ERG30, a VAP-33–related Protein, Functions in Protein Transport Mediated by COPI Vesicles

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Abstract. Intracellular transport of newly synthesized and mature proteins via vesicles is controlled by a large group of proteins. Here we describe a ubiquitous rat protein—endoplasmic reticulum (ER) and Golgi 30-kD protein (ERG30)—which shares structural characteristics with VAP-33, a 33-kD protein from *Aplysia californica* which was shown to interact with the synaptic protein VAMP. The transmembrane topology of the 30-kD ERG30 corresponds to a type II integral membrane protein, whose cytoplasmic NH_2 terminus contains a predicted coiled-coil motif. We localized ERG30 to the ER and to pre-Golgi intermediates by biochemical and immunocytochemical methods. Consistent with a role in vesicular transport, anti-ERG30 antibodies specifically inhibit intra-Golgi transport in vitro, leading to significant accumulation of COPI-coated vesicles. It appears that ERG30 functions early in the secretory pathway, probably within the Golgi and between the Golgi and the ER.

Key words: endoplasmic reticulum • Golgi • coated vesicles • secretion • transport intermediates

TRANSPORT of proteins between different organelles of eukaryotic cells is mediated by coated vesicles that bud from one membrane compartment and are targeted to and fuse with an appropriate acceptor organelle (Palade, 1975; Rothman, 1994).

Budding of vesicles is regulated by small GTPases and mediated by cytosolic coat proteins (COP)[1] that assemble on the donor membrane (Rothman and Wieland, 1996; Schekman and Orci, 1996). COPII vesicles mediate transport of proteins from the ER to the Golgi apparatus (Barlowe et al., 1994; Campbell and Schekman, 1997), whereas COPI vesicles are implicated in transport through the Golgi (Malhotra et al., 1989; Ostermann et al., 1993; Orci et al., 1997) as well as in retrograde transport of proteins from the Golgi back to the ER (Cosson and Letourneur, 1994; Letourneur et al., 1994; Orci et al., 1997).

Docking of vesicles to the appropriate target membrane involves interaction between integral membrane proteins located on the vesicle, called v-SNAREs, and the target membrane proteins, t-SNAREs (Söllner et al., 1993b; Pfeffer, 1996). Before this pairing event, initial docking of a vesicle with its specific target organelle is mediated by a peripheral membrane protein, p115 (Nakamura et al., 1997), originally identified as a protein needed for intra-Golgi transport (Waters et al., 1992). P115 interacts with two integral membrane proteins, giantin, located on COPI vesicles, and GM130, a Golgi matrix protein, thus providing a bridge between vesicles and their target membrane (Sonnichsen et al., 1998). In yeast, Uso1p (the homologue of mammalian p115) acts together with the small GTPase Ypt1p before the formation of SNARE pairs (Lupashin et al., 1996; Sapperstein et al., 1996; Lupashin and Waters, 1997), and initiates docking of ER-derived COPII vesicles to the Golgi (Cao et al., 1998).

Following pairing, the v/t-SNARE complex binds two soluble factors: NEM-sensitive fusion protein (NSF) and soluble NSF attachment protein (SNAP). These, in turn, catalyze the disassembly of the SNARE complex (Söllner et al., 1993a) after a round of fusion, thereby allowing a new round of transport (Mayer et al., 1996; Otto et al., 1997; Ungermann et al., 1998). However, the events that lead to membrane fusion following the docking of vesicles are still being investigated. It has been argued that the

1. *Abbreviations used in this paper:* βCOP, β subunit of COPI; BFA, brefeldin A; COP, cytosolic coat protein; ERG30, ER and Golgi 30-kD protein; MBP, maltose binding protein; NSF, NEM-sensitive fusion protein; PDI, protein disulfide isomerase; SNAP, soluble NSF attachment protein; VAMP, vesicle-associated membrane protein; VAP-33, VAMP-associated protein of 33 kD; VSVG, vesicular stomatitis virus G protein.

SNARE complex allows the cell to overcome the energy barrier required for membrane fusion (Fasshauer et al., 1997; Hanson et al., 1997). Furthermore, Weber et al. (1998) used liposomes reconstituted with t- or v-SNAREs to show that the v/t-SNARE complex per se fulfills the minimal requirement for lipid mixing (hemi-fusion) and possibly fusion between two membranes. Yet, Ungermann et al. (1998), using an in vitro system that reconstitutes homotypic fusion of yeast vacuoles, hypothesized that the formation of the SNARE complex is only an intermediate step in the overall fusion reaction. According to this view, SNARE molecules are involved in docking and possibly in hemi-fusion between donor and acceptor membranes, but another set of proteins is involved in subsequent stages of the fusion process. This notion is supported by Peters and Mayer (1998), who suggested that calmodulin and other yet-unidentified factors are involved in mediating late stages of vacuolar fusion.

The vesicle-associated membrane protein (VAMP)–associated protein of 33 kD (VAP-33) was identified in *Aplysia califorca* (Skehel et al., 1995b). This protein is conserved in evolution with homologues found in *Saccharomyces cerevisiae* (*SCS2*) (Nikawa et al., 1995), *Caenorhabditis elegans* (Klass et al., 1984), *Arabidopsis thaliana* (Galaud et al., 1997), and humans (Weir et al., 1998). Antibodies directed against VAP-33 blocked neurotransmission when injected into cultured neurons, suggesting that in *A. califorca* this protein is involved in neurotransmitter release. The yeast *SCS2* gene has been cloned as a suppressor of inositol auxotrophy of *CSE1* and *hac/ire*15 mutants (Nikawa et al., 1995) and was recently characterized as an integral membrane protein of the ER (Kagiwada et al., 1998). The *C. elegans* major sperm protein, a 127–amino acid long protein, also exhibits significant homology with the NH_2 terminus of VAP-33. Major sperm protein is thought to be involved in sperm pseudopodial movement, by forming a cytosolic filamentous network that translocates vesicles to the plasma membrane (Italiano et al., 1996).

In the present study we describe a VAP-33–related protein which we denote ERG30, and demonstrate its type II transmembrane topology. We found that ERG30 is localized in the ER and in pre-Golgi intermediates. Functional in vitro assays attribute to ERG30 a role in COPI vesicle transport. We put forward the hypothesis that ERG30 is involved in intra-Golgi transport and in retrograde transport of proteins between the Golgi and the ER.

Materials and Methods

Construction of Bait Plasmids

ERG30 was cloned by the two-hybrid system using the cytosolic portion of Neu differentiation factor β4a ($NDFβ4a_{290-662}$) as a bait. $NDFβ4a_{290-662}$ was generated by PCR with EcoRI and BamHI ends using the following primers: 5′-CCGGAATTCACCAAGAAGCAGCGGCAG-3′ and 5′-CGCGGATCCTTATACAGCAATAGGGTC-3′. The resulting PCR product was digested with EcoRI and BamHI and cloned into the appropriate sites in the pGBT9 vector (Clontech) downstream from the GAL4 DNA binding domain. This plasmid was transformed into the two-hybrid strain HF7c reporter strain (Clontech), and tested for expression of the fusion protein by Western analysis. The inserted fragment was sequenced to verify that no mutation had occurred because of PCR and to confirm the correct reading frame of the resultant fusion protein.

Construction of a Rat Brain cDNA Library in the pACT Vector

A cDNA library was constructed from 5 μg of oligo (dT)-selected mRNA, using a Stratagene kit. RNA was prepared from rat brain by the guanidinium thiocyanate-phenol-chloroform extraction method. The mRNA was purified and used as a template for cDNA synthesis. The resulting dscDNA was methylated by XhoI methylase and ligated to an EcoRI linker, thus generating an EcoRI site at the 5′ end of the cDNA and an XhoI site at the 3′. The average size was ∼2.5 kb. The purified dscDNA was ligated to λACT. The library titer was ∼1.4×10^7 pfu. The titer of the library after amplification was 3×10^9 pfu/ml. In vivo excision was performed from the phagemid λACT to pACT.

