Proteomics of Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) Membranes from Brefeldin A-treated HepG2 Cells Identifies ERGIC-32, a New Cycling Protein That Interacts with Human Erv46*§

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Cycling proteins play important roles in the organization and function of the early secretary pathway by participating in membrane traffic and selective transport of cargo between the endoplasmic reticulum (ER), the intermediate compartment (ERGIC), and the Golgi. To identify new cycling proteins, we have developed a novel procedure for the purification of ERGIC membranes from HepG2 cells treated with brefeldin A, a drug known to accumulate cycling proteins in the ERGIC. Membranes enriched 110-fold over the homogenate for ERGIC-53 were obtained and analyzed by mass spectrometry. Major proteins corresponded to established and putative cargo receptors and components mediating protein maturation and membrane traffic. Among the uncharacterized proteins, a 32-kDa protein termed ERGIC-32 is a novel cycling membrane protein with sequence homology to Erv41p and Erv46p, two proteins enriched in COPII vesicles of yeast. ERGIC-32 localizes to the ERGIC and partially colocalizes with the human homologs of Erv41p and Erv46p, which mainly localize to the cis-Golgi. ERGIC-32 interacts with human Erv46 (hErv46) as revealed by covalent cross-linking and mistargeting experiments, and silencing of ERGIC-32 by small interfering RNAs increases the turnover of hErv46. We propose that ERGIC-32 functions as a modulator of the hErv41-hErv46 complex by stabilizing hErv46. Our novel approach for the isolation of the ERGIC from BFA-treated cells may ultimately lead to the identification of all proteins rapidly cycling early in the secretary pathway.

Protein transport from the ER† to Golgi is mediated by the ER-Golgi intermediate compartment (ERGIC), a collection of tubulovesicular membrane clusters (1) in the vicinity of ER exit sites (2, 3). ERGIC membranes enriched in the marker protein ERGIC-53 exhibit a protein pattern that is different from that of ER and Golgi (4), but only a few ERGIC-associated proteins have been identified and characterized. Morphologically, the ERGIC can most reliably be identified by the cycling membrane protein ERGIC-53 (1, 5) (p58 in rat (6)) in conjunction with the COPI coat subunit β-COP (7). ERGIC-53 is a mannos-lectin assisting efficient ER export of some glycoprotein cargo including cathepsin C (8), cathepsin Z (9), and the blood coagulation factors V and VIII (10). Other membrane proteins enriched in the ERGIC (and cis-Golgi) include the KDEL receptor (KDEL-R (11)), p24 family members (12–14), and SNARE proteins such as syntaxin 5 (15, 16), rBet1 (17), Sec22 (18), and syntaxin 18 (19). Moreover, the small GTPases Rab1 and Rab2 are peripherally associated with the ERGIC (20–22).

The ERGIC is the first anterograde/retrograde sorting station in the secretary pathway. Proteins cycling in the early secretary pathway are sorted in the ERGIC from secretory cargo with different efficiencies depending on the features and the expressed amounts of the individual proteins. ERGIC-53 is efficiently sorted into the retrograde pathway, and only a minor fraction escapes to the cis-Golgi (7), whereas a larger fraction of the KDEL-R also cycles via the cis-Golgi (11, 21).

It appears unlikely that protein sorting is the only function of the ERGIC, but other presumed functions are less clear. The ERGIC appears to play some role in quality control of newly synthesized proteins as indicated by the presence of the KDEL-R, which cycles chaperones and folding enzymes to the ER that have inadvertently escaped from the ER. In general, only fully folded and oligomerized proteins are transport-competent and can leave the ER (23). It is presently unclear, however, to what extent protein folding can also extend to the ERGIC in different cells (24–27). The ERGIC has also been postulated to be involved in retrotranslocation to the cytosol of permanently misfolded proteins (28).

A particularly striking feature of the ERGIC is its resistance to the fungal metabolite brefeldin A. BFA blocks ADP-ribosylation factor (Arf) in an inactive GDP-bound conformation and thereby prevents binding of COPI coats to ERGIC and Golgi membranes (29, 30). Upon BFA treatment the Golgi rapidly tubulates and fuses with the ER by an energy-, temperature-, and microtubule-dependant process (31–33). In contrast, the

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§ The on-line version of this article (available at http://www.jbc.org) contains supplementary Fig. 1.

The nucleotide sequence reported in this paper has been submitted to the Swiss Protein Database under Swiss-Prot accession no. Q969X5.

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† The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; KDEL-R, KDEL-receptor; siRNA, small interfering RNA; SNARE, soluble NSF attachment protein receptor; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption time-of-flight; DSP, dithiobis(succinimidyl propionate).
HepG2 cells (one 150-mm diameter dish/gradient) were treated with 10 Nycodenz gradients (Nycomed, Oslo, Norway). 5 days postconfluent Erv41 (hErv41)-Myc was induced by incubation for 16 h with 10 mM recommendations (Roche Diagnostics). Stable cell lines were obtained and characterized in yeast and functioning in ER to Golgi protein transport. ERGIC-32 was produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immunizing rabbits with a recombinant peptide encompassing amino acids 45–200 of the luminal domain of ERGIC-32 were produced by immu...
processed for immunofluorescence in 1% BSA containing PBS. Confocal laser-scanning microscopy pictures were acquired with a TCS NT microscope (Leika). To determine the topology of ERGIC-32, cells were fixed with 3% paraformaldehyde on ice, and the plasma membrane was permeabilized with 20 μg/ml digitonin (Calbiochem-Novabiochem) for 5 min on ice and washed with PBS. The cells were then further permeabilized or not with 0.1% saponin and processed for immunofluorescence microscopy. Immunofluorescence was recorded with a Zeiss Axiovert 135M microscope coupled to a charge-coupled device camera.

