Volume 280, number 1, 61–64 FEBS 09460 March 1991

ADONIS 001457939100170Q

Antibodies against the cytoplasmic tail can differentiate between the quaternary forms of the M_r 46000 mannose 6-phosphate receptor

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Received 18 December 1990; revised version received 14 January 1991

An antiserum against a peptide of the cytoplasmic tail of the M_r 46000 mannose 6-phosphate receptor is described which recognizes preferentially the tetrameric versus the dimeric form of this receptor. This indicates that the conformation of the cytoplasmic tail, which harbours signals necessary for the trafficking of the receptor, depends on the quaternary structure of the receptor.

Mannose 6-phosphate receptor; Peptide-specific antibody; Quaternary structure; Receptor recycling; Transport signal

1. INTRODUCTION

The M_r 46000 mannose 6-phosphate receptor (MPR 46) is one of the receptors that recognize mannose 6-phosphate residues on lysosomal proteins and mediate the transport of these proteins to the lysosomes (for review see [1,2]). The MPR 46 is a transmembrane protein with a single membrane-spanning domain. The extracytoplasmic domain is sufficient to bind the ligands and to form dimers [3], while the cytoplasmic domain contains transport signals necessary for the routing of the receptor [4–6]. Monomeric, dimeric and tetrameric forms of the receptor have been isolated. The equilibrium between these forms is controlled by pH, ligand binding and receptor concentration [7]. These factors are subject to variations during the cycling of the receptor between the site, where ligands bind to the receptor (presumably Golgi complex) and the site, where the ligands dissociate (presumably a prelysosomal organelle linked to the endocytotic route). This raises the possibility that the quaternary structure of the MPR 46 not only changes during the cycling between different compartments, but contributes to the trafficking of the receptor [8].

In the present study we have analyzed antibodies raised against peptides of the cytoplasmic tail of MPR 46 for their reactivity with dimeric and tetrameric forms of the MPR 46. One of the peptide-specific antisera recognized preferentially the tetrameric receptor, indicating that the conformation of the cytoplasmic tail depends on the quaternary structure of MPR 46. This observation lends further support to the notion that the trafficking of the receptor may be controlled by its quaternary structure.

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Abbreviations: MPR 46, M_r 46000 mannose 6-phosphate receptor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Peptide-specific antisera*

Peptides corresponding to residues 7–21 (peptide A) and 38–52 (peptide B) of the cytoplasmic tail of MPR 46 (the numbering of the tail starts with Arg-212, [9]) were synthesized using F-moc-protected, PyBOP- (benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate-) activated amino acids and an automatic peptide synthesizer (Milligen 9050, Eschborn, Germany). The peptides were purified by reverse-phase chromatography and conjugated to keyhole limpet hemocyanin using glutaraldehyde as described [10]. The conjugate (corresponding to 0.25 mg peptide) was mixed with Freunds complete (first injection) or incomplete (booster injections) adjuvant and subcutaneously injected into rabbits. Booster injections were given 4 weeks after the first injection in biweekly intervals. For purification of peptide-specific antibodies, 7.6 and 6.4 mg of peptide A and B, respectively, was coupled to 1 ml of Affigel 10 (Bio-Rad, München, Germany) according to the manufacturers instructions. Affinity purification of antibodies was performed as described [11].

2.2. *Separation of dimeric and tetrameric [^{125}I]MPR 46*

MPR 46 purified from human liver was iodinated, and dimeric and tetrameric forms were separared by sucrose density centrifugation as described [7]. The quaternary structure was controlled by cross-linking of the dimeric and tetrameric forms with 0.6 mM disuccinimidyl suberate followed by SDS-PAGE [7].

