Characterization of phosphorylation sites in the cytoplasmic domain of the 300 kDa mannose-6-phosphate receptor

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The human 300 kDa mannose-6-phosphate receptor (MPR 300) is phosphorylated *in vivo* at serine residues of its cytoplasmic domain. Two-dimensional separation can resolve tryptic phosphopeptides into four major species. To identify the kinases involved in MPR 300 phosphorylation and the phosphorylation sites the entire coding sequence of the cytoplasmic tail was expressed in *Escherichia coli*. The isolated cytoplasmic domain was used as a substrate for four purified serine/threonine kinases [casein kinase II (CK II), protein kinase A (PKA), protein kinase C and Ca²⁺/calmodulin kinase]. All kinases phosphorylate the cytoplasmic tail exclusively on serine residues. Inhibition studies

using synthetic peptides, partial sequencing of isolated tryptic phosphopeptides and co-migration with tryptic phosphopeptides from MPR 300 labelled *in vivo* showed that (i) PKA phosphorylates the cytoplasmic MPR 300 domain at Ser²⁰ and at a non-identified site, neither of which are phosphorylated *in vivo*, and that (ii) the two sites phosphorylated by CK II *in vivo* and *in vitro* are Ser⁸² and Ser¹⁵⁷. The results indicate that the human MPR 300 is a physiological substrate of either CK II or a related kinase which may play a role in the transport function of MPR 300 and/or interaction with other proteins.

INTRODUCTION

The 300 kDa mannose-6-phosphate receptor (MPR 300) cycles continuously between the trans-Golgi network (TGN) and endosomes as well as between plasma membranes and endosomes. This allows the MPR 300 to transport mannose-6-phosphate-containing ligands from the TGN and cell surface to endosomes [1]. It has been shown that signal structures in the cytoplasmic tail are necessary for the cellular routing of MPR 300. For example, a stretch of five amino acids containing two tyrosine residues was shown to mediate rapid internalization of MPR 300 [2]. In addition, post-translational modifications like phosphorylation/dephosphorylation may be involved in MPR 300 trafficking [3,4]. Thus, a casein kinase II (CK II) purified from Golgi-derived clathrin-coated vesicles phosphorylates the bovine MPR 300 on two serine residues, which might be involved in the interaction of MPR 300 with HA I adaptor proteins [5,6].

In the present study we used the cytoplasmic tail of human MPR 300 (MPR 300-CT) expressed in *Escherichia coli* as a substrate for different purified protein kinases to identify potential phosphorylation sites. We find that CK II, protein kinase A (PKA), protein kinase C (PKC) and Ca²⁺/calmodulin kinase (CMK) phosphorylate MPR 300-CT *in vitro*. Comparative phosphopeptide analysis of cytoplasmic tail phosphorylated *in vitro* and MPR 300 phosphorylated *in vivo* showed that MPR 300 is phosphorylated *in vivo* at the two major CK II sites, whereas the two PKA phosphorylation sites are not used *in vivo*.

MATERIALS AND METHODS

Materials

Tosylphenylalanylchloromethane (TPCK)-treated trypsin (66

units/mg) and restriction enzymes were from Boehringer-Mannheim, Germany. Cellulose-coated thin-layer plates were obtained from Merck, Darmstadt. Pansorbin was purchased from Calbiochem and pGEX-3x plasmid from Pharmacia (Uppsala, Sweden). Coloured marker proteins, [γ -³²P]ATP and [³²P]P_i were from Amersham. The cDNA for the human MPR 300 was kindly provided by Dr. W. S. Sly (St. Louis University, St. Louis, MO, U.S.A.). The expression vector pQE11 and the nickel-chelate column were obtained from Diagen (Düsseldorf, Germany). Poly(vinylidene fluoride) (PVDF) membranes were from Schleicher and Schüll (Dassel, Germany). Phosphoserine, phosphothreonine and phosphotyrosine were from Sigma.

