An abundant 58-kDa (p58) homodimeric and hexameric microsomal membrane protein has been biochemically characterized and localized to tubulo-vesicular elements at the endoplasmic reticulum-Golgi interface and the cis-Golgi cisternae in pancreatic acinar cells (Lahtinen, U., Dahllof, B., and Saraste, J. (1992) J. Cell Sci. 103, 321–333). Here we report the purification of p58 by two-dimensional gel electrophoresis, and the cloning and sequencing of the rat and part of the Xenopus laevis cDNAs. The rat cDNA encodes a 517-amino acid protein having a putative signal sequence, a transmembrane domain close to the C terminus and a short cytoplasmic tail. The C-terminal tail contains a double-lysine motif (KKFF), known to mediate retrieval of proteins from the Golgi back to the endoplasmic reticulum. The rat p58 sequence was found to be 88% identical with those of ERGIC-53 and MR60, two previously identified human membrane proteins. Strong homology with the frog sequence was also observed indicating high evolutionary conservation. Overexpression of c-Myc-tagged p58 resulted in accumulation of the protein both in the endoplasmic reticulum and the cis-Golgi cisternae in pancreatic acinar cells (Lahtinen, U., Dahllof, B., and Saraste, J. (1992) J. Cell Sci. 103, 321–333). Here we report the purification of p58 by two-dimensional gel electrophoresis, and the cloning and sequencing of the rat and part of the Xenopus laevis cDNAs. The rat cDNA encodes a 517-amino acid protein having a putative signal sequence, a transmembrane domain close to the C terminus and a short cytoplasmic tail. The C-terminal tail contains a double-lysine motif (KKFF), known to mediate retrieval of proteins from the Golgi back to the endoplasmic reticulum. The rat p58 sequence was found to be 88% identical with those of ERGIC-53 and MR60, two previously identified human membrane proteins. Strong homology with the frog sequence was also observed indicating high evolutionary conservation. Overexpression of c-Myc-tagged p58 resulted in accumulation of the protein both in the endoplasmic reticulum and in an apparently enlarged Golgi complex, as well as its leakage to the plasma membrane. Immunolocalization using antibodies raised against a luminal peptide stained the total cellular pool of p58, while anti-tail peptide antibodies detected p58 only in a restricted Golgi region. This suggests that the C-terminal tail of p58 located in the endoplasmic reticulum and transport intermediates is hidden, but becomes exposed when the protein reaches the Golgi complex.

The endomembranes participating in the early stages of protein maturation and transport consist of the endoplasmic reticulum (ER), the cis-Golgi cisternae and tubulo-vesicular elements at the interface between these organelles (Palade, 1975). After their translocation across the membrane of the rough ER, newly synthesized secretory and membrane proteins undergo folding and post-translational modification reactions, before becoming competent to be transported out of the ER (Helenius et al., 1992; Gething and Sambrook, 1992; Helenius, 1994). Transport along this pathway occurs via an intermediate compartment which could originally be visualized in Semliki Forest virus-infected cells using low temperature as a reversible transport arrest (Saraste and Kuismanen, 1984). Identification and localization of two marker proteins, rat p58 (Saraste et al., 1987) and human p53 (Schweizer et al., 1988; renamed ERGIC-53, Schindler et al. (1993)), has provided additional information of the structural complexity of this compartment. The tubulo-vesicular elements, to which these proteins concentrate (Schweizer et al., 1991; Lahtinen et al., 1992), have been shown to be involved in the transport of viral glycoproteins to the Golgi complex (Schweizer et al., 1990; Saraste and Svensson, 1991; Plutner et al., 1992).

The removal of membrane from the ER, that accompanies the formation of vesicular carriers, is balanced by the counterflow of lipids and retrieval of membrane proteins from the Golgi (Wieland et al., 1987). This retrograde pathway serves as a mechanism to return escaped, KDEL-containing, resident proteins back to the ER (Pelham, 1988; Dean and Pelham, 1990) and maintain the localization of either KXXX- or KXXXX-containing ER membrane proteins by retrieval (Nilsson et al., 1989; J ackson et al., 1990; Jackson et al., 1993; Townsley and Pelham, 1994; Gaynor et al., 1994).