2.3. *Immunoprecipitation of [^{125}I]MPR 46*

[^{125}I]MPR 46, 10 000 cpm, was incubated at 4°C with 1 μl antiserum in 0.25 ml 0.1 M Tris-HCl, 1% Triton X-100, pH 7.5. Immunoprecipitation was performed overnight with preparations con-

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taining a mixture of the oligomeric forms of MPR 46, or for 1 h with purified dimeric or tetrameric forms of MPR 46. The immune complexes wer adsorbed for 0.5 h at 4°C to 20 µl Pansorbin (Calbiochem, Frankfurt a. M., Germany), sedimented and washed 3 times with 50 mM Tris-HCl, 1.2 M KCl, 1.2% Triton X-100, pH 7.5 and 2 times with 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5. The pelleted [^{125}I]MPR 46 was quantified, solubilized and separated by SDS-PAGE.

2.4. *Immunoprecipitation of [^{35}S]MRP 46*

BKH cells overexpressing MPR 46 [3] were metabolically labelled with [^{35}S]methionine, and [^{35}S]MPR 46 was immunoprecipitated from detergent extracts of the cells as described [3].

3. RESULTS

3.1. *Antisera against two peptides of the cytoplasmic tail of MPR 46*

Synthetic peptides corresponding to residues 7–21 (peptide A) and 38–52 (peptide B) of the cytoplasmic tail of MPR 46 were conjugated to hemocyanin and used for immunization of rabbits. For both peptides the algorithm of Jameson and Wolf [12] predicted a high antigenicity, and peptide A had earlier been shown to be antigenic [10]. Antibodies precipitating [^{125}I]MRP 46 were detectable after the third (peptide A) and sixth (peptide B) injection of the conjugates. Maximal antibody levels were obtained after one to two further booster injections. Affinity chromatography on peptide-Affigel 10 columns yielded 2 and 0.25 mg immunogobulin using 1 ml of antiserum against peptide A and B, respectively.

The specificity of the antisera against peptides A and B was controlled by immunoprecipitation of [^{125}I]MPR 46 in the presence of an excess of peptides. While an excess of the peptide which had been used for immunization inhibited the immunoprecipitation completely, the unrelated peptide did not interfere with immunoprecipitation (shown in Fig. 1 for the antiserum against peptide A). To test the monospecificity of the antisera, extracts from metabolically labelled BKH cells overexpressing human MPR 46 were incubated with the peptide-specific antisera, preimmune serum and a polyclonal rabbit antiserum raised against MPR 46 [13] (shown in Fig. 2). The peptide-specific antisera selectively immunoprecipitated a 46 kDa polypeptide, which corresponds to MPR 46. In the experiment shown in Fig. 2 only 88% and 56% of the labelled MPR 46 was precipitated by the peptide-specific antisera A and B, respectively. Control experiments showed that essen-

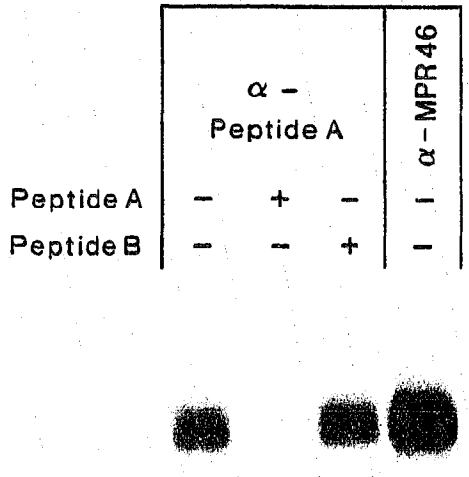

Fig. 1. Immunoprecipitation of [^{125}I]MPR 46. [^{125}I]MPR 46 was incubated with antiserum against peptide A (α-Peptide A) or MPR 46 (α-MPR 46) (see [13]). Where indicated 3.7 µg/ml peptide A or B were present. The immune complexes were separated by SDS-PAGE. The part of the fluorogram containing the band of MPR 46 is shown.

