

Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein

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Tail-interacting 47-kDa protein (TIP47) binds the cytoplasmic domains of the cation-dependent (CD) and cation-independent (CI) mannose 6-phosphate receptors (MPRs) and is required for their transport from endosomes to the Golgi complex. TIP47 recognizes a phenylalanine-tryptophan signal in the CD-MPR. We show here that TIP47 interaction with the 163-residue CI-MPR cytoplasmic domain is highly conformation dependent and requires CI-MPR residues that are proximal to the membrane. CI-MPR cytoplasmic domain residues 1–47 are dispensable, whereas residues 48–74 are essential for high-affinity binding. However, residues 48–74 are not sufficient for high-affinity binding; residues 75–163 alone display weak affinity for TIP47, yet they contribute to the presentation of residues 48–74 in the intact protein. Independent competition binding experiments confirm that TIP47 interacts with the membrane-proximal portion of the CI-MPR cytoplasmic domain. TIP47 binding is competed by the binding of the AP-2 clathrin adaptor at (and near) residues 24–29 but not by AP-1 binding at (and near) residues 160–161. Finally, TIP47 appears to recognize a putative loop generated by the sequence PPAPRPG and other hydrophobic residues in the membrane-proximal domain. Although crystallography will be needed to define the precise interaction interface, these data provide an initial structural basis for TIP47–CI-MPR association.

Mannose 6-phosphate receptors (MPRs) carry newly synthesized lysosomal hydrolases from the Golgi complex to prelysosomes and then return to the Golgi for additional cycles of lysosomal enzyme delivery (1, 2). Two MPRs have been identified to date. One is a dimer of 46-kDa subunits that requires divalent cations for ligand binding and is referred to as the cation-dependent (CD) MPR; the other is the 300-kDa cation-independent (CI) MPR.

Although the two MPRs are structurally distinct, they traverse common pathways of intracellular transport. We recently discovered a 47-kDa tail-interacting protein (TIP47) that binds to the cytoplasmic domains of both MPRs (3). TIP47 is essential for the transport of the receptors from endosomes to the Golgi complex, both *in vitro* and in living cells. We showed previously that TIP47 interacts with a Phe-Trp motif in the CD-MPR (3). Kornfeld and colleagues discovered this motif (4); they demonstrated that it was essential for diversion of CD-MPRs from lysosomes. Satisfyingly, cellular depletion of TIP47 protein triggered the same phenotype as mutation of the CD-MPR Phe-Trp residues; that is, MPRs were missorted to lysosomes (3). Together, these data suggest that productive binding of TIP47 to the Phe-Trp signal in the CD-MPR is needed for retrieval of MPRs from endosomes and delivery to the Golgi complex.

Although these results are very gratifying, they skirt the fact that TIP47 binds the CI-MPR despite its lack of a Phe-Trp motif. Thus we undertook a mutational analysis of the CI-MPR cytoplasmic domain in the hope of identifying sequences needed for TIP47 binding. We show here that TIP47 recognizes hydrophobic residues in the membrane-proximal region of the CI-MPR cytoplasmic domain. Moreover, recognition requires that these

residues be presented within the context of the fully folded CI-MPR cytoplasmic domain.

Materials and Methods

Mutagenesis of Glutathione S-Transferase (GST)–CI-MPR Cytoplasmic Domains. The CI-MPR cytoplasmic domain fused to GST in pGEX-3X (Pharmacia Biotech; ref. 3) was mutagenized using single-step and two-step PCR strategies. For C-terminal truncations, the 5'-pGEX primer was used in PCRs with primers that replaced the first codon to be deleted with two stop codons followed by an *EcoRI* site. N-terminal truncations were generated by using the 3'-pGEX primer in conjunction with a primer allowing the new N-terminal codon to be directly preceded by a *BamHI* site and two cytosines. For point mutations, primers carrying the corresponding mutation flanked by ≈ 20 bases on either side were used with the nearest pGEX primer. The resulting PCR product was used as a primer in a second PCR together with the other, most distant, pGEX primer. PCR products were digested with *BamHI* and *EcoRI* and subcloned into pGEX-3X. All mutagenesis was verified by sequencing.