Studies using in vitro assays and yeast secretion mutants have identified several cytosolic components needed for vesicle budding, transport, and fusion (reviewed by Rothman, 1994). Coatomer is a complex of seven proteins (a, b, b′, γ, δ, ε, and ζ-COP), which assemble on membranes in an ADP-ribosylation factor-dependent manner forming a cytoplasmic coat (Serafini et al., 1991; Waters et al., 1992; Ord et al., 1993; Ostermann et al., 1993). Coatomers are involved both in intra-Golgi (Rothman and Orci, 1992) as well as ER to Golgi transport (Pepperkok et al., 1993; Peter et al., 1993). Recently, coatomer subunits and double-lysine motifs have been shown to interact in vitro (Cossor and Letourneur, 1994). The functional significance of this interaction in membrane dynamics between the ER and Golgi was revealed by genetic studies in yeast (Letourneur et al., 1994) showing that several coatomer subunits were essential for protein retrieval.

As a first stage in trying to understand both the function and the molecular mechanism for trafficking of p58 we have cloned and expressed its cDNA and developed peptide antibodies against different domains of the protein. We report here that p58 is the rat homolog of the human ERGIC-53/MR60 protein (Schindler et al., 1993; Arar et al., 1995), which has been suggested to function as a membrane bound lectin in the early secretory pathway (Fiedler and Simons, 1994; Arar et al., 1995). Furthermore, immunolocalization using the anti-tail peptide antibodies detected p58 only in the Golgi complex, suggesting masking of the tail in earlier compartments.
EXPERIMENTAL PROCEDURES

Materials—Methionine and cysteine-free minimal essential medium were obtained from Sigma, all other cell culture media and reagents including Lipofectin® were from Life Technologies, Inc. Pro-Mix (10% foetal bovine serum and -cysteine) was from GIBCO-BRL. Polyclonal anti-c-Myc antibodies (9E10; Evan et al., 1992) were from Santa Cruz Biotechnology Inc. Polyclonal anti-HA antibodies (12CA5) were from Berkeley Antibody Co. The preparation and affinity purification of polyclonal anti-p58 antibodies against GST-p58 lethal class I membranes were performed as described elsewhere (Lahtinen et al., 1992), except that proteins were alkylated in 40 mM dithiothreitol before (Lahtinen et al., 1992), followed by SDS-PAGE in the second dimension as described by the manufacturer (GIBCO-BRL). For transfections with both the wild type and c-Myc- or HA-tagged p58, the cDNAs were transferred into the pSV1 vector (Liljestro¨m and Garoff, 1991).

Antibodies—Antiserum raised against the catalytic domain of v-erbB were obtained from TLIndia (Liljestro¨m et al., 1993) and affinity-purified monoclonal anti-c-Myc antibodies (9E10; Evan et al., 1992) were kindly provided by Dr. Marilyn Farquhar and Dr. Tommy Nilsson, respectively; monoclonal anti-HA antibodies (12CA5) were from Berkeley Antibody Co. The preparation and affinity purification of polyclonal anti-p58 antibodies against GST-p58 lethal class I membranes were performed as described elsewhere (Lahtinen et al., 1992), except that proteins were alkylated in 40 mM dithiothreitol before (Lahtinen et al., 1992), followed by SDS-PAGE in the second dimension as described by the manufacturer (GIBCO-BRL). For transfections with both the wild type and c-Myc- or HA-tagged p58, the cDNAs were transferred into the pSV1 vector (Liljestro¨m and Garoff, 1991).