Two-Hybrid Screen

The HF7c yeast strain carrying the bait plasmid $pGBT9-NDFβ4a_{290-662}$ was transformed with the rat brain cDNA library generated in pACT AD vector (Clontech). Transformation efficiency was assessed by plating small aliquots onto SCD plates lacking tryptophan, leucine, and histidine and supplemented with 10 mM 3-AT. The yeast colonies were transferred to nitrocellulose filters (BA85; Schleicher and Schuell), immersed in liquid nitrogen for 5 s, and incubated at 30°C on a 3-mm Whatman paper soaked with 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, and 1 mg/ml X-GAL. Colonies containing the interacting pair of proteins became blue within 2–6 h. From an initial screen of 200,000 colonies, we isolated 5 individual clones containing different cDNAs corresponding to the same mRNA. To isolate library plasmids from positive clones, cells were grown in SC liquid media lacking leucine (to allow loss of bait, but not of the library plasmid), and plasmid DNA was prepared and transformed at low dilution into HB101 competent *Escherichia coli* cells. The transformants were selected on a M9 minimal medium containing 50 μg/ml Amp. Plasmids isolated were then used to retransform SFY526 yeast cells either alone or with $pGBT9-NDFβ4a_{290-662}$. Transformants were assayed for β-galactosidase activity. cDNA isolated from the positive clones was subcloned into *p*Bluescript-II, and then sequenced using an Applied Biosystems 373A automated DNA sequencer and Applied Biosystems *Taq* Dye Deoxy™ Terminator cycle sequencing kit. Of the five colonies isolated by this screen, clone pACT17 contained the complete coding sequence of ERG30. RT-PCR performed on rat brain mRNA, using a specific oligonucleotide derived from pACT17, confirmed that the sequence obtained from the cDNA library was the full-length cDNA.

Construction and Expression of MBP-ERG30 Fusion Proteins

ERG30 and its truncated forms were tagged at their NH_2 terminus with a maltose binding protein (MBP) domain. For that purpose, full-length ERG30, ERG30 (Δcoiled-coil) (amino acids 1–140), or ERG30 (ΔNH_2 terminus) (amino acids 141–243) was ligated into the EcoRI BamHI site of pMAL-p2 vector (New England Biolabs). The different fusion proteins were overexpressed in the JM109 strain of bacteria by growing log phase bacteria in the presence of 1 mM IPTG for 4 h. Cell extracts were prepared by sonicating the bacteria in a buffer containing 50 mM Tris (pH 8.0), 50 mM KCl, 0.1 mM EDTA, and 1% Triton X-100. Bacterial extracts were centrifuged in a Ti60 rotor (Beckman) at 45,000 rpm for 60 min, and the supernatant was passed over a 1-ml amylose column at 4°C. The column was washed with 10 column volumes of 20 mM Tris-HCl (pH 7.4), 0.4 M NaCl, and 10 mM β-mercaptoethanol. The protein of interest was eluted with the above buffer plus 10 mM maltose.

Production of Anti-ERG30 Antibodies

An antiserum was raised in rabbits against a recombinant MBP-ERG30 protein purified from *E. coli*. The antiserum was first run through CNBr activated Sepharose column with covalently bound MBP, to remove anti-MBP antibodies. Anti-ERG30 antibodies were purified from the flow-through material of the first column by affinity chromatography on a CNBr-activated Sepharose column with covalently bound MBP-ERG30 fusion protein.

Subcellular Fractionation

Rat liver homogenates were fractionated over sucrose gradients as described previously (Taylor et al., 1997; Walter et al., 1998), with slight

modifications. Fresh rat livers (25 g) were mixed with 150 ml of ice-cold homogenization buffer (0.5 M sucrose, 0.1 M KPi, pH 6.8, 5 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotonin, 2 μM pepstatin A, and 0.5 μg/ml leupeptin) and the tissue was minced and homogenized by forcing it through a fine stainless steel mesh (aperture 150 μm). The homogenate was centrifuged in a SLA-1500 rotor (Sorvall) for 10 min at 3,000 rpm. The postnuclear supernatant was layered over a 0.86/1.25 M sucrose step gradient. The gradients were centrifuged at 4°C in a SW-28 rotor (Beckman) at 25,000 rpm for 90 min. The 0.86/1.25 M interface was adjusted to 1.6 M sucrose and layered into SW-28 tubes. This layer was overlaid with 1.25 M, 1 M, 0.86 M, and 0.5 M sucrose solutions. The gradient was centrifuged at 4°C in a SW-28 rotor (Beckman) at 25,000 rpm for 2.5 h. The samples were continuously collected from the top, tested for their sucrose concentration, and subjected to Western blot analysis with different antibodies.

Drug Treatment and Immunofluorescence Microscopy

NRK or CHO cells were seeded onto microscope slides 24 h before staining. For drug treatment before immunofluorescence microscopy, cells were incubated for 1 h in RPMI (NRK cells) or in α-MEM (CHO cells) medium containing 10 μg/ml brefeldin A (BFA). To fix cells for microscopy, the growth medium was removed and cells were incubated for 10 min in methanol at −20°C. Cell staining involved a 1-min incubation in acetone at −20°C for permeabilization followed by incubation in a blocking solution containing 10% FCS in PBS. Cells were incubated for 2 h with the primary antibodies in 2% FCS in PBS, then washed three times with PBS and incubated in an incubation solution containing affinity-purified fluorescein- or rhodamine-labeled antibodies against mouse or rabbit IgG. Slides were finally washed three times with PBS, and mounted beneath coverslips. The stained cells were analyzed with an MRC1024 confocal microscope (BioRad).

EM/Immunocytochemistry of CHO Cells

CHO cells were grown on glass coverslips that had been coated with carbon and gelatin (Olek et al., 1986). Preembedding immunogold-silver labeling was performed by a modification of the method described by Tanner et al. (1996). Cultures were rinsed with serum-free culture medium and fixed for 45 min in 4% paraformaldehyde, 4% sucrose, and 0.1 M sodium phosphate buffer, pH 7.4. The first 15 min of fixation were at room temperature, followed by 30 min at 4°C. Fixed cultures were washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS), then permeabilized for 1 h at room temperature in 0.05% saponin, 10% normal goat serum, and 1% BSA in DPBS. Cultures were then incubated for 1 h at room temperature with affinity-purified antibodies to ERG30 diluted to 10 μg/ml with 0.05% saponin, 1% BSA in DPBS. For controls, cultures were incubated without antibodies to ERG30 or with ERG30 antibodies that had been preabsorbed for 30 min at 36°C with a 50-fold molar excess of MBP-ERG30, and centrifuged to remove any precipitate. After several washes with the diluent, cultures were incubated for 1 h at room temperature with 1.4 nm gold-conjugated Fab′ fragments of goat anti–rabbit IgG (Nanoprobes) diluted 1:100, followed by washes with diluent and DPBS, and further fixation for 40 min in 2% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.4. The glutaraldehyde-fixed cultures were washed several times with DPBS, then with double-distilled water, before silver enhancement under a dark-red safelight for 4–8 min performed according to instructions provided by Nanoprobes with their HQ silver enhancement kit. After several washes with double-distilled water and DPBS, cultures were treated with 0.2% OsO$_4$ for 30 min at room temperature followed by en bloc staining with uranyl acetate, dehydration, and embedding in Epon, all as described (Olek et al., 1986). Thin sections were cut parallel to the plane of the culture substrate and stained with uranyl acetate and lead citrate. Observations were made by transmission electron microscopy on >100 cells from both the K1 (ATCC) and NDF-expressing CHO cell lines (Burgess et al., 1995).

Cis- to Medial-Golgi Transport Assay

The standard assay mixture (25 μl) contained 0.4 μCi UDP-[^{3}H] N-acetylglucosamine (America Radiolabeled Chemical), 5 μl of a 1:1 mixture of donor and acceptor CHO Golgi membranes, and crude bovine brain cytosol, as described (Balch et al., 1984).

