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ERGIC-53 Is a Functional Mannose-selective and Calcium-dependent Human Homologue of Leguminous Lectins

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Based on sequence homologies with leguminous lectins, the intermediate compartment marker ERGIC-53 was proposed to be a member of a putative new class of animal lectins associated with the secretory pathway. Independently, a promyelocytic protein, MR60, was purified by mannose-column chromatography, and a cDNA was isolated that matched MR60 peptide sequences. This cDNA was identical to that of ERGIC-53 and homologies with the animal lectin family of the galectins were noticed. Not all peptide sequences of MR60, however, were found in ERGIC-53, raising the possibility that another protein associated with ERGIC-53 may possess the lectin activity. Here, we provide the first direct evidence for a lectin function of ERGIC-53. Overexpressed ERGIC-53 binds to a mannose column in a calcium-dependent manner and also co-stains with mannosylated neoglycoprotein in a morphological binding assay. By using a sequential elution protocol we show that ERGIC-53 has selectivity for mannose and low affinity for glucose and GlcNAc, but no affinity for galactose. To experimentally address the putative homology of ERGIC-53 to leguminous lectins, a highly conserved protein family with an invariant asparagine essential for carbohydrate binding, we substituted the corresponding asparagine in ERGIC-53. This mutation, as well as a mutation affecting a second site in the putative carbohydrate recognition domain, abolished mannose-column binding and co-staining with mannosylated neoglycoprotein. These findings establish ERGIC-53 as a lectin and provide functional evidence for its relationship to leguminous lectins. Based on its monosaccharide specificity, domain organization, and recycling properties, we propose ERGIC-53 to function as a sorting receptor for glycoproteins in the early secretory pathway.

INTRODUCTION

Numerous sorting receptors of animal cells, many of which operate in endocytosis, recognize carbohydrate moieties on glycoproteins (Drickamer and Taylor, 1993). The best characterized sorting receptors of the secretory pathway are the mannose-6-phosphate receptors, which are involved in trafficking of lysosomal enzymes (Kornfeld, 1992). In the trans-Golgi network, the mannose-6-phosphate receptors recognize proteins bearing N-linked carbohydrates with terminal mannose 6-phosphate residues and sort them into clathrin-coated vesicles destined for endosomes. After release of the ligand in endosomes, the receptors recycle back to the Golgi for the next round of transport. Such a recycling feature is common to most sorting receptor proteins. Furthermore, these receptors share

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a general domain organization: a lumenal (exoplasmic) domain that contains one or multiple carbohydrate recognition domains (CRD)[1], a single transmembrane domain, and a cytoplasmic domain with sorting signals that can interact with cytosolic coat components. The interaction with cytosolic coats is believed to drive these receptors into budding vesicles (Pearse and Robinson, 1990; Schmid, 1992; Pelham, 1994).

Two proteins, ERGIC-53 (Schweizer *et al.*, 1988; Schindler *et al.*, 1993) and VIP-36 (Fiedler *et al.*, 1994), have recently been postulated to belong to a new class of sorting receptors in the secretory pathway (Fiedler and Simons, 1994). Both proteins have a type I transmembrane topology, and a stretch of about 200 amino acids in their luminal domain shows 19–24% identity to leguminous lectins. Their short cytoplasmic domains contain related sorting signals that are likely to interact with cytoplasmic coats, either coatomer or clathrin adaptors (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994; Itin *et al.*, 1995a,b). The two proteins are differently distributed in the endomembrane system. ERGIC-53 is associated with the ER-Golgi intermediate compartment (ERGIC; Hauri and Schweizer, 1992) and continuously recycles between the endoplasmic reticulum (ER) and the ERGIC (Lippincott-Schwartz *et al.*, 1990; Foguet, Clausen, Tang, Hong, and Hauri, unpublished data), while VIP36 is presumed to recycle between the Golgi apparatus and the plasma membrane (Fiedler *et al.*, 1994). Together these features of ERGIC-53 and VIP36 would fit the predictions for intracellular sorting receptors. However, it is unknown if their putative carbohydrate-binding domains are indeed functional.

