The carboxy-terminal domain of the receptor-associated protein binds to the Vps10p domain of sortilin

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Abstract Binding of the receptor-associated protein (RAP) to the newly identified putative sorting receptor, sortilin, was analyzed by surface plasmon resonance analysis of recombinant RAP and sortilin domains and compared with binding to megalin and low density lipoprotein receptor-related protein (LRP). The data show that the RAP-binding site in sortilin is localized in the cysteine-rich lumenal part homologous to yeast vacuolar protein-sorting 10 protein (Vps10p), and the sortilin-binding site in RAP is localized in the carboxy-terminal domain III of the three homologous domains in RAP. Whereas sortilin bound only RAP domain III, megalin and LRP bound all RAP domains with the functional affinity order: domain III > domain I > domain II.

Key words: Sortilin; Receptor-associated protein; Chaperone; Sorting; Brain

1. Introduction

Receptor-associated protein (RAP) is a 39–40 kDa intracellular protein that binds to the cysteine-rich ligand-binding low density lipoprotein (LDL) receptor class A repeats of LDL receptor-related receptors [1–4]. RAP is mainly located in the endoplasmic reticulum (ER) (about 70%) and Golgi (about 24%) compartments, and only minor amounts are located in endosomes [5]. Consistent with this, RAP has a C-terminal HNEL motif that functions as an ER retention signal [6]. RAP competes for ligand binding to all the LDL receptor family proteins [7].

Gene knock-out studies have shown that cells lacking RAP exhibit an approximately 75% reduction of the expression of the α_2-macroglobulin receptor/LDL receptor-related protein (LRP), presumably because RAP prevents premature binding of newly synthesized ligands to LRP and precipitation of the receptor within the ER [8]. In addition, it has been reported that soluble recombinant minireceptors comprising the clusters of ligand-binding repeats in LRP were only secreted in transfected cells when the cells were cotransfected with RAP cDNA [9]. The retention of disulfide-bonded receptor oligomers in ER was reduced when the cells were cotransfected with RAP.

RAP is a three-domain protein with an internal triplicate sequence homology [4,10]. The autonomous regions of human RAP comprise domain I (18–112), domain II (113–218), and domain III (219–323). The solution structure of domain I (18–112) was recently determined [11]. Domains I and III bind independently to LRP [4,10,12] and domain III has been reported to promote the correct folding and subsequent secretion of the RAP-binding soluble ligand-binding regions of LRP [4].

We have recently identified and isolated the cDNA of a RAP-binding 95 kDa type I receptor designated sortilin [13], which has no similarity to LDL receptor family receptors. Sortilin consists of a large lumenal domain homologous to each of two lumenal domains in yeast vacuolar protein-sorting 10 protein (Vps10p) and a cytoplasmic tail with similarity to that of the cation-independent mannose-6-phosphate receptor. One function of Vps10p is to mediate the lysosomal sorting of carboxypeptidase Y in yeast [14]. The Vps10p domain is also present in sorLA [15], a recently described hybrid receptor of unknown function, which also contains a cluster of 11 LDL receptor type A repeats. The identical sequence of the nine C-terminal residues of sortilin and the cation-independent mannose-6-phosphate receptor as well as the colocalization of the two receptors in Golgi [13] and on the plasma membrane further suggest a role of sortilin in protein sorting.

The aim of the present study was to define whether RAP binds to the suggested ligand-binding lumenal domain, and secondly to identify the RAP domain(s) involved in this interaction. The three homologous RAP domains were analyzed and compared with the binding to the giant LDL receptor family receptors, megalin and LRP.

2. Materials and methods

2.1. Sortilin, RAP, megalin and LRP

The extracellular domain of sortilin was expressed by stable transfection of Chinese hamster ovary (CHO) K-1 cells. The cDNA encompassing the N-terminal lumenal Vps10p domain plus a 6×His tag was amplified by PCR using the forward primer 5′-CGCCTCGAGCTGGCACACTCCACAGACC and the reverse primer 5′-GCGCGGCCGCCTAATGATGATGATGATGAGAATTTGACTTGGAAT. This PCR product, which contains an internal *Bsp*MII restriction site, was cut with *Xho*I and *Not*I and ligated into the pcDNA 3.1/Zeo− vector from Invitrogen (San Diego, CA, USA). Full-length sortilin cDNA in pBK-CMV [13] was cut out with *Xba*I and *Bsp*MII and ligated into the same sites in the pcDNA 3.1/Zeo− vector containing the PCR product. This created a cDNA encoding the extracellular sortilin domain and a 6×His tag. Stably transfected CHO cell clones were established by limited dilution of transfected cells using Zeocin (500 μg/ml) for selection. The clone with highest secretion was subcloned and grown in serum-free CHO cell medium (HyQ-CCM 5 from Hyclone, Utah, USA). Secretion of the extracellular sortilin domain into the medium of the clones was tested by Western blotting using a rabbit polyclonal antibody generated against an N-terminal peptide [13]. The extracellular domain was secreted as a soluble protein and was efficiently purified by RAP affinity chromatography as previously described for the purification of megalin [16].

