

Export of Cellubrevin from the Endoplasmic Reticulum Is Controlled by BAP31

Wim G. Annaert,* Bernd Becker,‡ Ute Kistner,* Michael Reth,‡ and Reinhard Jahn*

*Howard Hughes Medical Institute and Department of Pharmacology and Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and ‡Max-Planck-Institut für Immunologie, D-79108 Freiburg, Germany

Abstract. Cellubrevin is a ubiquitously expressed membrane protein that is localized to endosomes throughout the endocytotic pathway and functions in constitutive exocytosis. We report that cellubrevin binds with high specificity to BAP31, a representative of a highly conserved family of integral membrane proteins that has recently been discovered to be binding proteins of membrane immunoglobulins. The interaction between BAP31 and cellubrevin is sensitive to high ionic strength and appears to require the transmembrane regions of both proteins. No other proteins of liver membrane extracts copurified with BAP31 on immobilized recombinant cellubrevin, demonstrating that the interaction is specific. Synaptobrevin I bound to BAP31 with comparable affinity, whereas only weak binding was detectable with synaptobrevin II. Furthermore, a fraction of BAP31 and cellubrevin was complexed when each of them was quantitatively immunoprecipitated from detergent extracts of fibroblasts

(BHK 21 cells). During purification of clathrin-coated vesicles or early endosomes, BAP31 did not cofractionate with cellubrevin. Rather, the protein was enriched in ER-containing fractions. When BHK cells were analyzed by immunocytochemistry, BAP31 did not overlap with cellubrevin, but rather colocalized with resident proteins of the ER. In addition, immunoreactive vesicles were clustered in a paranuclear region close to the microtubule organizing center, but different from the Golgi apparatus. When microtubules were depolymerized with nocodazole, this accumulation disappeared and BAP31 was confined to the ER. Truncation of the cytoplasmic tail of BAP31 prevented export of cellubrevin, but not of the transferrin receptor from the ER. We conclude that BAP31 represents a novel class of sorting proteins that controls anterograde transport of certain membrane proteins from the ER to the Golgi complex.

EXOCYTOTIC membrane fusion is mediated by a complex of evolutionary-conserved membrane proteins. In neurons, these proteins include the synaptic vesicle protein synaptobrevin (VAMP) and the synaptic membrane proteins syntaxin and synaptosome-associated protein (SNAP)-25.¹ These proteins undergo regulated

protein-protein interactions that are controlled by soluble proteins including *N*-ethylmaleimide-sensitive factor (NSF) and soluble *N*-ethyl maleimide-sensitive factor attachment (SNAP) proteins (Söllner et al., 1993b). Relatives of all of these proteins have been discovered in many eukaryotic cells including yeast, suggesting that intracellular membrane fusions may, at least to a large extent, be mediated by common mechanisms (Ferro-Novick and Jahn, 1994; Rothman, 1994; Scheller, 1995). Although the molecular details of membrane fusion are not yet understood, it is becoming clear that the components of the fusion apparatus operate by conformation-dependent assembly and disassembly reactions which ultimately lead to the rearrangement of membrane phospholipids (Söllner et al., 1993a; Calakos et al., 1994). For these reasons, the interactions between synaptobrevin, SNAP-25, and syntaxin have received considerable attention (for review see Südhof, 1995). These proteins form a tight and stable ternary complex as soon as they have access to each other. Binding probably occurs before or during vesicle docking in preparation for fusion. Incubation with the ATPase NSF and SNAP proteins reversibly disassembles this complex, an

W.G. Annaert's current address is Experimental Genetics Group, Center for Human Genetics, Herestraat 49, B-3000 Leuven, Belgium.

U. Kistner's current address is Institut für Anatomie, Medizinische Fakultät (Charité), Humboldt-Universität, Schumannstr. 20-22, D-10098 Berlin, Germany.

Address all correspondence to Reinhard Jahn, Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany. Tel.: (49) 551.201.1635. Fax: (49) 551.201.1639. E-mail: rjahn@gwdg.de

1. *Abbreviations used in this paper:* aa, amino acids; AP, alkaline phosphatase; COP, coat proteins; DTAF, 5-(4,6-dichlorotriazinyl) amino fluorescein; ECL, enhanced chemiluminescence; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; MTOC, microtubule organizing center; PDI, protein disulfide isomerase; PNS, postnuclear supernatant; SCAMP, secretory carrier membrane proteins; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25,000 kD.

event thought to precede membrane fusion (Söllner et al., 1993a,b).

It is less well understood to what extent synaptobrevin, SNAP-25, and syntaxin interact with other proteins, particularly during stages of their life cycle when they are not bound to each other. It is conceivable that companion proteins exist that assist in sorting to the correct compartment or in positioning at the site of release and that control the availability for entering the fusion complex. For syntaxin, interactions with several other proteins were reported, including synaptotagmin munc-18/rbSEC-1, and the N-type Ca²⁺-channel (Südhof, 1995). For synaptobrevin, it has recently been observed that most of the protein is associated with synaptophysin, an integral membrane protein of yet unknown function that resides alongside synaptobrevin in the synaptic vesicle membrane (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). Although the binary interaction of synaptobrevin with synaptophysin is weaker than its ternary interaction with syntaxin and SNAP-25, synaptophysin-bound synaptobrevin is not available for binding to these proteins (Edelmann et al., 1995). Thus, synaptobrevin participates at least in two different complexes that are mutually exclusive: one with its partners syntaxin and SNAP-25 during membrane fusion, and another with synaptophysin during vesicle recycling and probably also during biogenesis, i.e., during transport of the proteins from the ER to the nerve terminal.

It remains to be established whether cellubrevin, a non-neuronal synaptobrevin homologue with widespread distribution, forms partnerships with other proteins with properties similar to the synaptobrevin-synaptophysin complex. Like synaptobrevins, cellubrevin is a small integral membrane protein with a single transmembrane domain at the COOH-terminal end of the molecule. Cellubrevin colocalizes with the transferrin receptor in fibroblasts and is enriched in purified clathrin-coated vesicles (McMahon et al., 1993), suggesting that it resides in constitutive trafficking vesicles shuttling mainly between the plasmalemma and the endosomal compartment (Daro et al., 1996). Like its neuronal counterparts, cellubrevin is selectively cleaved by clostridial neurotoxins including tetanus toxin. Toxin cleavage impairs exocytosis of recycling vesicles in fibroblasts (Galli et al., 1994), whereas fusion of early endosomes appears not to be affected (Link et al., 1993; Jo et al., 1995).

Here we report that cellubrevin interacts specifically with a recently characterized integral membrane protein, BAP31. BAP31 and a related protein (BAP29) were first identified as membrane proteins copurifying with membrane-bound immunoglobulin from lysates of β lymphocytes (Kim et al., 1994). Cloning of human and murine BAP31 cDNA showed that BAP31 is an evolutionary-conserved protein which is ubiquitously expressed in all tissues (Adachi et al., 1996). Several open reading frames encoding for proteins with a similar structure and a significant degree of homology are present in the genome of the yeast *Saccharomyces cerevisiae*, suggesting that BAP31 represents an ancient protein family with basic functions (EMBL/GenBank/DBJ accession numbers Z28065, Z74120, and Z48502). BAP31 has a hydrophobic NH₂ terminus with three potential transmembrane domains and a charged α -helical COOH terminus that is exposed to the cytoplasm. The COOH terminus ends with a KKXX sequence motif typical for proteins

transported back to the ER. Indeed, an immunocytochemical analysis revealed that BAP31 exhibits an ER-like staining pattern (Becker, B., and M. Reth, unpublished observations). We show that BAP31, as a resident of the ER and of ER-derived trafficking vesicles, may control the export of cellubrevin from the ER.

Materials and Methods

Antibodies

To generate cellubrevin antibodies, a cDNA was constructed encoding the NH₂-terminal cytoplasmic part of cellubrevin (amino acids [aa] 1–81) devoid of its transmembrane anchor (ceb-cyt). The PCR product was ligated into pTrcHis (Invitrogen, Carlsbad, CA), resulting in a fusion protein containing the amino terminal His(6) tag. After expression in *E. coli*, the protein was extracted and purified on a nickel resin (Probond; Invitrogen) as described in Chapman et al. (1994). Bound proteins were eluted with a gradient of 0–500 mM imidazole. Fractions containing the fusion protein were pooled, dialyzed against PBS, and concentrated before immunization.

The same domain of cellubrevin was expressed as glutathione-S-transferase (GST)-fusion protein and purified on glutathione-Sepharose. 20 mg of purified fusion protein were coupled to 1 g (dry weight) CNBr-Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech., Piscataway, NJ), and used for affinity purification of the antibody. Bound IgGs were eluted with 0.1 M glycine (pH 2.7), neutralized with 1 M Tris (pH 9), dialyzed against PBS, and concentrated (Centricon 30; Amicon Inc., Bedford, MA) to yield a final protein concentration of 2–4 mg/ml. For some experiments, the affinity-purified antibodies were biotinylated using sulfo-NHS-Lc biotin (EZ-Link; Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Rabbit antibodies against BAP31 were raised using the COOH-terminal cytoplasmic half of the protein (BAP31, aa 137–246) fused to GST as the antigen and then purified with Rivanol (Hoechst, Frankfurt, Germany; Franek, 1986; Adachi et al., 1996). To remove antibodies reacting with GST, the antibody (10 μ l) was diluted (1:2,000) in TBS with Tween-20 [0.1%] and 5% dry milk and then incubated overnight with strips of nitrocellulose loaded with 125 μ g of recombinant GST by means of SDS-PAGE and electrotransfer.

The following antibodies have been described previously: monoclonal antibodies against Rab3 (clone 42.1, Matteoli et al., 1991), and Rab 5 (clone 621.1–3, Fischer von Mollard et al., 1994). Monoclonal antibodies against the transferrin receptor, secretory carrier membrane proteins (SCAMP) (Brand et al., 1991), protein disulfide isomerase (PDI) (1D3, Vaux et al., 1990), and endoplasmic reticulum-Golgi intermediate compartment (ERGIC)-53 (Schweizer et al., 1988) were given by I. Trowbridge (Salk Institute, San Diego, CA), J.D. Castle (University of Virginia, Charlottesville, VA), S. Fuller (EMBL, Heidelberg, Germany), and H.-P. Hauri (Biocenter, University of Basel, Basel, Switzerland), respectively. The polyclonal antibody against p58 was a gift of J. Saraste (University of Bergen, Bergen, Norway). Antibodies to β -coat proteins (COP) were provided by T. Kreis (University of Geneva, Geneva, Switzerland). The polyclonal antibody against calnexin was a gift of A. Helenius (Yale University, New Haven, CT). Monoclonal anti-*myc* (9E10) ascites fluid was purchased from Berkeley Antibody Co. (Berkeley, CA). All donkey anti-rabbit or donkey anti-mouse secondary antibody- and streptavidin-conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Expression Vectors and Recombinant Proteins

cDNAs encoding rat synaptobrevin I, II, and cellubrevin were provided by T.C. Südhof (University of Texas, Dallas, TX). Full-length or truncated (see above) coding regions were amplified using the PCR with oligonucleotides containing BamHI and EcoRI restriction sites. The PCR products were further cloned into the BamHI-EcoRI sites of the pGex-2T vector (Pharmacia Biotech, Inc.). Fusion proteins were expressed in *E. coli* strain JM109 and purified as described in Chapman et al. (1994). Immobilized proteins were analyzed by SDS-PAGE and Coomassie blue staining and then the concentration of the bound protein was determined by comparison with GST (3–4 μ g/ μ l beads). Recombinant fusion proteins were always used in subsequent binding assays.

An expression vector coding for full-length cellubrevin in pCMV2 (McMahon et al., 1993) was provided by T.C. Südhof. cDNA encoding a *myc*-tagged full-length cellubrevin was constructed using a sense primer including a BamHI restriction site, the nucleotide sequence encoding *myc*, and 12 nucleotides of the cellubrevin sequence and then amplified using PCR. The PCR product was subcloned in pCDNA3 and used for transfection of BHK-21 cells.