Isolation of COPI Vesicles

Golgi-derived COPI vesicles were isolated as described previously (Stamnes et al., 1995). In brief, Golgi membranes were isolated from wild-type CHO cells as described and prewashed with 250 mM KCl. The membranes (0.25 mg/ml) were then incubated with crude bovine brain cytosol (16 mg/ml) in 0.5 ml transport assay buffer (see above) for 15 min at 37°C. After incubation, KCl was added to a final concentration of 250 mM, and the Golgi membranes were pelleted as above. The supernatant was mixed with 1 ml of 70% (wt/wt) sucrose and overlayed in 5 ml SW 55 ultracentrifugation tubes with 0.5 ml 45% (wt/wt) sucrose, and 1 ml 40%, 35%, and 30% (wt/wt) sucrose in 20 mM Tris, pH 7.4. The gradient was centrifuged for 14 h at 50,000 rpm at 4°C and fractionated into 7 fractions of 0.7 ml each. The indicated fractions were diluted twofold in double distilled water and membranes were pelleted by ultracentrifugation for 40 min at 4°C in a TLA 100.1 rotor at 100,000 rpm. The membrane pellets were dissolved in SDS-PAGE sample buffer, after which the proteins were separated by electrophoresis and blotted onto nitrocellulose filters. The β subunit of COPI (βCOP) was detected by Western blot analysis using monoclonal anti-βCOP antibodies M3A5 as described (Palmer et al., 1993).

Formation of 20S Particles

For the isolation of 20S SNARE particles we used the procedure described by Söllner et al. (1993b). In brief, crude rat brain membranes were incubated with 10 mM Hepes/KOH, pH 7.8, 100 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, and 4% (vol/vol) Triton X-100 on ice for 45 min and the suspension was clarified by ultracentrifugation for 60 min at 50,000 rpm in a Ti-60 rotor. The supernatant was dialyzed against 100 vol of 25 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM DTT, 1% Triton X-100. The dialyzed material was ultracentrifuged in a Ti-60 rotor for 60 min at 50,000 rpm. This rat brain extract was incubated with His$_6$-αSNAP and NSF-Myc in a buffer containing 25 mM Hepes-HCl, pH 7.0, 75 mM KCl, 1 mM DTT, 2 mM EDTA, 0.75% Triton X-100 and 0.5 mM ATPγS, 1% (wt/vol) polyethyleneglycol (PEG 400), and 0.5 mM PMSF for 30 min at 4°C. Mouse anti-Myc monoclonal antibodies coupled to protein G–Sepharose were added and the incubation continued for an additional 2 h with constant agitation. The beads were then washed with 10 vol of buffer A (25 mM Hepes-HCl, pH 7.0, 100 mM KCl, 1 mM DTT, 2 mM EDTA, 0.5% Triton X-100, and 0.5 mM ATPγS), followed by elution with buffer A containing 8 mM MgCl$_2$ (nonspecific elution), or with buffer A containing 8 mM MgCl$_2$ and 0.5 mM ATP to allow ATP hydrolysis (specific elution). The eluted fractions were precipitated with trichloroacetic acid, boiled, and analyzed by Western blotting using the appropriate antibodies.

Western Blot Analysis

To prepare rat tissue extracts, frozen organs were washed in cold PBS and lysed with a homogenizer (PCU Kinematica) in ice-cold protein extraction buffer containing 0.5 M β-glycerophosphate, 15 mM EGTA, 10 mM EDTA, 1 mM ortho-vanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM DTT, at pH 7.4. Lysates were cleared by 15 min spin at 20,000 rpm at 4°C. Supernatants were then mixed with sample buffer and heated to 95°C for 2 min. Equal amounts of tissue extracts were subjected to SDS-PAGE (15% acrylamide), transferred to nitrocellulose (Sartorius), and probed with anti-ERG30 antibodies using HRP-coupled secondary antibodies and ECL reagent (Amersham).

Immunoprecipitation

Rat brain membranes were isolated as described previously (Söllner et al., 1993b), and solubilized with 4% Triton X-100 as described above. This membrane extract was diluted 1 to 4 (vol/vol) with a buffer containing 10 mM Hepes/KOH, pH 7.8, 100 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, and incubated with protein A–agarose coupled to different antibodies for 2 h at 4°C. Immune complexes were washed five times with the immunoprecipitation buffer and subjected to Western blot analysis.

Fractionation by Triton X-114

Rat liver Golgi fractions were washed once with Tris-salt buffer (10 mM Tris-HCl, pH 7.6) and resuspended to a protein concentration of 4 mg/ml. Triton X-114 was added at 4°C to a final concentration of 2% (wt/vol). Solubilized membranes were incubated on ice for 4 min and then centri-

A

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  1 CAC GAG GTG CGT GCC GTG AGC TCG CCC GGC ACC GCG GCC TCG CCC TCC GCC CCT GTG CCC

 61 CGC CAC CCA GGC CGC CGC CCC CCG CCC GCG CCG CCG CCC CCG ACG GGG AGG ACC ATG GCG
                                                                              M   A     2
121 AAG GTG GAG CAG GTC CTG AGT CTC GAG CCA CAG CAC GAG CTC AAG TTC CGA GGT CCC TTC
     K   V   E   Q   V   L   S   L   E   P   Q   H   E   L   K   F   R   G   P   F   22
181 ACT GAT GTT GTC ACT ACC AAC CTA AAG CTG GGC AAC CCG ACA GAC CGG AAC GTG TGT TTC
     T   D   V   V   T   T   N   L   K   L   G   N   P   T   D   R   N   V   C   F   42
241 AAA GTG AAG ACC ACA GCG CCG CGC AGG TAC TGC GTG CGG CCC AAC AGT GGG GTC ATT GAT
     K   V   K   T   T   A   P   R   R   Y   C   V   R   P   N   S   G   V   I   D   62
301 GCC GGG GCC TCT CTC AAT GTG TCT GTG ATG TTA CAG CCT TTC GAT TAT GAC CCC AAT GAG
     A   G   A   S   L   N   V   S   V   M   L   Q   P   F   D   Y   D   P   N   E   82
361 AAA AGT AAA CAC AAG TTT ATG GTT CAG TCT ATG TTT GCT CCG CCC GAC ACT TCC GAT ATG
     K   S   K   H   K   F   M   V   Q   S   M   F   A   P   P   D   T   S   D   M   102
421 GAG GCA GTA TGG AAG GAG GCA AAA CCG GAA GAC CTT ATG GAT TCA AAA CTT AGA TGT GTG
     E   A   V   W   K   E   A   K   P   E   D   L   M   D   S   K   L   R   C   V   122
481 TTT GAA TTG CCA GCA GAA AAC GCT AAA CCA CAT GAT GTA GAA ATA AAT AAA ATT ATG CCT
     F   E   L   P   A   E   N   A   K   P   H   D   V   E   I   N   K   I   M   P   142
                                                                                  c   d
541 ACA AGT GCA TCC AAG ACA GAA GCA CCG GTG GCC GCC AAG CCC CTC ACA TCG CCC CTT GAT
     T   S   A   S   K   T   E   A   P   V   A   A   K   P   L   T   S   P   L   D   162
         e   f   g   a   b   c   d   e   f   g   a   b   c   d   e   f   g   a
601 GAC CCG GAA GTA AAG AAG GTG ATG GAA GAG TGC AGG CGG CTG CAG GGG GAG GTG CAG AGG
     D   A   E   V   K   K   V   M   E   E   C   R   R   L   Q   G   E   V   Q   R   182
         d   e   f   g   a   b   c   d   e   f   g   a
661 CTT CGG GAG GAG AGC AGG CAG CTC AAG GAA GAA GAC GGG CTT CGG GCG AGG AAG GCA CTG
     L   R   E   E   S   R   Q   L   K   E   E   D   G   L   R   A   R   K   A   L   202
721 CCG AGC AAC AGC CCC ATG GCG GCT CTG GCT GCC AGC GGG AAG GAG GAG GGC CTC AGC GCG
     P   S   N   S   P   M   A   A   L   A   A   S   G   K   E   E   G   L   S   A   222
781 CGG CTG CTG GCT CTG GTG GTG CTG TTC TTT ATT GTT GGG GTC ATT ATA GGG AAG ATC GCC
     R   L   L   A   L   V   V   L   F   F   I   V   G   V   I   I   G   K   I   A   242
841 TTG TAG
     L   *
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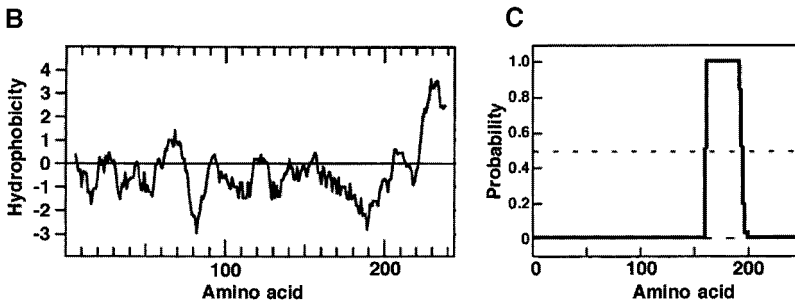