**RNA Interference**—ERGIC-32 gene expression was silenced using synthesized small interfering RNA duplexes (Eurogentech, Liege, Belgium). The targeted sequences of ERGIC-32 were AACGAGCTCTATATGGCAGGAGC and AACATCGTGCCTACAGCACCA for si1 and si2, respectively. Their specificity for the ERGIC-32 mRNA was confirmed by a BLASTn search in NCBI database. Similarly the oligonucleotide AGCCAGGATTTTTCATACATG was designed to interfere specifically with ERGIC-53. Immunofluorescence was recorded with a Zeiss Axiovert 135M microscope coupled to a charge-coupled device camera.

**RESULTS**

**Purification of ERGIC Membranes from BFA-treated Cells**—To identify new cycling proteins in the early secretory pathway, we developed a procedure for the purification of ERGIC membranes from HepG2 cells treated with BFA. The rationale of using BFA is 2-fold. BFA leads to an accumulation of rapidly cycling proteins in the ERGIC, as exemplified by ERGIC-53 and KDEL-R, and to the fusion of the Golgi apparatus with the ER. The latter effect facilitates the purification of ERGIC membranes which, in the absence of BFA, largely cofractionate with trans-Golgi membranes on density gradients (7). In a first step, a postnuclear supernatant of BFA-treated HepG2 cells was separated by Nycodenz gradient centrifugation. ER, Golgi, and plasma membrane distributed to the bottom fractions of the Nycodenz gradient, whereas ERGIC membranes, identified by ERGIC-53 and KDEL-R, floated to the top fractions (Fig. 1A; also see supplementary Fig. 1). The ERGIC-containing top fractions were still contaminated by endosomes (transferrin receptor, not shown), lysosomes (LAMP-1), trans-Golgi network (TGN46), and some ER (BAP31) (Fig. 1B, lane T). In a second step, these contaminating membranes were removed by immunopurification. The pooled top fractions were incubated with magnetic beads to which an antibody against a cytoplasmically exposed epitope of the KDEL-R was coupled (Fig. 1). The resulting membrane fraction was devoid of detectable transferrin receptor, LAMP-1, TGN46, and BAP31 but contained some antibodies that had leaked from the immunobeads. The immunopurified membranes were then subjected to the classical carbonate, pH 11.5, treatment to remove the antibodies as well as peripheral proteins. The final ERGIC fraction was 110 ± 19.5-fold (mean ± S.E., n = 3) enriched over the homogenate.

**Proteomics of ERGIC Membranes**—To determine the protein composition of the purified ERGIC membrane fraction we scaled-up the purification and separated the proteins by 6–12% SDS-PAGE. One-dimensional instead of two-dimensional gels were used because of the unresolved difficulty of solubilizing membrane proteins by isoelectric focusing (50). 30 bands in a molecular mass range of 20 to more than 300 kDa were reproducibly detected on silver-stained gels (Fig. 2). Bands were excised, trypsin-digested in gel, and analyzed in parallel by MALDI-TOF MS and nano-electrospray tandem mass spectrometry techniques. 19 proteins were unambiguously identified by screening NCBI databases. Several proteins were identified with both techniques, whereas some could only be detected with one. A refinement of the gel analysis using SDS gels containing urea, to better solubilize membrane proteins and resolve low molecular weight proteins, led to the identification of five more proteins, bringing the total number to 24 identified proteins (Table I).

The identified proteins can essentially be grouped into four classes: cargo receptors, proteins involved in membrane traffic, chaperones involved in protein maturation, and uncharacterized proteins (Table I). ERGIC-53 is apparently the most abundant protein of the purified ERGIC fraction. VIP36, another lectin that is related to ERGIC-53 (51, 52), was also identified, as well as p24A, Gp25L2, Tmp21, and CGI-100, four members of the p24 protein family. In accord with these results, previous studies have shown that VIP36 cycles between the ER and Golgi (53), although it may also operate at a post-Golgi level in some cells (54). P24 family members are known to cycle in the early secretory pathway, both in higher eukaryotes and in yeast, and to operate as cargo receptors linking cargo to COPI and COPII coats (13, 55–58).

Among the proteins having functions in membrane trafficking, the small GTPase Rab1 and the t-SNARE Sec22b are already known to be associated with the ERGIC (18, 21, 22). The largest protein in the ERGIC fraction was identified as giantin, a cis-medial Golgi protein implicated in intra-Golgi traffic (39, 59, 60). By immunofluorescence microscopy we confirmed that giantin indeed colocalizes in part with ERGIC elements in BFA-treated HepG2 cells (not shown) as previously shown for other cells (61).