For expression, mouse BAP31 cDNA (full length) was cloned in the pA vector (Zhang et al., 1996). COOH-terminal-truncated or -mutated BAP31 coding sequences were obtained using the same sense primer with a SalI restriction site and the following antisense primers: 5' GCGG-GATCCTTAGACTGAGGGACCACGTAC 3' for the BAP31 minus 4 last amino acids (BAP31-KKKEE); 5' GCGGGATCCTTATTCTTTGG-TAAGGCCCTC 3' for the BAP31 minus 24 last amino acids (BAP31-24aa); 5' GCGGGATCCTTACTCCTCGTCTGCTGACTGAGGGACC-ACGTAC 3' for the BAP31 in which the two COOH-terminal lysines at -3 and -4 positions were changed in two series (BAP31 KK/SS). Finally, a construct was made encoding only the NH₂-terminal half of BAP31(aa 1–137) that contained a *myc* epitope at the COOH-terminal end (residue 137; *myc*-BAP31TMR). This cDNA was obtained by PCR using the following antisense primer: 5' GCGGGATCCTTAGTTCAGGTCCTCCT-CGCTGATCAGCTTCTGCTCAAAGGCTTCATT 3'. All antisense primers contain a BamHI restriction site. PCR products were subcloned into the SalI-BamHI sites of the expression vector pA and used for transfection in BHK-21 cells.

Recombinant wild-type and mutant BAP31 proteins were expressed by *in vitro* translation in the presence of radiolabeled methionine. cDNAs encoding full-length BAP31 and the NH₂ terminal half of BAP31 (*myc*-BAP31TMR) were placed under control of the T7 promoter by subcloning into the SalI-BamHI restriction site of pBlueScript SK+. Radiolabeled protein was generated by coupling *in vitro* transcription translation using the TnT system (Promega Corp., Madison, WI) in the presence of [³⁵S]methionine and microsomes according to the manufacturer's instructions. Vector without insert was used as a control. Aliquots (5 µl) were diluted 40-fold in extraction buffer (1% Triton X-100 in 75 mM KCl, 2 mM EDTA, and 20 mM Hepes-KOH, pH 7.4 [unless indicated otherwise]) and incubated for 1 h at 4°C. Extracts were cleared by centrifugation (at 90,000 *g*_{max}), for 20 min in a rotor (model TL100.3; Beckman and Dickinson Co., Mountain View, CA) and then used for binding assays with recombinant synaptobrevin fusion proteins (see below).

Binding Assays and Immunoprecipitation of Protein Complexes

For preparation of microsomal extracts, rat liver was homogenized in homogenization buffer (75 mM KCl [unless indicated otherwise] containing 20 mM Hepes-KOH, pH 7.3, and 2 mM EDTA) using a teflon-glass homogenizer and centrifuged at 15,000 *g*_{max} for 15 min. The low speed supernatant was spun at 230,000 *g*_{max} for 60 min in a Beckman TL100.3 rotor, and the resulting pellet was used for extraction. When BHK-21 cells were used, homogenization was done with a cell cracker (clearance 0.0010"). Pellets were stored at -70°C until use.

For the preparation of detergent extracts, the pellets were extracted for 1 h at 4°C in ice-cold homogenization buffer containing 1% Triton X-100 (1 mg/ml of protein) and centrifuged at 90,000 *g*_{max} for 20 min in a Beckman TL100.3 rotor to remove unsolubilized material. 40 µl of glutathione-Sepharose (60% suspension in extraction buffer) containing the immobilized fusion proteins were added to 1 ml extract and incubated overnight (4°C) under slow rotation. The beads were washed four times in extraction buffer and resuspended in a small volume of PBS. To elute bound proteins, the immobilized fusion proteins were cleaved from the beads using two NIH U/thrombin (Sigma Chemical Co., St Louis, MO) per 100 µl beads for 2 h at room temperature. The supernatant was adjusted to 1 mM phenylmethylsulfonyl fluoride, and 1:10 vol was analyzed by SDS-PAGE followed by silver staining or immunoblotting.

For immunoprecipitation, BHK-21 cells (control or transfected) were homogenized in homogenization buffer and centrifuged at 1,300 *g*_{max} for 15 min. The postnuclear supernatant (PNS) was extracted by adding Triton X-100 (final concentration 1%, vol/vol) for 1 h at 4°C and then centrifuging at 90,000 *g*_{max} for 20 min in a Beckman TL100.3 rotor. 10 µl of anti-*myc* ascites, 15 µl of affinity-purified anti-cellubrevin, or 25 µl of anti-BAP31 (whole IgG fraction) were added to 200–250 µl of extract (1 mg protein/ml), followed by overnight incubation (4°C). These amounts of antibody were sufficient for quantitative depletion of the antigen. Next, 30–40 µl of protein G-Sepharose slurry (Pharmacia Biotech Inc.) were

added and then the incubation was continued for 1.5 h at 4°C. The beads were washed four times, resuspended in SDS-sample buffer free of reducing agents, and analyzed by SDS-PAGE and immunoblotting.

Subcellular Fractionation

All fractionation steps occurred at 4°C and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin A) was added freshly to all homogenates.

Clathrin-coated vesicles were purified from rat liver as described previously (Maycox et al., 1992). For enrichment of early endosomes, BHK-21 cells were homogenized in 0.25 M sucrose containing 3 mM imidazole, pH 7.4 (HB) using a ball-bearing homogenizer (eight passages, 0.0009" clearance). Fractionation was carried out according to Gorvel et al. (1991).

Fractions enriched in intermediate compartment and ER were obtained from BHK-21 cells exactly as described by Schweizer et al. (1991), except that Nycodenz instead of Metrizamide (both from GIBCO BRL, Gaithersburg, MD) was used in the final gradient centrifugation step.

Cell Culture, Transfection, and Immunocytochemistry of BHK-21 Cells

BHK-21 cells were routinely grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine, Pen/Strep (1:100 final dilution; GIBCO BRL, Gruenberg et al., 1989). Tissue culture reagents were from GIBCO BRL. For immunocytochemistry, confluent cells were split (1:10), plated on glass coverslips, and allowed to grow for 18–20 h.

For transfection experiments BHK-21 cells were split (1:10) 1 d before transfection. The next day, cells were cotransfected with the vectors, encoding for *myc*-tagged cellubrevin and the different BAP31 constructs, or for full-length cellubrevin and *myc*-BAP31TMR using Lipofectamine (GIBCO BRL) exactly as described by the manufacturer. After transfection, cells were grown for 24 h in normal medium, plated on glass coverslips, and grown for another 18–20 h. When indicated, cells were treated with 33 µM nocodazole (Sigma Chemical Co.) for 2 h before fixation. Nontransfected cells were grown on glass coverslips to 50–60% confluency. Cells were rinsed three times for five min with PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ (PBS²⁺), fixed for 5 min in methanol at -20°C and then followed by treatment with acetone for 30 s at -20°C. The coverslips were then rinsed several times in PBS²⁺ and processed immediately for double labeling immunocytochemistry. All subsequent blocking and incubation steps with primary and secondary antibodies were done in PBS²⁺ containing 8% goat serum. For double labeling with mouse monoclonal and rabbit polyclonal antibodies, the cells were incubated with a mixture of both primary antibodies followed by washing three times in PBS²⁺ and then subsequently incubated with both secondary antibodies (5-(4,6-dichlorotriazinyl) amino fluorescein (DTAF) in combination with CY3- or lissamine-rhodamine conjugates). When biotinylated rabbit anti-cellubrevin antibody was used for double labeling, the cells were first incubated with polyclonal anti-BAP31 and CY3-conjugated donkey anti-rabbit antibodies, followed by washing and blocking with PBS²⁺ supplemented with 10% rabbit serum for 1 h. After rinsing with PBS²⁺, the cells were incubated in biotinylated anti-cellubrevin followed by FITC-conjugated streptavidin. Controls were included where either one of the first or second antibodies was omitted. At the final step cells were rinsed in PBS and distilled water and mounted in VectaShield (Vector Labs, Inc., Burlingame, CA). Confocal laser scanning microscopy was performed on a MRC-600 system (Bio-Rad Laboratories, Hercules, CA) attached to a compound microscope (Axiovert; Carl Zeiss, Inc., Thornwood, NY). Image files were converted using the Confocal Assistant software, and finally processed and annotated using Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA) and PowerPoint 6.0 (Microsoft Corporation, WA).

Miscellaneous Methods

SDS-PAGE (Laemmli, 1970) and immunoblotting (Towbin et al., 1979) was done according to established procedures. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) kit (Pierce Chemical Co.), DAB reaction in the case of HRP-conjugated second antibodies (Bio-Rad Laboratories) or by the alkaline phosphatase (AP) reaction when AP-conjugated second antibodies (Bio-Rad Laboratories) were used. The method of Heukeshoven and Dernick (1985) was used for

silver staining of the minigels. Proteins were quantitated according to the method of Bradford (1976), following the manufacturer's instructions (Bio-Rad Laboratories).

Results

Identification of BAP31 As a Major Binding Protein for Cellubrevin

To search for proteins interacting with cellubrevin, recombinant GST–cellubrevin fusion protein was immobilized on glutathione–Sepharose and incubated with Triton X-100 extracts of BHK-21 cells. After washing, cellubrevin was released by thrombin cleavage to recover bound proteins. The eluted proteins were analyzed by SDS-PAGE and silver staining. A band with an apparent molecular mass of ~30,000 eluted from immobilized GST–cellubrevin (Fig. 1 *a*, arrow). This band specifically interacts with cellubrevin because it was not detected when beads containing bound GST–synaptobrevin II (Fig. 1 *a*) or bound GST (data not shown) were used. No binding was observed in 450 mM NaCl, similar to the synaptobrevin–synaptophysin interaction (Edelmann et al., 1995). To isolate larger quantities, we incubated a Triton X-100 extract of liver membranes with immobilized GST–cellubrevin and eluted bound proteins with 450 mM NaCl. SDS-PAGE of the concentrated eluate, followed by Coomassie blue staining, revealed that the apparent molecular mass 30,000 band was the only major protein eluted from the column under these conditions (Fig. 1 *b*). The band was excised, further concentrated by electrophoresis (Lombard-Platet and Jalinot, 1993), and digested by trypsin followed by peptide analysis using HPLC and microsequencing. Two peptide sequences were obtained, VNLQNNPGAMEHFHML and AENEVLAMRK. Database searches revealed that the sequences matched with BAP31, an ubiquitously expressed integral mem-

brane protein that has previously been identified as a member of a group of proteins associated with B cell antigen receptor in β lymphocytes (Kim et al., 1994; Adachi et al., 1996).

Characterization of the BAP31–Cellubrevin Interaction

The interaction between cellubrevin and BAP31 was further characterized using antibodies specific for BAP31. To determine the influence of ionic strength on the interaction more precisely, immobilized GST–cellubrevin was incubated with extracts of BHK-21 cells or rat liver containing increasing concentrations of KCl. Binding of BAP31 decreased at KCl concentrations >140 mM and was no longer detectable at 450 mM (Fig. 2 *a*). Next, we tested BAP31 binding to GST-fusion proteins of synaptobrevin I, synaptobrevin II, cellubrevin, and to a cellubrevin-deletion mutant lacking the transmembrane domain (ceb-cyt). The latter mutant was chosen since we found previously that the binding of synaptobrevin to synaptophysin requires the presence of its transmembrane region (Edelmann et al., 1995).