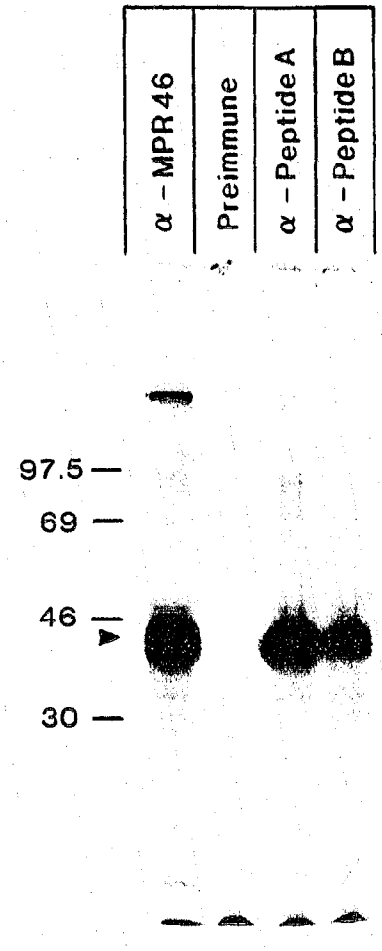

Fig. 2. Immunoprecipitation of [^{35}S]MPR 46. Extracts of metabolically labelled BHK cells overexpressing MPR 46 were incubated with antiserum against MPR 46 (α-MPR 46), peptide A or B (α-Peptide A or B) or preimmune serum. The immune complexes were separated by SDS-PAGE. The position of ^{14}C-methylated molecular mass standards (New England Nuclear, Dreieich, Germany), given in kDa, and of MPR 46 (arrowhead) are indicated.

Table I

Immunoprecipitation of dimeric and tetrameric [^{125}I]MPR 46 by antisera against MPR 46 and the tail peptide A

Antiserum against	MPR 46		Tail peptide A	
Form of [^{125}I]MPR 46	Dimer	Tetramer	Dimer	Tetramer
	(Percentage of radioactivity recovered in the immunoprecipitate)			
Exp. I	42	89	8	31
II	20	42	2	11
III	39	74	4	19
Mean of exp. I–III	34	68	5	20

Dimeric or tetrameric [^{125}I]MPR 46, 10 000 cpm, were incubated with 1 μl of antiserum. The immune complexes were analyzed by SDS-PAGE followed by quantification of MPR 46.

tially all of the [^{35}S]MPR 46 can be precipitated with higher amounts of the peptide-specific antisera (not shown).

3.2. *Differential recognition of dimeric and tetrameric MPR 46 by antibodies against the tail peptide A*

MPR 46 monomers reversibly associate to dimers and tetramers. In cells the dimeric form prevails, while in preparations of purified receptor the fraction of tetrameric form increases [7,8]. Preliminary experiments with mixtures of dimeric and tetrameric MPR 46 revealed that compared to antisera against MPR 46 and tail peptide B, the antiserum against the tail peptide A reacted preferentially with the tetrameric form of MPR 46. Dimeric and tetrameric [^{125}I]MPR 46 were therefore separated by sucrose density centrifugation and their binding to antisera against MPR 46 and tail peptide A was compared. The data of 3 independent binding experiments are summarized in Table I, and one of these experiments is shown in Fig. 3. Using the antiserum against MPR 46, twice as much radioactivity was found in the immunoprecipitate of tetrameric MPR 46 compared to that of dimeric MPR 46. Since the tetrameric MPR 46 contains twice as much radioactivity per mol than the dimeric MPR 46, comparable numbers of dimeric and tetrameric receptor forms were bound by the antiserum against MPR 46. Under the same conditions, using the antiserum against tail peptide A, 4 times more radioactivity was found in the immunoprecipitate of tetrameric MPR 46 compared to that of dimeric MPR 46. This indicates that on a molar basis, twice as many antibody-receptor complexes were formed between the peptide-specific antiserum and tetrameric MPR 46 than between the peptide-specific antiserum and dimeric MPR 46.