Figure 1. (A) Nucleotide and amino acid sequences of ERG30. The complete primary structure and key elements of ERG30 are indicated. The hydrophobic motif is boxed. The predicted coiled-coil motif at positions 161–194 is registered starting in c. (B) Hydropathy profile of ERG30 gene product. Kyte-Doolittle hydrophobicity profile of ERG30 indicates the position of a hydrophobic domain from residues 223 to 238, the putative transmembrane domain. (C) The coiled-coil prediction of ERG30 protein. Coiled-coil prediction was performed with the computer algorithm PAIRCOIL using a scoring cutoff of 0.50 as positive. Maximum probability (1.0) was found at positions 161–194. (D) Alignment of amino acid sequence of ERG30 with related proteins. The ERG30 amino acid sequence was aligned with those of human (hVAP33), *A. californica* (aVAP33), and *S. cerevisiae* (Scs2p). Residues identical in three sequences are boxed; shading highlights similarities between sequences.

fuged for 10 min in a Beckman TLA100.1 rotor at 37,000 *g*. The supernatant was layered over a cushion of 0.25 M sucrose in a Tris-salt buffer containing 0.06% Triton X-114, and incubated at 30°C for 5 min. After centrifugation at 2,500 *g* in a benchtop centrifuge, the Triton X-114 phase was saved. The aqua phase was brought to 0.5% Triton X-114, and layered once more on a cushion, incubated at 30°C for 5 min, and centrifuged as described before. The detergent layers were combined and the supernatant kept separately. Before the SDS-PAGE separation the detergent was removed by Biobeads SM-2 (Bio-Rad).

Results

Cloning cDNA Encoding ERG30

We have serendipitously cloned by the two-hybrid screen system a cDNA from a rat brain cDNA library that encodes a 30-kD protein. Because our analysis indicated that ERG30 is located in the ER and Golgi (see below), we have tentatively termed it ERG30. The nucleotide and deduced amino acid sequence of the cDNA are shown in Fig. 1 A. The hydrophobicity profile of ERG30 suggests that its COOH-terminal region is highly hydrophobic [residues 224–243 (Kyte and Doolittle, 1982)] (Fig. 1 B). In addition, we identified a predicted coiled-coil motif at positions 161–194 (Fig. 1, A and C). The amino acid sequence of ERG30 exhibits a high degree of homology to three integral membrane proteins: Scs2p, found in *S. cerevisiae*; the VAMP-associated protein, VAP-33, of *A. californica*; and the homologous hVAP-33 of humans (Nikawa et al., 1995;

Skehel et al., 1995b; Weir et al., 1998) (Fig. 1 D). Compared with hVAP-33, aVAP-33, and Scs2p, ERG30 displays 62%, 52%, and 27% identity and an overall 86%, 70%, and 50% similarity, respectively.

ERG30 Is a Type II Integral Membrane Protein

The expression pattern of ERG30 in various rat tissues was examined by immunoblot analysis using affinity-purified anti-ERG30 antibodies. As evident from Fig. 2 A, the 30-kD ERG30 protein is expressed in all tissues studied with a smaller 28-kD isoform, or a proteolytic product in the kidney, liver, and heart.

Because the amino-acid sequence of ERG30 suggested that it might contain a COOH-terminal transmembrane domain (Fig. 1 B), we examined its putative transmembrane topology by using anti-ERG30 antibodies for immunoblot analyses of cytosolic and membrane fractions. In rat brain, ERG30 was detected almost exclusively in the membrane fraction (Fig. 2 B). The association of ERG30 with the membrane fraction was resistant to washing with 1 M KCl (data not shown) and the protein could be extracted entirely into a detergent phase upon solubilization with Triton X-114 (Fig. 2 B). Using an in vitro translation system, we demonstrated that ERG30 was translocated into the microsomal membranes (Fig. 2 C). To exclude the possibility of peripheral association with the microsomal membranes, the translated products were incubated with

D

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ERG30    1  MAKVEQVLSLEPQHELKFRGPFTDVVVTNLKLG 33
hVAP33   1  MANDEQILVLDPPTDLKFKGPFTDVVVTNLKLR 33
aVAP33   1  MASHEQALILEPAGELRFKGPFTDVVVTADLKLS 33
SCS2     1  MSAVE----TSPD-VLVYKSPLTEQSTEYASIS 28

ERG30   34  NPITDRNVCFKVKTTAPRRYCVRPNSGVIDAGAS 66
hVAP33  34  NPSDRKVCFKVKTTAPRRYCVRPNSGIDPGST 66
aVAP33  34  NPITDRRICFKVKTTAPKRYCVRPNSGILEPKTS 66
SCS2    29  NNSDQTIAFKVKTTAPKFYCVRPNAAVVAPGET 61

ERG30   67  LNVSVMLQPFDYDPNE--KSKHKFMVQSMFAPP 97
hVAP33  67  VTVSVMLQPFDYDPNE--KSKHKFMVQSMYAPD 97
aVAP33  67  IAVAVMLQPFNYDPNE--KNKHKFMVQSMYAPD 97
SCS2    62  IQVQVIFLGLTEBPAADFKCRDKFLVITLPSPY 94

ERG30   98  DTSD--MEAVWKEAKPEDLMDSKLRCVFFELP-- 126
hVAP33  98  NTSD--MEAVWKEAKPDELMDSKLRCVFEMP-- 126
aVAP33  98  HVVES-QELLWKDAPPESLMDTKLRCVFEMPDG 129
SCS2    95  DLNGKAVADVWSDLEAEFKQQAISKKIKVKY-- 125

ERG30  127  ----------AENAKPHDVEINKIMPTSAS-K 147
hVAP33 127  ----------NENDKLNDMEPSKAVPLNAS-K 147
aVAP33 130  SHQAPASDASRATDAGAHFSESALEDPTVASRK 162
SCS2   126  ----------LISPDVHPAQNQNIQENKETVE 147

ERG30  148  TEAPVAAKPLTSPLDDAEVKKVMEECRRLQGEV 180
hVAP33 148  QDGP-MPKPHSMSLNDTETRKLMEECKRLQGEM 179
aVAP33 163  TETQSPKRVGAVGSAGEDVKKLQHELKKAQSEI 195
SCS2   148  PVVQ-DSEPKEVPAVVNE--KEVPAEPETQPPV 177

ERG30  181  QRLREESRQLKEEDGLRARKALPSN--SPMAAL 211
hVAP33 180  MKLSEENRHLRDE-GLRLRKVAHSD--KPGSTS 209
aVAP33 196  TSLKGENSQLKDE-GIRLRKVAMTDTVSPTPLN 227
SCS2   178  QVKKEEVPPVVQKTVPHENEKQTSN-STPAPQN 209

ERG30  212  AASGKEEGLSAR---LLALVVLFFIVGVIIGKL 241
hVAP33 210  TASFRDNVTSPLP--SLLVVIAAIFIGFFLGKF 240
aVAP33 228  PSPAPAAAVRAFP--PVVVVVAAIILGLIIGKF 258
SCS2   210  QIKEAATVPAENESSSMGIFILVALLILVLGWF 209