Cells

Mouse L-cells, deficient for the murine MPR 300 and transfected with the cDNA of human MPR 300, were maintained in 135-mm-diam. or 160-mm-diam. plates in Eagle's minimal essential medium (MEM), supplemented with 5% (v/v) fetal-calf serum and G 418 (0.8 mg/ml). The mouse embryonal carcinoma F9 cell line (A.T.C.C. CRL 1720) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal-calf serum and 1% (w/v) penicillin/streptomycin (all reagents from GIBCO).

Immunoprecipitation after ³²P labelling

Fibroblasts were incubated for 1 h in phosphate-free MEM containing 0.5% BSA. Then the cells were labelled with [³²P]P_i in the same medium (1 mCi/ml) for 4 h at 37 °C. After washing, the cells were scraped in 10 mM Tris buffer, pH 7.0, containing 1.5% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% SDS, 1% (w/v) BSA, 0.5 M NaCl, 5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.3 μ M leupeptin,

Abbreviations used: MPR 300, 300 kDa mannose-6-phosphate receptor; MPR 300-CT, cytoplasmic tail of MPR 300; MEM, minimal essential medium; PMSF, phenylmethanesulphonyl fluoride; CK II, casein kinase II; PKA, protein kinase A; PKC, protein kinase C; CMK, Ca²⁺/calmodulin-dependent kinase; TGN, trans-Golgi network; TPCK, tosylphenylalanylchloromethane; PVDF, poly(vinylidene fluoride); rh-CK II α , recombinant human CK II α .

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0.15 $\mu\text{g/ml}$ chymostatin, 0.2 μM pepstatin, 5 mM iodoacetic acid, 0.05 trypsin-inhibitory unit/ml aprotinin, 50 mM sodium pyrophosphate, 100 μM sodium vanadate and 50 mM NaF (lysis buffer). The MPR 300 was immunoprecipitated as described [4], analysed by SDS/PAGE [5% (w/v) acrylamide] under non-reducing conditions and visualized by autoradiography (exposing gels to Kodak X-Omat films for 16 h at -70°C).

Plasmid construction

Recombinant DNA techniques were generally those described previously [7]. Plasmid pGEMINI, containing the whole human MPR 300 receptor cDNA, was cleaved with *Bam*H1, and the fragment encoding positions 6911–8600 was subcloned into M13mp18. A *Xho*I restriction site was created at positions 7123–7129, the N-terminus of the cytoplasmic domain, by site-directed mutagenesis [8]. The *Xho*I–*Bam*H1 fragment containing the entire cytoplasmic tail sequence (the first two amino acids of the tail are changed from Lys/Lys to Ser/Arg by the mutagenesis) was then inserted into plasmid pGEX-3x digested with *Sma*I by blunt-end ligation. This construct was digested with *Bam*H1 and *Pst*I and ligated in the expression plasmid pQE11 which had been digested with the same enzymes. The structures of all plasmid constructs were verified by restriction mapping and dideoxynucleotide sequencing.

Expression and purification of recombinant MPR 300-CT

The cytoplasmic tail of MPR 300 encoded by the plasmid pQE11-CT was expressed and purified from cultures (typically 400 ml) of *E. coli*. TG1 containing the expression plasmid was grown in Luria–Bertani broth containing ampicillin (50 $\mu\text{g/ml}$) at 37°C . At an A_{600} value of 0.6 induction of expression was initiated by adding isopropyl β -D-thiogalactoside to a final concentration of 1 mM. After 4 h further growth the cells were harvested by centrifugation and resuspended in 10 mM Tris/HCl, pH 8.0, containing 4% (w/v) sucrose, 100 mM KCl and 2 mM PMSF. Lysozyme and Tris/HCl, pH 8.0, were added at final concentrations of 1 mg/ml and 60 mM respectively. After 10 min on ice the cells were lysed by adding LiCl and Nonidet P-40 at final concentrations of 50 mM and 0.25% (w/v) respectively followed by sonification at 4°C . The suspension was centrifuged for 30 min at 20000 *g*. Supernatants were loaded on to a nickel-chelate agarose column, equilibrated with 50 mM Tris/HCl, pH 8.0, 17 mM KCl, 500 mM LiCl, 24% (w/v) sucrose and 0.25% Nonidet P-40. The column was washed with 15 bed volumes of the same buffer. The MPR 300-CT was eluted using a pH gradient over the range 7–3.8 in the same buffer. The fractions were analysed by SDS/PAGE [12.5% (w/v) acrylamide] followed by Coomassie Blue staining. Protein concentrations were determined according to the method of Bradford using BSA as the standard [9]. Protein sequencing of the isolated protein was done using an Applied Biosystems model 470A gas-phase sequencer using 200 pmol of protein transferred to PVDF membranes.

