Memapsin 2 (β-secretase) cytosolic domain binds to the VHS domains of GGA1 and GGA2: implications on the endocytosis mechanism of memapsin 2

Xiangyuan He[a], Wan-Pin Chang[a], Gerald Koelsch[a,b], Jordan Tang[a,c,*]

[a] *Protein Studies Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104, USA*
[b] *Zapaq, Inc., Oklahoma City, OK 73104, USA*
[c] *Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA*

Received 27 May 2002; revised 22 June 2002; accepted 24 June 2002

First published online 9 July 2002

Edited by Gianni Cesareni

Abstract Memapsin 2, or β-secretase, is a membrane-anchored aspartic protease that initiates the cleavage of β-amyloid precursor protein (APP) leading to the production of β-amyloid peptide in the brain and the onset of Alzheimer's disease. Memapsin 2 and APP are both endocytosed into endosomes for cleavage. Here we show that the cytosolic domain of memapsin 2, but not that of memapsin 1, binds the VHS domains of GGA1 and GGA2. Gel-immobilized VHS domains of GGA1 and GGA2 also bound to full-length memapsin 2 from cell mammalian lysates. Mutagenesis studies established that Asp^{496}, Leu^{499} and Leu^{500} were essential for the binding. The spacing of these three residues in memapsin 2 is identical to those in the cytosolic domains of mannose-6-phosphate receptors, sortilin and low density lipoprotein receptor-related protein 3. These observations suggest that the endocytosis and intracellular transport of memapsin 2, mediated by its cytosolic domain, may involve the binding of GGA1 and GGA2.

Key words: Alzheimer's disease; Memapsin 2; β-Secretase; Endocytosis; GGA1; GGA2

1. Introduction

Memapsin 2 [1], also called BACE [2] or ASP-2 [3,4], is a membrane-associated aspartic protease that has long been known as β-secretase. Memapsin 2 hydrolyzes a membrane protein, β-amyloid precursor protein (APP), and together with another protease, γ-secretase, release a 40/42 residue fragment called β-amyloid (Aβ). Since the accumulation of Aβ in the brain is a central event leading to Alzheimer's disease (see [5] for a recent review), memapsin 2 is generally regarded as a major therapeutic target for the development of inhibitor drugs. For a better understanding of this target, there is a great deal of current interest in the intracellular activities of memapsin 2.

*Corresponding author. Fax: (1)-405-271 7249.
E-mail address: jordan-tang@omrf.ouhsc.edu (J. Tang).

Abbreviations: APP, β-amyloid precursor protein; Aβ, β-amyloid peptide; GST, glutathione *S*-transferase; CI-MPR, cation-independent mannose-6-phosphate receptor; CD-MPR, cation-dependent mannose-6-phosphate receptor

The newly synthesized pro-memapsin 2 is known to be processed in the secretory pathway by furin [6–8] and transported to the cell surface where APP is also present [9]. Both APP and memapsin 2 are endocytosed into the early endosomes where APP is cleaved by memapsin 2 at the β-secretase site. Although APP cleavage by β-secretase has been reported also for endoplasmic reticulum and Golgi, endosomes are likely the major site for β-secretase processing owing to an acidic pH activity of the enzyme [1–4]. Endosomal memapsin 2 has been shown to recycle back to the cell surface [9], possibly via the *trans*-Golgi network although this has not been demonstrated. The endocytosis of membrane proteins is in general mediated by their cytosolic domains (see [10,11] for review). For memapsin 2, two leucine residues at positions 499 and 500 (Table 1) in its cytosolic domain have been shown to be essential for its endocytosis [9,12].

Recently, the involvement of a family of GGA (Golgi-localized γ-ear-containing ARF binding) proteins in the regulation of intracellular transport of membrane proteins has become clear (see [13] for a recent review). The N-terminal VHS (Vps-27, Hrs and STAM) domains of GGA proteins were shown to bind the cytosolic domain of cation-independent and cation-dependent mannose-6-phosphate receptors (CI-MPR and CD-MPR respectively) [14–16], sortilin [16,17] and the low density lipoprotein receptor-related protein 3 [17]. This binding is believed to be the first step in the recruitment of these membrane proteins to the Golgi membrane for packaging into the vesicles targeting to endosomes [14–17]. Although memapsin 2 has not been shown to be transported from *trans*-Golgi to endosomes, we however found sequence similarity of its C-terminal region to that of GGA binding proteins mentioned above (Table 1, A). Here we report that the C-terminal region of the cytosolic domain of memapsin 2 binds to human GGA1 and GGA2.

