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The 'cation-dependent' mannose 6-phosphate receptor binds ligands in the absence of divalent cations

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The requirement of divalent cations for binding of the 46 kDa mannose 6-phosphate receptors to phosphomannan and pentamannose 6-phosphate-substituted bovine serum albumin was examined. Receptors from human liver and human brain bound to both affinity ligands in the absence or presence of divalent cations with similar efficiency. The requirement for divalent cations therefore appears not to be necessary for the binding.

Lysosomal enzyme; Enzyme receptor; Mannose 6-phosphate receptor

1. INTRODUCTION

Two mannose 6-phosphate receptors (MPR) are known which differ in molecular mass, binding specificity and function. The larger of the two (MPR 215) has an apparent molecular mass of 215 kDa (this receptor has recently been cloned and shown to be identical with the IGF II receptor; the cDNA predicts a size of about 275 kDa for the receptor polypeptide [15]) and binds mannose 6-phosphate-containing ligands in the absence of divalent cations [1]. Binding of the ligands to the smaller receptor (MPR-46) with an apparent molecular mass of 46 kDa was reported to have an absolute requirement for divalent cations [2]. This property has led to the designation of the smaller receptor as cation-dependent MPR (MPR^{CD}) and has been employed to separate MPR-46 and MPR-215 from lysates of Chinese hamster ovary cells [3].

We had previously noted that binding of a mannose 6-phosphate-neoglycoprotein to MPR-46-bearing membranes of Morris hepatoma 7777 cells was largely independent of divalent cations [4]. The lack of cation requirement for binding was attributed to the unusually large amount of mannose 6-phosphate in the neoglycoprotein (≥ 30 mol per mol polypeptide) [5]. We were later unable to separate MPR-215 from MPR-46 on the basis of their differential requirement of cations for high-affinity binding. This prompted us to reinvestigate the effect of divalent cations on the binding of MPR-46 with annose 6-phosphate-containing ligands.

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2. MATERIALS AND METHODS

2.1. *Materials*

MPR-46 purified from human liver [6] was [^{125}I]iodinated with the aid of iodogen (Pierce) according to Parker et al. [7] to a specific activity of 2500 cpm/ng receptor. Pentamannose 6-phosphate-substituted bovine serum albumin (PMP-BSA) was kindly provided by Dr T. Braulke of this institute [5]. Phosphomannan-Sepharose 4B, *Dictyostelium discoideum* lysosomal enzyme-Sepharose 4B and rabbit antisera against human liver MPR-215 and MPR-46 were those previously described [6,8].

2.2. *Binding of ^{125}I-MPR-46 to phosphomannan-Sepharose 4B*

^{125}I-MPR-46 (40 000 cpm in 100 μl binding buffer: 50 mM imidazole/150 mM NaCl/5 mM Na β-glycerophosphate/0.05% Triton X-100/0.05% BSA) containing either 10 mM $MgCl_2$ or 2 mM EDTA was loaded on phosphomannan-Sepharose 4B columns (500 μl bed volume) at 4°C and incubated for 5 min. Each

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column was washed under its loading conditions with 8 ml binding buffer followed by 5 ml binding buffer/2 mM EDTA. The receptor was eluted with 6 ml of 0.5 mM mannose 6-phosphate in binding buffer. The fraction was assayed for radioactivity, pooled as indicated in fig.1, dialyzed against 10 mM NH_4 acetate, pH 7.0/0.05% Triton X-100 and lyophilized. Samples were dissolved in H_2O and precipitated with 80% acetone. One tenth of each sample was applied to SDS-PAGE [9]. The ^{125}I-MPR was visualized by autoradiography.