Fig. 2 *b* shows that BAP31 binds not only to cellubrevin but also to synaptobrevin I. No binding to synaptobrevin II (in agreement with the data shown above) or ceb-cyt was observed. The lack of binding to synaptobrevin II is not because of inactivation of the protein, since binding of synaptophysin as well as SNAP-25 and syntaxin was observed when incubated with brain extracts (data not shown; Edelmann et al., 1995). Also, less BAP31 bound to synaptobrevin I when BHK21 cell extract was used instead of rat liver extract, possibly indicating some species difference between rat and hamster BAP31. To confirm the specificity of the interaction, we tested for several other membrane-bound proteins including the transferrin receptor, SCAMP (Brand et al., 1991), the small GTPases Rab3

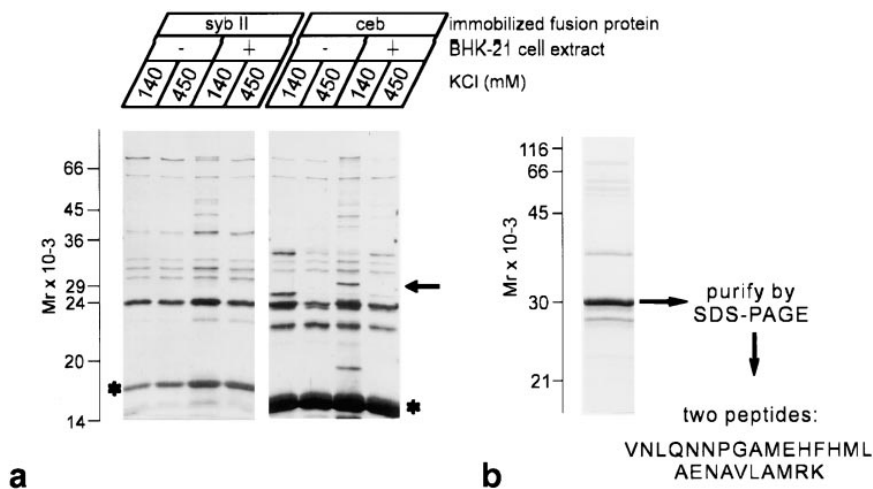


Figure 1. Identification of a major cellubrevin binding protein as BAP31. (A) Electrophoretic analysis of proteins of a Triton X-100 extract derived from BHK-21 cell membranes that bind to immobilized GST–synaptobrevin II (left) or GST–cellubrevin (right). Equal amounts of GST–fusion proteins were immobilized on glutathione–Sepharose and incubated with BHK-21 cell extracts (adjusted to 140 and 450 mM KCl, respectively) at 4°C overnight. After washing, the fusion proteins were released by thrombin cleavage and 10% of each sample was analyzed by SDS-PAGE (13% gel) and silver staining. Asterisks indicate the positions of synaptobrevin II and cellubrevin, respectively. The arrow points to a protein of 30 kD that eluted specifically from the cellubrevin

column only when extracts in 140 mM KCl were used. (b) Purification of the 30-kD protein by cellubrevin affinity chromatography using elution in high salt buffer. 2.5 ml of glutathione–Sepharose were saturated with GST–cellubrevin and incubated overnight at 4°C with 10 ml of a Triton X-100 extract of rat liver membranes (2 mg protein/ml). After washing, the bound protein was eluted with high salt buffer containing 1% Triton X-100, dialyzed against extraction buffer, and concentrated by ultrafiltration (Centricon 10). The purification procedure was repeated several times using fresh extract and the same column. The pooled and concentrated eluates were separated by preparative SDS-PAGE (15% gel). The bands corresponding to 30-kD protein were visualized by staining with Coomassie blue, excised, and further concentrated using a funnel web SDS-PAGE system (Lombard-Platet and Jalinot, 1993). The main band was excised and subjected to trypsin digestion, followed by peptide separation using RP-HPLC and microsequencing. Two peptide sequences were obtained as indicated.

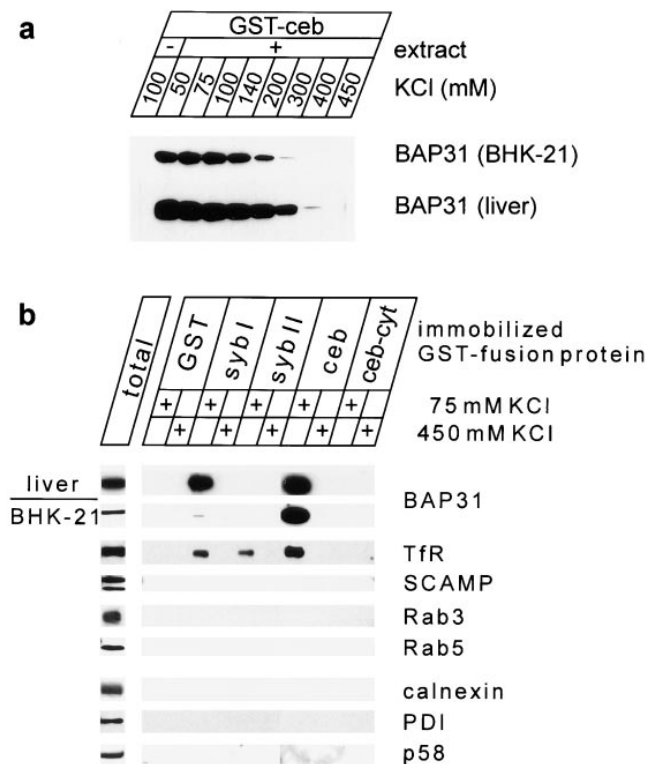


Figure 2. Properties and specificity of the cellubrevin–BAP31 interaction. (a) The interaction of BAP31 to GST–cellubrevin (*ceb*) is sensitive to high KCl concentrations. Triton X-100 extracts of BHK-21 cells or of rat liver membranes were prepared in the KCl concentrations indicated. Binding and washing (using the appropriate KCl buffers) were performed as described in Fig. 1. Bound proteins were released by thrombin cleavage and 10% of the eluates were extracted with SDS sample buffer for electrophoresis. The figure shows immunoblot analysis for BAP31. (b) BAP31 binds selectively to synaptobrevin I and cellubrevin, and requires the transmembrane domain of cellubrevin for binding. Binding assays using immobilized fusion proteins of synaptobrevin I (*syb I*), synaptobrevin II (*syb II*), cellubrevin (*ceb*) and the NH₂-terminal cytoplasmic part of cellubrevin (*ceb-cyt*) were performed as described in Fig. 1, except that the material eluted after thrombin cleavage was analyzed by immunoblotting (10% of each eluate/lane). The eluates were tested for the following proteins: transferrin receptor (*TfR*), secretory carrier associated membrane protein (*SCAMP*), Rab3 (all isoforms), Rab5, calnexin, *PDI*, and p58.

and Rab5, the ER residents calnexin, PDI, and the markers for the intermediate compartment, p58 and ERGIC-53. With exception of small quantities of the transferrin receptor, none of these proteins bound to the immobilized synaptobrevins.

To further study the binding of BAP31, recombinant [³⁵S]methionine-labeled BAP31 was generated by in vitro translation. As shown in Fig. 3 a, recombinant BAP31 bound to immobilized cellubrevin and this binding was salt dependent, very similar to the native protein. Furthermore, the recombinant protein showed the same preference for cellubrevin and synaptobrevin I, although in this case weak binding to GST–synaptobrevin II was detectable. Analogous to the native protein, recombinant BAP31 did not bind to cellubrevin lacking its transmembrane domain or to GST alone (Fig. 3 b, top). To investigate which domain of BAP31 is responsible for the interaction we con-

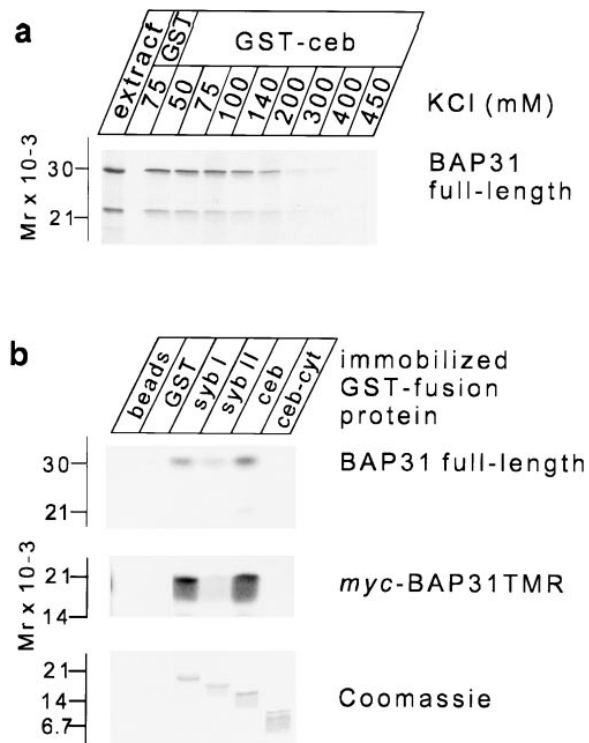


Figure 3. Binding of recombinant full-length and truncated BAP31 to cellubrevin and synaptobrevins. Recombinant [³⁵S]methionine labeled BAP31, either full-length or COOH-terminally truncated (*myc-BAP31TMR*), was generated by in vitro transcription translation. The translation mix was diluted ~20–25-fold in extraction buffer, and binding to immobilized fusion proteins was performed as in Fig. 1, except that bound [³⁵S]methionine labeled BAP31 was detected by autofluorography. (a) Binding of recombinant BAP31, like that of native BAP31, to GST–cellubrevin is sensitive to ionic strength. For binding, the translation mix was diluted in extraction buffer containing the indicated concentrations of KCl. Equal proportions of the starting material (load) and the bound material were analyzed. Note that a band with higher mobility was generated in addition to full-length BAP31, probably corresponding to a truncated version of BAP31. (b) Binding of full-length (top) and COOH-terminally truncated, myc-tagged BAP31 (middle) to immobilized GST–synaptobrevin fusion proteins. Fig. 2 shows details. (Bottom) A Coomassie blue-stained gel of GST–fusion protein eluted after thrombin cleavage, demonstrating that comparable amounts of immobilized fusion proteins were used in the binding assays.

structed, a BAP31 mutant which lacks the COOH-terminal cytoplasmic portion of the protein (*myc-BAP31TMR*). The interactions of the mutant protein were very similar to full-length BAP31, suggesting that the transmembrane regions of BAP31 are required for binding (Fig. 3 b, middle).

Comparison of the Subcellular Membrane Pools Containing BAP31 and Cellubrevin

The experiments described above demonstrate that the interaction between cellubrevin and BAP31 is very similar to that of synaptobrevin and synaptophysin suggesting that, analogous to the synaptophysin–synaptobrevin complex, BAP31 may serve as a companion for cellubrevin. However, cellubrevin is known to be concentrated on recycling vesicles distal of the Golgi complex, whereas BAP31 con-

tains a KKXX motif and, in preliminary experiments, appeared to reside primarily in the ER. Interestingly, BAP31 also contains a sequence motif (YDRL) that is known to be responsible for binding the medium chain of AP-2, the adaptor complex involved in recruiting clathrin molecules at the plasma membrane (Ohno et al., 1995), raising the possibility that at least a pool of BAP31 may reach post-Golgi compartments and colocalize with cellubrevin. We investigated, therefore, to what extent the subcellular localization of BAP31 overlaps with that of cellubrevin by means of subcellular fractionation and immunocytochemistry.

Subcellular fractionation was used as enrichment for recycling organelles in communication with the plasma membrane which are known to contain cellubrevin (McMahon et al., 1993; Galli et al., 1994). First, clathrin-coated vesicles were purified from rat liver (Maycox et al., 1992). When the enrichment of cellubrevin and BAP31 during fractionation was monitored by immunoblotting, a clear dissociation between the two proteins was observed (Fig. 4 *a*). Cellubrevin is highly enriched in clathrin-coated vesicles, in agreement with earlier observations (McMahon et al., 1993). However, virtually no BAP31 was detected in the coated vesicle fraction. Second, fractions enriched in early endosomes were prepared by flotation density gradient centrifugation (Gorvel et al., 1991). As expected, cellubrevin and two additional constituents of early endosomes, the small GTPase Rab5 and the transferrin receptor, were enriched in the early endosomal fraction (Fig. 4 *b*). In contrast, most of the BAP31 was recovered in the low density interface of the gradient.