Purification of GST–CI-MPR Cytoplasmic Domains. GST fusion proteins were expressed in *Escherichia coli* XL-1 Blue cells, induced with 0.2 mM isopropyl β -D-thiogalactoside for 3 h at 30°C. To reduce breakdown, induction was started at an OD_{600} of 0.10–0.12. Cells from 2 liters of culture were broken by one pass through a French press in buffer A (50 mM Hepes, pH 8.0/50 mM KCl/2 mM EDTA) containing DNase I, 3 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (10 μ g/ml leupeptin, 40 μ g/ml aprotinin, and 1 μ M pepstatin A). GST fusion proteins were collected on 0.5 ml of glutathione-Sepharose 4B (Pharmacia Biotech), washed with 50 ml of buffer A, and eluted by two incubations for 10 min, each with 0.5 ml of buffer B (50 mM Tris-HCl, pH 8.0/100 mM NaCl/20 mM glutathione). The pooled eluates from two glutathione columns were fractionated on a 45-ml high-resolution Sephacryl S100 column (Pharmacia Biotech) for glutathione removal, separation from free GST, and exchange into buffer C containing 10% glycerol (to increase the stability of the proteins) [50 mM Hepes, pH 7.5/150 mM KCl/1 mM $MgCl_2$ /10% (vol/vol) glycerol].

Quantitation of GST Fusion Proteins. The concentrations of GST–CI-MPR cytoplasmic domain fusion proteins were measured

Abbreviations: MPR, mannose 6-phosphate receptor; CD, cation-dependent; CI, cation-independent; AP-1, clathrin adaptor 1; AP-2, clathrin adaptor 2; GST, glutathione S-transferase; TIP47, 47-kDa tail-interacting protein.

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with Bio-Rad reagent and BSA as a standard. More exact determinations were obtained by densitometric quantitation of three different concentrations of each fusion protein on polyacrylamide gels stained with Coomassie brilliant blue. To determine whether binding of the proteins to glutathione-Sepharose 4B was the same for different constructs, equal volumes of the eluates obtained in the binding assay were also quantitated on Coomassie blue-stained polyacrylamide gels.

Purification and Texas Red Labeling of His-Tagged TIP47. His-tagged TIP47 was expressed in XL-1 Blue cells, induced at an OD_{600} of 0.7 with 0.1 mM isopropyl β -D-thiogalactoside at room temperature for 12–16 h. The cells from a 1-liter culture were broken by one pass through a French press in buffer T1 (50 mM Hepes, pH 7.5/150 mM KCl) containing the same additions as for the purification of GST fusion proteins. His-tagged proteins were purified by using a 1-ml HiTrap column (Pharmacia Biotech), according to the manufacturer's instructions. The eluate was extensively dialyzed against buffer C, the protein concentration was determined, and TIP47 was immediately labeled with Texas Red sulfonyl chloride (Molecular Probes) according to the manufacturer's instructions. The resulting degree of substitution was 2.2 Texas Red molecules per TIP47 molecule.

TIP47 Binding to GST-CI-MPR. Texas Red-labeled TIP47 was rotated at room temperature with increasing concentrations of GST-CI-MPR constructs, in a total volume of 0.5 ml of buffer C containing 0.6 mg/ml BSA and 25 μ l of glutathione-Sepharose 4B. The reaction mixture was collected in columns made of 1-ml syringes, washed two times with buffer C, and eluted by incubation for 15 min in buffer B. Bound TIP47 was quantitated and binding constants were calculated with Kaleidagraph (Synergy Software, Reading, PA) as described. Adaptor purification and binding to GST-CI-MPR were as described (5); binding to GST-CI-MPR constructs was determined by immunoblot with commercially available antibodies [clathrin adaptor 1 (AP-1), using 100/3 anti- γ adaptin; clathrin adaptor 2 (AP-2), using 100/2 anti- α adaptin; Sigma]. For competition studies, Texas Red-labeled TIP47 (800 nM) was incubated with 200 nM GST-CI-MPR in the presence or absence of 800 nM AP-1 or AP-2. Binding was then determined as described above.