Preparation of protein kinases

Recombinant human CK II α (rh-CK II α) was produced in *E. coli* using the expression vector pT7-7 and purified as described previously [10]. The specific activity was 70.8 units/mg of protein with the synthetic peptide substrate (RRRDDDSDDD). The catalytic subunit of PKA was prepared from bovine heart muscle according to [11]. The specific activity was 100 m-unit/mg of protein using histone II A as the substrate. CMK was purified

from rabbit muscle as described [12,13]. The specific activity was 68 m-unit/mg of protein using synapsin I as substrate. PKC was prepared from rat brain as described by Walton et al. [14] except for the substitution of the protamine chromatography by phosphatidylserine-affinity chromatography [15]. The specific activity was 90 m-unit/mg using histone III as substrate.

Preparation of cytosolic extracts

The cell suspension was sonicated (3×10 s) on ice and centrifuged at 10000 *g* for 10 min at 4°C . The supernatant (10 mg of protein) was loaded on to a MA7Q column (30 ml, Bio-Rad) equilibrated in 25 mM Tris buffer, pH 8.5, containing 300 mM NaCl, 7 mM 2-mercaptoethanol and 0.5 mM PMSF. The proteins were eluted by a 40 ml linear gradient of NaCl (300–1500 mM) in the same buffer. Fractions (2.5 ml) were collected and tested for CK II activity using both the synthetic peptide substrate and MPR 300-CT. Aliquots (1 ml) of each fraction were dialysed against 50 mM ammonium hydrogen carbonate buffer, lyophilized, boiled in SDS sample buffer for 5 min, separated on SDS/PAGE [10% (w/v) acrylamide] and blotted on to nitrocellulose membranes. The membranes were blocked and incubated with a monoclonal antibody (1AD9) directed against rh-CK II α (1:1000). The blots were developed using goat anti-(mouse antibody) alkaline phosphatase conjugates (1:1000, Dianova) as described [16].

In vitro phosphorylation

Purified MPR 300-CT (1 μg) was resuspended in 20 μl of phosphorylation buffer containing 25 mM Hepes, pH 7.5, and 10 mM magnesium acetate. For PKA 0.20 m-unit of the enzyme, for PKC 1 mM CaCl_2 , 0.5 mg/ml phosphatidylserine/10 $\mu\text{g/ml}$ dioleoin and 0.03 m-unit of the enzyme and for CMK 1 mM CaCl_2 , 50 $\mu\text{g/ml}$ calmodulin and 0.03 m-unit of the enzyme were added. For CK II 20 mM Mes buffer, pH 6.9, containing 10 mM MgCl_2 , 130 mM KCl and 2 mM dithiothreitol was used and either 57 m-unit of the enzyme or 5 μl of cytosol extracts. The phosphorylation reactions were started immediately by addition of [γ - ^{32}P]ATP (0.1 mM; 3 $\mu\text{Ci/nmol}$). After 30 min at 30°C the reaction was stopped by the addition of Laemmli sample buffer. After boiling for 5 min the samples were analysed by SDS/PAGE [12.5% (w/v) acrylamide] and autoradiography at -70°C using DuPont Cronex Lightning Plus intensifying screens.

Phosphoamino-acid analysis and phosphopeptide mapping

Phosphorylated MPR 300-CT peptides were cut from the gel and swollen in 50 mM ammonium bicarbonate, pH 8.5. TPCK-treated trypsin (2 μg) was added for 12 h at 37°C , and after additional incubation with 1 μg of trypsin for a further 8 h at 37°C and centrifugation the supernatants were dried under vacuum and twice resolved in water. For phosphoamino-acid analysis the peptides were hydrolysed in 200 μl of 6 M HCl for 3 h at 110°C after which the hydrolysates were dried and resuspended in 10 μl containing 2 μg each of phosphoserine, phosphothreonine and phosphotyrosine. Electrophoresis was run on the thin-layer cellulose plates for 30 min at 1300 V, using a running buffer of acetic acid/pyridine/water (10:1:189, by vol.). After drying the phosphoamino acids were visualized by ninhydrin staining and autoradiography at -70°C .