To enrich the ER and intermediate compartment, we used consecutive Percoll and Nycodenz gradient centrifugation steps (*F*₃; Schweizer et al., 1991). As shown in Figure 4 *c*, BAP31 cofractionated with proteins p58 and ERGIC-53, two constituents of the intermediate compartment, and with ER proteins PDI and calnexin. In contrast, cellubrevin, SCAMP, and the transferrin receptor, proteins known to localize mainly in compartments at the proximal side of the Golgi complex, were enriched in the *F*₂ rather than in the *F*₃ fraction.

Together, these results suggest that despite their biochemical interaction, the majority of BAP31 and cellubrevin reside on different organelles. To identify these organelles more precisely, we used immunocytochemistry to compare the localization of BAP31 and cellubrevin in BHK-21 cells with each other, as well as with PDI and the transferrin receptor, established markers for the ER and recycling endosomes, respectively. PDI mediates the folding of newly synthesized proteins in the lumen of the ER. It is characterized by the COOH-terminal KDEL ER retention signal and, consequently, its distribution is restricted to the ER (Vaux et al., 1990).

In methanol-fixed BHK-21 cells, BAP31 immunolabeling resulted in a reticular staining pattern that overlapped perfectly with the staining obtained for PDI (Fig. 5, *first row*). However, BAP31 staining was extended to a dense cluster of dots in a paranuclear region devoid of PDI staining. Treatment with 33 μ M nocodazole, a drug that depolymerizes microtubules, for 2 h at 37°C led to the disappearance of the paranuclear cluster and an almost complete colocalization of BAP31 with PDI (Fig. 5, *second row*). The transferrin receptor did not colocalize with BAP31,

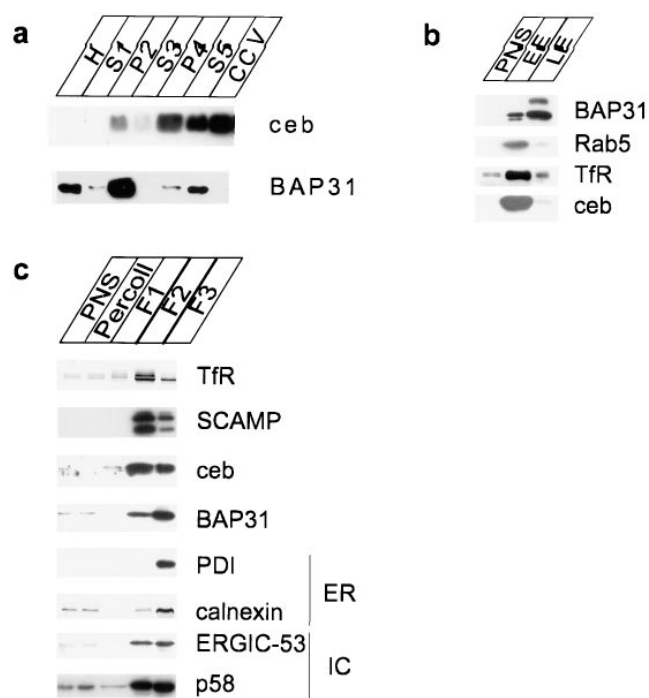


Figure 4. Subcellular fractionation reveals differential distribution of BAP31 and cellubrevin. (*a*) BAP31 does not coenrich with cellubrevin (*ceb*) during purification of clathrin-coated vesicles from rat liver. Homogenates (*H*) were centrifuged for 20 min at 20,000 g_{max} . The supernatant (*S*₁) was centrifuged again at 55,000 g_{max} for 1 h and then the pellet (*P*₂) was resuspended and recentrifuged after adding ficoll and sucrose. The supernatant (*S*₃) was diluted and centrifuged at 100,000 g_{max} for 1 h. This pellet, *P*₄, was again resuspended and centrifuged at 20,000 g_{max} for 20 min. The resulting supernatant *S*₅ was layered on top of a D₂O–sucrose density gradient. The pellet obtained after centrifugation at 110,000 g_{max} for 2 h contained purified coated vesicles (Maycox et al., 1992). Of each fraction, 10 μ g of protein were analyzed by SDS-PAGE and immunoblotting for cellubrevin and BAP31. (*b*) BAP31 does not coenrich with cellubrevin during purification of early endosomes (*EE*). Analysis of endosomal fractions. A *PNS* of BHK-21 cells was subjected to flotation gradient centrifugation on a discontinuous D₂O–sucrose gradients (Gorvel et al., 1991), resulting in a fraction enriched in *EE* and a fraction enriched in late endosomes and carrier vesicles (*LE*). The gradient fractions were diluted and pelleted by centrifugation. Of each fraction, 10 μ g of protein were analyzed by SDS-PAGE and immunoblotting for cellubrevin and BAP31, and for the *EE* markers *TfR* and *Rab5*. (*c*) BAP31 coenriches with markers of the intermediate compartment and the ER. Fractionation of ER and intermediate compartment. A *PNS* was mixed with Percoll to give a final density of 1.129 g/ml, and then centrifuged. A midportion of the gradient was pooled (*Percoll*), adjusted to 30% Nycodenz, and overlaid with 27 and 18.5% (wt/wt) of Nycodenz. After equilibrium density gradient centrifugation, three interfaces were collected (*F*₁, *F*₂, *F*₃) and analyzed by SDS-PAGE and immunoblotting (10 μ g of protein per lane). ER markers (*PDI* and *calnexin*) are highly enriched in the *F*₃ fraction, together with markers for the *IC* (*ERGIC-53* and *p58*). BAP31 immunoreactivity was recovered mainly in this interface, in contrast to cellubrevin, SCAMP, and the transferrin receptor, which were enriched in the *F*₂ interface.

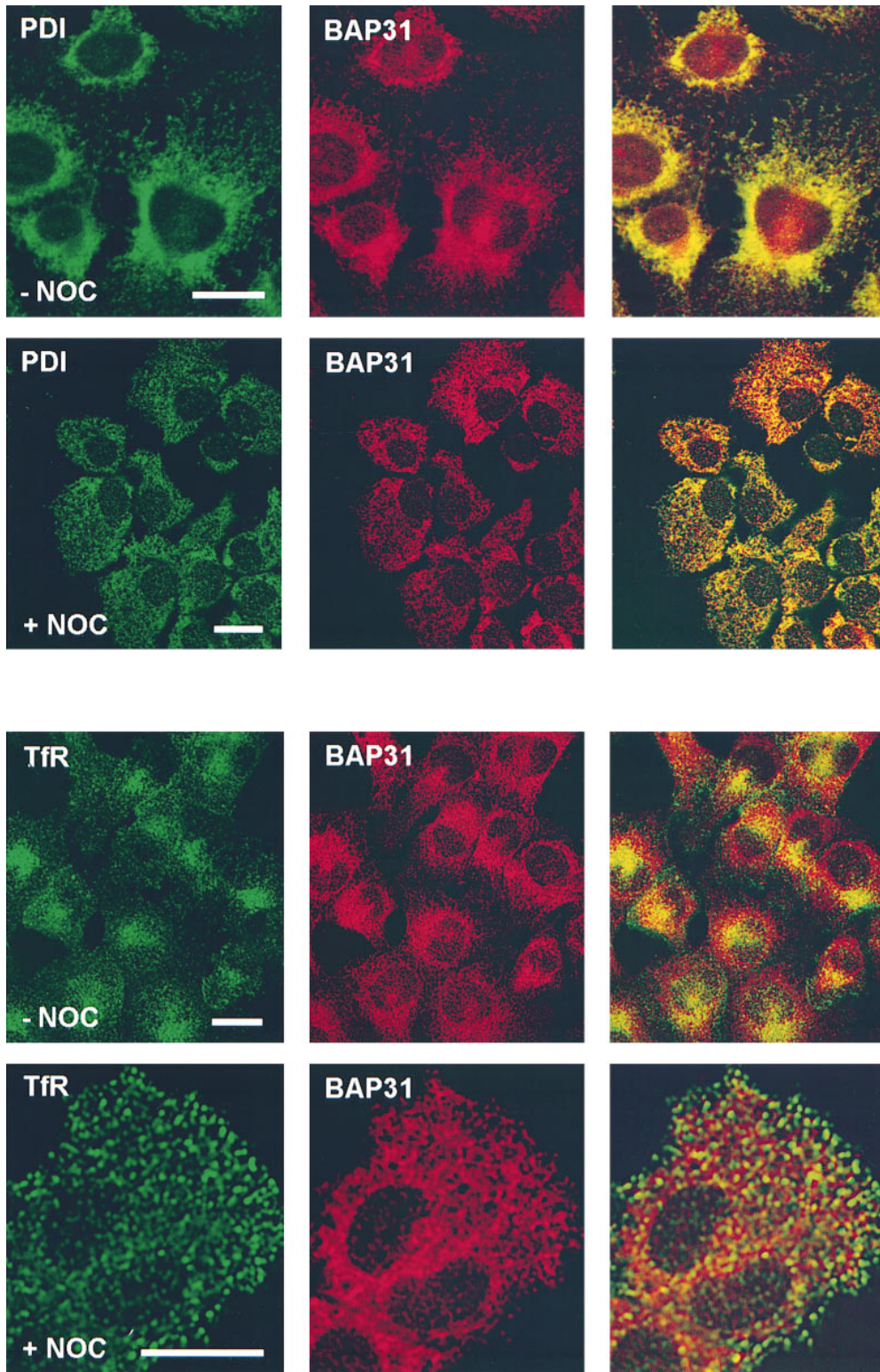


Figure 5. Localization of BAP31 in BHK-21 cells in comparison to markers of the ER (*PDI*) and the endosomal recycling compartment (*TfR*). Before fixation, cells were incubated for 2 h in the incubator in the absence (*-NOC*) or presence (*+NOC*) of nocodazole (33 μ M) to depolymerize microtubules. Cells were double stained with rabbit anti-BAP31 antibodies and mouse *PDI* antibodies (1D3 monoclonal antibody, *top rows*) or with rabbit anti-BAP31 antibodies and mouse anti-TfR monoclonal antibody (*bottom rows*), using DTAF-labeled donkey anti-mouse antibody (*green column*) and CY3-labeled donkey anti-rabbit antibody (*red column*) as secondary antibodies, respectively. Analysis was performed by confocal laser scanning microscopy. The third column in each row shows color overlays. Note that BAP31 staining colocalizes with *PDI* in the cell periphery, but extends to a paranuclear region where TfR-positive dots also accumulate, corresponding to vesicle clusters around the MTOC. This accumulation disappears upon nocodazole treatment. Bars, 20 μ m.

with exception of the paranuclear cluster where extensive overlap was observed. (Fig. 5, *third row*). This cluster probably corresponds to vesicles accumulating around the microtubule organizing center (MTOC) where the transferrin receptor is known to be concentrated (Trowbridge et al., 1993; Daro et al., 1996). To establish whether BAP31 and transferrin receptor are indeed overlapping in the same organelle population, cells were again treated with no-

codazole to disrupt the MTOC. Clearly different patterns were obtained: a punctate reticular staining for BAP31, and dispersed dots positive for the transferrin receptor. Taken together, we assume that BAP31 is an ER resident that probably shuttles between the ER and the intermediate compartment/*cis*-Golgi complex. The accumulation of BAP31-containing organelles may reflect accumulation of vesicles en route from the ER to the Golgi apparatus, a