Some luminal chaperones associated mainly with the ER were also identified, including BIP and protein disulfide isomerase. These proteins carry a COOH-terminal KDEL motif that allows them to cycle between ER and Golgi (62). Class 1A aminopeptidase N is an enzyme involved in the maturation of glycoproteins (63), and CBP1/Hsp47 is a procollagen-binding lectin that is related to ERGIC-53 (51, 52), was also identified, as well as Surf-4 being more enriched in the ER compared with COPII vesicles and operates as a transport receptor for specific cargo in yeast (66, 68). Surf-4 encodes a 30-kDa protein localized in the early secretory pathway, but its function is unknown (69). KIAA1181 is a putative protein of 290 amino acids the cDNA of which was cloned by the Kasuza project. The protein has not been characterized yet (47).

hYif1p and Surf-4 have not been described as cycling proteins before and are mainly uncharacterized. hYif1p is the human ortholog of yeast Yif1p a protein enriched in COPII vesicles (66). Surf-4 (surfeit locus 4) gene belongs to a cluster of highly conserved housekeeping genes (67), and the corresponding protein is the human ortholog of Erv29p of yeast. Erv29p is also enriched in COPII vesicles and operates as a transport receptor for specific cargo in yeast (66, 68). Surf-4 encodes a 30-kDa protein localized in the early secretory pathway, but its function is unknown (69). KIAA1181 is a putative protein of 290 amino acids the cDNA of which was cloned by the Kasuza project.
update our procedure regarding its suitability for identifying cycling proteins of the early secretory pathway (also see enrichment of ERGIC-32 along the purification procedure in supplementary Fig. 1).

**ERGIC-32 Is Homologous to Erv46p and Erv41p**—For the remainder of this report we will focus on the characterization of KIAA1181. The corresponding gene is localized on human chromosome 5 at locus 5q35.2 and comprises 10 exons. The cDNA was cloned by the Kasuza DNA Research Institute from human adult brain (clone hf00712a) and contains an open reading frame of 800 base pairs and 5'- and 3'-untranslated regions (47). Conceptual translation yields a protein of 290 amino acid residues and a predicted molecular mass of 32.5 kDa in agreement with the apparent molecular mass on SDS gels (Fig. 2).

We designate the protein ERGIC-32 (Swiss-Prot accession number Q969X5) referring to its molecular weight and its enrichment in ERGIC membranes (see below). ESTs corresponding to ERGIC-32 were found in NCBI EST databases for most tissues, indicating that ERGIC-32 is ubiquitously expressed in agreement with the RT-PCR enzyme-linked immunosorbent assay data provided by the Kasuza human cDNA project (www.kazusa.or.jp/huge/). Data base searches also revealed orthologs of ERGIC-32 in mouse and *Caenorhabditis elegans* with sequence similarities of 98 and 55%, respectively (Fig. 4A). The function of these proteins is unknown. Moreover, two human proteins, CGI-54 (also named “putative 43.2-kDa protein”) and CDA14, with sequence identity scores of 34 and 21% were identified (Fig. 4B). These proteins are the human orthologs of Erv46p and Erv41p, two proteins enriched in ER-derived COPII vesicles (66). ERGIC-32 shares higher homologies with Erv46p (34 and 26% identity with human and yeast Erv46, respectively) than with Erv41p (21 and 18% identity)

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**Fig. 1. Purification of ERGIC membranes.** A, flow diagram of the procedure used to purify ERGIC membranes from cells treated with BFA. B, analysis of the purification by immunoblotting. Aliquots of the cell homogenate (H, 25 μg of proteins), the pooled bottom fractions 1–8 from the Nycodenz gradient (B, 20 μg of proteins), the pooled top fractions 9–12 (T, 20 μg of proteins), and the immunopurified membranes (M, 0.4 μg of proteins) were analyzed by SDS-PAGE and immunoblotting. Top fractions are enriched in ERGIC-53 and KDEL-R, and ER and Golgi are mostly found in the bottom fractions. Immunopurified membranes are specifically enriched in ERGIC markers, and TGN (TGN46) and lysosomal (LAMP-1) membranes are depleted. The weak signal obtained with anti-BAP31 in the M lane is because of the anti-KDEL light chain and not BAP31.
According to TopPred2 predictions (70), the NH₂- and COOH- termini of ERGIC-32, Erv46p and Erv41p belong to the same protein family (Fig. 4D). Molecular Characterization of ERGIC-32—The hydrophobicity profile of ERGIC-32 suggests two hydrophobic domains at the NH₂ and COOH termini, each of sufficient length to span the membrane bilayer, but the predicted protein sequence lacks a typical NH₂-terminal signal sequence (Fig. 4, A and C). According to TopPred2 predictions (70), the NH₂- and COOH-terminal ends of the protein are on the cytoplasmic side, whereas the greater part of the protein in between the two transmembrane domains is luminally exposed. The luminal domain carries a consensus site for N-glycosylation (Fig. 4A). To further characterize ERGIC-32, we raised a rabbit polyclonal antibody against a recombinant GST-ERGIC-32 fusion protein, which immunoprecipitated a protein with the expected Mr from HepG2 cells metabolically labeled with [35S]methionine (Fig. 5A, lane 1). The reaction could be quenched with purified recombinant GST-ERGIC-32 (Fig. 5A, lane 3), and as expected, a stronger signal with a slightly higher Mr was obtained with cells expressing HA-tagged ERGIC-32, confirming the specificity of the antibody (Fig. 5A, lane 2). ERGIC-32 is N-glycosylated as probed with endoglycosidase H but does not appear to undergo complex glycosylation as revealed by pulse-chase experiments (Fig. 5B). ERGIC-32 is a bona fide membrane protein because it remains membrane-associated after carbonate, pH 11.5, treatment (Fig. 5C) like the type 1 membrane protein ERGIC-53 (5) but unlike the soluble receptor-associated protein RAP (71).