Peptides. Synthesized peptides were purified by gel filtration over Sephadex G10 (Sigma). Their concentrations were determined by averaging results from two methods: absorption at 205 nm (6) and the difference between absorption at 215 and absorption at 225 nm (7).

Results

We have devised a quantitative binding assay using Texas Red-labeled TIP47 and used it to show that TIP47 binds to CI-MPR and CD-MPR cytoplasmic domains with K_d values of 1 μ M and 3 μ M, respectively (5). These experiments are carried out with a constant, limiting amount of TIP47 and increasing amounts of receptor cytoplasmic domains in the form of GST fusion proteins. K_d values are then determined by fitting the experimental data to theoretical curves obtained from the equation describing bimolecular reversible binding.

Because TIP47 binds to aromatic residues in the CD-MPR cytoplasmic domain, we first tested whether the association of TIP47 with the CI-MPR cytoplasmic domain is also based on hydrophobic interactions. Texas Red-labeled, N-terminally His-tagged TIP47 was mixed with the CI-MPR cytoplasmic domain (fused to the C terminus of GST); protein complexes were collected on glutathione-Sepharose and quantified with a fluorimeter after elution (5). In the presence of 0.7 M KCl or 1 M urea, the affinity of TIP47 for CI-MPR was indistinguishable from that measured under physiological buffer conditions (Fig.

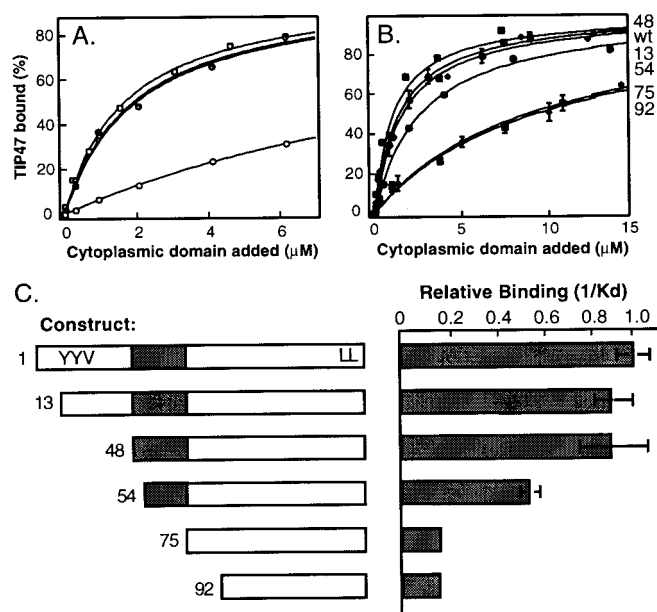


Fig. 1. The TIP47–CI-MPR interaction is hydrophobic in nature and lies between residues 48 and 75. (A) Binding of TIP47 to a GST fusion of the CI-MPR wild-type cytoplasmic domain was measured and fitted as described in *Materials and Methods*. Binding was measured without any additions (\square) and in the presence of 0.75 M KCl (\bullet) or 1 M urea (\blacklozenge) or 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (\circ). The solid lines represent theoretical curve fits to the acquired data. (B and C) Binding curves for association to TIP47 (B) and the calculated relative binding constants (C) are shown for several membrane-proximal deletions of the CI-MPR cytoplasmic domain. The numbers to the right of the curves in B indicate the residue at which the corresponding cytoplasmic domain begins. In C Left, the tyrosine motif (for binding of AP-1 and AP-2) and dileucine motif (for AP-1 binding) are depicted; the numbers to the left of each construct indicate the number of the cytoplasmic domain residue that is fused to GST. The darkened region represents the probable location of the TIP47 binding site, between residues 48 and 75. Binding for each of the proteins is presented in C Right as the inverse of its binding constant relative to the K_d for wild type. Error bars represent standard deviation. The actual relative K_d values are presented in Table 1.

1A). However, when 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate was added to the reaction, binding was reduced significantly and possibly even nonspecifically, as it did not appear to display saturation. The sensitivity of the association to detergent, and not to salt, is consistent with a hydrophobic interaction.