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The yield was ~2 μg sortilin domain/ml medium. The identity of the purified expression product was verified by microsequencing. Approximately 5 μg of soluble sortilin was subjected to SDS-gel electrophoresis in a 8–16% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Problot, Applied Biosystems). The electroblotted band was cut out and subjected to Edman degradation using an Applied Biosystems 477 A sequencer equipped with a 120 A online chromatograph.

The sortilin cytoplasmic tail construct was made by PCR using full-length sortilin cDNA as template for PWO polymerase (Boehringer Mannheim) and 5′-CACGGATCCATCGAGGGTAGGAAGAAAT-ATGTCTGTG-3′ and 5′-TCAAGCTTATTCCAAGAGGTCCTCA-TC-3′ as NH_2-terminal and COOH-terminal primer, respectively. Using the newly generated *Bam*HI and *Hin*dIII sites, the PCR product was subcloned into the *Escherichia coli* T7 expression vector, sequenced and subsequently expressed in *E. coli* Bl21(DE3) cells. The expressed 7.5 kDa hexa-His-tagged sortilin tail protein (MGSHHHHHHSIEGRKYVCGGRFLVHRYSVLQQHAEANGV-DGVDALDTASHTNKSGYHDDSDEDLLE) was purified on a Ni^{2+} nitrilotriacetic acid column as described previously [12].

Human RAP and the RAP constructs encompassing amino acid residues 18–112 (domain 1), amino acid residues 113–218 (domain II), amino acid residues 219–323 (domain III), domain I+II and domain II+III were produced as 6×His-tagged constructs in *E. coli* as previously described [10]. Megalin [16] and LRP [17] were purified from rabbit kidney and human placenta, respectively, as described.

2.2. Surface plasmon resonance analysis

Analysis of the binding of RAP to purified recombinant sortilin was performed by surface plasmon resonance measurements on a BIAcore 2000 instrument (Biosensor, Uppsala, Sweden). The BIAcore sensor chips (type CM5, Biosensor) were activated with a 1:1 mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxysuccinimide in water. RAP, sortilin, megalin and LRP were immobilized at a concentration of 10–40 μg/ml in 10 mM sodium acetate, pH 3.5–4.5, and the remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. The estimated density of RAP, sortilin, megalin and LRP was 24, 45, 37 and 24 fmol ligand/mm^2, respectively. The flow cells were regenerated with 1.6 M glycine-HCl, pH 3.0 (LRP and megalin) or 10 mM glycine-HCl, 20 mM EDTA, 500 mM NaCl, pH 4.0 (sortilin and RAP). The flow buffer was 10 mM HEPES, 150 mM NaCl and 1.5 mM $CaCl_2$, 1 mM EGTA pH 7.4. The binding data were analyzed using the BIAevaluation program. The number of ligands bound per immobilized receptor was estimated by dividing the ratio '$RU_{ligand}/mass_{ligand}$' with '$RU_{receptor}/mass_{receptor}$'.

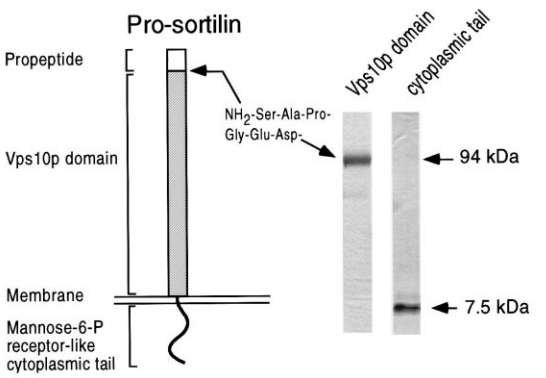

Fig. 1. Reducing SDS-gel electrophoresis of the recombinant Vps10p domain and cytoplasmic tail of sortilin. The cytoplasmic tail was expressed as a 6×His-tagged protein in *E. coli* and purified by Ni^{2+} affinity chromatography. The Vps10p sortilin domain was expressed as a secreted protein in CHO-K1 cells and purified by RAP affinity chromatography. The N-terminal sequence Ser-Ala-Pro-Gly-Glu-Asp of the Vps10p domain demonstrates that the propeptide of the Vps10p domain had been cleaved off.

