The sorLA cytoplasmic domain interacts with GGA1 and -2 and defines minimum requirements for GGA binding

Linda Jacobsen[a,*], Peder Madsen[b], Morten S. Nielsen[b], Wijnand P.M. Geraerts[a], Jørgen Gliemann[b], August B. Smit[a], Claus M. Petersen[b]

[a] *Department of Molecular and Cellular Neurobiology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands*
[b] *Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark*

Received 19 October 2001; revised 10 December 2001; accepted 10 December 2001

First published online 28 December 2001

Edited by Ulrike Kutay

Abstract We report that the Vps10p domain receptor sorLA binds the adaptor proteins GGA1 and -2, which take part in Golgi–endosome sorting. The GGAs bind with differential requirements via three critical residues in the C-terminal segment of the sorLA cytoplasmic tail. Unlike in sortilin and the mannose 6-phosphate receptors, the GGA-binding segment in sorLA contains neither an acidic cluster nor a dileucine. Our results support the concept of sorLA as a potential sorting receptor and suggest that key residues in sorLA and sortilin conform to a new type of motif (Ψ–Ψ–X–X–∅) defining minimum requirements for GGA binding to cytoplasmic receptor domains.

Key words: SorLA; Sortilin; GGA; Sorting adaptor

1. Introduction

SorLA/LR11 is a highly conserved putative sorting receptor located mainly in the *trans*-Golgi network (TGN). SorLA predominates in the brain, but is also expressed in non-neuronal tissues such as testis, ovary, and lymph nodes ([1,2] and references herein). Taken from the N-terminus, the lumenal part comprises a Vps10p domain with homology to the yeast sorting receptor Vps10p [3] and the mammalian receptors sortilin [4] and sorCS [5], elements typical of the low density lipoprotein receptor (LDLR) family including a cluster of LDLR class-A repeats, and six fibronectin type III repeats also found in neural cell adhesion molecules. The 54-residue cytoplasmic domain (cd) comprises a putative internalization motif (F^{12}ANSHY^{17}), an acidic cluster (D^{30}DLGEDDED^{38}), and most C-terminally a patch of hydrophobic residues (V^{49}PMVIA^{54}) preceded by two acidic ($D^{47}D^{48}$) residues (Fig. 1) [1].

SorLA binds certain neuropeptides, e.g. the sortilin ligand neurotensin, to the Vps10p domain [2,6], as well as ligands of the LDLR family, e.g. apolipoprotein E and lipoprotein lipase, to the cluster of class-A repeats [2]. Thus, sorLA is structurally and functionally related both to sortilin, a putative receptor for Golgi–endosome transport [7], and to the endocytic and signalling receptors of the LDLR family [8,9], and might accordingly be involved in both ligand sorting and signal transduction.

To identify cytoplasmic binding partners, we used the sorLA-cd as bait in a yeast two-hybrid assay. Here we report interaction with the VHS (Vps27/Hrs/STAM homology) domains of the Golgi-localized, γ-adaptin ear homologous Golgi-localizing γ-adaptin ear homologous ADP-ribosylation factor (ARF)-binding proteins (GGAs), GGA1 and GGA2. The GGAs constitute a newly discovered family of monomeric adaptor proteins with three mammalian members. The domain organization comprises an N-terminal VHS domain, a GAT domain, a hinge domain, and a C-terminal domain (GEAH) with homology to the γ-adaptin ear [10–16]. Clathrin is mobilized to the membrane via binding to the hinge and GEAH domains [15], and the VHS domains were recently shown to mediate binding of GGAs to C-terminal acidic cluster-dileucine (ac-LL) motifs in the sorting receptors sortilin, cation-independent (CI) mannose 6-phosphate receptor (MPR), cation-dependent (CD) MPR, as well as the LDLR-related protein 3 (LRP3) [7,17–19]. As GGAs can facilitate clathrin-mediated transport of selected cargo from the TGN to the endosomal–lysosomal system [7,18,19], our result supports the hypothesis that sorLA may function in sorting of protein in the late biosynthetic pathway. Moreover, we demonstrate that binding of GGAs to the C-terminus of the sorLA-cd depends primarily on a single methionine and a pair of preceding acidic residues, which appear to constitute a motif defining minimum requirements for GGA binding to receptor-cds.