2.3. *Solid-phase binding assay*

Flexible microtiter wells were incubated for 6 h with 200 or 500 ng PMP-BSA in 50 μl of 25 mM Tris-HCl, pH 7.0. The wells were washed 5 times with 50 mM Hepes/150 mM NaCl/0.1% Tween 20/0.1% Triton X-100/0.1% BSA and saturated overnight in the same buffer. ^{125}I-MPR-46 (20 000 cpm in 20 μl of 50 mM Na phosphate, pH 7.0/150 mM NaCl/5 mM Na β-glycerophosphate/0.1% Triton X-100/0.1% BSA, supplemented with EDTA, $MgCl_2$ and mannose 6-phosphate as indicated in table 1) was added. After 3 h incubation the wells were washed 5 times with the phosphate buffer/6 mM $MgCl_2$, dried, cut and counted.

2.4. *Purification of MPR-46 from human brain*

MPR-46 from human brain was purified in the presence and absence of divalent cations: typically 10 g human brain (pooled material from cortex and white matter obtained after brain surgery) was homogenized in 20 ml of 50 mM imidazole, pH 7.0/150 mM NaCl/5 mM Na β-glycerophosphate/0.1 TIU/ml aprotinin/5 mM iodoacetamide/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (buffer A) and centrifuged at 1000 × *g*. The pellet was resuspended in buffer A and spun again at 1000 × *g*. The 1000 × *g* supernatants were pooled and centrifuged for 1 h at 100 000 × *g*. The pellet was extracted overnight in 30 ml buffer A substituted with 1% Triton X-100/0.1% Na deoxycholate. After centrifugation for 1 h at 100 000 × *g*, the supernatant was applied to the *D. discoideum* lysosomal enzyme-Sepharose 4B column [6] either directly or after adjusting to 10 mM $MnCl_2$. The flow-through (50 ml) was applied to the phosphomannan-Sepharose 4B column (15 ml bed volume). The phosphomannan-Sepharose 4B was washed with 3 l of either buffer A/5 mM EDTA or buffer A/10 mM $MnCl_2$ prior to elution with 120 ml buffer A/2 mM mannose 6-phosphate. The eluents were analyzed by Western blotting [10] with a 1:1 mixture of antisera against MPR-215 and MPR-46.

3. RESULTS

3.1. *Binding of ^{125}I-MPR-46 to phosphomannan-Sepharose 4B*

MPR-46 purified to homogeneity from human liver was iodinated and assayed for binding to phosphomannan-Sepharose 4B in the presence of either 10 mM $MgCl_2$ or 2 mM EDTA. More than 70% of the receptor bound to the column under both conditions (fig.1). Receptor that was bound to the column in the presence of 10 mM $MgCl_2$ could not be eluted with 2 mM EDTA and required 0.5 mM mannose 6-phosphate for release from the affinity matrix. This indicated that binding of ^{125}I-MPR-46 to phosphomannan-Sepharose 4B did not depend on divalent cations.