The TIP47 Recognition Site in the CI-MPR Is Membrane Proximal. We next sought to localize the TIP47 binding site within the CI-MPR cytoplasmic domain. For this purpose, a number of N-terminal truncations of the cytoplasmic domain were generated. Removal of the first 75 residues of the cytoplasmic domain led to a major decrease in TIP47 binding capacity (Fig. 1B and C). A truncation lacking only the first 48 residues displayed binding at almost wild-type levels (Fig. 1B and C). These data point to the importance of residues 48–75 in TIP47 binding to CI-MPR.

An independent approach was taken to verify that TIP47 interacts with membrane-proximal sequences. The CI-MPR cytoplasmic domain is also recognized by the clathrin adaptor complexes AP-1 and AP-2 (8, 9). AP-2 binds to a CI-MPR tyrosine motif Y²⁴Y²⁶V²⁹, whereas AP-1 binds both to this motif and to a dileucine motif located at position 161–162 (10–13). Neither of these motifs is needed for TIP47 interaction (3). If AP-1 and AP-2 can compete for TIP47 binding, they might offer clues to the location of the TIP47 interaction site.

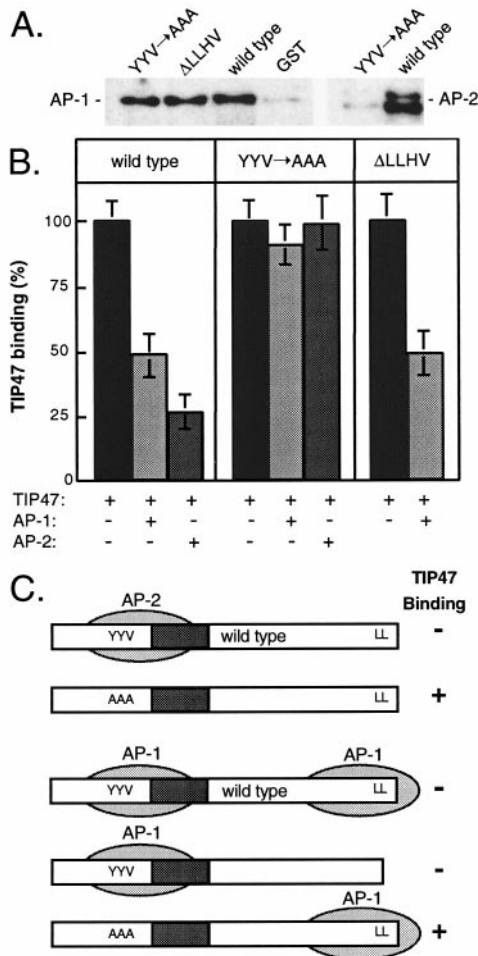


Fig. 2. AP-1 and AP-2 inhibit TIP47 association when bound to the membrane-proximal portion of the CI-MPR cytoplasmic domain. (A) Immunoblot analysis of clathrin-adaptor binding to GST-CI-MPR constructs confirms the recognition of tyrosine and/or dileucine motifs by AP-1 and AP-2. (Left) AP-1 (detected with anti- γ adaptin). (Right) AP-2 (detected with anti- α adaptin). (B) The amount of TIP47 binding to the CI-MPR cytoplasmic domain is shown for several mutations, in the presence or absence of AP-1 or AP-2, as indicated. Error bars represent standard deviations. (C) Summary diagram. Occupation of the membrane-proximal YVY motif by either AP-1 or AP-2 inhibits TIP47 binding, but binding of AP-1 to the membrane-distal dileucine does not. Shaded bars represent residues 48–75.

We first verified that AP-1 and AP-2 bind to GST-CI-MPR cytoplasmic domain constructs as expected. As shown in Fig. 2A, AP-1 bound to a CI-MPR cytoplasmic domain in which $Y^{24}Y^{26}V^{29}$ was converted to alanines, or to a CI-MPR cytoplasmic domain in which the C-terminal dileucine motif was deleted. In contrast, AP-2 failed to bind to the $Y^{24}Y^{26}V^{29} \rightarrow A^{24}A^{26}A^{29}$ mutant, as expected from previous published studies. These results confirm the expected specificity of interaction for the clathrin adaptor complexes.