ERG30  242  AL 243
hVAP33 241  IL 242
aVAP33 259  LL 260
SCS2   243  YR 244
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Figure 1 (continued)

full length ERG30 was able to self-oligomerize in vivo, whereas the truncated forms, i.e., ERG30 (Δcoiled-coil) or ERG30 (ΔNH₂ terminus), were inactive. Our results indicate that ERG30 can self-oligomerize and both the coiled-
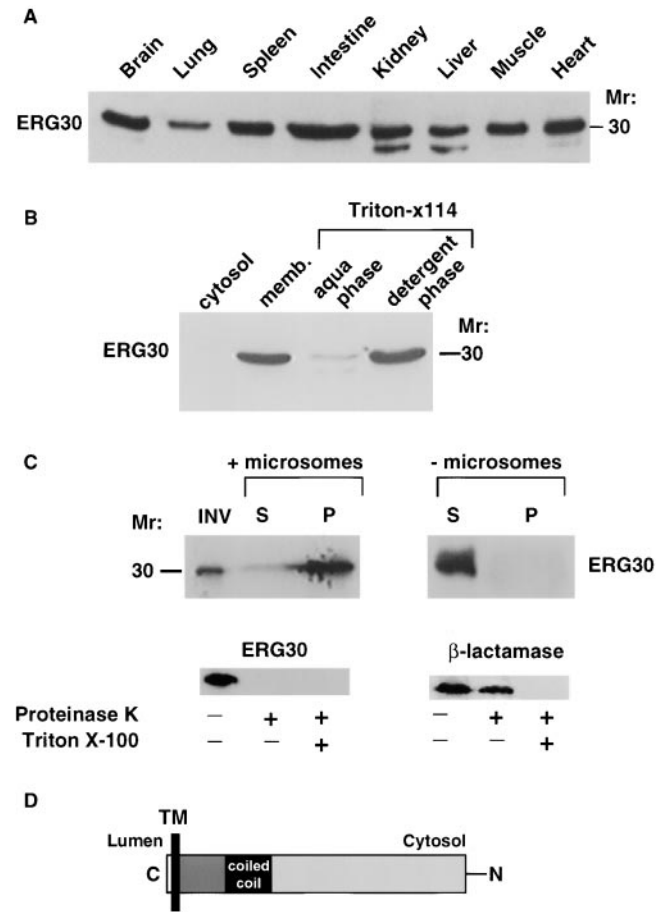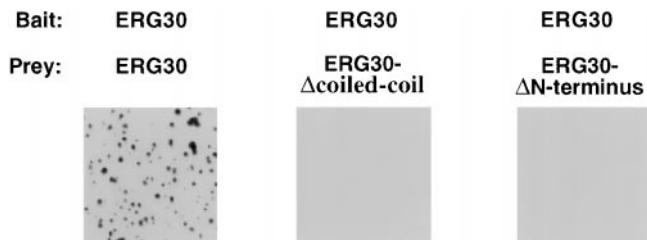
Figure 2. (A) Tissue distribution of ERG30 protein. Equal amounts (42-μg protein) of total extracts from the indicated tissues were analyzed by immunoblot with anti-ERG30 specific antibodies. No differences were observed in the total amount of protein between the different samples, as determined by amido-black staining of the nitrocellulose filters. (B) ERG30 is an integral membrane protein. Rat brain membrane extract and cytosol (prepared as described in Materials and Methods) were subjected to immunoblot analysis with antiserum specific to ERG30. The Golgi membranes were fractionated by Triton X-114 (see Materials and Methods), with the aqua phase representing the proteins found in the buffer fraction and the detergent phase proteins fractionated with Triton X-114. (C) ERG30 resides on the cytoplasmic surface of the membrane. ERG30 was translated in vitro in the presence or absence of canine pancreatic microsomes (in the minus microsomes experiment, microsomes were added at the end of the translation reaction). The translation mixture was exposed to alkaline pH and the microsomal pellet (P) and supernatant (S) were separated by centrifugation as described in Materials and Methods. To determine the membrane topology of the protein, ERG30 and β-lactamase were translated in vitro in the presence of microsomes and then were incubated for 60 min with 1 mg/ml proteinase K with or without addition of Triton X-100 to a final concentration of 1% as indicated. The proteolysis was stopped by adding 4 mM phenylmethylsulfonyl fluoride and the samples were analyzed on 15% SDS-PAGE. (D) Schematic representation of ERG30 topology.

100 mM Na₂CO₃, pH 11. Under these conditions ERG30 remained associated with the membrane fraction (Fig. 2 C). We next addressed the topology of ERG30 with respect to the cytosol by using a protease protection assay. Treatment of the in vitro translated ERG30 (in the presence of microsomes) with proteinase K fully digested the translated protein, whereas the mature form of *E. coli* β-lactamase, serving as a control protein translocated into the microsomes lumen, was fully protected (Fig. 2 C). Taken together, these results indicate that ERG30 is a type II integral membrane protein, with an NH₂-terminal domain facing the cytoplasm and a very short COOH-terminal hydrophobic domain located inside the membrane (Fig. 2 D).

Self-oligomerization of ERG30

ERG30 shares with SNAREs a similar domain organization, including a predicted membrane-proximal coiled-coil domain, a motif common to various self-oligomerizing proteins involved in protein-protein interactions (Weimbs et al., 1997). To test whether ERG30 is capable of self-oligomerization we employed the yeast two-hybrid system. Two ERG30 truncation mutants were prepared: ERG30 (ΔNH₂ terminus) containing residues 141–243, and its complementary coiled-coil deletion mutant (Δcoiled-coil) containing residues 1–140. Yeast cells were cotransfected with a construct of ERG30 fused to the Gal4 DNA binding domain, and ERG30 fused with pACT Gal4-activation domain. Only cells that contained both constructs were able to grow on a selective medium and exhibited significant βGal activity. Furthermore, as shown in Fig. 3, only the

Bait:	ERG30	ERG30	ERG30
Prey:	ERG30	ERG30-Δcoiled-coil	ERG30-ΔN-terminus

Figure 3. Self-oligomerization of ERG30 detected by the two-hybrid system. Yeast cells were cotransfected with a construct of ERG30 fused with the Gal4 DNA binding domain, and with either full-length ERG30, ERG30 (Δcoiled-coil) or ERG30 (ΔNH$_2$ terminus), fused with pACT Gal4-activation domain. The intense β-galactosidase staining of the yeast colonies observed on the left panel indicates an interaction between the different ERG30 fusion proteins.

coil and the NH$_2$-terminal motifs appear indispensable for oligomerization.

ERG30 Is Localized to the ER and the Pre-Golgi Intermediates

Intracellular targeting of a protein to a particular organelle often provides a valuable insight into its specific biological role. To examine the subcellular localization of ERG30 in NRK cells, we performed indirect immunofluorescence analysis using affinity-purified polyclonal anti-ERG30 antibodies. As illustrated in Fig. 4 A (panels 2 and 4), ERG30 is localized in a juxta-nuclear crescent resembling the Golgi complex and possibly in parts of the ER. No labeling was observed when anti-ERG30 antibodies were incubated with excess recombinant ERG30 in the form of a MBP fusion protein (data not shown). To identify the subcellular localization of ERG30, we performed a double labeling experiment using monoclonal antibodies directed against βCOP, a commonly used marker for the Golgi, together with anti-ERG30 antibodies. Using confocal microscopy, we found that labeling with the anti-ERG30 antibodies partially coincided with that of βCOP, indicating that in NRK cells ERG30 is localized in the vicinity of the Golgi complex (Fig. 4 A, panels 3 and 4). Treating cells with the drug BFA was shown previously to specifically disrupt the Golgi complex in vivo (Lippincott-Schwartz et al., 1989). Here we showed that in the presence of BFA, the labeling observed by anti-βCOP spread throughout the cytoplasm, whereas labeling with anti-ERG30 formed a characteristic ER pattern (Fig. 4 A, panels 6 and 8). To confirm the localization of ERG30 in ER membranes, the cells were double labeled with monoclonal antibodies directed against protein disulfide isomerase (PDI), an ER resident protein, and with anti-ERG polyclonal antibodies. As shown in Fig. 4 A (panels 1 and 2), part of the labeling observed with the anti-ERG30 antibodies coincided with that of PDI, indicating that the two proteins might share the same compartment. Upon treatment with BFA, the colocalization of PDI and ERG30 increased significantly (Fig. 4 A, panels 5 and 6).

