction (≤20% of total) of the mannose 6-phosphate specific receptors is expressed at the cell surface. These receptors undergo rapid internalization and recycling, but ligands are internalized only via the M6P/IGF II receptor. Inspite of its recycling, cell surface associated MPR-46 does not bind and internalize ligands (Stein et al., 1987c). This suggests that not yet recognized factors can control the ligand binding activity of MPR-46.

We have shown in the present study that MPR-46 exists in solution as a mixture of dimeric and tetrameric forms. Due to their higher valency, the tetrameric receptors bind more avidly ligands, while the pH dependence of binding is similar for the two receptor forms. The equilibrium between dimeric and tetrameric forms is dependent on receptor concentration, pH, and occupation with ligands. Higher receptor concentration, near-neutral pH, and occupation with ligands favor the tetrameric receptor conformation. The dissociation of tetrameric receptors is induced by lowering the pH and impeded at neutral pH by the presence of ligands.

It should be noted that the dissociation of the receptors from the affinity ligands and the dissociation of tetramers show a different pH dependence. Ligand binding decreases sharply and profoundly upon lowering the pH from 5 to 4.5, while the tetramer/dimer ratio decreases steadily and much less steep upon lowering the pH from 6.5 to 4.5. This clearly indicates that the dissociation of ligands and tetramers is not coupled and that different ionizable groups ensure ligand binding and tetramer stabilization. This explains also why the binding to the multivalent affinity matrix depends on the quaternary structure of the receptor only at near-neutral pH but not below pH 5.

It would be hard to envisage from the present results that cycling of the receptor between the Golgi, where it binds its ligands, and the acidic prelysosomal compartment, where it releases its ligands, is coupled to changes of the quaternary structure. The kinetics of recycling would require that these changes occur within minutes, rather than within days. Furthermore, only dimeric receptors have so far been observed in membranes of cells (Stein et al., 1987a). In preliminary experiments initiated by the present findings, we observed that at 37 °C solubilized receptor exists as a mixture of monomeric dimeric and tetrameric receptors and that the effects of pH and receptor concentration on the quaternary structure are much more pronounced and faster than at 4 °C; e.g., acid pH induced dissociation of the receptor is complete within 2 min, and a significant change of the quaternary structure within the same time period is observed by varying the receptor concentration within 1 order of magnitude (3–30 nM). Furthermore, by optimizing the cross-linking procedure, we were able to demonstrate that in BHK-21 cells and in membranes of BHK-21 cells transfected with MPR-46 cDNA about 10% of MPR-46 exist as trimeric and tetrameric receptors and 20% as a monomeric receptor (Waheed, unpublished results).

These preliminary results indicate that the receptor exists in vivo in the different quaternary structures that were observed under in vitro conditions and the rate constants of association and dissociation as well as the effects of variables such as pH and receptor concentration are greatly enhanced near the physiological temperature of 37 °C. The changes of the quaternary structures of MPR-46 may therefore occur under in vivo conditions as fast that they could contribute to the function and trafficking of MPR-46.

Many receptors exist as homo- or heterooligomers (Bishop et al., 1987; Loeb & Drickamer, 1987; Olson et al., 1988; Turkewitz et al., 1988; Schlessinger, 1986). A ligand-induced change of the quaternary structure has been reported for the EGF receptor (Böni-Schetzler & Pilch, 1987; Yarden & Schlessinger, 1987). Binding of EGF to monomeric EGF receptors favors the formation of dimeric EGF receptors, which have a higher affinity for EGF and cluster in clathrin-coated pits and undergo rapid internalization. In an allosteric activation model, it has been postulated that dimerization of the receptor potentiates the tyrosine kinase activity of EGF receptor and is therefore also important for the signal transduction pathway initiated by the tyrosine kinase activity of the EGF receptor (Schlessinger, 1988). It should be noted, however, that as for MPR-46 oligomerization is an intrinsic property of the EGF receptor, which does not require ligand binding, and tyrosine kinase activity is displayed also by the monomeric EGF receptor (Northwood & Davis, 1988; Böni-Schetzler & Pilch, 1987; Yarden & Schlessinger, 1987). Ligand- and/or pH-induced changes of the quaternary structure of receptors may therefore be a more general means to influence the function of these receptors.

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