Cell Culture, Transfection, and Metabolic Labeling—BHK-21 cells were grown in supplemented minimal essential medium containing 5% of both fetal calf serum and tryptophanase and supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected using the Senlifex kit virus-based expression system (Liljestro¨m and Garoff, 1991). Subconfluent plates were first preincubated in Opti-MEM for 10 min and 9 µg of Lipofectin was mixed with 2.3 µg of RNA in 1 ml of Opti-MEM by vortexing. The cells were incubated with Lipofectin-RNA mixture for 2 h at 37°C and thereafter the transfection mixture was replaced with BHK growth medium.

For metabolic labeling, the cdls were first preincubated with methionine and cysteine-free minimal medium containing 10% dialyzed fetal calf serum for 1 h and then labeled with Pro-Mix (500 µCi/ml).

Preparation and Affinity Purification of Peptide Antibodies—Two peptides corresponding to amino acids 158–170 (FFDSFDNGKKN, luminal) and 507–517 (TQEEAAAKKFI, tail) were synthesized with an additional cysteine at the N terminus. For immunochemical, peptides were coupled via the cysteine residue to keyhole limpet hemocyanin using SPDP as a heterobifunctional cross-linker. 10 µg of keyhole limpet hemocyanin was incubated in the presence of a 20 times molar excess of SPDP in 100 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl for 1 h at room temperature. The reaction mixture was then passed through a NAP-25 column to separate uncoupled SPDP. Five times molar excess of peptide was added and the reaction was continued for 1 h at room temperature or overnight at 4°C depending on the solubility of the peptide.

Due to the relatively high background observed in immunofluorescence staining, Immunopure Ag/Ab Immobilization Kit #2 (Pierce) was used for affinity purification of the anti-tail antibodies according to the manufacturer’s instructions.

Preparation of Microsomes—Preparation of rat pancreas microsomes was carried out as described in detail elsewhere (Saraste et al., 1995). Microsomes were extracted with sodium carbonate (pH 11.3) according to the manufacturer’s instructions. For transfections with both the wild type and c-Myc- or HA-tagged p58, the cDNAs were transferred into the pSV1 vector (Liljestro¨m and Garoff, 1991).

For transfections with both the wild type and c-Myc- or HA-tagged p58, the cDNAs were transferred into the pSV1 vector (Liljestro¨m and Garoff, 1991).

RESULTS

Purification of p58—Two-dimensional gel analysis of rat pancreatic microsomal proteins demonstrated that p58 was a major membrane protein in this subcellular fraction (see Fig. 3, B and D, in Lahtinen et al., 1992). This observation allowed us to purify the protein to homogeneity. Total microsomes were
isolated from rat pancreatic homogenates and membrane proteins were extracted with alkaline sodium carbonate, resulting in a considerable enrichment of p58. The proteins were separated by isoelectric focusing followed by SDS-PAGE, transferred onto nitrocellulose filters, and after staining with proteinase digestions in situ with either trypsin or endoproteinase Lys-C, pooled p58 from 40 blots was used. Peptides were separated by reverse-phase high performance liquid chromatography. Lys-C, pooled p58 from 40 blots was used. Peptides were separated by reverse-phase high performance liquid chromatography and four major peptides were microsequenced.

 Conservation of p58—Sequence comparison (Fig. 2) in the Swissprot and EMBL data bases shows 89% identity (94% similarity) with the human intermediate compartment ERGIC-53 protein (Schindler et al., 1993) and the human mannospecific lectin MR60 (Arar et al., 1995). The sequences of ERGIC-53 and MR60 are identical, differing only in 1 amino acid residue. The similarity in the rat and human proteins is evenly distributed along the sequences, but in the rat sequence an 8-amino acid insertion is found close to the N terminus of the mature protein. In the cytoplasmic tail the two lysines at positions –3 and –4 are conserved with only a serine at position –11 being replaced by a threonine.