2. Materials and methods

2.1. cDNA cloning and protein expression

cDNAs of human GGA1 and GGA2 [18] were kindly provided by Dr. M.S. Robinson, University of Cambridge. For the construct of glutathione *S*-transferase (GST)–VHS fusion proteins, the cDNAs encoding the VHS domain from GGA1 (corresponding to residues 1–147) and GGA2 (residues 13–72) were separately amplified by PCR and cloned into plasmid pGEX2T (Amersham-Pharmacia, Piscataway, NJ, USA) and expressed in *Escherichia coli* strain BL21. Three hours after the induction with isopropyl-β-D-thiogalactoside, the bacterial cells were collected and lysed by sonication. The fusion

0014-5793/02/$22.00
PII: S0014-5793(02)03052-1

proteins in the supernatant of the lysate were purified by affinity chromatography using a glutathione-Sepharose 4B column.

2.2. Peptides

The nomenclature and sequences of the peptides used are shown in Table 1. These peptide sequences were derived from the C-terminal regions of the cytosolic domains of the proteins. A cysteine was added to the N-terminus of peptide CI-MPR for gel attachment. All peptides were synthesized at Research Genetics (Huntsville, AL, USA) except CI-MPR, which was synthesized at Synpep (Dublin, CA, USA).

2.3. Binding experiments

The peptides were covalently linked by their thiol groups to Sulfolink Coupling Gel (Pierce) using the procedure provided by the manufacturer. Gel bearing immobilized peptide (150 μl) and individual GST–VHS proteins (200 μg) were incubated in 1.5 ml phosphate-buffered saline (PBS) at room temperature for 2 h. The gel beads were pelleted by centrifugation at 750×*g* for 1 min and washed three times with PBS. The proteins on the gel beads were eluted by sodium dodecylsulfate (SDS)-containing sample buffer and subjected to SDS–PAGE electrophoresis.

2.4. Pull-down experiments

The constructs of expression vectors of human memapsin 2 and Swedish mutant of APP cDNAs and their transfection into HEK 293 cells will be described elsewhere. Transfected cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), 250 μg/ml Geneticin (G418 from Gibco BRL) and 5 μg/ml zeocin (Invitrogen). Cells were collected and lysed on ice in buffer A (50 mM Tris–HCl, pH 7.4, containing 300 mM NaCl and 1% Nonidet P-40). A mixture of 100 μl of centrifuged supernatant of the lysate, 900 μl of buffer A and 100 μg of GST–VHS fusion protein was incubated overnight at 4°C. An aliquot of 80 μl of glutathione-Sepharose (Pharmacia) suspension was added and the incubation continued for 6 h. The glutathione-Sepharose beads were then recovered by centrifugation, washed three times with buffer A at 4°C and resuspended in SDS-containing sample buffer for SDS–PAGE. The presence of memapsin 2 in the electrophoresis was identified by Western blot using rabbit polyclonal antibodies (Covance, Denver, PA, USA) against recombinant pro-memapsin 2 [1]. The antibodies were affinity-purified using Affigel (Bio-Rad)-immobilized memapsin 2 protease domain [1].

3. Results

3.1. The cytosolic domain of memapsin 2, but not memapsin 1, binds the VHS domains of GGA1 and GGA2

The binding of the VHS domains from GGA1 and GGA2 to peptides derived from the C-terminal regions of the cytosolic domains of memapsin 2 and memapsin 1 was studied. In these experiments, VHS proteins bound to peptides immobilized on gel beads were eluted and visualized on SDS–PAGE. Fig. 1 shows that purified GST–VHS fusion proteins from GGA1 and GGA2 appeared as single bands. A peptide from the C-terminal region of CI-MPR was used as the positive control and cysteine-blocked gel as the negative control. The peptide from memapsin 2 produced clear bands corresponding to GST–VHS from both GGA1 (Fig. 1A) and GGA2 (Fig. 1B). The peptide derived from memapsin 1, however, did not bind to either VHS domain. These observations indicate that the cytosolic domain of memapsin 2, but not that of memapsin 1, binds to GGA1 and GGA2.