For phosphopeptide mapping the tryptic phosphopeptides were dissolved in 10 μl of 1% (w/v) ammonium bicarbonate buffer and spotted on to a thin-layer cellulose plate. The peptides were separated by electrophoresis in the same buffer at 400 V (50 mA) for 1 h, followed by ascending chromatography in

butanol/pyridine/acetic acid/water (375:250:75:300, by vol.). The plates were dried and exposed to X-ray film at -70°C .

RESULTS

Phosphorylation of MPR 300 *in vivo*

For the analysis of the phosphorylation of MPR 300 *in vivo*,

Figure 1 Purification of MPR 300-CT from *E. coli*

Total cell lysates of bacteria transformed with the MPR 300-CT plasmid (1), the fraction eluted at pH 4.5 from the nickel-chelate affinity matrix (2) and molecular-mass marker proteins (3) (14; 21.5; 31; 46; 68 kDa) were separated by SDS/PAGE [12.5% (w/v) polyacrylamide] and stained with Coomassie Brilliant Blue.

mouse L-cells overexpressing human MPR 300 were used. The cells were labelled with $[\text{P}^{32}]\text{P}_i$ for 4 h followed by immunoprecipitation of MPR 300. As with the bovine receptor [5], human MPR 300 is phosphorylated on serine residues [4] in its cytoplasmic domain (C. Körner, O. Rosorius, K. von Figura and T. Braulke, unpublished work). ^{32}P -labelled MPR 300 was digested with trypsin and the resulting peptides were resolved by two-dimensional peptide mapping. Four major (I–IV) and one minor (V) labelled peptides were identified (see Figure 3). The same pattern was obtained by phosphopeptide mapping of ^{32}P -labelled MPR 300 from human fibroblasts (results not shown). The peptides I, II and V were characterized by high mobility during electrophoresis at pH 8.0. In some experiments (four out of ten) phosphopeptides I and II were not separated.

Phosphorylation of MPR 300-CT *in vitro*

To obtain sufficient quantities of the cytoplasmic tail of MPR 300 for the analysis of its phosphorylation sites the entire coding sequence of the C-terminal 164 amino acids of the cytoplasmic tail of the human MPR 300 was cloned into the bacterial expression vector pQE11. The 'cytoplasmic tail' plasmid was used to transform *E. coli* (TG1) cells. The MPR 300-CT peptide was extracted and purified to homogeneity in a single-step nickel-chelate affinity chromatography process yielding about 1 mg of cytoplasmic tail peptide per litre of bacterial culture (Figure 1).

The purified peptide has an apparent molecular mass of 25 kDa, which is about 5 kDa higher than that predicted from the cDNA sequence. The identity of the purified peptide with the cytoplasmic tail of MPR 300 was confirmed by amino-acid sequencing, which was carried out for the N-terminal 29 residues by Edman degradation.

The cytoplasmic tail was used as a substrate for four highly purified protein kinases (CK II, PKA, PKC and CMK). All of the kinases were able to phosphorylate MPR 300-CT (Figure 2a). In each case $[\text{P}^{32}]\text{phosphoserine}$ was the only detectable phosphoamino acid (Figure 2b). The shift in the apparent molecular mass of MPR 300-CT after incubation with PKC by

Figure 2 Phosphorylation of MPR 300-CT by purified protein kinases

(a) MPR 300-CT (1 μg) was incubated with the purified α -subunit of CK II, PKA, PKC and CMK and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as described in the Materials and methods section. The reaction was stopped by the addition of Laemmli sample buffer and samples were separated by SDS/PAGE. An autoradiograph of the dried polyacrylamide gel is presented. Molecular-mass markers (in kDa) and the position of the phosphorylated MPR 300-CT peptide (P-MPR 300-CT) are indicated at the left-hand side. (b) MPR 300-CT phosphorylated by different protein kinases was excised from the gel and hydrolysed in 6 M HCl after trypsin digestion. The phosphoamino acids were separated by thin-layer electrophoresis and visualized by autoradiography. Positions of $[\text{P}^{32}]\text{P}_i$ and the non-radioactive phosphoserine [Ser(P)], phosphothreonine [Thr(P)] and phosphotyrosine [Tyr(P)] standards detected by the ninhydrin reagent are indicated.