The membrane topology of ERGIC-32 was probed in situ by antibody accessibility in cells permeabilized with digitonin alone or digitonin plus saponin. Digitonin permeabilizes the plasma membrane but leaves internal membranes intact, permitting the selective detection of cytoplasmic epitopes on transmembrane proteins of internal membranes (72). Fig. 5D shows that digitonin permeabilization allowed the detection of giantin with an antibody against its cytoplasmic domain (73), whereas an antibody against the luminal domain of GPP130 (74) gave no reaction (upper panels). In contrast, permeabilization with digitonin and saponin allowed the detection of both cytoplasmic and extracytoplasmic (luminal) epitopes (Fig. 5D, lower panels). HA epitopes attached to either the NH₂ or the COOH terminus of ERGIC-32 were detected with both conditions of permeabilization, indicating that both ends of the molecule are exposed to the cytoplasm. We conclude that ERGIC-32 has two transmembrane domains and that the greater part of the protein is luminally exposed, whereas the NH₂ and COOH termini are cytoplasmic. Consistent with this topology, the (used) consensus sequence for N-glycosylation is located between the two transmembrane domains.

Endogenous ERGIC-32 Localizes to the ERGIC and Overlaps with the Distribution of hErv41 and hErv46.—Next, we examined the localization of endogenous ERGIC-32 in HeLa and HepG2 cells by immunofluorescence microscopy using affinity-purified anti-ERGIC-32 (Fig. 6). In both cell lines ERGIC-32 localizes to dots scattered throughout the cytoplasm and in the Golgi area. The dots also co-stain for ERGIC-53. In BFA-treated cells ERGIC-32 localizes to the typical ERGIC-53-positive punctate elements (Fig. 6, bottom panels). Consistent with the morphological data, ERGIC-32 co-fractionated largely with ERGIC-53 on Nycodenz gradients (Fig 7B). Unlike ERGIC-53, ERGIC-32 was almost excluded from fraction 9, which is enriched in the cis-Golgi marker GPP-130. We conclude that ERGIC-32 is an ERGIC marker.

The homology of ERGIC-32 with Erv41p and Erv46p proteins prompted us to compare the three proteins. We first compared the localization of ERGIC-32 and hErv46 by confocal immunofluorescence microscopy. Mouse Erv46 localizes to the cis-Golgi and the ERGIC (41). Using the rabbit anti-mouse Erv46 antibody, which cross-reacts with the human antigen, we found prominent colocalization of C-terminally HA-tagged ERGIC-32 with hErv46 in the Golgi area. In contrast, most of the peripheral ERGIC clusters were positive for ERGIC-32 only (Fig. 7A, upper panels). This localization was confirmed by Nycodenz gradient centrifugation. ERGIC-32 was more enriched in ERGIC fractions than hErv46, which was enriched in cis-Golgi fractions (Fig. 7B). To visualize hErv46, we isolated the cDNA of human hErv41 (CDA14/hErv41) by RT-PCR using mRNA from HepG2 cells and stably expressed NH₂- and COOH-terminally tagged hErv41 in HepG2 cells. Both hErv41 variants were found to colocalize with the cis-Golgi marker GPP-130 (data not shown). Double labeling experiments revealed strong colocalization of hErv41-Myc and hErv46 in the Golgi (Fig. 7A, lower panels). Collectively, these results indicate that ERGIC-32, hErv41, and hErv46 partially colocalize in the ERGIC/Golgi area, although ERGIC-32 is more localized to the ERGIC.

ERGIC-32 Interacts with hErv46—In yeast, Erv41p and Erv46p form a complex and cycle between the ER and Golgi. Complex formation is required for the stability of the two proteins and their co-transport to the Golgi (75). We tested whether ERGIC-32 can interact with hErv46 in a similar way. To this end ERGIC-32HA was immunoprecipitated with an anti-HA from lysed membranes of HepG2 cells that had been treated with the membrane-permeable thiol-cleavable cross-linker DSP and probed for the presence of hErv46. Fig. 8A (lane 3) shows that some hErv46 specifically co-precipitated with ERGIC-32HA but not in absence of the cross-linker (not shown). As a further control, hErv46 was not detectable in immunoprecipitates from nontransfected HepG2 cells (Fig. 8A, lane 2).