When TIP47 binding to the wild-type CI-MPR cytoplasmic domain was measured in the presence of either AP-1 or AP-2, the adaptors interfered with TIP47 binding (Fig. 2B Left). AP-2 was more potent than AP-1 in this competitive binding experiment, although both adaptors were inhibitory. In contrast, neither AP-1 nor AP-2 inhibited TIP47 binding in reactions using a CI-MPR mutant in which the tyrosine motif was changed to alanines ($Y^{24}Y^{26}V^{29} \rightarrow A^{24}A^{26}A^{29}$) (Fig. 2B Center). Under these conditions, AP-1 was capable of binding to the $Y^{24}Y^{26}V^{29} \rightarrow A^{24}A^{26}A^{29}$ mutant exclusively via the C-terminal dileucine

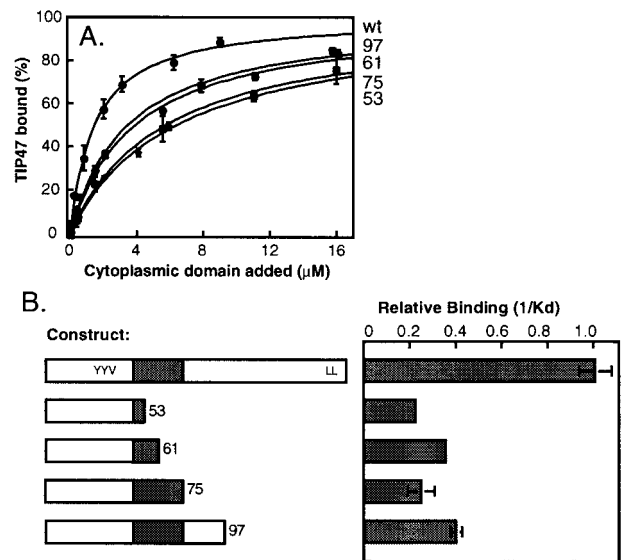


Fig. 3. Membrane-distal residues are required for proper presentation of membrane-proximal determinants. TIP47 binding curves (A) and the calculated inverse relative binding constants (B) are shown for C-terminal truncations of the CI-MPR cytoplasmic domain. The numbers to the right of the curves in A indicate the residue at which the corresponding MPR cytoplasmic domain ends. The actual relative K_d values are presented in Table 1.

motif (Fig. 2A), despite its failure to interfere with TIP47 binding. With a truncation lacking the dileucine motif, AP-1 bound to the cytoplasmic domain via $Y^{24}Y^{26}V^{29}$ (Fig. 2A), and under these conditions it also competed for TIP47 binding (Fig. 2B Right). These data show that adaptors can interfere with TIP47 binding only when they bind at or near tyrosines 24 and 26 (Fig. 2C). Thus these findings are consistent with a model in which the TIP47 binding site is located adjacent to the membrane-proximal site occupied by AP-2 and is accessible to TIP47 binding, even when AP-1 binds to the membrane-distal dileucine motif.

An alternative possibility is that AP-1 or AP-2 binding at the membrane-proximal $Y^{24}Y^{26}V^{29}$ sequence changes the conformation of the CI-MPR cytoplasmic domain to one that does not favor TIP47 binding. This is an equally interesting possibility when one considers the selective recognition of the CI-MPR by different sorting machineries, depending upon the receptor's subcellular localization.

Membrane-Distal Residues Are Required for Proper Folding. N-terminal truncations of the CI-MPR cytoplasmic domain suggested that the binding site is located between residues 48 and 75. To determine if these residues were sufficient for TIP47 binding, we generated a series of C-terminal truncations and measured their affinity for TIP47 (Fig. 3A and B). To our surprise, constructs containing residues 48–75 but lacking more distal sequences showed decreased affinity for TIP47. Constructs containing either cytoplasmic domain residues 1–75 or 1–97 showed significantly less binding than the full-length cytoplasmic domain (Fig. 3). Because these distal sequences alone do not contain significant, independent binding information (Fig. 1B and C), we reasoned that membrane-distal sequences are needed for the proper folding of the cytoplasmic domain and the correct presentation of membrane-proximal sequences.