Figure 3 Tryptic-peptide mapping of MPR 300-CT phosphorylated by purified kinases and *in vivo* phosphorylated MPR 300

[32 P]MPR 300-CT phosphorylated by purified kinases as shown in Figure 2 were excised from the gel, treated with trypsin and 50% of the samples were processed for two-dimensional phosphopeptide mapping as described in the Materials and methods section. (a) Phosphopeptide mapping of MPR 300-CT phosphorylated by CK II; (b) phosphopeptide mapping of MPR 300-CT phosphorylated by PKA and (c), phosphopeptide maps derived from MPR 300 phosphorylation catalysed by CMK. (d) The tryptic phosphopeptides from (a), (b) and (c) were mixed and subjected to peptide mapping, resulting in phosphopeptides 1 to 4. The exposure time of the phosphopeptide autoradiographs were 2–8 h. For comparison, a 3 day autoradiograph of phosphopeptides (I–V) from *in vivo* 32 P-labelled MPR 300 is shown. The human MPR 300 was immunoprecipitated from receptor-overexpressing mouse L-cells, separated by SDS/PAGE and analysed by autoradiography and two-dimensional mapping.

up to 10 kDa could be due to a hyperphosphorylation (see below). CK II, PKA and PKC but not CMK mediated the phosphorylation of MPR 300 in membranes derived from human fibroblasts also (results not shown).

MPR 300-CT phosphorylated by the purified kinases was excised from the gel and digested by trypsin. The analysis of the tryptic-phosphopeptide maps revealed that two or three major phosphopeptides were generated from MPR 300-CT phosphorylated by CK II, PKA and CMK (Figure 3). After phosphorylation by PKC at least 11 phosphopeptides were detectable (not shown). Further analysis was restricted to the phosphopeptides obtained after phosphorylation by CK II, PKA and CMK.

After mixing the tryptic peptides obtained from MPR 300-CT

phosphorylated separately by CK II, PKA and CMK four major phosphopeptides (1–4) were observed (Figure 3d). Further mixing experiments of phosphopeptides derived from different phosphorylations *in vitro* indicate that the two sites phosphorylated by CK II or PKA are different, whereas CMK phosphorylates two sites also phosphorylated by CK II (peptide 1 and 2) and one site which is also phosphorylated by PKA (peptide 3) (results not shown).

MPR 300 is phosphorylated by CK II *in vivo*

The two tryptic phosphopeptides derived from MPR 300-CT phosphorylated by CK II *in vitro* were isolated and mixed with phosphopeptides derived from MPR 300 phosphorylated *in vivo*. The CK II phosphopeptides 1 and 2 co-migrated with MPR 300-derived phosphopeptides I/II and III respectively (Figure 4). Due to the variable separation of phosphopeptides I and II their correlation to the CK II phosphopeptide 1 could not be further resolved. Our results show, however, that MPR 300 is phosphorylated *in vivo* at the same sites as by CK II *in vitro*, indicating that one of the kinases which phosphorylates MPR 300 physiologically is either CK II or a highly related kinase. This is confirmed by high MPR 300-CT kinase activity in CK II-enriched cytosolic fractions from cultured F9 cells. The kinase activity was abolished completely in the presence of heparin (20–200 μ g/ml) (Figure 5). Peptide mapping of MPR 300-CT phosphorylated by cytosolic fraction 6 revealed two phosphopeptides corresponding to the phosphopeptides 1 and 2 of MPR 300-CT phosphorylated by rh-CK II (results not shown).