To further analyze the interaction between ERGIC-32 and hErv46, we took advantage of the observation that transfected Myc-hErv46 is trapped in the ER and does not concentrate in ERGIC upon BFA treatment (Fig. 8B) but co-precipitates HA-tagged ERGIC-32 to the same extent as in nontreated cells (not shown). This mislocalization is most likely a result of limited
availability of the partner hErv41 and not to the Myc tag, because nontagged, overexpressed hErv46 also accumulates in the ER. Interestingly, coexpression of ERGIC-32HA changed the ER pattern of Myc-hErv46 to a Golgi-like pattern (Fig. 8C).

To confirm the relocalization to the Golgi we performed triple labeling with the KDEL-R in cells cotransfected with Myc-hErv46 and ERGIC-32HA. In HepG2 cells the KDEL-R is localized in the cis-Golgi and to a more minor extent to the ERGIC. Confocal analysis showed that Myc-hErv46 indeed relocalizes to KDEL-R positive structures, confirming that

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**TABLE I**

Proteomic analysis of the ERGIC

For each identified protein, the percentage of total protein coverage (MALDI-TOF) or the number of identified peptides (MS/MS) is indicated. The commonly accepted names are reported together with the accession number, theoretic molecular weight, and the known or inferred function.

| Protein name | NCBI accession no. | Theoretical mass | Mass spectrometry | Known or inferred function |
|--------------|--------------------|------------------|-------------------|---------------------------|
| Giantin      | gi[28201804]       | 373              | MS/MS (1),        | Membrane trafficking      |
| FN precursor | gi[2506872]        | 260              | MALDI-TOF (4%)    | Cargo                     |
| Aminopeptidase N | gi[29840829]   | 109              | MALDI-TOF (12%)   | Cargo                     |
| Bip/GRP78    | gi[14916999]       | 72               | MS/MS (2),        | Protein maturation        |
| Man9-mannosidase Class 1A member 1 | gi[17380426] | 70               | MS/MS (1)         | Protein maturation        |
| PDI          | gi[2507460]        | 57               | MALDI-TOF (11%)   | Protein maturation        |
| ERGIC-53     | gi[22261801]       | 54               | MS/MS (2),        | Cargo receptor            |
| Nucleobindin (CALNUC) | gi[12803105] | 54               | MALDI-TOF (14%)   | Calcium homeostasis       |
| Nucleobindin2 | gi[17367404]      | 50               | MS/MS (2),        | Protein maturation        |
| PDI-related protein 5 (hCaBP1) | gi[5031973]   | 48               | MALDI-TOF (17%)   | Calcium homeostasis       |
| CBP1/Hsp47   | gi[30585081]       | 46               | MS/MS (1)         | Protein maturation        |
| ERP44        | gi[31077035]       | 44               | MS/MS (2),        | Protein maturation        |
| GP36B        | gi[21264108]       | 40               | MS/MS (2)         | Cargo receptor            |
| KIAA1181     | gi[15215343]       | 32               | MS/MS (3)         | Unknown                   |
| Yif1p        | gi[12654907]       | 30               | MS/MS (1)         | Membrane trafficking      |
| SURF-4       | gi[12644052]       | 30               | MS/MS (3)         | Unknown                   |
| CGI-100      | gi[12585534]       | 28               | MS/MS (1)         | Membrane trafficking      |
| gp25L2       | gi[37580006]       | 25               | MS/MS (2)         | Cargo receptor            |
| TMP21        | gi[3915893]        | 25               | MS/MS (2)         | Cargo receptor            |
| KDEL-R       | gi[119543]         | 25               | MALDI-TOF (32%)   | Cargo receptor            |
| Rab37        | gi[20139581]       | 25               | MALDI-TOF (27%)   | Membrane trafficking      |
| SEC22b       | gi[12655033]       | 24               | MS/MS (4)         | Membrane trafficking      |
| p24A         | gi[3914237]        | 23               | MS/MS (1)         | Cargo receptor            |
| Rab1A        | gi[131786]         | 23               | MS/MS (5)         | Membrane trafficking      |

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**FIG. 3.** Effect of BFA on the localization of hYif1p, hSurf-4, and KIAA1181. HA-tagged versions of human Yif1p, Surf-4, and KIAA1181 were stably expressed in HepG2 cells. Cells were treated with BFA for 90 min (BFA) or left untreated (Control) and processed for indirect immunofluorescence and analysis by confocal microscopy. Cells were double-labeled with anti-HA (red in overlay) and the KDEL-R (green in overlay). Arrows indicate colocalization of Yif1p, Surf-4, and KIAA1181 with KDEL-R. Bar, 20 μm.
overexpression of ERGIC-32HA pulls Myc-hErv46 out of the ER. Collectively these observations indicate that ERGIC-32 interacts with hErv46 in the ER and that this interaction leads to transport of the two proteins to ERGIC and cis-Golgi.