3.2. Residue requirements of memapsin 2 cytosolic region for VHS binding

The residues in the memapsin 2 C-terminal sequence required for binding to the VHS domains of GGA1 and GGA2 were studied by selectively replacing them with alanines (Table 1, B) and the binding of VHS proteins to the peptides immobilized on gel was determined. First, a series of modified M2 peptides was designed, each containing a substitution of two alanines for an adjacent pair of residues, span-

Table 1
Sequence alignment for the C-terminal regions of proteins and peptide nomenclature

Protein source	Peptide name	Sequence
A. Alignment of C-terminal sequences[a]		
Memapsin 2	M2	-CLRQQHDDFADD ISLLK
Mannose-6-phosphate receptor		
Cation-Independent	CI-MPR	-CT KLVS FHDDSDEDLLH I
Cation-Dependent		-DDQLGEESEERDDHLLPM
Sortilin		-TNKSGYHDDDSDEDLLE
LRP3		-PPCSPMLEASDDEALLVC
Memapsin 1	M1	-CQRRQRDPEVVNESSLVRHRWK
B. Residue replacements in memapsin 2 peptides[b]		
		490 500
Memapsin 2	M2	-CLRQQHDDFADD ISLLK
	M2(D491A/D492A)	-CLRQQHAAFADD ISLLK
	M2(F493A)	-CLRQQHDDAADD ISLLK
	M2(D495A/D496A)	-CLRQQHDDFAAA ISLLK
	M2(I497A/S498A)	-CLRQQHDDFADDAALLK
	M2(L499A/L500A)	-CLRQQHDDFADD ISAAK
	M2(D495A)	-CLRQQHDDFAAD I SLLK
	M2(D496A)	-CLRQQHDDFADA I SLLK
	M2(L499A)	-CLRQQHDDFADD ISALK
	M2(L500A)	-CLRQQHDDFADD ISLAK

[a]Three conserved residues are shown in boldface.
[b]Substituent alanine residues are shown in boldface.

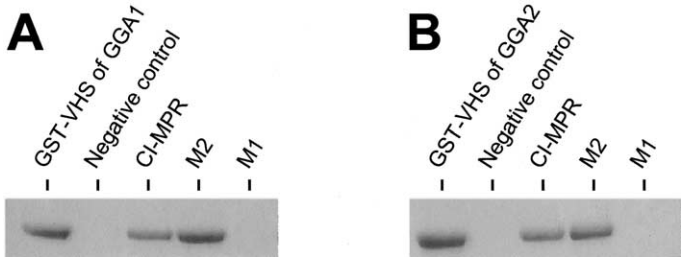

Fig. 1. Binding of VHS domain of GGA1 (A) and GGA2 (B) to peptide M2 (Table 1) from the C-terminal of human memapsin 2 (see Table 1, A). Peptides M2, M1 (from the C-terminal region of human memapsin 1) and CI-MPR (positive control) were immobilized on beaded agarose gel. After binding of GST–VHS fusion proteins, the bound protein was determined by SDS–PAGE. Cysteine-blocked gel was used as negative control.

ning from Asp^{491} to Leu^{500} with exception for residue Ala^{494}. Fig. 2 shows binding of these peptides to the VHS domain of GGA1 (panel A) and GGA2 (panel B). In both cases, the replacement of either residues Asp^{495} and Asp^{496} or Leu^{499} and Leu^{500} abolished binding. The replacement of other residues, however, did not have an effect. Peptides with single residue replacement then established that, in both GGA1 (panel C) and GGA2 (panel D), the change of either Asp^{496}, Leu^{499} or Leu^{500} abolished the binding. The replacement of Asp^{495} did not affect the binding. These results showed that Asp^{496}, Leu^{499} and Leu^{500} are essential residues for VHS/GGA binding.

3.3. *Binding of VHS domains of GGA1 and GGA2 to memapsin 2 from cell lysate*

To show that the VHS domains of GGA1 and GGA2 can bind to the full-length memapsin 2, we performed 'pull-down' experiments to test the binding of memapsin 2 in cell lysates to gel-immobilized GST–VHS fusion proteins. Clear bands corresponding to full-length memapsin 2 were present in Western blot from the eluents of both VHS domains from GGA1 and GGA2 (Fig. 3). Immobilized GST, as a negative control, did not bind to memapsin 2 in the cell lysate. These observations indicate that the VHS domains also bind the full-length memapsin 2.