2. Materials and methods

2.1. Yeast two-hybrid analysis

The yeast two-hybrid screening was conducted according to the manufacturers instructions (Clontech, Palo Alto, CA, USA; Stratagene, La Jolla, CA, USA). For the bait construction, the PCR-amplified wild type (wt) sorLA-cd (nucleotides 6688–6842, GenBank accession number U60975) was inserted into the *Eco*RI–*Sal*I-digested pBD-GAL4 vector (Stratagene). The bait construct was then cotransformed into the yeast strain PJ69-2a with a human brain Matchmaker cDNA

*Corresponding author. Fax: (31)-20-444 7112.
E-mail address: lija@bio.vu.nl (L. Jacobsen).

Abbreviations: ac-LL, acidic cluster-dileucine (motif); cd, cytoplasmic domain; CD, cation-dependent; CI, cation-independent; GGA, Golgi-localizing γ-adaptin ear homologous ADP-ribosylation factor (ARF)-binding protein; GAEH, γ-adaptin ear homology; GST, glutathione *S*-transferase; IL2R, interleukin 2 receptor-α (Tac, CD25); LDLR, low density lipoprotein receptor; MPR, mannose 6-phosphate receptor; TGN, *trans*-Golgi network; wt, wild type; VHS, Vps27/Hrs/STAM homology

0014-5793/02/$22.00
PII: S0014-5793(01)03299-9

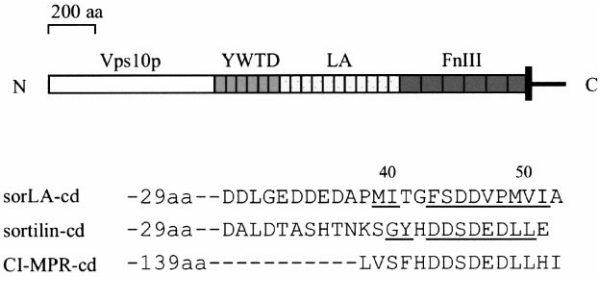

Fig. 1. Schematic representation of sorLA showing the vacuolar protein sorting 10 protein (Vps10p) homology domain, the YWTD β-propeller repeats (YWTD), the LDLR class-A repeats (LA) and the fibronectin type III repeats (FnIII). The vertical bar signifies the transmembrane domain followed by the cd. Parts of the amino acid sequences of the sorLA-cd and the sortilin-cd are presented, and residues substituted for mutational analyses are underlined. The last 15 residues of the 154 amino acid CI-MPR are shown for comparison.

library in pACT2 (Clontech). The bait did not exhibit any intrinsic reporter activity. Transformed cells (6.4×10^6) were selected on plates supplemented with 3 mM 3-amino-1,2,3-triazole and lacking Leu, Trp, and His (low stringency selection). After replica plating onto Leu-, Trp-, His- and adenine-depleted synthetic media (high stringency selection), the colonies were assayed for β-galactosidase activity by the filter lift method. Yeast plasmids were rescued in *Escherichia coli*. Sequence analysis was performed on an ABI Prism™ Genetic Analyser 310. Direct two-hybrid analysis of interactions between GGA1 or -2 (in pACT2) and the pBD-GAL4 alone or fused with the sorLA wt-/mutant-cd, the LDLR-cd [20], or the control lamin C (Stratagene), was performed by sequential transformation. Transformants were grown (−Leu, −Trp, +His) for 2 days after which the colonies were picked, diluted in water (0.1 OD, A_{600}), spotted (5 μl) on plates with double (−Leu, −Trp, +His), triple (−Leu, −Trp, −His) or quadruple (−Leu, −Trp, −His, −Ade) selection and scored for growth and β-galactosidase activity after 2–3 days. Mutations were introduced into the sorLA-cd by a two-step PCR strategy using sorLA primers with specific base substitutions. The mutant products were inserted into pACT2 as described above. Expression of the various baits was examined by Western blot analysis of soluble protein extracts of transformed yeast (as described in the Clontech manual) and probed with GAL4 DNA-BD antibody (Santa Cruz Biotechnology).

Yeast two-hybrid analysis of the sortilin-cd (Matchmaker LexA two-hybrid system, Clontech) and construction of sortilin-cd mutants was performed as described by Nielsen et al. [7].