ERGIC-32 Silencing Affects hErv46 Stability—To study the function of ERGIC-32 we reduced its expression by RNA interference (76). Synthetic duplexes specific for ERGIC-32 were transfected twice into HeLa cells, and the expression of ER- 

![Figure 4](http://www.jbc.org/)

**Fig. 4.** ERGIC-32 is a putative transmembrane protein homologous to yeast Erv41p and Erv46p. A, protein sequence alignment of ERGIC-32 orthologs. Human (hERGIC-32, gi/15215343), mouse (mERGIC-32, gi/12835932), and C. elegans (ceERGIC-32, gi/13775507) ERGIC-32-related sequences were aligned using ClustalW. Identical residues are shaded, putative transmembrane domains are boxed as deduced by the Toppred2 prediction program (84), and a consensus site for N-glycosylation is underlined. B, ERGIC-32 protein sequence identity with Erv41p and Erv46p proteins. Alignment of human and C. elegans ERGIC-32 with Erv46p (gi/63235573) and Erv41p (gi/63235573) of yeast and their human homologs hErv46 (CGI-54/putative protein of 43.2 kDa, gi/4923577), hErv41 (CDA14, gi/706105) was obtained by ClustalW analysis. The numbers indicate percentages of identity (BLOSUM matrix analysis). C, ERGIC-32 transmembrane profile (Kyte and Doolittle). Two amino acid stretches of sufficient length and hydrophobicity are predicted to be transmembrane domains near the N and C termini according to the Toppred2 program. D, dendogram of the ERGIC-32/Erv41/Erv46 protein family. Multiple alignment (ClustalW program) and tree drawing (PHYLIP program) were performed using the Biology Workbench interface (workbench.sdsc.edu). Mouse Erv46 (mErv46, gi/12844094), Erv41 (mErv41, gi/12841082), and C. elegans Erv46 (ceErv46, gi/3878484), Erv41 (ceErv41, gi/20509204) sequences were also included.

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modifies the half-life of hErv46. Fig. 9B shows that this is indeed the case. The half-life of hErv46 was reduced from 3 to 1 h in cells treated with ERGIC-32 siRNA (si1) but was not affected by control siRNA (Fig. 9C). After 3 h of chase only 35.2% (± 3.5 S.D.) of newly synthesized protein remained in si1-treated cells, whereas 54.7% (± 2.8 S.D.) remained in cells.
transfected with control RNA. Silencing hErv41 by siRNA also reduced the protein levels of hErv46 (Fig. 9A), and by silencing hErv46, hErv41 was not detectable after transfection, presumably because it is rapidly degraded (not shown). In contrast, reduced levels of either hErv46 or hErv41 had no effect on ERGIC-32. The results indicate that hErv41 and hErv46 stabilize each other as in yeast but that the \( \text{rv41-Erv46 complex} \) is further stabilized by ERGIC-32.

**DISCUSSION**

In this study we present the first comprehensive characterization of ERGIC membranes from BFA-treated cells. The new procedure we have developed for the isolation of ERGIC membranes is superior to previous methods (4, 77) and results in a 2–3-fold higher purity. The enrichment factor is comparable with that of highly enriched Golgi membrane fractions used for proteomic analysis (78). Unlike the present method, the previously published procedures for the purification of the ERGIC could not completely remove contaminating membranes from other organelles. The new procedure was specifically designed to identify new cycling proteins and takes advantage of two different effects of BFA. First, the \( \text{in situ} \) merging of Golgi with ER by BFA allows efficient separation of the ERGIC from Golgi membranes by density gradient centrifugation. Second, the BFA-induced accumulation of KDEL-R in ERGIC membranes facilitates their subsequent affinity isolation by magnetic immunobeads. No membrane marker is known to exclusively localize to the ERGIC, rendering it difficult to purify these membranes from untreated cells. In BFA-treated cells, however, KDEL-R and ERGIC-53 almost quantitatively redistribute to the ERGIC and can therefore serve as ERGIC-specific markers under these conditions. The low level of contaminating proteins and the identification of a number of known and new cycling proteins in the final fraction clearly document the value of our approach. It should be noted that the new BFA-based procedure does not provide quantitative information on the number of individual proteins present in the ERGIC of untreated cells. For instance, KDEL-R and ERGIC-53 almost quantitatively redistribute to the ERGIC and can therefore serve as ERGIC-specific markers under these conditions. The low level of contaminating proteins and the identification of a number of known and new cycling proteins in the final fraction clearly document the value of our approach. It should be noted that the new BFA-based procedure does not provide quantitative information on the number of individual proteins present in the ERGIC of untreated cells.

**FIG. 7. Localization of ERGIC-32, hErv41 and hErv46 in HepG2 cells. A.**

- Cells expressing ERGIC-32HA (upper panels) or hErv41-Myc (lower panels) were processed for double-labeling immunofluorescence microscopy. ERGIC-32HA (red in overlay) colocalizes partially with hErv46 in the Golgi area (green in overlay). hErv41-Myc (red in overlay) displays a Golgi-like pattern and colocalizes with hErv46 (green in overlay). Bar, 20 μm. **B.** HepG2 membranes were fractionated on a 29–13% Nycodenz gradient. Fractions (numbering is from bottom to top) were analyzed by Western blotting with antibodies against GPP130, ERGIC-53, mErv46, and ERGIC-32. Although hErv46 mainly co-fractionates with the \( \text{cis-Golgi marker GPP130} \), ERGIC-32 largely co-distributes with ERGIC-53. *, nonspecific band.
availability that the purified membranes also contain some ER exit site membranes, although ER exit sites do not merge with the ERGIC in BFA-treated cells (79). Currently, there is no accepted membrane marker for ER exit sites with which to test this issue.