4. Discussion

We have demonstrated above that the C-terminal region of memapsin 2 binds to the VHS domain of GGA1 and GGA2. Similar binding of GGAs to the cytosolic domains of CI-MPR, CD-MPR [14–16] and sortilin [16,17] have been shown recently and such binding is considered to be a recognition step in the targeting of these receptors for intracellular transport. In this mechanism, the remaining GGA domains following the VHS interact with adapter proteins, such as ARF-1, and clathrin to package the VHS-bound membrane proteins into transport vesicles for specific destinations. The current results, therefore, suggest that the GGA/VHS recognition of the cytosolic domain of memapsin 2 is also a recognition step that facilitates the intracellular transport of memapsin 2. It is interesting to note, however, that the GGA involvement in MPRs and sortilin is mainly for the transport of these membrane receptors from *trans*-Golgi to endosomes (see [19,20] for

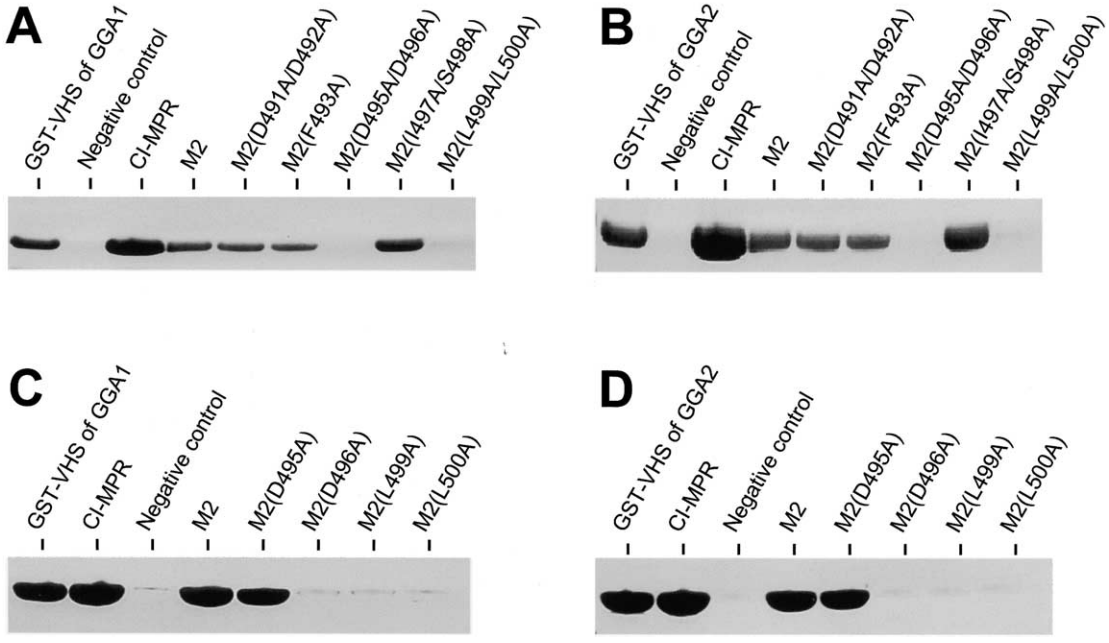

Fig. 2. Binding of alanine-substituted memapsin 2 C-terminal peptide (M2) to the VHS domains of GGA1 (A,C) and GGA2 (B,D). The sequences of the peptides are shown in Table 1, B. The binding experiments were carried out under similar conditions as those in Fig. 1.

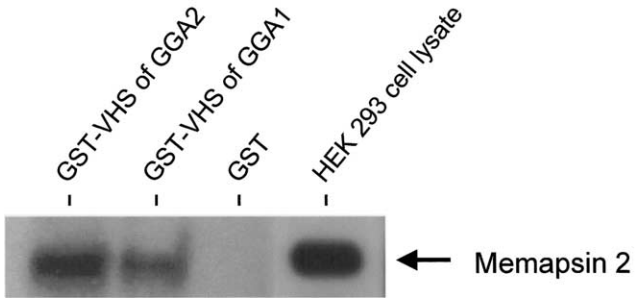

Fig. 3. Binding of immobilized GST–VHS domains from GGA1 and GGA2 to memapsin 2 from the lysate of HEK 293 cells transfected for stable expression of memapsin 2. The bound memapsin 2 was visualized by Western blot.