A CK II associated to HA I adaptors phosphorylates bovine MPR 300 at serine residues [5] equivalent to Ser⁸² and Ser¹⁵⁷ of the human MPR 300 [17,18]. Both Ser⁸² and Ser¹⁵⁷ are surrounded by acidic amino acids forming a characteristic consensus sequence recognized and phosphorylated by CK II [19]. In addition, both Ser⁸² and Ser¹⁵⁷ are localized in stretches of amino acids highly conserved in bovine, rat and human MPR 300 [17]. In order to determine whether CK II phosphorylates Ser⁸² and Ser¹⁵⁷ in the MPR 300-CT, phosphopeptides 1 and 2 were isolated from the thin-layer plate and processed by automated Edman degradation. The 32 P release from CK II peptides 1 and 2 was maximal in the eighth and twelfth cycle, respectively (results not shown). The only tryptic peptides derived from the MPR 300-CT having serine residues at position 8 or 12 extend from residue 150 to residue 164 (LVSFHDDS¹⁵⁷DEDLHI) and from residue 71 to residue 93 (ALSSLHGDDQDS⁸²TDEVLTIPEVK), respectively and hence comprise Ser⁸² and Ser¹⁵⁷.

To examine the sites phosphorylated by PKA different synthetic 16-mer peptides, in which serine residues were partially substituted by alanine, were used as competing peptides for MPR 300-CT phosphorylation. These data and Edman-degradation analysis of the isolated phosphopeptide 3 showed that PKA phosphorylates serine residue 20 *in vitro*. The serine residue phosphorylated in phosphopeptide 4 could not be identified. Neither the *in vitro* phosphopeptide 3 nor 4 co-migrated with phosphopeptides derived from MPR 300 phosphorylated *in vivo* (results not shown). This indicated that *in vivo* the MPR 300 is unlikely to become phosphorylated by PKA.

DISCUSSION

The present results show that the cytoplasmic domain of human MPR 300 can be phosphorylated *in vitro* by different serine/threonine protein kinases such as CK II, PKA, PKC and

Figure 4 Co-migration of tryptic peptides of MPR 300 phosphorylated *in vivo* and CK II peptides phosphorylated *in vitro*

Human MPR 300 immunoprecipitated from [32 P]-labelled receptor-overexpressing mouse L-cells was digested with trypsin and analysed as described in Figure 3. The MPR 300 phosphopeptides I/II (420 c.p.m.) and III (180 c.p.m.) were eluted from the thin-layer plate and mixed with either CK II phosphopeptide 1 (480 c.p.m.) or 2 (130 c.p.m.) (Figure 3). The mixture was re-analysed by two-dimensional peptide mapping followed by autoradiography for 4 days.

Figure 5 MPR 300-CT phosphorylated by CK II from cytosolic extracts

The CK II was partially purified by ion-exchange chromatography on an MA 76 column as described in the Materials and methods section. The absorbance of the collected fractions was recorded (a), and the CK II activity was measured under standard conditions in the absence (○) or presence (●) of 20 μ g/ml heparin (b). Aliquots of fractions 6 to 8, containing the peak of CK II activity, were tested in a Western blot with anti-CK II α -subunit antibody (c) or for MPR 300-CT kinase activity (d). Abbreviation: Co, MPR 300-CT phosphorylated by rh-CK II α *in vitro*.

CMK. With the exception of CMK all other kinases catalyse the phosphorylation of MPR 300 also in membranes isolated from human fibroblasts (results not shown). All kinases phosphorylate serine residues exclusively. This agrees with the phosphorylation

in vivo of human MPR 300, which is restricted to serine residues in the cytoplasmic domain [4]. Also the bovine and hamster MPR 300s are phosphorylated exclusively at serine residues [5,20], while in the rat receptor serine and threonine residues are phosphorylated [21]. In addition, only CK II, not PKA or PKC, was able to catalyse the phosphorylation of rat MPR 300 *in vitro* [21]. These differences may be explained by a higher number of serine residues in the human MPR 300 cytoplasmic tail (20 in the human receptor versus 11 in the rat receptor), the different positions of the threonine residues [19,20] and the use of different receptor tails as substrates (MPR 300-CT expressed in *E. coli* versus MPR 300 isolated from rat tissue).