By mass spectrometry we have identified 24 proteins enriched in the ERGIC membranes, many of which are already well characterized and their function known or inferred from proteins of the same family. The identified proteins represent rather abundant proteins, but the methodology can also be applied directly for a more refined analysis of minor proteins in view of the purity of the membrane fraction. The inclusion of a sodium carbonate, pH 11.5, treatment favors the identification

FIG. 8. ERGIC-32 interacts with hErv46. A, total membranes prepared from HepG2 cells, transfected with ERGIC-32HA (1, 3) or non-transfected (2), were subjected to cross-linking with DSP, and the protein was immunoprecipitated with anti-HA. Input lysate (1:10, lane 1) and immunoprecipitates (lanes 2 and 3) were analyzed by SDS-PAGE and Western blotting using anti-HA and anti-Erv46. Note that hErv46 co-immunoprecipitates with ERGIC-32HA (lane 3) as opposed to TGN46, which was used as a negative control in the same experiment. B, Myc-hErv46 fails to concentrate in ERGIC clusters in the presence of BFA. HepG2 cells stably expressing Myc-hErv46 were treated with BFA for 90 min and processed for indirect immunofluorescence and analysis by confocal microscopy. Cells were double labeled with anti-Myc antibody and anti-ERGIC-32 polyclonal antibody. Upon BFA treatment, ERGIC-32 is enriched in ERGIC clusters throughout the cytoplasm, whereas Myc-hErv46 still displays an ER pattern. C, ERGIC-32HA overexpression re-localizes ER-locked Myc-hErv46 to the cis-Golgi. HepG2 cells stably expressing Myc-hErv46 were transiently transfected with ERGIC-32HA, fixed, and processed for triple-labeling immunofluorescence microscopy. Myc-hErv46 (green in overlay) exhibits a classical ER pattern (asterisks, cells expressing Myc-hErv46 alone). In cells co-expressing recombinant ERGIC-32HA (red in overlay) Myc-hErv46 is re-localized to structures positive for KDEL-R (blue in overlay). White in the overlay indicates colocalization of Myc-hErv46, ERGIC-32HA, and KDEL-R (arrowheads). Bar, 20 μm.

FIG. 9. Knock-down of ERGIC-32 affects the stability of hErv46. A, HeLa cells were transfected with control or ERGIC-32 (si1 and si2)- or hErv41 (si)-specific siRNA oligonucleotides for 1 week. Equal amounts of total proteins were analyzed by SDS-PAGE and Western blotting using antibodies against BAP31, TFR, hErv46, and ERGIC-32. ERGIC-32 depletion reduces hErv46 expression. hErv46 is strongly reduced by silencing hErv41. Similar results were obtained with two other oligonucleotides targeting hErv41 mRNA. B, HeLa cells transfected with control and ERGIC-32 siRNA (si1) were pulsed and chased for the indicated times. hErv46 was immunoprecipitated and analyzed by SDS-PAGE (fluorogram). Relative amounts of hErv46 were quantified and expressed as the percentage of time 0 chase. C, HeLa cells treated with siRNA oligonucleotides were pulsed and chased for 3 h as in B. The amount of hErv46 was quantified and expressed as the percentage remaining after 3 h of chase compared with the amount present at the beginning of the chase (mean ± S.D., n = 3; Student’s t test for control versus si1, p < 0.01).
of integral membrane proteins at the expense of peripheral membrane proteins and soluble proteins, although some soluble chaperones were still present, the reason for which is not entirely clear. We do not know to what extent the substantial amount of these chaperones is due to a BFA effect or whether the ERGIC of nontreated cells also contains high levels of these chaperones in HepG2 cells. However, it is noticeable that the chaperone CBP1/HSP47 is known to be associated with procol-lagen in the ERGIC from where it is retrieved to the ER via its RDEL motif (64, 80).

Interestingly, some proteins we have identified in the ERGIC fraction have orthologs or homologs in yeast where they are enriched in COPII vesicles (66). These human proteins are p24 family members, Surf-4, Yif1p, and ERGIC-32. Data base searches reveal the existence of at least eight human p24 proteins, four of which we have identified, namely p24A, gp25L2, TMP21, and CGI-100. The reason the other p24 family members have escaped our analysis may be the differential cycling properties of the individual p24 proteins (36), low expression levels, or difficulties in detecting them by mass spectrometry. Surf-4 has been described as an ER protein (69). The present study indicates that Surf-4 can cycle in the early secretory pathway and may therefore function as a cargo receptor similar to its yeast ortholog Erv29p. Yif1p binds the transport GTPase Yipt1p and is required for fusion competence of ER-derived vesicles with the Golgi membranes in yeast (81, 82). Its presence in ERGIC membranes together with Rab1 (the mammalian homolog of Yipt1p) may suggest that Yif1p controls vesicle fusion at the ERGIC and/or Golgi. Further studies are required to uncover the functions of Surf-4 and Yif1p.