2.2. Construction of the interleukin 2 receptor-α (IL2R)/sorLA-cd chimeras

The cDNA construct containing the lumenal and transmembrane parts of the IL2R was transferred from pCMV-IL2R [21] by *Nhe*I–*Xba*I digestion and ligated into pcDNA 3.1/Zeo(+) (Invitrogen, Carlsbad, CA, USA). The wt sorLA-cd was amplified by PCR and inserted into the *Hin*dIII–*Xho*I-cleaved IL2R-pcDNA 3.1/Zeo(+) to produce an IL2R/sorLA-cd chimeric receptor construct comprising the IL2R lumenal and transmembrane domains and the sorLA-cd. A chimera containing a deletion (Fig. 1) of the C-terminal 14 residues of the sorLA-cd (Δ14) was generated by the same procedure using the pACT2 construct as a template.

2.3. Cell culture and transfection

The L2R/sorLA-cd chimeric constructs were transfected (Fugene, Roche Molecular Biochemicals) into CHO-K1 cells, and stable transfectants were selected in serum-free HyQ-CCM5 CHO medium (HyClone, Logan, UT, USA) containing 500 μg/ml zeocin. CHO transfectants expressing full-length sortilin were generated and grown as previously described [6].

2.4. Glutathione S-transferase (GST) fusion proteins and pull-down experiments

GGA1, GGA1-VHS and GGA1-GAEH nucleotides 16–1935, 16–519 and 1429–1935 respectively [10], were amplified by PCR using primers containing *Bam*HI and *Xho*I sites, and cloned into pGEX4T-1 (Amersham Pharmacia Biotech). The construction of GGA2s, GGA2-VHS and GGA2-GEAH and the expression and purification of the GST fusion proteins were carried out as described previously [7]. For pull-down experiments, lysates of CHO transfectants were prepared and incubated with GST fusion proteins as described [7]. Precipitated proteins were identified by Western blotting using goat anti-IL2R (Roche Molecular Biochemicals, Mannheim, Germany) or rabbit anti-sortilin as primary antibodies and HRP-labelled rabbit anti-goat Ig or swine anti-rabbit Ig as secondary antibodies (DAKO, Glostrup, Denmark).

3. Results and discussion

3.1. The sorLA-cd binds GGA1 and GGA2

To identify cytosolic proteins potentially involved in sorLA functions, 6.4×10^6 clones of a human brain cDNA library were screened in a yeast two-hybrid system with the sorLA-cd as bait. From 242 clones isolated by high stringency nutritional selection, library plasmids of 100 transformants were rescued, and sequencing demonstrated that 11 cDNA inserts (all approximately 2.8 kb) represented GGA1 and one (∼2.6 kb) represented GGA2. GGA3 was not identified in the screen. The specificity of the interaction with GGA1 or GGA2 was confirmed in a direct assay with the empty bait and lamin C as negative controls (data not shown). Similar experiments were performed with the cds of the LDLR and of sortilin, a member of the Vps10p domain receptor family recently shown to bind GGA1 and -2 [7,17] and to provide Golgi–endosome sorting of cargo [7]. The LDLR-cd did not bind, whereas the sortilin-cd, taken as a positive control, bound both GGA1 and -2 (data not shown).

3.2. The sorLA-cd binds to the VHS domains of GGA1 and GGA2

The interactions determined by the yeast two-hybrid assays were verified by pull-down experiments performed on lysates of CHO transfectants expressing the IL2R/sorLA-cd (Fig. 2) or full-length sortilin (not shown). As exemplified with the IL2R/sorLA-cd, both receptors were readily precipitated by GST-GGA1 and by fusion proteins containing the N-terminal VHS domain of GGA1 or GGA2 (Fig. 2). Pull-down was also achieved with the VHS domain containing a differentially spliced short variant of GGA2 (GGA2s; accession number

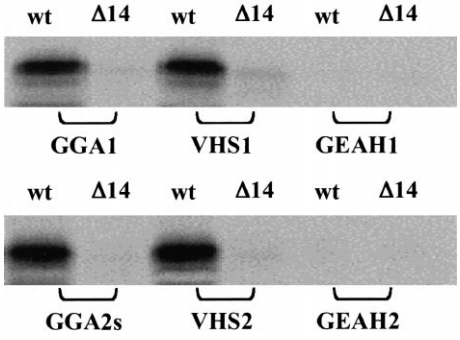

Fig. 2. Analysis of the interaction between the sorLA-cd and GGAs. Lysates of CHO cells expressing IL2R/sorLA chimeras containing the wt cd (wt) or the truncated sorLA-cd (Δ14) were incubated with the indicated GST fusion proteins overnight at 4°C and subsequently precipitated with glutathione–Sepharose. The precipitates were subjected to reducing SDS–PAGE and analyzed by immunoblotting using goat anti-IL2Rα as primary antibody.