4. DISCUSSION

The cytoplasmic tail of MPR 46 is essential for the trafficking of this receptor. Truncated receptors lacking a cytoplasmic tail accumulate at the cell surface, indicating the sequences necessary for endocytosis reside in the cytoplasmic tail [4–6]. Other signals required for the cycling of this receptor between the Golgi apparatus

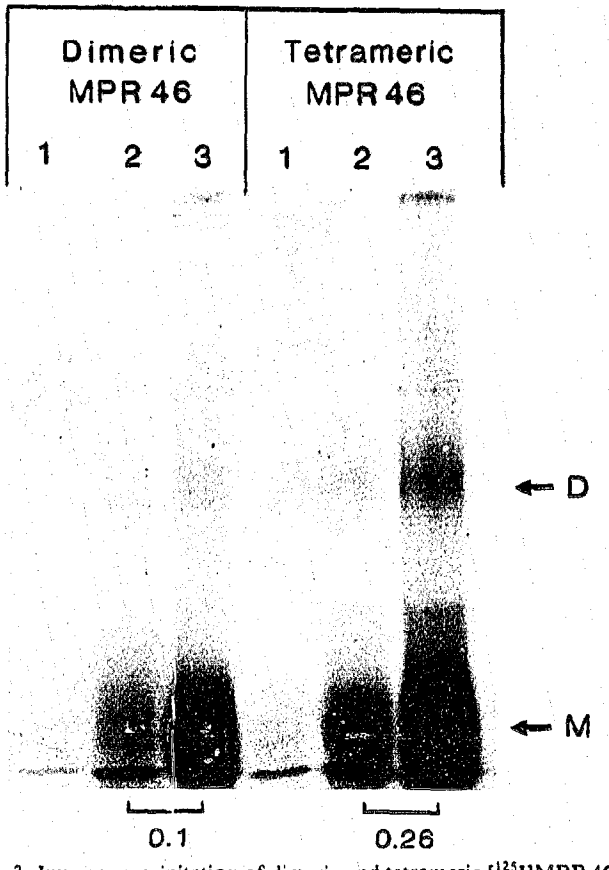

Fig. 3. Immunoprecipitation of dimeric and tetrameric [^{125}I]MPR 46. Dimeric or tetrameric [^{125}I]MPR 46 was incubated with preimmune serum (1), antiserum raised against peptide A (2), or against MPR 46 (3). The immune complexes were analysed by SDS-PAGE. The numbers below the lane give the amount of [^{125}I]MPR 46 precipitated by the peptide-specific antiserum as a fraction of the [^{125}I]MPR 46 precipitated by the antiserum against MPR 46. All receptor preparations contain small amounts of receptor homodimers which are resistant to boiling in SDS and dithiothreitol (see [14]). The position of monomeric (M) and dimeric (D) MPR 46 is indicated.

and prelysosomal organelles linked to the endocytic route are also suspected to residue in the cytoplasmic tail. It is therefore of interest to note that the reactivity of a tail-specific antibody depends on the quaternary structure of the receptor. The preferential binding of the antibody against tail peptide A to tetrameric MPR 46 indicates that the epitope on tail residues 7–21 either becomes more accessible to antibodies or changes its conformation, when dimeric MPR 46 associates to tetrameric MPR 46. This indicates that a change of the quaternary structure of MPR 46 affects the structure of its cytoplasmic tail and may hence expose or disclose signals required for the trafficking of the receptor.

Previous studies have suggested that changes of the quaternary structure of MPR 46, which in turn could affect its signal-mediated trafficking, can occur during the cycling of the receptor between its ligand-binding site in the Golgi apparatus and its ligand delivery site in prelysosomes. At 37°C the quaternary structure of MPR 46 is highly sensitive to changes in pH, presence of ligands and changes of receptor concentration in a manner that would favour the association of the receptor in the Golgi apparatus and the dissociation in acidic prelysosomal organelles [8]. In summary, this study provides the first evidence that the structure of the cytoplasmic tail, which itself is dispensible for oligomerization of MPR 46, depends on the quaternary structure of the receptor.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB 236) and the Fonds der Chemischen Industrie. Siva Kumar Nadimpalli was recipient of a fellowship of the 'Deutscher Akademischer Auslandsdienst'.

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