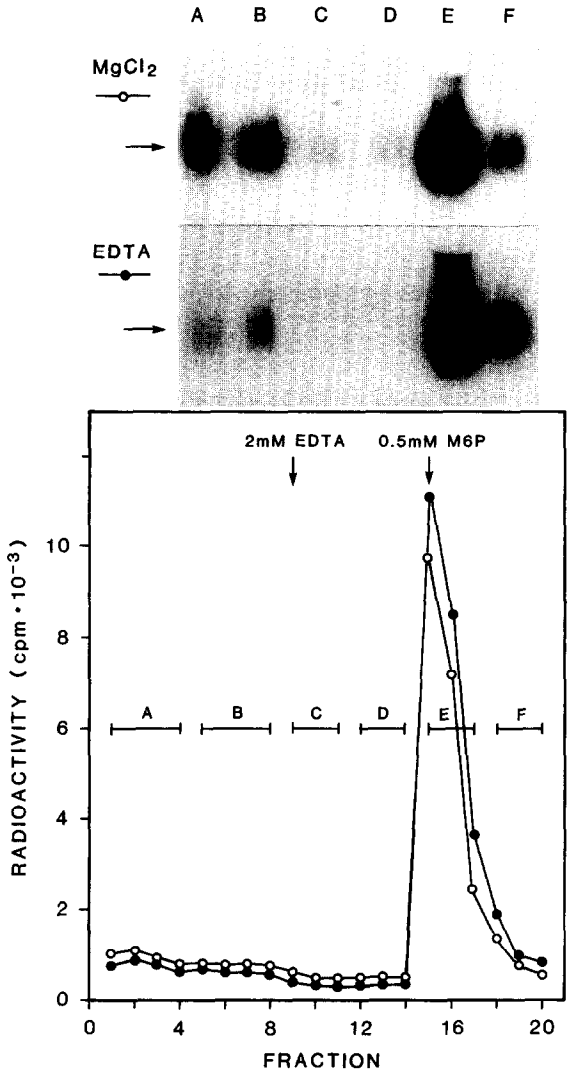

Fig.1. Binding of ^{125}I-MPR-46 to phosphomannan-Sepharose 4B. ^{125}I-MPR-46 was loaded on the column in the presence of 10 mM $MgCl_2$ (○—○) or 2 mM EDTA (●—●). After washing under loading conditions the columns were eluted sequentially with 2 mM EDTA and 0.5 mM mannose 6-phosphate (M6P) as indicated by arrows. Fractions of 1 ml were collected, assayed for radioactivity (bottom), pooled as indicated by horizontal bars and analyzed by SDS-PAGE followed by autoradiography (top). The position of MPR-46 is shown. A compression effect on MPR-46 by the high amounts of BSA in the sample can be noted.

3.2. *Binding of ^{125}I-MPR-46 to PMP-BSA*

To examine the binding of ^{125}I-MPR-46 to immobilized PMP-BSA, microtiter wells coated with PMP-BSA were incubated with ^{125}I-MPR-46 in the presence of 6 mM $MgCl_2$ or 2 mM EDTA. Under both conditions, low but similar amounts of ^{125}I-MPR-46 bound to the solid phase (table 1). The binding was specific as indicated by the sensitivity to 5 mM mannose 6-phosphate.

3.3. *Isolation of MPR-46 from human brain by affinity chromatography in the absence of divalent cations*

The previous experiments had shown that binding of iodinated MPR-46 with mannose 6-phosphate-containing ligands did not require divalent cations. To exclude the possibility that the lack of cation requirement was a result of secondary modifications of the receptor during storage or iodination, we investigated whether purification of MPR-46 on a phosphomannan-Sepharose 4B column could be achieved in the absence of divalent cations.

A membrane extract from human brain was passed through a *D. discoideum* lysosomal enzyme-Sepharose 4B column to remove MPR-215 [2] and then over a phosphomannan-Sepharose 4B column (an affinity matrix for both types of MPR, see [2]). After extensive washing the columns were eluted with 2 mM mannose 6-phosphate. The eluents were analyzed for the presence of the two receptors by Western blotting with a mixture of antisera against MPR-46 and MPR-215. In eluents of the *D. discoideum* lysosomal enzyme-Sepharose 4B column, only MPR-215 (and fragments thereof) was detectable (not shown). MPR-46 was detectable only in the eluent from the phosphomannan-Sepharose 4B column (fig.2). Substitution of the binding and washing buffers with either 10 mM $MnCl_2$/1 mM EDTA or 1 mM EDTA yielded similar amounts of MPR-46 polypeptides. The monomeric forms of MPR-46 were accompanied by a small fraction of dimeric receptor forms, which was resistant against boiling in the presence of SDS and dithiothreitol. Dimeric forms were noted earlier in receptor preparations from bovine [2] and human [6] liver. In contrast to liver, several

Table 1

Binding of ^{125}I-MPR-46 to microtiter wells coated with PMP-BSA

Addition	Bound ^{125}I-MPR (cpm)	
	A	B
2 mM EDTA	108 ± 1	132 ± 8
2 mM EDTA + 5 mM M6P	22	
6 mM $MgCl_2$	92 ± 4	135 ± 23
6 mM $MgCl_2$ + 5 mM M6P	3	21