To obtain further information of the degree on evolutionary conservation of p58, we screened a X. laevis UNI-ZAP XR cDNA library with a 1.3-kilobase rat p58 cDNA fragment. Two positive overlapping clones were isolated having a long open reading frame and a stop codon. The Xenopus protein was estimated to have the same size as the rat p58 since anti-p58 antibodies recognized a protein co-migrating with the rat p58 in immunoblots of frog liver homogenates (data not shown). The C-terminal 420-amino acid sequence was 71% identical (85% similar) to the rat sequence.

 Membrane Insertion and Topology of in Vitro Translated p58—Membrane insertion and signal sequence cleavage were analyzed by translating the in vitro transcribed p58 mRNA in the presence or absence of canine microsomal membranes followed by immunoprecipitations (Fig. 3). In the presence of microsomes p58 was inserted into the membrane and co-migrated with the endogenous protein, immunoprecipitated from metabolically labeled BHK cells (Fig. 3A, lanes 1 and 3). In the absence of microsomes the mobility of the protein was slightly slower (lane 2), indicating the presence of a functional and cleavable signal sequence. The protein could not be post-translationally inserted into the membranes (data not shown). Both the c-Myc- and HA-tagged forms of p58 behaved as the wild type (lanes 4–7).

When membranes were subjected to extraction with sodium carbonate (pH 11.3) followed by centrifugation, the in vitro translated p58 was almost quantitatively recovered in the resulting pellet (Fig. 3B), confirming proper membrane integration.

**Fig. 1. Nucleotide sequence of p58 and the deduced amino acid sequence.** The putative N-terminal signal sequence (medium bar), the four microsequenced peptides (thin bar), and the predicted C-terminal transmembrane segment (thick bar) are underlined.
Previous experiments have shown that p58 remained largely protected in microsomes after protease treatments (Lahtinen et al., 1992). To probe the membrane topology of in vitro synthesized p58, anti-tail peptide antibodies (see below) were used. Both anti-tail and polyclonal anti-p58 antibodies precipitated the intact protein (Fig. 3C, lanes 1 and 2). After digestion with proteinase K, anti-tail antibodies failed to precipitate the protein, indicating that the short tail was accessible to the enzyme.
whereas anti-p58 antibodies precipitated a slightly faster migrating protected form (lane 4). Together these results show that p58 is a type I integral membrane protein, having the bulk of the protein on the luminal side and a short C-terminal cytoplasmic tail in agreement with the topology deduced from the amino acid sequence (see above).

Peptide Antibodies Reveal Different Populations of p58—Antisera were raised against two different peptides, one from the luminal N-terminal region (corresponding to amino acids 158–170; anti-lumenal) and the other corresponding to the C-terminal tail (amino acids 507–517; anti-tail). Both antibodies gave a strong signal in immunoblots of rat pancreatic microsomal proteins (Fig. 4, lanes 1 and 3), which could be competed out with the corresponding peptide (not shown). They also specifically precipitated a 58-kDa protein from metabolically labeled BHK cells, although precipitation with the anti-tail antibodies from cell lysates was considerably less efficient (lanes 2 and 4).

When tested by immunofluorescence a distinct difference in the staining patterns obtained with the two peptide antibodies was observed in BHK cells. The staining with the anti-lumenal antibodies was similar to the staining obtained with the previously described affinity-purified anti-p58 antibodies (Saraste and Svensson, 1991); the Golgi region as well as vesicular elements concentrated in the Golgi area, but also scattered throughout the cell, were seen (Fig. 5, panels A and B). In contrast, the anti-tail antibodies stained a perinuclear, often ring-shaped structure typical of the Golgi complex (panel C). No scattered punctate elements could be visualized even in transfected cells having higher expression levels of p58 (data not shown). This suggests that in the vesicular elements the epitope(s) in the C-terminal tail may be hidden and therefore not accessible to the anti-tail antibodies.