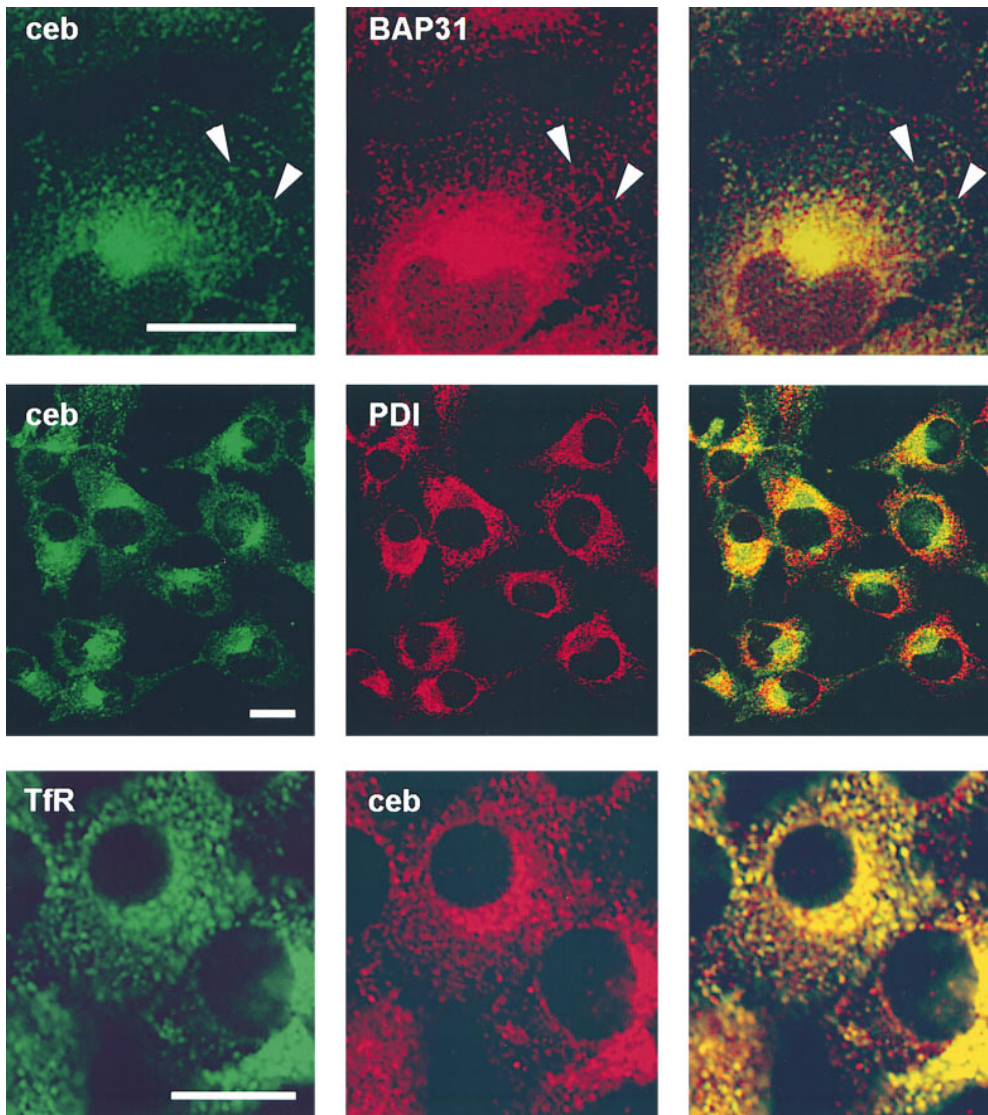


Figure 6. Localization of cellubrevin in BHK-21 cells in comparison to BAP31 and markers of the ER (*PDI*), and the endosomal recycling compartment (*TfR*). Fig. 5 shows details. For double labeling of BAP31 and cellubrevin (*top*), we used biotinylated cellubrevin antibodies that were affinity purified from rabbit serum. Visualization was with CY3-conjugated donkey anti-rabbit for BAP31 (*top*) and cellubrevin (*bottom*), or donkey anti-mouse for *PDI* (*middle*; red column) and with DTAF-labeled streptavidin for biotinylated cellubrevin antibodies (*top*), donkey anti-rabbit for cellubrevin (*middle*), and donkey anti-mouse for transferrin receptor (*TfR*, *bottom*; green column). Color overlays are on the right. Cellubrevin immunostaining is mainly clustered in a region devoid of *PDI* immunostaining. At higher magnification, peripheral localized cellubrevin immunostained dots colocalize with the peripheral reticular staining for BAP31 (*top*, arrowheads). Bar, 20 μ m.

pathway that is disrupted by nocodazole. Indeed, we observed some overlap between the staining patterns of BAP31 and β -coat protein (COP), a component of COP-coated vesicles mainly accumulating in the *cis*-Golgi network (Oprins et al., 1993; data not shown).

To perform double labeling for cellubrevin and BAP31, rabbit antibodies for cellubrevin were affinity purified and biotinylated. Cellubrevin immunoreactivity was concentrated in the area of the MTOC. Here it overlaps with BAP31, but in peripheral areas of the cell the staining patterns were different (Fig. 6, *top*), even though a weakly stained reticulum positive for cellubrevin with the reticular ER pattern typical for BAP31 staining was noticeable (Fig. 6, *top row*, arrowheads). Double labeling for cellubrevin and *PDI* (Fig. 6, *middle row*) confirms that the proteins have different distributions, with *PDI* being virtually excluded from the region of the MTOC. Only a minor overlap was observed between cellubrevin and β -COP in control and nocodazole-treated cells (data not shown). All spots positive for the transferrin receptor were also positive for cellubrevin (Fig. 6, *bottom*), in agreement with earlier results (McMahon et al., 1993; Galli et al., 1994; Daro et al., 1996).

Association of Endogenous BAP31 and Cellubrevin

The experiments described so far provided evidence that native BAP31 binds with high specificity and efficiency to recombinant cellubrevin, but also showed that the major pools of the two proteins are localized to different compartments. Therefore, we investigated whether complexes of native cellubrevin and BAP31 can be directly isolated from cell extracts using immunoprecipitation with affinity-purified antibodies specific for either cellubrevin or BAP31. Equal amounts of detergent extracts derived from untreated and nocodazole-treated BHK-21 cells were incubated with excess amounts of antibodies, resulting in the disappearance of cellubrevin and BAP31, respectively, from the extract (Fig. 7 *b*; data not shown). As shown in Fig. 7 *a* (*right lanes*), a considerable amount of cellubrevin coimmunoprecipitated with the anti-BAP31 antibody. This amount, however, constituted only a relatively small fraction of the cellubrevin pool in these extracts (compare the amount of cellubrevin precipitated with the anti-cellubrevin and anti-BAP31 antibodies, respectively) which probably reflects cellubrevin pools in the ER that are freshly synthesized

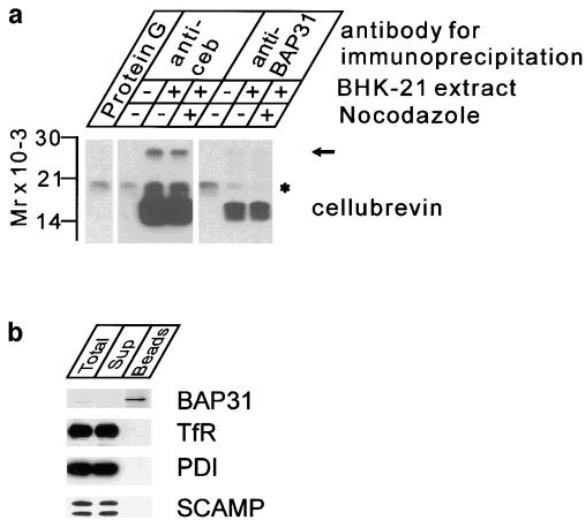


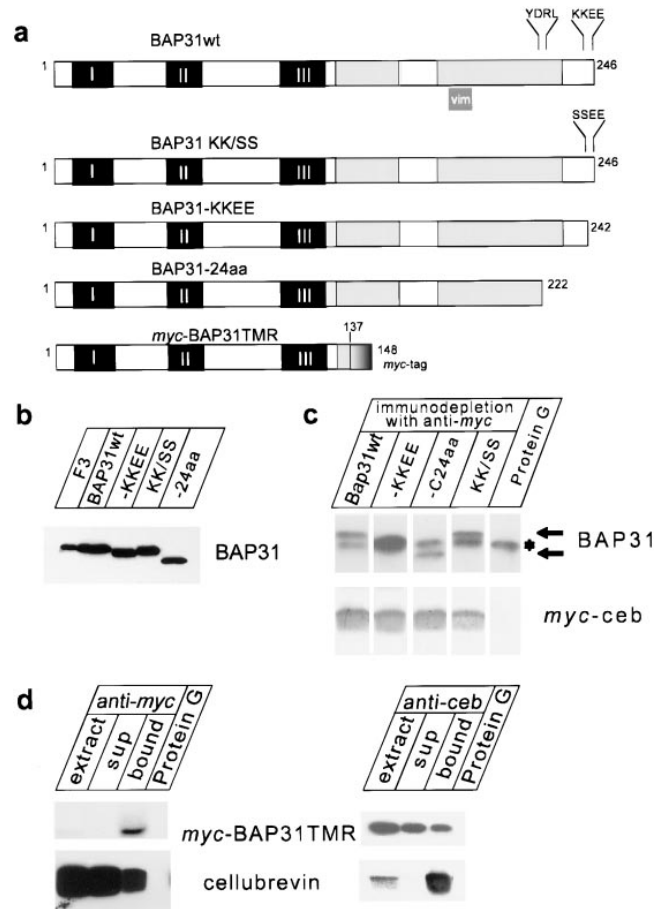
Figure 7. Cellubrevin coimmunoprecipitates with BAP31 in detergent extracts of BHK-21 cells. (a) Excess amounts of antibodies specific for cellubrevin (*anti-ceb*) and for BAP31 (*anti-BAP31*) were added to Triton X-100 extracts of control (–) and nocodazole (+)-treated BHK-21 cells before isolation of immune complexes using protein G–Sepharose. Equal proportions of each sample were analyzed for cellubrevin by immunoblotting using biotinylated affinity-purified anti-cellubrevin followed by HRP-conjugated streptavidin and visualization by ECL. The asterisk denotes a nonspecific band recognized by the detection system. No binding was observed when extracts were incubated with only protein G–Sepharose beads (*Protein G*). (b) Coimmunoprecipitation of cellubrevin with BAP31 is specific. Immune complexes were isolated from untreated BHK cell extracts as above using anti-cellubrevin antibodies. 20 μ g protein each of total (starting) extract (*Total*) and unbound supernatant (*Sup*), and 15% of the bead-bound immune complexes (*Beads*) were analyzed as above and probed for the TfR, PDI, and SCAMP. For BAP31, reducing agents were omitted for SDS-PAGE and visualization was performed with the AP method instead of the ECL method used for the other antigens.

and en route to the Golgi apparatus. Coprecipitation was specific since neither PDI nor TfRn or SCAMP, the latter two being integral membrane proteins colocalizing with cellubrevin, were detectable on the beads (Fig. 7 b). Also, no differences were observed when the cells were treated with the microtubule-disrupting agent nocodazole before extraction (Fig. 7 a), demonstrating that disruption of the MTOC does not affect the amount of the cellubrevin–BAP31 complex.