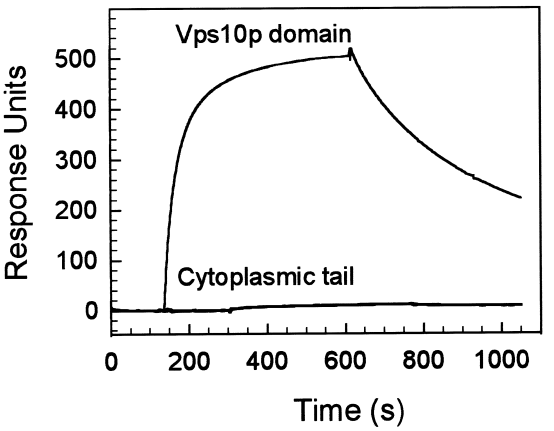

Fig. 2. Surface plasmon resonance analysis of the binding of the Vps10p sortilin domain (10 μM) and the cytoplasmic sortilin tail (10 μM) to immobilized RAP. The binding curves of the Vps10p domain were obtained in a concentration range of 0.01–10 μM and fitted to one-binding-site kinetics. The following constants were estimated: $k_{ass} = 1.8 \times 10^4$ M^{-1} s^{-1}, $k_{diss} = 1.39 \times 10^{-3}$ s^{-1}, $K_d = 78$ nM.

3. Results

3.1. Expression of the Vps10p domain of sortilin as a RAP-binding protein

Fig. 1 shows SDS-gel electrophoresis of the mannose-6-phosphate receptor-like cytoplasmic tail of sortilin expressed in *E. coli* and the cysteine-rich lumenal Vps10p domain of sortilin expressed by stable transfection in CHO-K1 cells. The lumenal sortilin domain was secreted into the medium as a soluble protein. Binding of full-length RAP to this protein was readily demonstrated, since RAP affinity chromatography turned out to be an efficient one-step procedure for purification of the protein. Amino-terminal sequencing of the secreted Vps10p sortilin domain revealed the sequence Ser-Ala-Pro-Gly-Glu-Asp, thus demonstrating that the propeptide had been cleaved off by cleavage at the putative furin recognition site preceding the recognized peptide sequence.

Surface plasmon analysis showed high-affinity binding ($K_d = 76$ nM) of the sortilin domain to immobilized RAP, whereas no significant binding of the cytoplasmic tail was evident (Fig. 2).

3.2. Binding of RAP domains to sortilin, megalin and LRP

In order to analyze the sortilin-RAP interaction, we performed a comparative surface plasmon resonance analysis of the binding of RAP and RAP domains to the Vps10p domain of sortilin and purified LRP and megalin.

Fig. 3 shows the analysis of wild-type recombinant RAP and RAP domains I, II and III. Only domain III displayed binding to sortilin, whereas all domains bound to LRP and megalin with the affinity order: domain III > domain I > domain II. Table 1 shows the stoichiometry of the binding of RAP, the single domains and the two-domain constructs. The two-domain RAP constructs (domains I+II and II+III) exhibited an increased functional affinity for both megalin and LRP (not shown) as compared to single domains, thus suggesting simultaneous binding of both domains. This is also evident from the dissociation curves (Fig. 3) showing that RAP dissociates at a lower rate compared to the single domains. Each of the tree RAP domains binds to multiple sites in LRP and