Expression of c-Myc-tagged p58—Due to the high, endogenous background of p58 staining in murine and other cell lines tested, a c-Myc-epitope tag (Evan et al., 1985) was introduced at the N terminus downstream from the predicted signal sequence cleavage site. To ensure that the insertion of a foreign peptide sequence did not interfere with proper signal sequence cleavage or cellular localization of p58, we also tested an influenza virus HA-tagged (Field et al., 1988) version of p58. In vitro translation experiments with these constructs showed that signal sequence cleavage occurred normally in both cases (Fig. 3A, lanes 4–7).

For in vivo expression, both the c-Myc- and HA-tagged p58 cDNAs were cloned into a Semliki Forest virus-based pSFV1-vector (Liljestrom and Garoff, 1991), the DNA was transcribed in vitro, and transfected into BHK cells by lipofection. Four hour post-transfection, cells were treated for 1 h with 50 μg/ml cycloheximide and then fixed and processed for immunofluorescence. Staining with anti-c-Myc (Fig. 6, panel A) antibodies showed a punctate, Golgi-concentrated pattern resembling the distribution of endogenous p58. Double-staining with antmannosidase II antibodies (panel B) showed overlapping distributions of the two antigens in the Golgi area as previously observed both in BHK and normal rat kidney cells (Saraste and Svensson, 1991). In cells having a higher expression level, the protein could also be clearly detected in reticular ER membranes. This is in good agreement with previous immunolocalization studies and the quantitation of p58 in subcellular fractions (Saraste and Svensson, 1991; Lahtinen et al., 1992). The same distribution was observed with the HA-tagged p58 (data not shown). In addition, c-Myc-tagged p58 was shown to form...
both homodimers and oligomers as well as heterodimers and oligomers with the endogenous protein indicating that the oligomerization (Lahtinen et al., 1992) was not affected by the introduction of a foreign peptide sequence at the N terminus (not shown).

Overexpression of the c-Myc-tagged p58 resulted in the accumulation of the protein in the intracellular membranes. No increase in punctate elements could be detected while the ER network was heavily stained, and the Golgi complex, identified by double-staining with anti-mannosidase II antibodies (not shown), appeared enlarged (Fig. 6, panel C). When non-permeabilized cells were stained with anti-c-Myc antibodies, plasma membrane patches could be visualized (panel D), indicating that overexpression of the c-Myc-tagged p58 led to its mislocalization and leakage from the intracellular membranes to the plasma membrane.

DISCUSSION

During the last several years p58 (Saraste et al., 1987; Saraste and Svensson, 1991; Lahtinen et al., 1992) and p53 (Schweitzer et al., 1988, 1990; renamed ERGIC-53, Schindler et al. (1993)), two membrane proteins identified in rat and human cells, respectively, have been widely used as markers for the intermediate compartment at the ER-Golgi interface. These proteins have very similar intracellular distributions and biochemical properties, but their structural and functional relationship has been unknown. The abundance of p58 in pancreatic microsomal membranes (Lahtinen et al., 1992) allowed us to study the protein to homogeneity, obtain peptide sequences, and clone the cDNA. The sequence reveals that rat p58 and human ERGIC-53 (Schindler et al., 1993) are homologous proteins sharing 89% identity at the amino acid level. Furthermore, the observed 71% identity between the isolated partial X. laevis p58 sequence and the rat sequence indicates a high degree of evolutionary conservation of the protein among different species.

The cDNA sequence, together with biochemical data, suggest that p58 is synthesized as a 517-amino acid protein having a predicted and cleavable signal sequence and a single transmembrane domain close to the C terminus. This type I membrane topology was confirmed by using peptide antibodies generated against luminal and cytoplasmic domains of p58. Although p58 has been suggested to contain immature N-glycans (Hendricks et al., 1991), no consensus sites for N-glycosylation were found.