reviews). This is consistent with the observation that the highest concentration of GGAs in the cell is found in the *trans*-Golgi membrane. Memapsin 2 has been shown to be endocytosed into endosomes while its transport from *trans*-Golgi directly to endosomes has not been demonstrated. In view of the similarity in the GGA binding motifs for memapsin 2 and MPRs, the possibility exists that memapsin 2 may also be transported from *trans*-Golgi to endosomes. The question if GGAs are involved in the endocytosis of memapsin 2 is also intriguing. Although VHS-containing proteins are known to participate in the endocytosis mechanism (see [21] for review), the involvement of GGAs in this process has not been shown. However, MPRs are known to also be present on the cell surface where they bind and transport the secreted lysosomal enzymes to the endosomes. The cell surface MPRs may possibly utilize the same cytosolic pool of GGAs for its endocytosis mechanism, which would be very similar to that for the endocytosis of memapsin 2 and transport to endosomes.

The residues essential for the binding of memapsin 2 C-terminal region to VHS are Asp^{496}, Leu^{499} and Leu^{500}. The same three residues in identical relative positions are also found for CI-MPR and CD-MPR binding to VHS domains [14,15] (see Table 1 alignment). The crystal structures of the VHS domains of GGA1 [22] and GGA3 [23] bound to peptides of CI-MPR C-terminal region have been reported recently. Substitution of the CI-MPR peptide in the VHS of GGA1 structure with a C-terminal peptide of memapsin 2 by modeling produced an excellent fit (results not shown). The interactions of all three essential residues of the peptide with VHS residues are retained. Although the memapsin 2 peptide is one residue shorter at the C-terminus when aligned with the CI-MPR peptide (Table 1), the C-terminal carboxyl group of Lys^{501} is free to interact with the side chain of Lys^{101} and Tyr^{102} of VHS and thus be stabilized. Ile^{497} of memapsin 2 is an interesting residue in this structural comparison. The corresponding residue in CI-MPR is a Glu located away from the binding interface and having contact only with solvent molecules but not with the VHS residues. Ile^{497} is a hydrophobic residue that seems unsuited for such a position. However, the side chain of Ile^{497} in the model of M2 complex with VHS [21] is not within the distance for hydrophobic interaction with any residue of the protein. Whether this surface isoleucine residue is involved in other recognition functions remains to be seen.

In contrast to memapsin 2, the cytosolic peptide of memapsin 1 did not bind GGA1 or GGA2. Although memapsin 1 is the closest homologue of memapsin 2 (about 50% residue identity) and also a class I membrane protein, the absence of GGA binding is not unexpected in view that its C-terminal region does not contain the essential dileucine/acidic cluster motif (Table 1). These observations suggest that memapsin 1 may have a mechanism of recognition for intracellular transport different from that of memapsin 2. Even though these two proteases have very close specificities [24] and are both present in many cell types [1,2], the possible difference in transport destination may result in different cellular localizations and roles.

Since memapsin 2 performs the first cleavage in APP leading to the production of Aβ and the pathogenesis of Alzheimer's disease, it seems possible that the interaction of memapsin 2 C-terminal region with GGAs can be a target for drugs which disrupt this process and thus the production of Aβ. However, there are serious questions concerning this drug target. It is clear that GGAs interact with many membrane proteins so selectivity for such disruption may indeed be very challenging. Although the transport of memapsin 2 and APP to endosomes has been shown to lead to the β-secretase cut and Aβ production [9], the production of Aβ in the endoplasmic reticulum and Golgi has also been reported [25,26]. Such reaction sites would not require the GGA-mediated memapsin 2 transport. However, as discussed in Section 1, endoplasmic reticulum and Golgi do not have sufficiently high acidity to support a significant memapsin 2 activity. At any rate, the validity of this interaction as a drug target will need further verification.

Acknowledgements: We thank Marcus Dehdarani and Li Liu for technical assistance, Dr. M.S. Robinson for the cDNA clones of GGA1 and GGA2 and Dr. Cai Zhang for helpful discussions of this work. This work was in part supported by NIH Grant AG-18933 and the Pioneer Award from the Alzheimer's Association to J.T. G.K. is a Scientist Development Grant Awardee of the American Heart Association. J.T. is holder of the J.G. Puterbaugh Chair in Biomedical Research at the Oklahoma Medical Research Foundation.