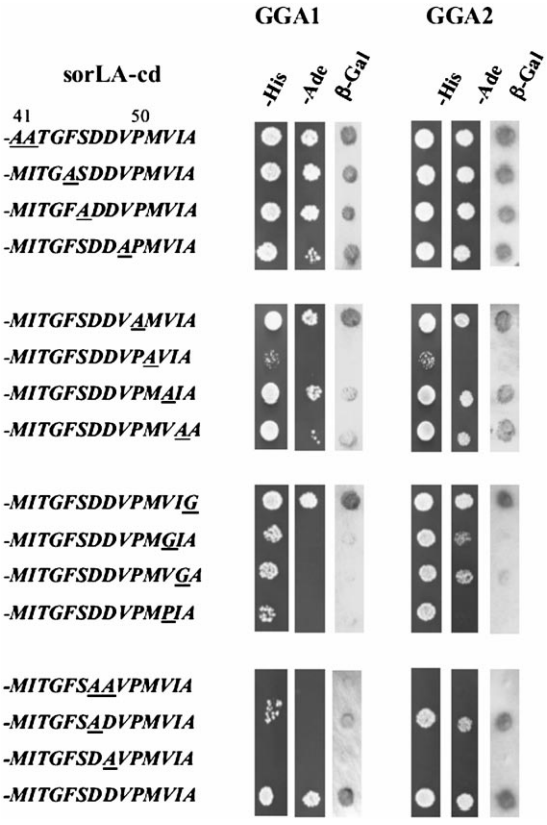

Fig. 3. Two-hybrid analysis of GGA binding to the sorLA-cd. Yeast was cotransformed with the sorLA-cd (wt or the indicated mutants, in pBD-GAL4), and GGA1 or GGA2 (in pACT2) and spotted on control plates (−Trp, −Leu, +His, not shown), triple selection/low stringency plates (−Trp, −Leu, −His) and quadruple selection/high stringency plates (−Trp, −Leu, −His, −Ade). Filter β-galactosidase activity (β-gal) was performed on the control plates.

AF323754) [7], whereas fusion proteins containing only the C-terminal GAEH domains (Fig. 2) or GST (not shown) had no effect. In accordance with previous reports, full-length GGA2 could not be purified as a uniform GST fusion protein [7,10] and was therefore not tested.

3.3. GGA binding is conditioned by C-terminal acidic and hydrophobic residues in the sorLA-cd

To identify sites involved in binding of the GGAs, truncated mutants of the sorLA-cd were tested for interaction with GGAs in yeast. Deletion of the C-terminal 14 amino acids completely abolished interactions with GGA1 and -2 (not shown). Accordingly, GST fusion proteins of GGA1 and -2 that mediated pull-down of the IL2R/sorLA-cd wt chimera did not interact with chimeras containing the sorLA-cd Δ14 (Fig. 2). An alanine scanning of the segment was then performed. As shown in Fig. 3 this established $D^{47}D^{48}$ and M^{51} as the functionally important residues. Thus, the $D^{47}A/D^{48}A$ mutant sorLA-cd was unreactive with both GGA1 and -2, and the substitution of M^{51} resulted in markedly reduced growth as well as loss of β-galactosidase activity. Single mutations of either D^{47} or D^{48} further revealed that whereas D^{48} is essential for reaction with both GGAs, only the binding of GGA1 was seriously affected by substituting D^{47} with Ala. Since replacement of either of the two hydrophobic residues following M^{51} with Ala appeared to cause a minor reduction in growth and β-galactosidase activity, in particular in combination with GGA1 under high stringency conditions, substitution with glycine and proline was also tested. As can be seen (Fig. 3), $V^{52}G$ and $I^{53}G$ both resulted in loss of β-galactosidase activity and in reduced or lack of growth (at low and high stringency conditions, respectively) with GGA1, and in a weakening of the β-galactosidase staining and of growth at high stringency with GGA2. This result, and the observation that neither GGA1 nor -2 induced any response in combination with the $V^{52}P$ mutant (Fig. 3) indicates that the conformation of the extreme C-terminus is also important for the interaction even though V^{52} and I^{53} may not participate directly.