The present paper describes the identification of the isolated peptides 1, 2 and 3 obtained after phosphorylation *in vitro* of MPR 300-CT by CK II, PKA and CMK. The sites phosphorylated by CK II are Ser⁸² (peptide 2) and Ser¹⁵⁷ (peptide 1) which are surrounded in MPR 300-CT by highly conserved acidic amino acids. It has been shown that in bovine MPR 300 the serine residues homologous to Ser⁸² (Ser²⁴²¹) and Ser¹⁵⁷ (Ser²⁴⁹²) are phosphorylated by a CK II-like MPR 300 kinase [5]. Co-migration of peptides 1 and 2 obtained after phosphorylation *in vitro* of MPR 300-CT by CK II with the tryptic phosphopeptides I/II and III derived from human MPR 300 phosphorylated *in vivo* showed that Ser¹⁵⁷ (peptide 1) and Ser⁸² (peptide 2) are phosphorylation sites for CK II or a related kinase *in vivo*. Furthermore, chromatographed extracts from F9 cells enriched in heparin-sensitive CK II activity phosphorylated efficiently the MPR 300-CT, demonstrating that CK II may be the cellular MPR 300 kinase responsible *in vivo*. The different electrophoretic behaviour of tryptic phosphopeptides I and II in some experiments could be due to another modification. Thus, it is likely that in MPR 300 labelled *in vivo* both Ser¹⁵⁷ and Ser¹⁵² (-S¹⁵²FHDDSS¹⁵⁷DED-) are phosphorylated by a CK II-like enzyme.

Ser²⁰, which is part of the consensus sequences for PKA and CMK [19], was phosphorylated by both enzymes *in vitro* (peptide 3) but not *in vivo*.

The location of the phosphopeptides IV and V isolated from

MPR 300 phosphorylated *in vivo* are still unknown. In preliminary studies, the tryptic peptides from an MPR 300 deletion mutant (stop at residue 119) ^{32}P -labelled *in vivo* were shown to lack phosphopeptide V in addition to peptide I/II (C. Körner, unpublished work). This suggests that the corresponding phosphorylated serine residue may be localized between residues 119 and 164. There might be two possible tryptic-peptide candidates stretching the residues 137–143 (-SSSAQK-) and 144–149 (-TVSSTK-). In these peptides the potential phosphoacceptor serines are neighboured by basic amino acids which form PKC consensus sequences (-K $\underline{\text{G}}$ K $\underline{\text{S}}$ 137 SS-; -K $\underline{\text{T}}$ V $\underline{\text{S}}$ 148 STK-).

The physiological role of MPR 300 phosphorylation remains largely obscure. It has been observed that the redistribution of MPR 300 to the plasma membrane of rat adipocytes, in response to insulin, is accompanied by a marked decrease in serine and threonine phosphorylation of MPR 300 localized in clathrin-enriched plasma membrane fractions [22]. In contrast, the redistribution of MPR 300 to the plasma membrane of human fibroblasts in response to insulin-like growth factor II does not change the phosphorylation state of the receptors in either the plasma membrane or in internal membranes [4]. Furthermore, MPR 300 which redistributed to the plasma membrane following the activation of PKC by phorbol ester showed an increased phosphorylation stoichiometry [23,24], which seems to be secondary to the PKC translocation [24]. All these results argue against a role for MPR 300 phosphorylation in receptor cycling between the plasma membrane and internal membranes. Finally, mutagenesis of four potential phosphorylation sites in bovine MPR 300 [25] or of CK II-like sites in human MPR 300 (C. Körner, unpublished results) showed no detectable effects on lysosomal enzyme sorting. However, phosphorylation could be important for long-term MPR 300 functions during cell proliferation, growth and differentiation which are accompanied by an increased CK II activity [26,27]. Preliminary results indicate that cross-linking of MPR 300-CT to a cytosolic brain protein depends on phosphorylation by CK II, while no cross-linked product was obtained with a MPR 300-CT phosphorylated by PKA (O. Rosorius, O.-G. Issinger and T. Bräulke, unpublished work). Further studies directed toward characterizing other kinases that phosphorylate MPR 300 and their regulation, as well as the MPR 300 substrate availability by binding to different ligands, will be of great interest.

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