Identification of Hydrophobic Residues Involved in TIP47 Binding. The TIP47 recognition site appears to be hydrophobic in nature and to be located between CI-MPR cytoplasmic domain residues 48

bovine (49-75)	PPAPRPGKEGQENGVAAKSVRAADTL
human	L P Q ITT K ---
rat/mouse	V L D T K EA-

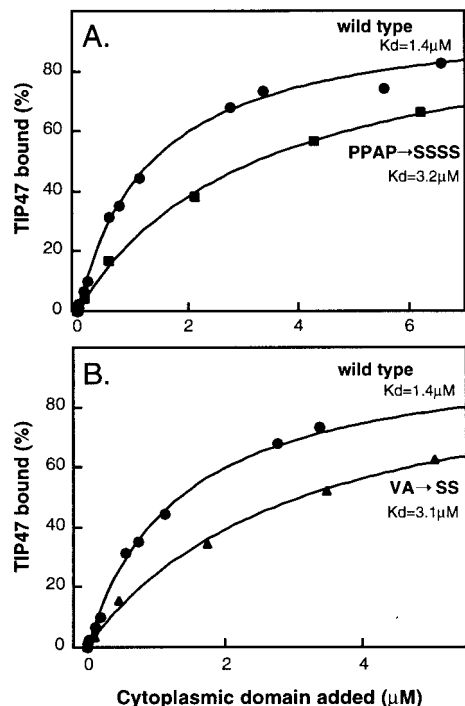


Fig. 4. Two hydrophobic sequences contribute to TIP47 binding. Residues 49–75 of bovine, human, and rat and mouse CI-MPR cytoplasmic domains are shown at the *Top*. Replacement of P⁴⁹P⁵⁰A⁵¹P⁵² (A) or V⁶⁹A⁷¹ (B) with serines in the full-length CI-MPR cytoplasmic domain decreases the affinity for TIP47.

and 75. In this region, only a few hydrophobic amino acids are fully conserved between the human, bovine, rat, and mouse sequences (Fig. 4 *Top*). Two of these conserved residues are P⁵⁰ and P⁵². Although the adjacent amino acids at positions 49 and 51 are not identical across species, they remain hydrophobic. Thus we replaced P⁴⁹P⁵⁰A⁵¹P⁵² of the bovine sequence with four serine residues to change their hydrophobic character and tested this mutant for its ability to recognize TIP47 (Fig. 4A). The K_d for the mutant is significantly higher than the wild-type K_d (2.3-fold, Table 1), although this decrease in affinity cannot account for full TIP47 recognition.

The only other conserved hydrophobic amino acids are V⁶⁹ and A⁷¹. Replacement of these residues with serines yielded a 2.2-fold decrease in affinity, comparable to the P⁴⁹P⁵⁰A⁵¹P⁵² → S⁴⁹S⁵⁰S⁵¹S⁵² construct.

As the effects of these mutations were not very strong, we also mutated numerous other conserved residues in the region between residues 48 and 75, as well as other hydrophobic amino acids outside of this region. Most of these mutations did not significantly influence TIP47 recognition (Table 1). Two mutations are noteworthy. In CD-MPR, binding requires a Phe-Trp motif that is located halfway between the transmembrane domain and two cysteine residues. Palmitoylation of the cysteines might promote the presentation of the Phe-Trp motif for binding to TIP47, as proposed by Schweizer *et al.* (14). In the CI-MPR, this potential loop would contain the sequence M⁷V⁸M⁹ at its apex, of which the valine residue is conserved between species. In addition, the CI-MPR cytoplasmic domain contains only one tryptophan residue that is flanked by two conserved hydrophobic residues. The only phenylalanine residue in the bovine

Table 1. Binding of CI-MPR cytoplasmic domain mutant constructs to TIP47

CI-MPR construct	Relative K_d
1–163	1.0 ± 0.1
13–163	1.3 ± 0.2
48–163	1.0 ± 0.2
54–163	2.5 ± 0.3
75–163	6.4 ± 0.4
92–163	6.7 ± 0.9
1–163	1.0 ± 0.1
1–53	4.6 ± 0.4
1–61	2.8 ± 0.1
1–75	4.1 ± 1.0
1–97	2.5 ± 0.1
MVM ^{7–9} → SSS	1.7 ± 0.5
Y ²⁴ V ²⁶ V ²⁹ → AAA	1.1
WLM ^{42–44} → SSS	1.5 ± 0.4
PPAP ^{49–52} → SSSS	2.3 ± 0.2
ENG ^{60–62} → AAA	1.2 ± 0.02
K ⁶⁷ → A	1.1
V ⁶⁹ A ⁷¹ → SS	2.2 ± 0.1

Construct 1–163 represents the full-length, wild-type form of this protein domain. Relative K_d values were determined based on a K_d of 1.4 μ M for the 1–163, wild-type CI-MPR cytoplasmic domain.