3.4. The sorLA-cd C-terminus represents the minimum requirements for GGA1 and -2 binding

The finding that interaction of GGAs with the sorLA-cd relies on a single pair of acidic residues and a nearby C-terminally located methionine shows that requirements for GGA binding are not confined to the ac-LL motifs previously ascribed to mediate the binding to CI-MPR, CD-MPR, sortilin and LRP3 [17–19]. We hypothesized that single residues within ac-LLs might be critical for the interaction with GGA1 and -2, and we therefore performed a detailed analysis of the sortilin-cd whose ac-LL ($GYHD^{45}DSDEDLL^{52}E$) is highly similar to that of CI-MPR (Fig. 1).

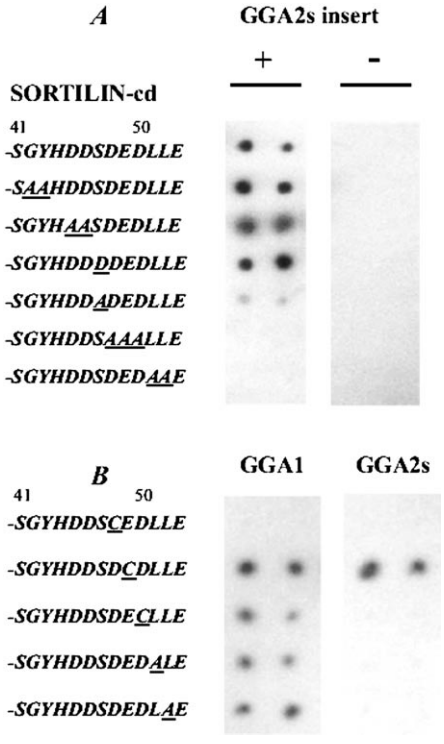

Fig. 4. Two-hybrid analysis of GGA binding to the sortilin-cd. cDNA encoding the sortilin-cd (wt or the indicated mutants) was inserted into the pLexA vector and tested against the pBAD42 plasmid with (+) or without (−) the GGA2s insert (A), and (B) against plasmid with GGA1-VHS (left panel) or GGA2s (right panel). Interaction between the expressed proteins was detected as stained colonies (in duplo) on induction plates containing galactose, raffinose and 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-gal).

Initial direct yeast two-hybrid analysis demonstrated that deletion of the acidic cluster (D^{45}–D^{50}) rendered the sortilin-cd unable to bind GGA1 and -2 (not shown), whereas replacement with Ala of residues preceding the acidic cluster, e.g. G^{42} and Y^{43}, did not perturb the response (Fig. 4A, results only shown for GGA2). Additional experiments revealed that the $D^{45}A/D^{46}A$ mutant interacted with GGA1 and -2, although the reaction was slightly delayed, whereas the $D^{48}A/E^{49}A/D^{50}A$ and the $L^{51}L^{52}$ mutants were completely unresponsive (Fig. 4A). Single mutations in the D^{48}–L^{52} segment established that D^{48}, L^{51} and L^{52} were essential for binding of GGAs (Fig. 4B). Thus, D^{48} was needed for interaction with both GGAs but, surprisingly, preservation of just one leucine (either one) was sufficient to yield a normal response with GGA1, whereas an intact dileucine was needed for interaction with GGA2. Substitution of Ser^{47} with Ala (Fig. 4A) or Cys (not shown) almost abolished interaction with GGA2, but not with GGA1 (not shown), while a normal response with GGA2 was obtained after replacement with Asp (Fig. 4A). Since Asp mimics a permanent phosphorylation, the results suggest that serine phosphorylation might regulate interactions with GGA subtypes.

The results with the sorLA-cd, which neither contains an acidic cluster nor a dileucine, establish that GGA binding depends only on two acidic and one hydrophobic residue ($D^{47}D^{48}$ and M^{51}) and, notably, only D^{48} and M^{51} are needed for GGA1 binding. Moreover, results with the sortilin-cd are surprisingly similar as D^{48}, in combination with either L^{51} or L^{52} suffice for interaction with GGA1, whereas targeting by GGA2 depends on S^{47}, D^{48} (in particular) as well as D^{50} and requires an intact dileucine. The requirements observed in the two receptor-cds conform to the motif Ψ–X–X–(X)–∅ for interaction with GGA1, and for interaction with GGA2 to the broader motif Ψ–Ψ–X–X/Ψ–∅–∅, where Ψ is D or E or phosphorylated S, X is any residue, and ∅ is a bulky hydrophobic residue, e.g. M or L, not followed by P or G. Both motifs are in accordance with previously reported data on sortilin, the two MPRs, and the LRP3 [17–19], and suggest Ψ–Ψ–X–X–∅ as a new putative motif defining minimum requirements for GGA binding.