In discussing potential functions of ERGIC-32, it may be informative to consider its relatives Erv41p and Erv46p previously studied in yeast. These proteins are selectively and efficiently packaged into COPII vesicles and cycle between the ER and Golgi (66). Their expression levels are interdependent, and deletion of the Erv41p-Erv46p complex influences the membrane fusion stage in a cell-free assay that reproduces transport between the ER and Golgi. However, the precise molecular mechanism by which these proteins function in membrane trafficking between the ER and Golgi is so far unknown. Although we did not find hErv41 and hErv46 by our proteomic approach, these proteins are also enriched in the ERGIC of BFA-treated cells (41)2 and are bona fide cycling proteins in higher eukaryotes. Like ERGIC-32, hErv41 and hErv46 lack a cleavable signal sequence and are predicted to have large luminal and short NH2- and COOH-terminal cytosolic domains. Transport signals present in the COOH-terminal part of Erv41p and Erv46p proteins and acting in trans are responsible for the COPII-dependent exit of the complex from the ER and its cycling between the ER and Golgi in yeast (75). Two hydrophobic residues, Ile-349 and Leu-350 in positions –4 and –3 of the COOH terminus of Erv41p, constitute a transport motif required for the recruitment of the Erv41p-Erv46p complex into COPII vesicles. However, the Ile-Leu motif is not sufficient for ER export. The Erv46p tail contains a Phe-Tyr sequence in positions –13 and –14 from the COOH terminus cooperating with the Ile-Leu motif in Erv41p for ER export of the complex. The combined presence of both motifs in a specific orientation is a prerequisite for efficient packaging. These motifs appear to be conserved in mammalian Erv41 and Erv46. A di-leucine motif is present in positions –6 and –7 of mammalian hErv41 and an Ile-Tyr sequence is located at the same distance from the membrane in hErv46 as the Phe-Tyr sequence in yeast Erv46p. A di-lysine motif in positions –3 and –4 of the Erv46p may mediate the Golgi to ER retrograde trafficking of the Erv41p-Erv46p complex (66, 75). Only the lysine in position –3 is conserved in mammalian Erv46. However, in some instances a lysine at position –3 is sufficient for retaining type 1 membrane proteins in the ER (83). Extrapolating from the similarities of transport motifs, the mechanisms of trafficking of Erv41 and Erv46 are most likely similar in yeast and mammals. Moreover, the interdependence and identical localization of hErv46 and hErv41 suggest that these two proteins presumably form a stable complex as described in yeast.

ERGIC-32 has many similarities to hErv46. Apart from its overall sequence similarity, its C-terminal tail is closely related and the transport motifs are conserved. This is the case for the two hydrophobic residues, Ile274 and Phe275, that may function in COPII packaging and the Lys288 in position –3 that might function in retrieval. However, there are notable differences between ERGIC-32 and hErv46. First, ERGIC-32 has a shorter luminal domain than hErv46. Second, ERGIC-32 localizes to the ERGIC, whereas hErv46 localizes more to the cis-Golgi as does hErv41. Third, overexpressed ERGIC-32 localizes correctly to the ERGIC as opposed to overexpressed hErv46 that is trapped in the ER unless ERGIC-32 is co-transfected. Fourth, silencing ERGIC-32 increases the turnover of hErv46 but not vice versa. Fifth, silencing hErv41 increases the turnover of hErv46 but not ERGIC-32. Sixth, ERGIC-32 is not present in yeast.

Obviously, ERGIC-32 does not require teaming up with either hErv46 or hErv41 for correct localization, nor does its stability depend on these two proteins. Together with the finding of different steady state localization it is unlikely that ERGIC-32 is permanently associated with the Erv41-Erv46 complex beyond the ER. Notably, correct localization of the Erv41-Erv46 complex does not require the presence of ERGIC-32. What then is the function of ERGIC-32? A likely scenario is that ERGIC-32 stabilizes monomeric hErv46 in the ER and thereby promotes optimal assembly of hErv46 with hErv41. In addition, or alternatively, ERGIC-32 may act as a transport chaperone facilitating ER to ERGIC transport of the Erv41- Erv46 complex. Such a function may not be required in yeast, where ERGIC-32 is absent, but in higher eukaryotes which have developed a more complex early secretory pathway.

The function of Erv41, Erv46 and ERGIC-32 in mammalian cells is so far unknown. Combined depletion of ERGIC-32 and hErv46 had no effect on protein or glycoprotein secretion in a pulse-chase approach (not shown). Because hErv46 and hErv41 are interdependent, this double knock down is in fact a triple knock down. Unless the function of these three proteins can be compensated by other proteins, the silencing data suggest that they are probably not essential for vesicular trafficking. Further studies are required to elucidate the function of these three proteins in mammalian cells.

The new procedure for purification of ERGIC membranes from BFA treated cells described in the present study should prove useful to ultimately identify all the proteins rapidly cycling early in the secretory pathway. Although the protein pattern of ERGIC membranes appears rather simple regarding the major proteins (Fig. 2), we anticipate many more proteins to cycle through the ERGIC.

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Proteomics of Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) Membranes from Brefeldin A-treated HepG2 Cells Identifies ERGIC-32, a New Cycling Protein That Interacts with Human Erv46
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