The intracellular localization of ERG30 was also determined by subcellular fractionation of bovine liver postnuclear supernatant on equilibrium density sucrose gradients (Fig. 4 B). Fractions were analyzed by immunoblotting with affinity-purified anti-ERG30 antibodies and with antibodies that recognize either Gos28, a marker of the Golgi apparatus, or PDI, an ER marker. Membranes that were concentrated at the 0.86/1.25 interface of the first gradient were harvested, adjusted to 1.6 M sucrose, and loaded onto the bottom of a second gradient (Fig. 4 B). Immunoblot analysis of the second gradient showed that ERG30 predominantly colocalizes with PDI and to some lesser extent with Gos28, indicating that it associates with both Golgi and ER membranes.

ERG30 subcellular localization was further analyzed by immunoelectron microscopy. Silver grains representing sites of immunoreactivity for ERG30 were localized predominantly on the cytoplasmic faces of the RER and on the outer leaflet of the nuclear membrane—both sites of synthesis for membrane and secreted proteins (Fig. 4 C, panel 1). Labeled cisternae of RER were found throughout the cytoplasm. Little immunoreactivity was localized to the Golgi apparatus. However, silver grains often were observed on RER cisternae that were very close to the Golgi, and on cisternae that appeared to be transitional between RER and the cis-Golgi (Fig. 4 C, panel 2 and inset). Very few silver grains were seen on other structures such as the plasma membrane, mitochondria, or the nucleus. These represented nonspecific labeling since they were not eliminated by absorption of ERG30 antibodies with excess antigen, whereas labeling of the RER, nuclear envelope, and RER-Golgi transitional structures was virtually eliminated (data not shown). Taken together, our results indicate that ERG30 is localized on the cytosolic surface of the ER and on the pre-Golgi intermediates.

ERG30 Is Not Part of the Synaptic SNARE Complex

It has been demonstrated in *Aplysia* that VAP-33 directly interacts with the synaptic v-SNARE, VAMP, suggesting its involvement in docking and fusion of synaptic vesicles (Skehel et al., 1995b). We used two approaches to examine whether ERG30 interacts with synaptic SNAREs. First, we used monoclonal antibodies against SNAP-25 and syntaxin-1 to coimmunoprecipitate ERG30 from rat brain membrane extract. As shown in Fig. 5 A, ERG30 did not precipitate with any of the known synaptic SNAREs. Next, we examined whether ERG30 interacts with synaptic SNAREs by testing its incorporation into 20S fusion particles (Söllner et al., 1993b). The 20S particles are formed when detergent extracts of membranes containing SNAP receptors are mixed with SNAPs and NSF in the presence of the nonhydrolyzable analogue of ATP, ATPγS. The 20S fusion particle assembly reaction can therefore be used as a cell-free read-out system to test whether candidate proteins are SNAREs, or specifically interact with a SNARE. The results of such a 20S particle experiment, using rat brain membrane extract as a source for SNAREs, are presented in Fig. 5 B. Specific (Mg-ATP) and nonspecific elutions (Mg-ATPγS) were analyzed by Western blots. As expected, the known synaptic membrane proteins VAMP, syntaxin, and SNAP-25 assembled into the 20S particle derived from brain membrane extract, but ERG30 did not. These results indicate that ERG30 is not part of the synaptic SNARE complex.
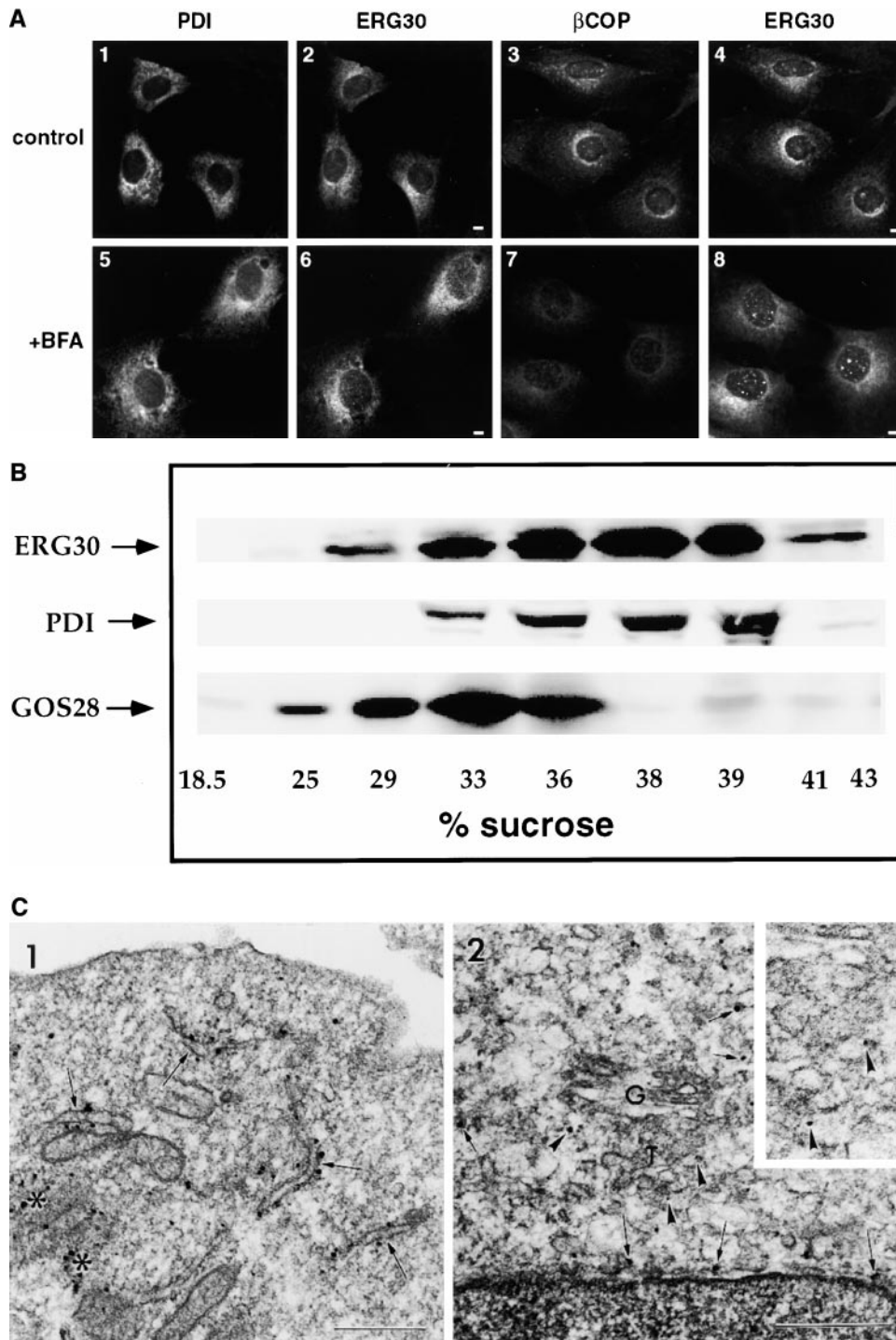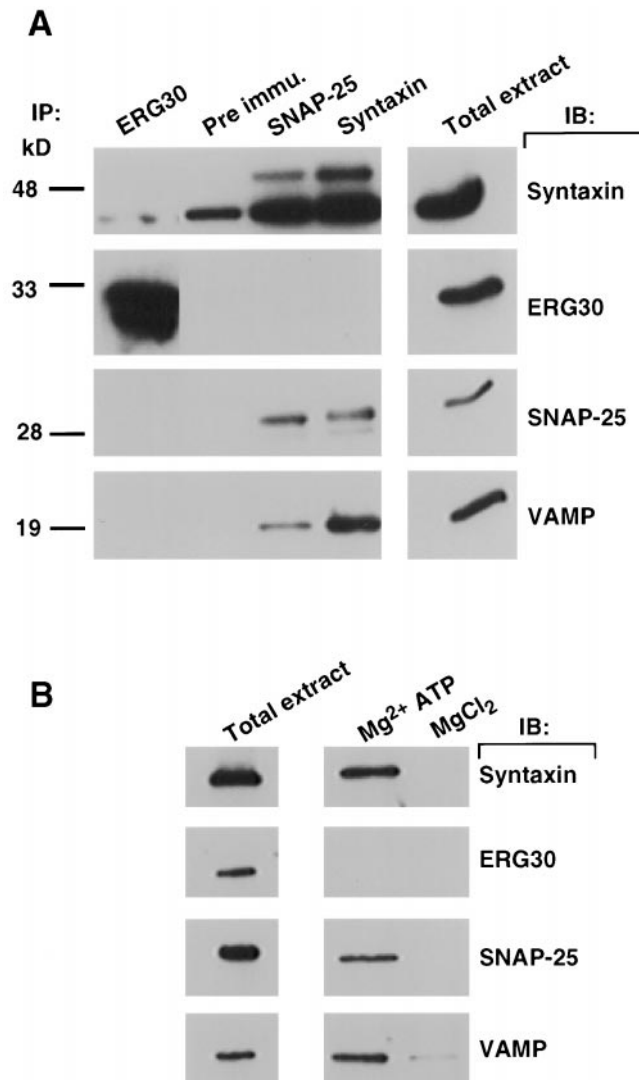
Figure 4. Localization of ERG30 in the Golgi and ER. (A) Indirect immunofluorescence microscopy of endogenous ERG30 with and without BFA treatment of NRK cells. Localization of ERG30, PDI, or β-COP was performed in NRK cells with and without BFA treatment (10 μg/ml, 1 h). PDI (1 and 5 plus BFA) and β-COP (3 and 7 plus BFA) immunoreactivity were detected with fluorescein-conjugated secondary antibodies; ERG30 immunoreactivity (2, 4 and 6, 8 plus BFA) was detected with rhodamine-conjugated secondary antibodies. (B) Determination of ERG30 localization by subcellular fractionation. Bovine liver postnuclear supernatant in 0.5 M sucrose was layered on top of a sucrose step gradient composed of 0.86/1.25 M sucrose layers. The gradients were centrifuged and the interfaces was collected (see Materials and Methods). The 0.86/1.25 M sucrose interface was adjusted to a 1.35 M sucrose and loaded at the bottom of a second step gradient with 1.25, 1.0, 0.86, and 0.5 M sucrose. After centrifugation, the different fractions were analyzed by SDS-PAGE and immunoblotting with affinity-purified anti-ERG30 antibodies or with anti-PDI or anti-Gos28 antibodies. (C) Immunogold-silver localization of ERG30 in CHO (K1) cells. (Panel 1) Most silver grains are located on the cytoplasmic face of RER cisternae (arrows). Some clusters of grains are found on indistinct structures in the cytoplasm, probably RER cisternae sectioned tangentially (asterisks). (Panel 2) Silver grains are often found on the cytoplasmic face of the outer leaflet of the nuclear envelope (arrows), on RER cisternae (small arrows) close to the Golgi (G), and on structures that appear to be transitional between the RER and the Golgi (T-arrowheads). The inset shows an area on the right hand side of the transitional zone and Golgi at twofold higher magnification, making it easier to see two small silver grains (arrowheads). Bars, 0.5 μm.