Wells were coated with 200 (A) or 500 (B) ng PMP-BSA. The values for EDTA and $MgCl_2$ give the mean and range of duplicates. All values were corrected for background binding to wells coated with BSA alone (19 ± 4 and 49 ± 7 cpm in series A and B, respectively)

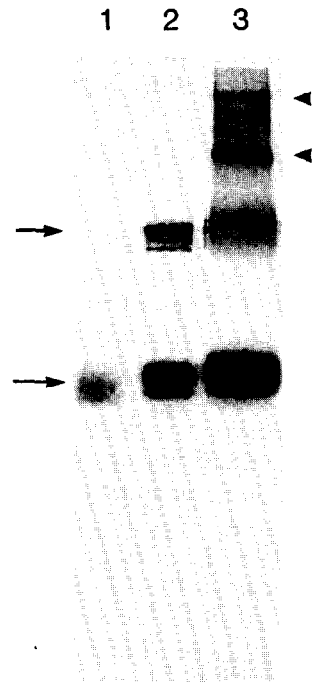

Fig.2. Western blot of MPR-46 from human brain. Brain extracts were chromatographed sequentially on *D. discoideum* lysosomal enzyme-Sepharose 4B and phosphomannan-Sepharose 4B columns in the presence of $MnCl_2$ (lane 2) or EDTA (lane 3). Aliquots of the mannose 6-phosphate eluents from phosphomannan-Sepharose 4B corresponding to 0.2 g fresh tissue and 200 ng human liver MPR-46 (lane 1) were separated by SDS-PAGE, transferred to nitrocellulose and analyzed with a mixture of antisera against MPR-46 and MPR-215. The monomeric and dimeric forms of MPR-46 are marked (→). MPR-215 polypeptides (◄) representing intact recepotor and an approx. 130 kDa fragment are present in lane 3 due to their incomplete absorption by the preceding *D. discoideum* lysosomal enzyme-Sepharose 4B column.

distinct forms of monomeric and dimeric forms of MPR-46 were discernible in brain. The molecular basis for this heterogeneity is presently under investigation.

4. DISCUSSION

Previous investigations had established an absolute requirement for divalent cations for high-affinity binding of MPR-46 [2,3,11,12]. Here, such a dependence was not detectable. The specificity of binding in the absence of divalent cations was sufficient to allow purification of the receptor from crude tissue extracts by affinity chromatography. Moreover, the yield in the absence or presence of divalent cations was comparable.

Cation dependence was established for MPR-46 from bovine liver [2]. It is possible that species variations account for the differences in cation requirement. However, the cation requirement may also depend on the type of affinity matrix. Cation dependence was observed for affinity matrices in which the phosphomannan was coupled directly to Sepharose 4B [2], while cation independence is observed with affinity matrices in which the phosphomannan is coupled via a six-carbon-long spacer [6]. Our initial attempts to prepare affinity matrices for MPR-46 by coupling phosphomannan directly to Sepharose 4B, yielded gels with unsatisfactory binding capacities (Stein, M. unpublished). It is therefore conceivable that high concentrations of divalent cations improve the interaction of MPR-46 with ligands if the accessibility of the latter is unfavorable.

The present observation of cation-independent binding of ligands to MPR-46 has precendents. Membranes of Morris hepatoma cells, which express only MPR-46, retain 60% or more of their mannose 6-phosphate-binding sites in the presence of 10 mM EGTA [4]. Distler and Jourdian [13,14] purified a cation-independent MPR from bovine testes of apparent molecular mass 42 kDa. At low β-galactosidase concentrations binding of β-galactosidase with the receptor was improved by high concentrations (20 mM) of $MnCl_2$.

The present results and earlier observations indicate the need for caution in attempts to discriminate between MPR-46 and MPR-215 solely on the basis of cation dependence. Furthermore, it appears more appropriate to designate the smaller of the two receptors according to molecular mass (MPR-46) than according to its cation requirement (MPR^{CD}).

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