Perturbation of the Sorting of Cellubrevin by Site-directed Mutagenesis of BAP31

The picture emerging from the data presented above suggests that cellubrevin interacts with BAP31 only during

Figure 8. Mutation or truncation of the COOH-terminal tail of BAP31 does not affect its ability to bind cellubrevin. (a) Diagram showing the expression constructs of BAP31 that were used for cotransfection of BHK-21 cells with full-length, myc-tagged (b and c) or untagged cellubrevin (d). The three transmembrane regions are indicated with I, II, and III; the two gray areas correspond to areas with a predicted propensity to form coiled coils. *vim* indi-



icates: region of homology with vimentin. (b) Detection of BAP31 expression in PNSs (10 μ g protein/lane) of transfected BHK cells. Under these assay conditions, the endogenous protein was below the detection limit. As reference, 10 μ g of protein obtained from a fraction enriched in ER (F_3 fraction; Fig. 4) was analyzed in parallel. The mutants can be distinguished by small differences in electrophoretic mobility. (c) BAP31 mutants containing small changes or deletions in the cytoplasmic tail coprecipitate with cellubrevin. BHK-21 cells cotransfected with cDNAs encoding myc-tagged cellubrevin and either the wild-type or mutant BAP31 were extracted in extraction buffer, followed by quantitative immunoprecipitation using excess amounts of anti-myc monoclonal antibody. BAP31 mutants and myc–cellubrevin were detected by ECL and the diaminobenzidine reaction, respectively. An additional band was detectable in the BAP31 blots that is nonspecific (*asterisk*), and is discriminated from the BAP31 mutants by slight differences in mobility. No binding was observed when only protein G–Sepharose beads were used (*protein G*). (d) Cellubrevin coprecipitates with a BAP31 mutant protein lacking the entire COOH-terminal cytoplasmic tail (*myc-BAP31TMR*). Quantitative immunoprecipitation was performed using excess amounts of anti-myc monoclonal antibody (*left*) or affinity-purified anti-cellubrevin antibody (*right*). Approximately 5% of extract and supernatant (*sup*) and one-third of the bead-bound proteins (*bound*) were analyzed by SDS-PAGE and immunoblotting. Cellubrevin was detected using affinity-purified and biotinylated rabbit antibody. The immunoprecipitating antibody was visualized using AP conjugated to second antibodies (*myc-BAP31TMR*, *top left*), or to streptavidin (cellubrevin, *bottom right*). The coimmunoprecipitating protein was visualized using HRP conjugated to second antibodies (*myc-BAP31TMR*, *top right*) or to streptavidin (cellubrevin, *bottom left*), followed by ECL. No binding was observed when only protein G–Sepharose beads were used (*Protein G*).

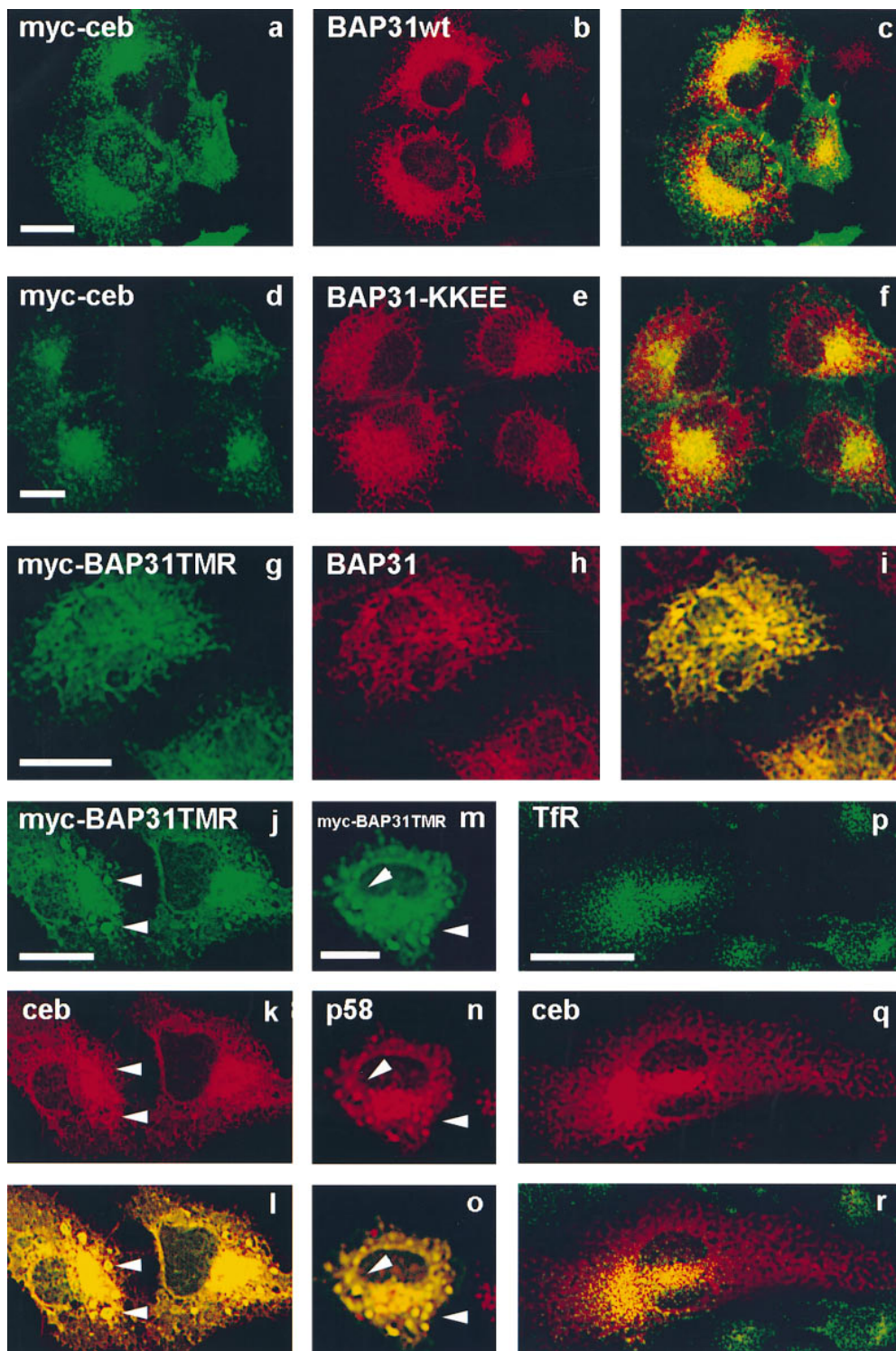


Figure 9. Truncation, but not any other mutation in the cytoplasmic tail of BAP31, results in selective retention of cellubrevin in the ER. BHK-21 cells were cotransfected with plasmids encoding myc-tagged cellubrevin (*myc-ceb*) and with wild-type BAP31 (*a-c*), BAP31-KKEE (*d-f*), or with plasmids encoding untagged cellubrevin (*ceb*) and myc-BAP31TMR (*g-r*). myc-tagged cellubrevin and myc-BAP31TMR were detected using mouse myc monoclonal antibodies. The secondary reagents were: DTAF-labeled donkey anti-mouse antibodies (*a, d, g, j, m* and *p*), lissamine-rhodamine-labeled donkey anti-rabbit (*b, e, h, k, n* and *q*). All images were analyzed by confocal laser scanning microscopy. Fields *c, f, i, l, o* and *r* are color overlays. Cellubrevin was only retained in the ER in cells cotransfected with *myc-BAP31TMR* (*j-l*). In these panels, intense immunoreactive spots (*arrowheads*) for *myc-BAP31TMR* are seen, as indicated by cellubrevin staining. These spots were also positive for p58 (*arrowheads* in *m-o*), and probably denote material accumulating at ER export sites. In such doubly transfected cells, no punctate colocalization was observed between cellubrevin and the TfR that appeared to be normally sorted (*p-r*). Bars, 20 μ m.

the early phases of its life cycle, i.e., during export out of the ER. We hypothesized, therefore, that BAP31 may regulate the export of cellubrevin from the ER, by serving as a sorting companion for cellubrevin which delivers cellubrevin to the Golgi and then returns to the ER by retrograde vesicular transport, for instance. To test this idea further, we generated mutants of BAP31 carrying alterations in the cytoplasmic tail, hoping that at least some of them would lead to missorting of the protein and, in turn, affect the distribution of cellubrevin.

As discussed above, the COOH terminus of BAP31 contains two different sorting motifs, a YDRL motif frequently involved in adaptin binding, and a KKXX motif. To disrupt these signals, the following mutants were constructed (Fig. 8, *top*): BAP31 KK/SS in which the penultimate lysines were replaced with serines; BAP31-KKEE in which the last four amino acids were deleted; BAP31-24 aa in which the last 24 amino acids (including both the YDRL and the KKXX motif) were deleted; and *myc-BAP31TMR* in which the entire cytoplasmic domain was deleted. In-

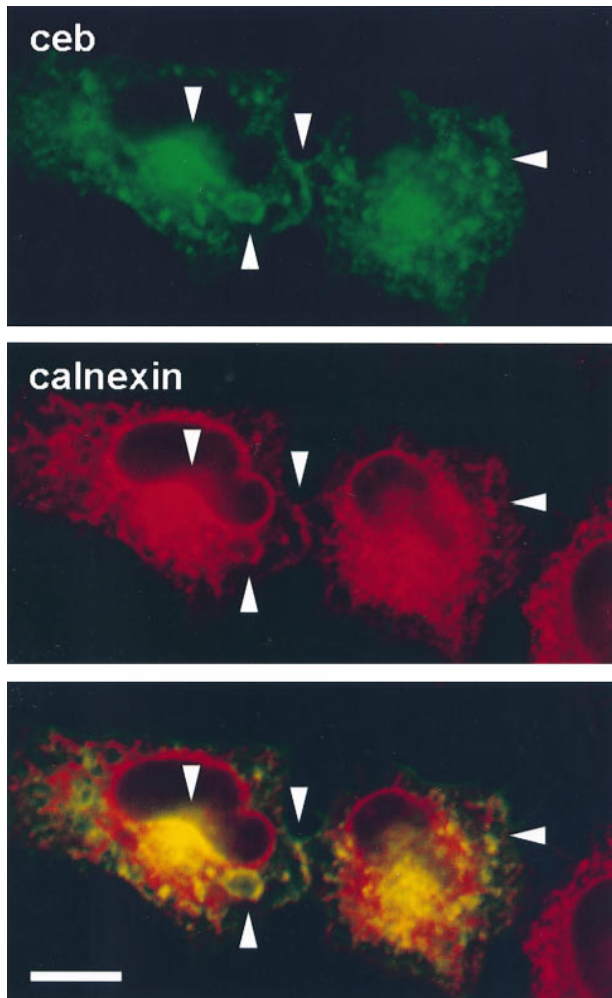


Figure 10. Overexpression of *myc*-tagged cellubrevin results in its partial retention within the ER. BHK-21 cells were transfected with plasmids encoding *myc*-tagged cellubrevin. After fixation, the cells were immunostained for cellubrevin using monoclonal *myc*-antibodies and a rabbit serum specific for calnexin. Fig. 9 shows detection procedures. Images were analyzed by confocal laser scanning microscopy. Arrowheads point to areas where cellubrevin is colocalized with calnexin. Bar, 20 μ m.

stead, a *myc*-epitope tag was added for detection since our BAP antibody binds to the cytoplasmic tail.

In the first series of experiments, we examined whether any of these mutations affects the interaction between BAP31 and cellubrevin. For this purpose, BHK-21 cells were cotransfected with cDNAs encoding the NH₂-terminal *myc*-tagged cellubrevin together with either full-length or mutant BAP31 constructs. Immunoblotting of cell extracts (Fig. 8 b) showed that all mutant proteins were recognized by the anti-BAP31 antibody and that expression levels were comparable. *myc*-BAP31TMR was also expressed as detected by the *myc*-antibody (data not shown). Equal amounts of each cell extract were immunodepleted using excess amounts of anti-*myc* monoclonal antibodies and then the immunoprecipitates were analyzed by immunoblotting (Fig. 8 c). All BAP31 mutants coprecipitated with *myc*-cellubrevin with no difference being observed between the various mutants (Fig. 8 c). This binding was disrupted upon SDS pretreatment of the cell extracts (data

not shown), and likewise for the synaptobrevin–synaptophysin interaction (Edelmann et al., 1995). To test binding of *myc*-BAP31TMR to cellubrevin, cells were cotransfected with cDNAs coding for untagged cellubrevin and *myc*-BAP31TMR. Again, equal quantitative immunoprecipitation from equal amounts of cell extracts were performed with either anti-cellubrevin or anti-*myc* and the immune complexes were analyzed by immunoblotting (Fig. 8 d). When anti-*myc* was used as the depleting antibody, a fraction of cellubrevin was found in the immunoprecipitate (Fig. 8 d, left panel, beads) but the supernatant was not depleted. Similarly, a fraction of *myc*-BAP31TMR coprecipitated with cellubrevin (Fig. 8 d, right panel, beads) but again, the majority stayed in the supernatant. These data show that all mutants interact with cellubrevin in a similar manner, but that large pools of both proteins exist in the transfected cells that do not interact with each other.