ERG30 Is Involved in Intra-Golgi Transport

The subcellular distribution of ERG30 indicates that it might play a role early in the secretory pathway. We used the cell-free intra-Golgi transport system (Balch et al., 1984) to examine this possibility. Affinity-purified anti-ERG30 antibodies specifically inhibited intra-Golgi transport by up to 90% of the control in a concentration-depen-

dent manner (Fig. 6 A, open squares); neither nonrelevant control antibodies (Fig. 6 A, open triangles) nor preimmune IgGs had any effect on this assay (data not shown). Recombinant ERG30 specifically reversed the inhibition by these antibodies (Fig. 6 C). Anti-ERG30 Fab′ fragments were also found to partially inhibit transport (Fig. 6 A, open rhombuses) excluding the possibility that the inhi-

A

| IP: | ERG30 | Pre immu. | SNAP-25 | Syntaxin | Total extract | IB: |

kD
48 — Syntaxin
33 — ERG30
28 — SNAP-25
19 — VAMP

B

| | Total extract | Mg²⁺ ATP | MgCl₂ | IB: |

Syntaxin
ERG30
SNAP-25
VAMP

Figure 5. ERG30 is not part of the synaptic SNARE complex. (A) Immunoprecipitation of ERG30 from rat brain membrane extract. Rat brain membrane extracts were incubated with protein A–agarose coupled to the indicated antibodies. The bound material was subjected to Western blot analysis. IP represents immunoprecipitation. IB represents immunoblot. (B) Formation of synaptic 20S particles. $MgCl_2$ represents nonspecific eluate from complete binding reaction with NSF-Myc and Mg-ATPγS; Mg^{2+} ATP represents specific eluate of the same column as for N.S. following the exchange of ATP for ATP-γS (in the presence of EDTA) and addition of $MgCl_2$ to allow ATP hydrolysis. Syntaxin, SNAP-25, VAMP, and ERG30 were detected with specific antibodies by immunoblot analysis.

bition brought about by the antibodies resulted from antibody-induced aggregation of ERG30 on the membrane. To determine the stage at which ERG30 is required, anti-ERG30 antibodies were added at different time points after initiation of transport. As shown in Fig. 6 B, transport was inhibited by addition of anti-ERG30 antibodies to the assay between time 0 and 10 min, establishing the involvement of ERG30 in the transport process per se rather than in the glycosylation of the VSV-G protein (i.e., after 10-min time points).

The involvement of ERG30 in intra-Golgi transport was also demonstrated by using soluble recombinant MBP-ERG30. The effect of recombinant full-length MBP-ERG30, MBP-ERG30 (Δcoiled-coil) or MBP-ERG30 (ΔNH₂ terminus) mutants on VSV-G transport in vitro was therefore examined. As shown in Fig. 6 D, addition of increasing amounts of recombinant MBP-ERG30 fusion protein inhibited transport in vitro by nearly 90%. No inhibition was detected in the presence of equivalent concentrations of MBP-ERG30-Δcoiled-coil, MBP-ERG30-ΔNH₂ terminus, or MBP-control. This result is in agreement with the fact that only the wild-type ERG30 can interact with the endogenous ERG30, whereas the truncated proteins could not (Fig. 3 B). We therefore concluded that inhibition observed in the presence of the soluble MBP-ERG30 results from blocking the function of the endogenous membranal ERG30.

Anti-ERG30 Antibodies Cause an Accumulation of COPI-coated Vesicles

Anti-ERG30 antibodies were used to establish the stage in the transport pathway at which ERG30 is required. For that purpose we used conditions that promote budding of COPI vesicles from Golgi membranes. Under such budding reactions, Golgi membranes were incubated with crude cytosol in the presence or absence of either GTPγS or anti-ERG30 antibodies. The Golgi cisternae were then pelleted at 14,000 rpm and the supernatant was fractionated on a sucrose gradient to isolate the COPI-coated vesicles. As shown in Fig. 7, incubation of Golgi membranes in the presence of GTPγS resulted in a significant accumulation of COPI-coated vesicles. A significant quantity of COPI vesicles also accumulated in the presence of affinity-purified anti-ERG30 antibodies. When both GTPγS and anti-ERG30 were present in the budding reaction, even more COPI vesicles accumulated (data not shown). Notably, the vesicles accumulated in the presence of anti-ERG30 antibodies appeared more homogeneous in comparison to those accumulated in the presence of GTPγS. These results show that ERG30 is not involved in the budding process but rather in the consumption of these vesicles. Most of the ERG30 remained associated with the Golgi cisternae and did not migrate with the accumulated COPI vesicles (data not shown).

Discussion

We show here that ERG30, the rat homologue of aVAP-33, is ubiquitously expressed and is localized primarily within the ER and the pre-Golgi intermediates. The localization of ERG30 suggests that it functions early in the secretory pathway rather than in the plasma membrane. This is further supported by the observation that ERG30 plays a role in intra-Golgi transport in vitro. We propose that ERG30 represents a novel integral membrane protein family which is involved in the process of vesicle fusion with target membranes.