In conclusion, we show that the sorLA-cd interacts with GGAs via three critical residues in a short segment proposed to represent a minimal binding motif. As interactions of GGAs with other receptor-cds can facilitate transport of cargo to endosomes, the results suggest that sorLA may function as a sorting receptor.

Acknowledgements: L.J. is supported by a Marie Curie Fellowship of the European Community Programme Improving the Human Research Potential and the Socio-Economic Knowledge Base under Contract number 2000-00549. J.G. and C.M.P. are supported by grants from the Danish Medical Research Council.

References

[1] Jacobsen, L., Madsen, P., Moestrup, S.K., Lund, A.H., Tommerup, N., Nykjær, A., Sottrup-Jensen, L., Gliemann, J. and Petersen, C.M. (1996) J. Biol. Chem. 271, 31379–31383.
[2] Jacobsen, L., Madsen, P., Jacobsen, C., Nielsen, M.S., Gliemann, J. and Petersen, C.M. (2001) J. Biol. Chem. 276, 22788–22796.
[3] Marcusson, E.G., Horazdovsky, B.F., Cereghino, J.L., Gharakhanian, E. and Emr, S.D. (1994) Cell 77, 579–586.
[4] Petersen, C.M., Nielsen, M.S., Nykjær, A., Jacobsen, L., Tommerup, N., Rasmussen, H.H., Røigaard, H., Gliemann, J., Madsen, P. and Moestrup, S.K. (1997) J. Biol. Chem. 272, 3599–3605.
[5] Hermey, G., Riedel, I.B., Hampe, W., Schaller, H.C. and Hermans-Borgmeyer, I. (1999) Biochem. Biophys. Res. Commun. 266, 347–351.
[6] Petersen, C.M., Nielsen, M.S., Jacobsen, C., Tauris, J., Jacobsen, L., Gliemann, J., Moestrup, S.K. and Madsen, P. (1999) EMBO J. 18, 595–604.
[7] Nielsen, M.S., Madsen, P., Christensen, E.I., Nykjær, A., Gliemann, J., Kasper, D., Pohlmann, R. and Petersen, C.M. (2001) EMBO J. 20, 2180–2190.
[8] Gliemann, J. (1998) Biol. Chem. 379, 951–964.
[9] Herz, J. (2001) Neuron 29, 571–581.
[10] Boman, A.L., Zhang, C.J., Zhu, X. and Kahn, R.A. (2000) Mol. Biol. Cell 11, 1241–1255.
[11] Hirst, J., Lui, W.W., Bright, N.A., Totty, N., Seaman, M.N. and Robinson, M.S. (2000) J. Cell Biol. 149, 67–80.
[12] Dell'Angelica, E.C., Puertollano, R., Mullins, C., Aguilar, R.C., Vargas, J.D., Hartnell, L.M. and Bonifacino, J.S. (2000) J. Cell Biol. 149, 81–94.
[13] Poussu, A., Lohi, O. and Lehto, V.P. (2000) J. Biol. Chem. 275, 7176–7183.
[14] Takatsu, H., Yoshino, K. and Nakayama, K. (2000) Biochem. Biophys. Res. Commun. 271, 719–725.
[15] Puertollano, R., Randazzo, P.A., Presley, J.F., Hartnell, L.M. and Bonifacino, J.S. (2001) Cell 105, 93–102.
[16] Robinson, M.S. and Bonifacino, J.S. (2001) Curr. Opin. Cell Biol. 13, 444–453.
[17] Takatsu, H., Katoh, Y. and Nakayama, K. (2001) J. Biol. Chem. 276, 28541–28545.
[18] Puertollano, R., Aguilar, R.C., Gorshkova, I., Crouch, R.J. and Bonifacino, J.S. (2001) Science 292, 1712–1716.
[19] Zhu, Y., Doray, B., Poussu, A., Lehto, V-P. and Kornfeld, S. (2001) Science 292, 1716–1718.
[20] Gotthardt, M., Trommsdorff, M., Nevitt, M.F., Shelton, J., Richardson, J.A., Stockinger, W., Nimpf, J. and Herz, J. (2000) J. Biol. Chem. 275, 25616–25624.
[21] LaFlamme, S.E., Thomas, L.A., Yamada, S.S. and Yamada, K.M. (1994) J. Cell Biol. 126, 1287–1298.