Next, we analyzed the distribution of the mutant proteins by immunocytochemistry using confocal laser scanning microscopy. To discriminate between endogenous and transfected BAP31, we used a less sensitive detection method than in the experiment shown in Figs. 5 and 6. Control stainings of nontransfected cells with this procedure confirmed that endogenous BAP31 was barely detectable and that BAP31 staining was dominated by the mutant protein. Transfections with wild-type BAP31 (BAP31wt) and *myc*-cellubrevin resulted in staining patterns indistinguishable from the respective endogenous proteins (Fig. 9, a–c, compare with Figs. 5 and 6). Thus, BAP31wt had a marked reticular distribution in addition to the accumulation around the MTOC (Fig. 9, a and c). *myc*-Cellubrevin colocalized with BAP31wt in this paranuclear region but was differentially distributed in the cell periphery (see above; Fig. 6). As described earlier, nocodazole treatment resulted in the disappearance of the MTOC and a dispersed punctate staining pattern for cellubrevin (data not shown). We then studied the distribution of the mutants of BAP31, in which the last four amino acids were modified or deleted (BAP31KK/SS and BAP31-KKEE). No differences in the staining pattern for BAP31 and cellubrevin were observed (Fig. 9, d and e; data not shown). Similarly, loss of the COOH-terminal 24 amino acids including both sorting motifs did not alter the distribution of the proteins (data not shown), suggesting that signals upstream in the BAP31 sequence contribute to ER retention.

In contrast, striking alterations were observed in cells transfected with *myc*-BAP31TMR and cellubrevin. When cells were stained for the mutant using *myc*-antibodies, the staining in the cell periphery was still mostly reticular but occasionally larger immunopositive blebs were observed, and the vesicle cluster around the MTOC was less conspicuous with a more fragmented appearance (Fig. 9, g and j). The staining pattern of endogenous BAP31 was identical (Fig. 9, h and i), demonstrating that the mutant-induced changes are dominant. Double labeling for *myc*-BAP31TMR and calnexin, a resident ER protein, resulted in virtually complete colocalization, confirming that *myc*-BAP31TMR is now restricted to the ER (data not shown). When these cells were analyzed for the distribution of cellubrevin, we noticed a dramatic change in the intracellular distribution of the protein (Fig. 9, j–l): cellubrevin fully colocalized with *myc*-BAP31TMR. In these cells, blebs positive for *myc*-

BAP31TMR seemed to be distinguished by cellubrevin staining (Fig. 9, compare *arrowheads* in *j* and *k* with color overlay in *l*). These immunoreactive structures probably mark the boundary between the ER and the intermediate compartment (ER export complexes; see Discussion), since they are also positive for p58, a marker protein of the intermediate compartment (Fig. 9, *arrowheads* in *m-o*).

These experiments suggest that intact BAP31 is required for exporting cellubrevin from the ER. However, they do not distinguish whether the retention of cellubrevin by *myc*-BAP31TMR is because of a general defect in ER to Golgi traffic, or whether the effect is specific for cellubrevin. To distinguish between these alternatives, we compared the distribution of cellubrevin with that of transferrin receptor in the cells transfected with *myc*-BAP31TMR and cellubrevin. In these cells (Fig. 9, *p* and *q*), the distribution of the transferrin receptor was strikingly different from the reticular pattern of cellubrevin (compare with Fig. 6, *bottom*). The transferrin receptor displayed a typical pattern of fine dots concentrated in the perinuclear region, documenting that it is not retained in the ER as is the case for cellubrevin.

If temporary association with BAP31 is needed for cellubrevin to be exported, it is possible that export becomes rate limiting when excess cellubrevin is produced, resulting in accumulation of cellubrevin in the ER because of saturation of BAP31. Using BHK-21 cells overexpressing *myc*-tagged cellubrevin, we examined the localization of the protein with respect to ER residents. As shown in Fig. 10, overexpression of cellubrevin resulted in accumulations of the protein in calnexin-positive reticular structures. These accumulations were significantly more pronounced than those observed with the endogenous protein (compare Fig. 6), although not all of the calnexin-positive structures were labeled. Taken together, we conclude that although both cellubrevin and the transferrin receptor are targeted to the same post-Golgi compartments, cellubrevin, but not the transferrin receptor, interacts with BAP31 before or while exiting the ER.

Discussion

In the present study, we have demonstrated that cellubrevin specifically interacts with BAP31. BAP31 is a resident of the ER that probably shuttles between the ER and the intermediate compartment and/or *cis*-Golgi complex. Our data suggest that BAP31 binds newly synthesized cellubrevin, and perhaps other proteins, to control their export to the Golgi apparatus where these transported proteins reach their final destinations.

As a resident of the ER, BAP31 does not colocalize with the major pools of cellubrevin. In addition, we found BAP31-positive membranes concentrated in a paranuclear region close to the Golgi apparatus and the MTOC, an area that is devoid of luminal ER proteins. Proteins involved in endosomal recycling, such as cellubrevin and the transferrin receptor, are also concentrated but, as our analysis indicates, they reside on different vesicle populations. This area apparently serves as a central relay station for trafficking vesicles of different origins and destinations.

Export from the ER, the first step in the vectorial transport of proteins, commences in specialized regions of the

ER. In some cells, e.g., the pancreatic acinar cells, these regions are juxtaposed to the *cis*-Golgi network and were originally referred to as transitional elements (Palade, 1975). In other cells, they appear to be distributed throughout the cytoplasm (Bannykh et al., 1996; Presley et al., 1997). They represent regions of the ER with many budding vesicles that are often adjacent to vesiculo-tubular clusters (Saraste and Svensson, 1991; Balch et al., 1994). Budding from the ER involves COPII coat proteins and results in the formation of COPII-coated transport vesicles (Barlowe et al., 1994). Before reaching the *cis*-Golgi, these transport vesicles pass through vesiculo-tubular clusters that may represent the intermediate compartment, functionally defined as the sorting compartment between the ER and the Golgi complex (Aridor and Balch, 1996). Here, ER resident proteins are probably sorted out and transported retrogradely to the ER, presumably involving COPI-coated transport vesicles (Aridor and Balch, 1996; Bannykh et al., 1996; Schekman and Orci, 1996). The accumulation of BAP31-containing vesicles around the MTOC, an area devoid of luminal ER proteins, demonstrates clearly that the protein exits the ER during its life cycle. However, it remains to be established whether it is transported all the way to the *cis*-Golgi. Since we found only minor colocalization with the *cis*-Golgi marker β -COP, its steady-state concentration in that compartment must be low and the time BAP31 resides in these cisternae very short. Additionally, the protein may be sorted out earlier and shipped back to the ER by retrograde transport. It should be emphasized, however, that the evidence that supports recycling of BAP31 from these compartments to the ER is indirect. Thus, we cannot exclude that BAP31 is directed to lysosomes where it is degraded instead of returning to the ER. We regard this as less likely because BAP31 does not exhibit a lysosomal staining pattern and it is completely absent from purified clathrin-coated vesicles (Fig. 4).

Upon nocodazole treatment, the BAP31 staining in the region of the MTOC disappeared and BAP31 was almost exclusively retained in the ER with no overlap with *cis*-Golgi markers (our unpublished observations). Thus, nocodazole blocks forward transport of BAP31-containing vesicles from the ER to the MTOC, in addition to its inhibition of retrograde transport that is known to be microtubule dependent (Lippincott-Schwartz et al., 1990). If only retrograde transport was inhibited by the drug, BAP31 would be expected to accumulate in the *cis*-Golgi area. Forward transport between the ER and the Golgi dependent on microtubules is in agreement with recent observations (Bannykh et al., 1996; Rowe et al., 1996; Presley et al., 1997). Nocodazole also disrupted the accumulation of vesicles containing transferrin receptor and cellubrevin in this area (Daro et al., 1996), making the differential distribution of BAP31 and cellubrevin more obvious. These findings highlight the role of the MTOC as a central relay station for microtubule-based intracellular vesicle traffic. Apparently, both ER-derived forward trafficking vesicles and plasmalemma- or endosome-derived endocytic vesicles are collected by microtubular transport from the cell periphery and then passage through the area of the MTOC before reaching their destinations at the *cis*- and *trans*-side of the Golgi complex, respectively.

It remains to be established which domains of BAP31

are responsible for its intracellular sorting. Deletion of the KKXX motif (which functions in the recruitment of COPI proteins), as well as deletion of a longer stretch (including the YDRL motif), had no obvious effects on the localization of the protein. Apparently, the KKXX motif is redundant to another as yet unknown sorting signal, and probably has a secondary signal function in assisting other proteins in the recruitment of COPI. It is possible, however, that mutant BAP31 is associated with endogenous wild-type BAP31 that still contains intact sorting signals. Interestingly, BAP31 remained in the ER even when the entire cytoplasmic tail was deleted, although upon extended culturing, abnormal vesicles were observed and cell viability decreased (see below).

Although cellubrevin and BAP31 are localized to different subcellular membranes, the interaction between these two proteins is highly specific. Like synaptophysin for synaptobrevin, BAP31 appears to be the dominant binding protein for cellubrevin, clearly exceeding the still elusive putative SNARE partners of the protein. Binding was observed with native as well as with recombinant proteins, suggesting that the interaction is direct and does not require intermediate proteins. Furthermore, binding appears to require the transmembrane domains of both BAP31 and cellubrevin, although electrostatic interactions must also be involved that can be shielded by high ion concentrations. Despite the specificity and affinity of the interaction, only a relatively small proportion of the proteins are complexed in cellular detergent extracts. This finding agrees well with the differential localization of the proteins and indicates they are associated with each other only during the early phase of the intracellular traffic of cellubrevin. Since cellubrevin, like synaptobrevin, is probably synthesized on free ribosomes (Kutay et al., 1995), we assume that it interacts with BAP31 after posttranslational insertion into the ER membrane, although an additional role of BAP31 in membrane insertion of cellubrevin cannot be excluded at present.

How does BAP31 influence the export of cellubrevin from the ER? Two explanations are possible. First, BAP31 may function as a negative regulator that retains (or even recruits) newly synthesized cellubrevin in the ER until it is released by an unknown regulatory mechanism. Second, BAP31 may function as a positive regulator to which cellubrevin needs to bind to reach the Golgi compartment. According to this scenario, cellubrevin would be unable to leave the ER unless it is recruited by BAP31 into export vesicles, i.e., being actively transported rather than passively sorted by bulk flow.