References

[1] Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A. and Tang, J. (2000) Proc. Natl. Acad. Sci. USA 97, 1456–1460.
[2] Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.C., Collins, F., Treanor, J., Rogers, G. and Citron, M. (1999) Science 286, 735–741.
[3] Hussain, I., Powell, D.J., Howlett, D.R., Chapman, G.A., Gilmour, L., Murdock, P.R., Tew, D.G., Meek, T.D., Chapman, C., Schneider, K., Ratcliffe, S.J., Tattersall, D., Testa, T.T., Southan, C., Ryan, D.M., Simmons, D.L., Walsh, F.S., Dingwall, C. and Christie, G. (1999) Mol. Cell. Neurosci. 14, 419–427.
[4] Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashier, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E., Carter, D.B., Tomasselli, A.G., Parodi, L.A., Heinrikson, R.L. and Gurney, M.E. (1999) Nature 402, 533–537.
[5] Selko, D. (2001) Physiol. Rev. 81, 741–766.
[6] Bennett, B.D., Denis, P., Haniu, M., Teplow, D.B., Kahn, S., Louis, J.C., Citron, M. and Vassar, R. (2000) J. Biol. Chem. 275, 37712–37717.
[7] Capell, A., Steiner, H., Willem, M., Kaiser, H., Meyer, C., Walter, J., Lammich, S., Multhaup, G. and Haass, C. (2000) J. Biol. Chem. 275, 30849–30854.
[8] Creemers, J.W., Ines Dominguez, D., Plets, E., Serneels, L., Taylor, N.A., Multhaup, G., Craessaerts, K., Annaert, W. and De Strooper, B. (2001) J. Biol. Chem. 276, 4211–4217.

[9] Huse, J.T., Pijak, D.S., Leslie, G.J., Lee, V.M. and Doms, R.W. (2000) J. Biol. Chem. 275, 33729–33737.
[10] Mellman, I. (1996) Annu. Rev. Cell Dev. Biol. 12, 575–625.
[11] Rothman, J.E. and Wieland, F.T. (1996) Science 272, 227–234.
[12] Pastorino, L., Ikin, A.F., Nairn, A.C., Pursnani, A. and Buxbaum, J.D. (2002) Mol. Cell. Neurosci. 19, 175–185.
[13] Lohi, O., Poussu, A., Mao, Y., Quiocho, F. and Lehto, V. (2002) FEBS Lett. 513, 19–23.
[14] Puertollano, R., Aguilar, R.C., Gorshkova, I., Crouch, R.J. and Bonifacino, J.S. (2001) Science 292, 1712–1716.
[15] Zhu, Y., Doray, B., Poussu, A., Lehto, V. and Kornfeld, S. (2001) Science 292, 1716–1718.
[16] Takatsu, H., Katoh, Y., Shiba, Y. and Nakayama, K. (2001) J. Biol. Chem. 276, 28541–28545.
[17] Nielsen, M.S., Madsen, P., Christensen, E.I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R. and Petersen, C.M. (2001) EMBO J. 20, 2180–2190.
[18] Hirst, J., Lui, W.W., Bright, N.A., Totty, N., Seaman, M.N. and Robinson, M.S. (2000) J. Cell Biol. 149, 67–80.
[19] Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330.
[20] Kirchhausen, T. (2002) Nature Struct. Biol. 9, 241–244.
[21] Lohi, O. and Lehto, V.-P. (1998) FEBS Lett. 440, 255–257.
[22] Shiba, T., Takatsu, H., Nogi, T., Matsugaki, N., Kawasaki, M., Igarashi, N., Suzuki, M., Kato, R., Earnest, T., Nakayama, K. and Wakatsuki, S. (2002) Nature 415, 937–941.
[23] Misra, S., Puertollano, R., Kato, Y., Bonifancino, J.S. and Hurley, J.H. (2002) Nature 415, 933–937.
[24] Turner III, R., Loy, J.A., Nguyen, C., Devasamudram, T., Ghosh, A., Koelsch, G. and Tang, J. (2002) Biochemistry 41, 8742–8746.
[25] Chyung, A.S.C., Greenberg, B.D., Cook, D.G., Doms, R.W. and Lee, V.M. (1997) J. Cell Biol. 138, 671–680.
[26] Xia, W., Zhang, J., Ostaszewski, B.L., Kimberly, W.T., Seubert, P., Koo, E.H., Shen, J. and Selkoe, D.J. (1998) Biochemistry 37, 16465–16471.