CI-MPR cytoplasmic domain is located at the C terminus. Thus, from comparisons with the CD-MPR, the potential contributions of M⁷V⁸M⁹ and W⁴²L⁴³M⁴⁴ seemed worth investigating. Interestingly, replacement of each of these sequences with serine residues resulted in a 50–70% decrease in affinity for TIP47 (Table 1), which was the highest effect for mutations other than P⁴⁹P⁵⁰A⁵¹P⁵² and V⁶⁹A⁷¹. Taken together, these data are best explained by a strict requirement for multiple residues in the cytoplasmic domain, some contributing to folding and others presenting a hydrophobic surface.

The P⁴⁹P⁵⁰A⁵¹P⁵² Region Interacts Directly with TIP47. P⁴⁹P⁵⁰A⁵¹P⁵² and V⁶⁹A⁷¹ might be part of a hydrophobic surface that interacts with TIP47. Alternatively, mutation of these residues could cause destabilization of a small hydrophobic core within the cytoplasmic domain and thus partial misfolding. To distinguish between these possibilities, we attempted to determine whether a 14-residue peptide centered on the P⁴⁹P⁵⁰A⁵¹P⁵² sequence would act as a competitive inhibitor of TIP47 binding. As a control, we used a peptide of scrambled sequence containing exactly the same amino acids.

Binding of the full-length wild-type cytoplasmic domain (1 μ M) was not influenced by the scrambled peptide (Fig. 5) but was decreased significantly in the presence of the P⁴⁹P⁵⁰A⁵¹P⁵² peptide in a concentration-dependent manner (Fig. 5). The decrease was pronounced, yielding a 70% reduction of binding of the full-length cytoplasmic domain at the highest concentration of peptide used. The high K_i (in the millimolar range) is not surprising, as probably only a small fraction of the peptide was present in a conformation that allowed it to bind to TIP47. A peptide of 14 residues centering on V⁶⁹A⁷¹ competed for TIP47 recognition in a similar manner (data not shown). The ability of these peptides to compete with the wild-type cytoplasmic domain indicates that the P⁴⁹P⁵⁰A⁵¹P⁵² and V⁶⁹A⁷¹ sequences interact directly with TIP47.

If TIP47 interacts directly with the PPAP sequence, why is the CI-MPR mutant lacking this sequence not more severely hindered in its ability to bind to TIP47? It is essential to keep in mind the fact that numerous residues contribute to the overall folded state of the CI-MPR cytoplasmic domain. If the PPAP residues

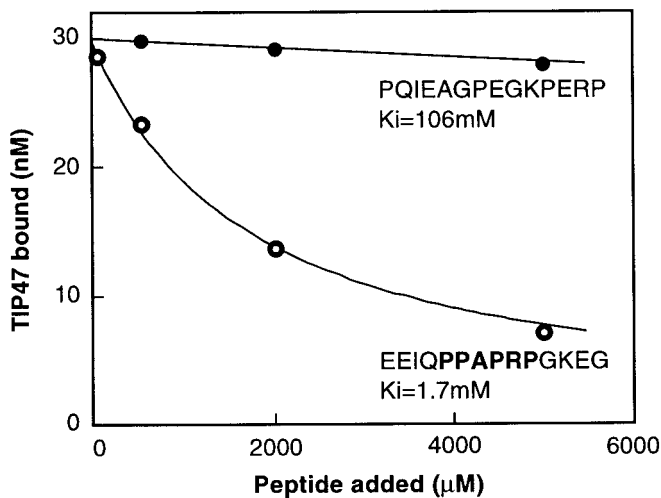


Fig. 5. The P⁴⁹P⁵⁰A⁵¹P⁵² region interacts directly with TIP47. A 14-residue-long peptide centered on the P⁴⁹P⁵⁰A⁵¹P⁵² sequence (●) and a peptide containing exactly the same residues in scrambled sequence (○) were tested as competitors of TIP47 interaction with the wild-type CI-MPR cytoplasmic domain. Binding reaction mixtures contained 1 µM GST-CI-MPR cytoplasmic domain. The data were fitted as described in *Materials and Methods*, and inhibition constants were estimated.