The expression of c-Myc-tagged p58 in BHK cells resulted in a distribution identical to the endogenous protein (Saraste and Svensson, 1991). A typical punctate, Golgi-concentrated pattern could be visualized by immunofluorescence microscopy. In cells overexpressing p58, the protein appeared to accumulate mostly in the Golgi complex and could also clearly be detected in the ER, but no increase in vesicular elements was observed. This is in contrast to the vesicular pattern and suggested enlargement of the intermediate compartment observed in Vero cells overexpressing ERGIC-53 (Schindler et al., 1993). In rat pancreas the staining of p58 in actively secreting acinar cells is more punctate and disperse, as compared to the reticular Golgi-staining pattern of β-cells (Lahtinen et al., 1992). It is thus possible that the distribution of p58 at the ER-Golgi interface correlates with the secretory status of the cell. In fibroblasts, where the level of secretion is relatively low, the amount of cargo proteins and/or cytosolic factors, e.g. coatmakers, could be limiting in cells overexpressing p58, and therefore the production of anterograde and/or retrograde transport vesicles would not be enhanced. This could result in the preferential accumulation of the protein in donor or acceptor organelles, respectively.

In non-permeabilized cells patchy staining of the cell surface, sometimes concentrated close to the cell edges, could also be visualized. Whether these structures represent dachrin-coated pits or cavedae is presently unknown. The same surface staining pattern has also been described for ERGIC-53 in overexpressing COS cells (Kappeler et al., 1994). This is likely to be due to saturation of the intracellular components needed for the retention of p58, resulting in leakage of the protein to the plasma membrane. The cytoplasmic tails of p58 and ERGIC-53 contain a double-lysine motif (Nilsson et al., 1989; Jackson et al., 1990), which mediates retrieval of membrane proteins from the Golgi to the ER (Jackson et al., 1993; Townsley and Pelham, 1994; Gaynor et al., 1994). Overexpressed ERGIC-53 has been shown to be endocytosed from the plasma membrane (Kappeler et al., 1994). Surprisingly, the C-terminal amino acid sequence K-K/R-F/Y-F/Y needed for internalization was found to be re-
lated to the KXXK ER retrieval signal (Itin et al., 1995). The immunofluorescence staining pattern obtained with the peptide antibodies made against the short cytoplasmic tail appeared to differ from the staining seen with antibodies made against a luminal sequence. Anti-tail antibodies recognized the subpopulation of p58 residing in the Golgi cisternae, indicating that the epitope is masked or not accessible in the vesicular elements, and exposed only in the Golgi complex. Knowing the distribution of p58 in the Golgi complex (Saraste et al., 1987), this is predicted to be the cis cisternae of the Golgi. Whether anti-tail antibodies can be used as a specific cis-Golgi marker has to be resolved at the electron microscopic level. The differential exposure of the double-lysine ER retrieval signal might regulate the trafficking of p58 by at least two mechanisms. First, the accessibility of the double-lysine to the retrieval machinery might correlate with different homo-oligomeric forms of p58 (Lahtinen et al., 1992). For example during the oligomeric assembly of the IgE receptor the α-chain is retained in the ER. Steric masking of the two lysines when the assembly with the γ-chain has been completed allows the receptor complex to be transported from the ER (Letourneur et al., 1995). Second, specific protein–protein interactions might be responsible for the transport to the Golgi complex, and recycling back to the ER. Some of the coatomer subunits interact directly with double-lysine containing peptides (Fiedler et al., 1995). MR60, p58 and VIP36 are non-glycosylated type I integral membrane proteins containing terminal mannoses might be needed for sorting and concentrating proteins into transport vesicles in the ER (Saraste et al., 1995). For example during the SFV-1 plasmid infection, the accessibility of the double-lysine to the retrieval signal might have to be resolved at the electron microscopic level. The differential exposure of the double-lysine ER retrieval signal might be followed by delivery of the ligand in the Golgi complex and recycling of unoccupied receptor back to the ER. Alternatively, p58 could have a function for retrieval of escaped, not fully matured, or folded glycoproteins back to the ER.

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Molecular Cloning and Expression of a 58-kDa cis-Golgi and Intermediate Compartment Protein
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