According to the first view, BAP31 would bind to cellubrevin and other to be exported proteins and keep them in the ER membrane until they are either assembled with other membrane components or properly folded. However, we found that under all experimental conditions, only a fraction of cellubrevin is associated with BAP31 in detergent extracts. Neither treatment with nocodazole (resulting in a minor increase of cellubrevin in the ER) nor expression of a BAP31 mutant lacking the cytoplasmic domain (resulting in retention of cellubrevin in the ER), led to a noticeable increase of cellubrevin-*myc*-BAP31-TMR complexes relative to the uncomplexed protein pools. Although artefacts can never be excluded when assessing mem-

brane protein complexes in detergent extracts, association of the proteins appears to be low even if they are both confined to the ER. This finding is difficult to reconcile with the negative regulator model. Rather, it suggests that BAP31 may serve as a sorting chaperone that recruits certain classes of membrane proteins into transport vesicles. BAP31 may directly interact with COPII budding components. Alternatively, it may bind (perhaps via its coiled coil regions) to integral membrane proteins that facilitate COPII-mediated export. In fact, there are precedents for protein-assisted export from the ER in yeast. For instance, the protein Erp25p forms a complex with Emp24p that is required for selective export of certain cargo molecules from the ER (Schimmöller et al., 1995; Belden and Barlowe, 1996). Another well-documented case is the yeast gene product Shr3p that, similar to BAP31, resides primarily in the ER and recruits specific amino acid permeases into transport vesicles (Kuehn et al., 1996).

Deletion of the cytoplasmic tail of BAP31 results in the retention of both BAP31 and cellubrevin in the ER, whereas other proteins such as the transferrin receptor still reach their normal destination beyond the Golgi complex. Moreover, the differences between transferrin receptor and cellubrevin localization are not caused by differences in the turnover rates of the proteins because preliminary observations suggest that their half lives are similar. Thus, these findings document that once the function of BAP31 is impaired, cellubrevin cannot reach its final destination and accumulates in the ER. Precisely how the function of BAP31 is affected by this deletion remains unclear. The deletion mutant still appears to be able to form a complex with cellubrevin, exhibiting properties that are not obviously different from the wild-type complex. We observed, however, that upon extended culturing cells developed abnormal membrane blebs, suggesting a delayed noxious effect of the mutant protein (Fig. 9, *j-l*). These blebs may represent membrane accumulations at the exit site of the ER (Bannykh et al., 1996), as suggested by the colocalization of *myc*-BAPTMR with p58 in these blebs (Fig. 9, *m-o*) or, alternatively, accumulation of membrane destined for degradation. It is possible that vesicle traffic out of the ER is affected to some extent, which may contribute to the phenotype.

We conclude that BAP31 is a representative of a novel class of proteins that regulates trafficking of certain membrane proteins out of the ER, either by retaining newly synthesized membrane proteins in the ER or by functioning as a conveyor belt for actively transporting these proteins from the ER to the Golgi complex. This class may include additional proteins such as BAP29 (Adachi et al., 1996) and other members of the BAP family which are specific for different membrane immunoglobulins (Kim et al., 1994; Terashima et al., 1994). Thus, trafficking of membrane proteins by means of such control proteins may be a common mechanism in eukaryotic cells.

We wish to thank A. Helenius, J.D. Castle, S. Fuller, J. Saraste, T. Kreis, H.-P. Hauri, and I. Trowbridge for their generous gifts of polyclonal and monoclonal antibodies and I. Mellman (all from Yale University) for critical comments to the manuscript. We are indebted to the W.M. Keck Foundation Biotechnology Resource laboratory at Yale University for their help in peptide purification and sequencing. We are especially grateful to L. Caron (Yale University) for invaluable advice in the use of the confocal microscope. We want to thank all the members of the Jahn lab

and the Department of Cell Biology at Yale University for many helpful discussions and technical assistance.

W.G. Annaert was supported by a D. Collen Fellowship and a Philips Fellowship of the Belgian American Educational Foundation, a Fulbright-Hays Award, and a North Atlantic Treaty Organization grant. U. Kistner was a recipient of a fellowship from the Boehringer-Ingelheim Foundation. This work was supported by the Deutsche Forschungsgemeinschaft by a grant to M. Reth through SFB364.

Received for publication 2 May 1997 and in revised form 6 October 1997.

References

- Adachi, T., W.W.A. Schamel, K.-M. Kim, T. Watanabe, B. Becker, P.J. Nielsen, and M. Reth. 1996. The specificity of association of the IgD molecule with the accessory proteins BAP31/BAP29 lies in the IgD transmembrane sequence. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1534–1541.
- Aridor, M., and W.E. Balch. 1996. Principles of selective transport: coat complexes hold the key. *Trends Cell Biol.* 6:315–320.
- Balch, W.E., J.M. McCaffery, H. Plutner, and M.G. Farquhar. 1994. Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell.* 76:841–852.
- Bannykh, S.I., T. Rowe, and W.E. Balch. 1996. The organization of endoplasmic reticulum export complexes. *J. Cell Biol.* 135:19–35.
- Barlowe, C., L. Orci, T. Yeung, M. Hosobuchi, S. Hamamoto, N. Salama, M.F. Rexach, M. Ravazzola, M. Armherdt, and R. Schekman. 1994. COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell.* 77:895–907.
- Belden, W.J., and C. Barlowe. 1996. Erv25p, a component of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. *J. Biol. Chem.* 271:26939–26946.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brand, S.H., S.M. Laurie, M.B. Mixon, and J.D. Castle. 1991. Secretory carrier membrane proteins 31–35 define a common protein composition among secretory carrier membranes. *J. Biol. Chem.* 266:18949–18957.
- Calakos, N., and R.H. Scheller. 1994. Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. *J. Biol. Chem.* 269:24534–24537.
- Calakos, N., M.K. Bennett, K.E. Peterson, and R.H. Scheller. 1994. Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science.* 263:1146–1149.
- Chapman, E.R., A. Seong, N. Barton, and R. Jahn. 1994. SNAP-25, at t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.* 269:27427–27432.
- Daro, E., P. van der Sluijs, T. Galli, and I. Mellman. 1996. Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. *Proc. Natl. Acad. Sci. USA.* 93:9559–9564.
- Edelmann, L., P.I. Hanson, E.R. Chapman, and R. Jahn. 1995. Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:224–231.
- Ferro-Novick, S., and R. Jahn. 1994. Everything that fuses must converge: proteins that mediate vesicle fusion from yeast to man. *Nature.* 370:191–193.
- Fischer von Mollard, G., B. Stahl, C. Walch-Solimena, K. Takei, L. Daniels, A. Khokhlatchev, P. De Camilli, T.C. Südhof. 1994. Localization of Rab5 to synaptic vesicles identifies endosomal intermediate in synaptic vesicle recycling pathway. *Eur. J. Cell Biol.* 65:319–326.
- Franek, F. 1986. Purification of IgG monoclonal antibodies from ascitic fluid based on Rivanol precipitation. *Methods Enzymol.* 121:631–638.
- Galli, T., T. Chilcote, O. Mundigl, T. Binz, H. Niemann, and P. De Camilli. 1994. Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J. Cell Biol.* 125:1015–1024.
- Gorvel, J.P., P. Chavrier, M. Zerial, and J. Gruenberg. 1991. Rab5 controls early endosome fusion in vitro. *Cell.* 64:915–925.
- Gruenberg, J., G. Griffiths, and K.A. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J. Cell Biol.* 108:1301–1316.
- Heukeshoven, J., and R. Dernick. 1985. Simplified method for silverstaining of proteins in polyacrylamide gels and the mechanism of silverstaining. *Electrophoresis.* 6:103–112.
- Jo, I., H.W. Harris, A.M. Amendt-Raduege, R.R. Majewski, and T.G. Hammond. 1995. Rat kidney papilla contains abundant synaptobrevin protein that participates in the fusion of antidiuretic hormone-regulated water channel-containing endosomes in vitro. *Proc. Natl. Acad. Sci. USA.* 92:1876–1880.
- Kim, K.-M., T. Adachi, P.J. Nielsen, M. Terashima, M.C. Lamers, G. Köhler, and M. Reth. 1994. Two new proteins preferentially associated with membrane immunoglobulin D. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3793–3800.
- Kuehn, M.J., R. Schekman, and P.O. Ljungdahl. 1996. Amino acid permeases require COPII components and the ER resident membrane protein Shr3p for packaging into transport vesicles in vitro. *J. Cell Biol.* 135:585–595.
- Kutay, U., G. Ahnert-Hilger, E. Hartmann, B. Wiedenmann, and T.A. Rapoport. 1995. Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:217–223.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.
- Link, E., H. McMahon, G. Fischer von Mollard, S. Yamasaki, H. Niemann, T.C. Südhof, and R. Jahn. 1993. Cleavage of cellubrevin by tetanus toxin does not affect fusion of early endosomes. *J. Biol. Chem.* 268:18423–18426.
- Lippincott-Schwartz, J., J.G. Donaldson, A. Schweizer, E.G. Berger, H.-P. Hauri, L.C. Yuan, and R.D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
- Lombard-Platet, G., and P. Jalinot. 1993. Funnel-well SDS-PAGE: a rapid technique for obtaining sufficient quantities of low abundance proteins for internal sequence analysis. *Biotechniques.* 15:669–672.
- Matteoli, M., K. Takei, R. Cameron, P. Hurlbut, P.A. Johnston, T.C. Südhof, R. Jahn, and P. De Camilli. 1991. Association of rab3A with synaptic vesicles at late stages of the secretory pathway. *J. Cell Biol.* 115:625–633.
- Maycox, P., E. Link, A. Reetz, S.A. Morris, and R. Jahn. 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *J. Cell Biol.* 118:1379–1388.
- McMahon, H.T., Y.A. Ushkaryov, L. Edelmann, E. Link, T. Binz, H. Niemann, R. Jahn, and T.C. Südhof. 1993. Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature.* 364:346–349.
- Ohno, H., J. Stewart, M.-C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science.* 269:1872–1875.
- Oprins, A., R. Duden, T.E. Kreis, H.J. Geuze, and J.W. Slot. 1993. β -COP localizes mainly to the cis-Golgi side in exocrine pancreas. *J. Cell Biol.* 121:49–59.
- Palade, G.E. 1975. Intracellular aspects of the process of protein transport. *Science.* 189:347–354.
- Presley, J.F., N.B. Cole, T.A. Schroer, K. Hirschberg, K.J.M. Zaal, and J. Lippincott-Schwartz. 1997. ER-to-Golgi transport visualized in living cells. *Nature.* 389:81–85.
- Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature.* 372:55–63.
- Rowe, T., M. Aridor, J.M. McCaffery, H. Plutner, C. Nuoffer, and W.E. Balch. 1996. COPII vesicles derived from mammalian endoplasmic reticulum microsome recruit COPI. *J. Cell Biol.* 135:895–911.
- Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.
- Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. *Science.* 271:1526–1533.
- Scheller, R.H. 1995. Membrane trafficking in the presynaptic nerve terminal. *Neuron.* 14:893–897.
- Schimmöller, F., B. Singer-Krüger, S. Schröder, U. Krüger, C. Barlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1329–1339.
- Schweizer, A., J.A.M. Fransen, T. Bächli, L. Ginsel, and H.-P. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J. Cell Biol.* 107:1643–1653.
- Schweizer, A., K. Matter, C.M. Ketcham, and H.-P. Hauri. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and cis-Golgi. *J. Cell Biol.* 113:45–54.
- Söllner, T., M.K. Bennett, S.W. Whiteheart, R.H. Scheller, and J.E. Rothman. 1993a. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell.* 75:409–418.
- Söllner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Gero-manos, P. Tempst, and J.E. Rothman. 1993b. SNAP receptors implicated in vesicle targeting and fusion. *Nature.* 362:318–324.
- Südhof, T.C. 1995. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature.* 375:645–653.
- Terashima, M., K.-M. Kim, T. Adachi, P.J. Nielsen, M. Reth, G. Köhler, and M.L. Lamers. 1994. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3782–3792.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
- Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* 9:129–161.
- Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotypic antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal. *Nature.* 345:495–502.
- Washbourne, P., G. Schiavo, and C. Montecucco. 1995. Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin. *Biochem. J.* 305:721–724.
- Zhang, Y., C. Riegerer, A.M. Ayrall, F. Sablitzky, T.D. Littlewood, and M. Reth. 1996. Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res.* 24:543–548.