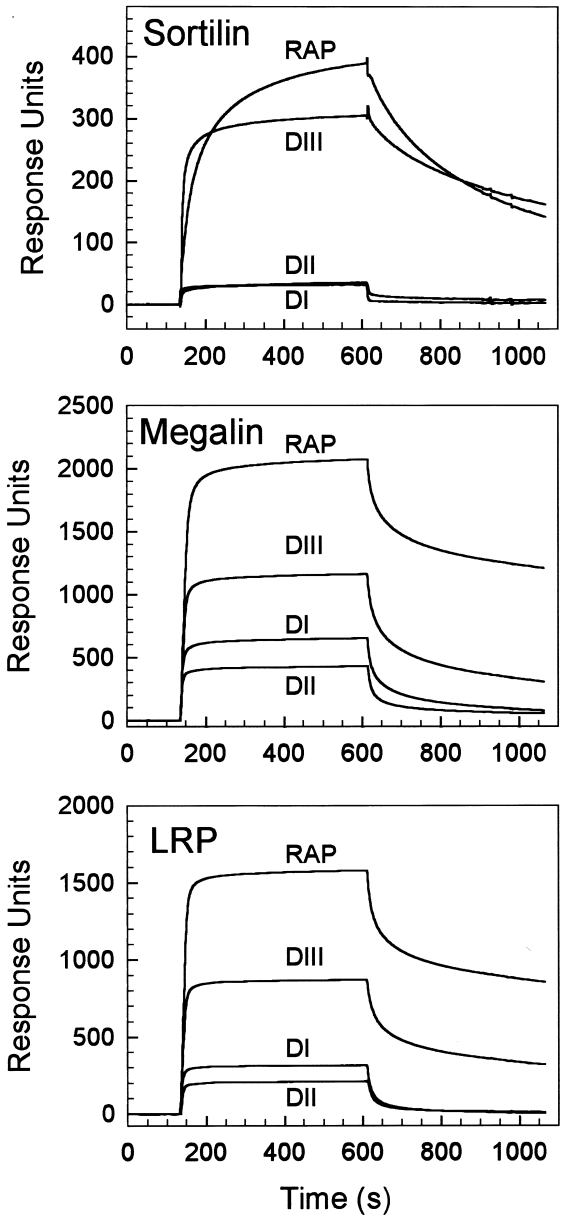

Fig. 3. Surface plasmon resonance analysis of the binding of recombinant human RAP and RAP domains I, II and III to the Vps10p domain megalin and LRP. The concentration of RAP and the RAP domains was 10 μM.

megalin. Accordingly, the curves for RAP binding to megalin and LRP did not fit to single-site kinetics.

Less than one RAP molecule was on average bound to immobilized sortilin (Table 1) thus suggesting a 1:1 stoichiometry. Accordingly, simple one-binding-site kinetics fitted to the binding data of RAP. The estimated affinity (K_d = 43 nM) was close to that estimated in the inverse assay (Fig. 2). Domain I+II had no affinity for sortilin and domain II+III had no increased affinity compared to domain III. The affinity of domain III was slightly higher (K_d = 30 nM) than wild-type RAP, indicating that binding of RAP to sortilin is entirely accounted for by domain III.

4. Discussion

The present study is a characterization of the binding of sortilin to RAP, the only molecule so far known to bind to the putative sorting receptor. The RAP-binding region was mapped to the extracellular Vps10p-like lumenal domain which was produced as a secreted and proteolytically processed recombinant protein in CHO-K1 cells and purified from conditioned medium by RAP affinity chromatography. The sortilin-binding domain of RAP was mapped to domain III by surface plasmon analysis of recombinant RAP domains.

Comparison with the binding of RAP to megalin and LRP showed that sortilin, in contrast to these receptors, forms a 1:1 complex with RAP and binds to only one RAP domain. Furthermore, our data and previous studies using radiolabeled ligands [10] [4] have established that domain II, like domains I and III, binds to both LRP and megalin and contributes to the high-affinity multisite interaction of single RAP molecules with these receptors.

The binding of RAP is important for the processing of LRP, and probably of megalin, as shown by Willnow et al. [18] who observed that RAP-deficient mice process LRP less efficiently than normal mice. The molecular details are not clear, but RAP is suggested to protect against ligand-induced aggregation of the receptors, and in addition, RAP domain III has been reported to be crucial for correct folding of LRP minireceptors [4]. We are now investigating the importance of RAP for processing of sortilin and assaying the RAP-binding lumenal sortilin domain as an affinity target for purification of novel sortilin ligands.

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Table 1
The binding stoichiometry for binding of RAP and RAP domains to sortilin, megalin and LRP as measured by surface plasmon resonance analysis after flow in 600 s with 10 μM ligand, as shown in Fig. 3

	kDa	Ligand/sortilin (mol/mol)	Ligand/megalin (mol/mol)	Ligand/LRP (mol/mol)
RAP domain I	16.1	0.04	1.09	1.42
RAP domain II	17.6	0.04	1.74	2.00
RAP domain III	17.4	0.35	2.77	3.50
RAP domain I+II	28.3	0.03	1.65	1.95
RAP domain II+III	29.9	0.28	2.85	3.51
RAP	37.7	0.23	2.06	2.82

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