Previous studies demonstrated that *A. californica* VAP-33 can interact with VAMP (synaptobrevin) and that it participates in the process of synaptic release (Skehel et al., 1995a,b). The yeast homologue, however, was local-
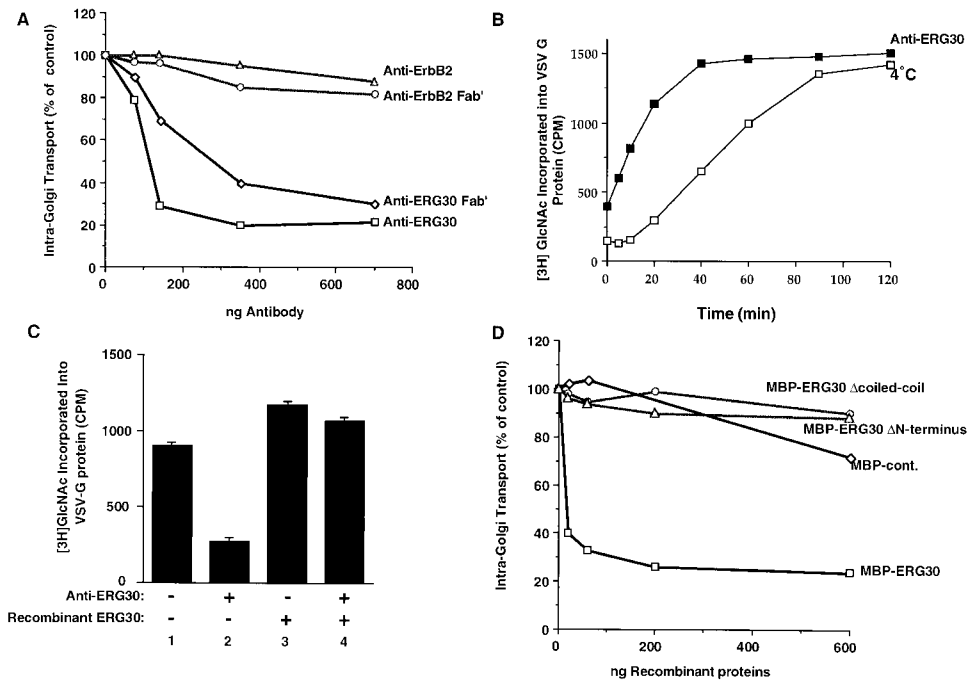
Figure 6. (A) Inhibition of intra-Golgi transport in vitro by anti-ERG30 antibodies. Transport activity of crude cytosol, measured by the standard transport assay (see Materials and Methods), was tested in the presence of the indicated concentrations of affinity-purified antibodies specific for ERG30 (open squares), Fab' fragments of anti-ERG30 (open rhombuses), or anti-ErbB2 (open circles) and anti-ErbB2 Fab' (open triangles) as controls. The Golgi membranes were preincubated with the different antibodies for 30 min on ice before being transferred to a 30°C bath to start the transport reaction. The extent of [³H]GlcNAc incorporated is expressed as the percentage of the control level of transport in reactions without added antibodies. (B) Temporal sensitivity of VSV-G transport to anti-ERG30 antibodies. At the time points indicated, the assays were shifted to ice (open squares) or the mixtures were supplemented with 1.0 μg of anti-ERG30 antibodies and incubated at 37°C for the remaining period up to 120 min (full squares). (C) Inhibition of vesicular transport by anti-ERG30 antibodies is reversed by recombinant ERG30. Bars 1 and 2 describe a standard transport reaction in the presence or absence of anti-ERG30 antibodies (0.5 μg); bars 3 and 4 describe the same transport conditions, respectively, in the presence of antibody-neutralizing amounts of MBP-ERG30 (1.0 μg). (D) Inhibition of vesicular transport by MBP-ERG30. Increasing concentrations of fusion proteins, MBP-ERG30, MBP-ERG30 (Δcoiled-coil) MBP-ERG30 (ΔNH₂ terminus), or MBP alone were added to the transport reaction. The extent of [³H]GlcNAc incorporated is expressed as the percentage of the control level of transport with no added recombinant proteins.

ized to the ER and was shown to be required for inositol metabolism (Kagiwada et al., 1998). Our data clearly demonstrate that the mammalian homologue of these proteins is localized in the ER and pre-Golgi intermediates, playing a role in protein transport in the early secretory pathway. We could not identify an interaction between ERG30 and synaptic SNAREs. Our immunolabeling experiments on neuronal cells indicate that ERG30 is localized on endomembranes, primarily the Golgi and the ER. No labeling of ERG30 was seen on or near the plasma membrane, where most of the VAMP labeling was observed (Soussan, L., and Z. Elazar, unpublished data). We clearly demonstrated here that ERG30 is not part of the synaptic SNARE complex. Our data, however, does not exclude the possibility that ERG30 interacts weakly with VAMP, in a manner that is not resistant to immunoprecipitation conditions. It is also possible that mammals carry other, yet-unidentified genes that encode VAP-33 homologues which function at later stages of the secretory pathway.

The accumulation of COPI vesicles caused by anti-ERG30 antibodies coupled with the localization of ERG30 both in the Golgi and ER suggest that this protein is involved in transport between these organelles, possibly in the retrograde direction. The accumulation of COPI vesicles may also indicate that ERG30 is involved in regulating the uncoating of these vesicles. It is not clear why the accumulated vesicles did not uncoat in the presence of the anti-ERG30 antibodies. ERG30 might be involved in trig-

gering ADP-ribosylation factor (ARF)-GTPase activating protein (GAP), an activity which, in turn, stimulates the uncoating process. Additional experiments are needed to determine whether uncoating of vesicles is affected by ERG30 directly, or is coupled to the vesicles' docking; the
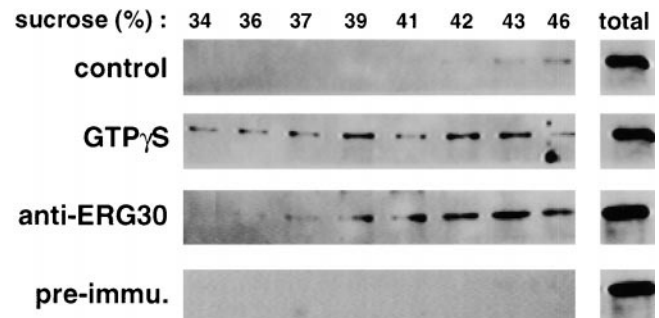
Figure 7. Accumulation of Golgi-derived COPI-coated vesicles. Golgi membranes were incubated with cytosol in the presence or absence of GTPγS or affinity-purified anti-ERG30 antibodies. Salt was added to a final concentration of 0.25 M KCl, Golgi was pelleted, and the supernatant (containing the vesicles) was fractionated on a sucrose gradient. Fractions corresponding to the indicated sucrose concentrations were analyzed for β-COP content by Western blotting. The bottom of the gradient where the original material was present is not shown. Total represents ∼20% of the original Golgi plus cytosol of each experiment.

latter would implicate a role for ERG30 in targeting vesicles to the appropriate organelle.

ERG30 is a type II integral membrane protein, most of which is presented to the cytosol. Examining the protein sequence by the Paircoil program revealed a strong probability for a coiled-coil domain at positions 161–194 followed by several basic residues. This structure resembles that of the SNARE family (Weimbs et al., 1998). We did not yet find an interaction between ERG30 and SNARE molecules, though we demonstrated here that ERG30 can self-oligomerize. We also demonstrated that the recombinant protein, which is capable of interacting with the endogenous membrane bound pool of ERG30, blocked the cell-free intra-Golgi transport assay possibly by inhibiting the function of the membrane-associated ERG30. Although it is not clear whether this interaction is direct or requires another membrane protein, similar to the interactions between v- and t-SNARES, the evidence presented above implicates ERG30 in the docking and fusion machinery.

We found that ERG30 functions in the early secretory pathway, predominantly within the ER and the Golgi complex. Our immunoelectron microscopy studies indicated that it is found mainly in the ER and in transitional elements found between this organelle and the cis-Golgi. These structures might represent the ER-Golgi intermediate compartment, known also as pre-Golgi intermediates (PGIs). It has been suggested by Balch and co-workers that ER-derived transport vesicles fuse to form vesicular-tubular clusters (VTCs) (Bannykh et al., 1996, 1998). These structures are the target for ER-derived COPII and Golgi-derived COPI vesicles. Considering the functional data presented in this study, specifically the involvement of ERG30 in COPI vesicle transport and its subcellular localization, we speculate that in addition to its intra-Golgi activity, ERG30 is involved in retrograde transport from the Golgi to the ER via PGIs.

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