contribute part of a binding interface, a competitive peptide should show significant inhibition for wild-type protein-protein interactions. In contrast, a mutant CI-MPR may still fold properly and provide other interface contact sites for the TIP47 protein.

Discussion

We have presented here the TIP47-binding properties of 17 CI-MPR cytoplasmic domain mutations, generated with the goal of identifying a TIP47 recognition sequence. Each of the cytoplasmic domain mutant proteins was purified in milligram quantities and tested quantitatively for TIP47 interaction. While binding of TIP47 to the CD-MPR relies in large part on a Phe-Trp motif, we have shown here that TIP47 binding to the larger CI-MPR cytoplasmic domain is more complex. Interaction of TIP47 with the CI-MPR cytoplasmic domain involves a distinct domain comprising residues 48–75. In addition, residues 75–163 are needed for proper presentation of this region.

Kornfeld and colleagues (10) found that efficient endocytosis of the CI-MPR required cytoplasmic domain residues 21–74. Interestingly, a mutant CI-MPR that terminated at cytoplasmic domain residue 74 was not absolutely wild type in terms of its cellular localization; a larger proportion was present at the cell

surface at steady state (10). This finding is consistent with our conclusion that the outermost residues contribute to the overall folding of the CI-MPR cytoplasmic domain.

Most nonsignaling endocytic receptors have rather short cytoplasmic domain sequences. For example, the cell surface receptors for mannose, transferrin, and low-density lipoprotein have cytoplasmic domains of 45, 61, and 50 residues, respectively. Even the CD-MPR cytoplasmic domain is significantly shorter than that of the larger CI-MPR (67 versus 163 residues). Relatively small cytoplasmic domains generally distinguish endocytic “nutrient” receptors from signaling receptors that often possess much larger cytoplasmic signaling domains. Schweizer *et al.* (14) have shown that the CD-MPR is reversibly palmitoylated on cysteine-34. This residue lies precisely in the middle of the CD-MPR cytoplasmic domain and, if modified, would generate a loop structure in this domain (14). In contrast, the CI-MPR palmitoylation sites lie only 15 residues from the transmembrane domain (15). It is possible that the additional mass of the CI-MPR cytoplasmic domain provides a structural scaffold to present sorting signals in this receptor, in the absence of conformational modulation by palmitoylation. Indeed, it seems likely that the cytoplasmic domain of the CI-MPR is used for protein-protein interactions distinct from those of other nutrient receptors. This could include a need for conformational modulation, depending upon its cellular localization.

Unlike most cell surface receptors, MPRs must be recognized by AP-1, AP-2, and TIP47. From this study and work from many other laboratories, each of these sorting complexes recognize MPRs by means of distinct sequences. AP-1 utilizes dileucine motifs (as well as a tyrosine-based signal in the CI-MPR; ref. 13); AP-2 recognizes a phenylalanine- or tyrosine-based signal (10–13), and TIP47 recognizes yet another domain of the cytoplasmic domains of MPRs (3). In addition, MPRs are likely to be phosphorylated (16, 17) and reversibly palmitoylated (14) at various stations within the biosynthetic and endocytic pathways. These modifications imply that MPR cytoplasmic domains may adopt distinct conformations depending upon their cellular localization. Such conformational modulation may contribute to MPR recognition by distinct sorting machines when MPRs are present in different cellular compartments.

Identification of the precise CI-MPR cytoplasmic domain residues that come into contact with TIP47 will require protein crystallography of the TIP47-MPR complex. Nevertheless, our findings predict the presence of key residues just downstream of the AP-2 binding site in the CI-MPR.

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