In an independent search for carbohydrate-binding proteins, an intracellular protein termed MR60 was purified from HL60 cells by mannose-column chromatography (Pimpaneau *et al.*, 1991). Based on peptide sequence information derived from MR60, a cDNA was isolated that was identical to that of ERGIC-53 (Arar *et al.*, 1995). However, not all peptide sequences obtained from the MR60 preparation were found in the ERGIC-53 sequence (Arar *et al.*, 1995; Roche and Monsigny, unpublished data), raising the possibility that an ERGIC-53–associated protein rather than ERGIC-53 itself may posssess the carbohydrate-binding activity. As it was not tested by expression whether the cloned cDNA indeed encodes a carbohydrate binding protein, the conclusion that ERGIC-53 is a lectin remained equivocal. Furthermore, it has not been established whether MR60 is indeed selective for mannose and which cations are required for binding. Both the monosaccharide and the cation specificities must therefore be considered unknown and require experimental analysis. An equally important issue to be clarified experimentally concerns the relationship to other lectins. At variance with the Fiedler and Simons (1994) hypothesis, Arar *et al.* (1995) noticed homology of ERGIC-53 to galectins, a mammalian lectin family with carbohydrate binding properties different from those of legume lectins.

Here we report that overexpressed ERGIC-53 indeed binds to the mannose column that was initially used for the purification of MR60. We show that overexpressed ERGIC-53 is selective for mannose and requires calcium for binding. These findings provide the first direct experimental evidence for a lectin function of ERGIC-53. To test the hypothesis that ERGIC-53 may be a functional homologue of leguminous lectins (Fiedler and Simons, 1994), we substituted N156 of ERGIC-53, which is conserved and essential in the carbohydrate binding site of leguminous lectins (Sharon and Lis, 1990; van Eijsden *et al.*, 1992; Mirkov and Chrispeels, 1993). A second mutation that also should affect the binding site was generated to confirm the first mutation. Both mutations in ERGIC-53 abolished binding of the mutant proteins to the mannose column, and no co-labeling with mannosylated bovine serum albumin (BSA) in a morphological binding assay could be detected. These findings identify ERGIC-53 as a functional animal homologue of leguminous lectins and suggest that it may function as a sorting receptor.

MATERIALS AND METHODS

Reagents

Antibodies used in this study were monoclonal antibody (mAb) G1/93 against ERGIC-53 (Schweizer *et al.*, 1988), mAb 9E10 against a c-myc epitope (Evan *et al.*, 1985; American Type Culture Collection, Rockville, MD: ATCC CRL 1729), and a rabbit polyclonal antiserum against ERGIC-53. Protease inhibitors (used at the following final concentrations: 1 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml benzamidin, 1 μg/ml antipain, and 0.2 mM phenylmethylsulfonyl fluoride), D-mannose, D-glucose, D-galactose, D-fucose, and *N*-acetylglucosamine were obtained from Sigma (St. Louis, MO).

Recombinant DNAs

ERGIC-53 in pECE expression vector (Ellis *et al.*, 1986) was as described by Schindler *et al.* (1993). ERGIC-53 myc/6xHis contains an N-terminal myc-epitope tag introduced after the signal sequence cleavage site and a C-terminal 6xHis tag replacing the C-terminal KKFF tetrapeptide. myc/6xHis was generated in two steps. First the 3′ end of ERGIC-53 was changed by polymerase chain reaction (PCR) into a modified ERGIC-53 termed pECE* (Itin *et al.*, 1995b) to encode six histidines in a row followed by a stop codon. The introduced PCR fragment was confirmed by sequencing, and then in a second step cloned as an *Eco*RI/*Xba*I fragment back to ERGIC-53 c-myc to remove the artificial *N*-glycosylation site present in pECE* (Itin *et al.*, 1995b). This recombination resulted in ERGIC-53 myc/6xHis. For expression, ERGIC-53 myc/6xHis was cloned as a

[1] Abbreviations used: BSA, bovine serum albumin; CRD, carbohydrate recognition domain; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

*Sal*I/*Xba*I fragment into pECE. Point mutations in the carbohydrate binding site were generated by PCR, cloned via the internal *Bgl*I and *Hin*dIII restriction sites into ERGIC-53 myc/6xHis, and confirmed by sequencing. AD120/121 was changed to VA. For reasons of simplicity we call this mutant D121. The codon for N156 was mutated to encode A.

Cell Culture and Transfection

COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 I.U./ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone. COS cells were transfected using the DEAE-Dextran method (Cullen, 1987). Two micrograms of DNA per 35-mm dish was used.

Binding of ERGIC-53 to the Mannose Column

The mannose column was prepared as described and the binding was adapted from Pimpaneau *et al.* (1991). COS cells were harvested on ice in 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$, 3 days after transfection with ERGIC-53 or mutants. All subsequent steps were carried out at 4°C. The cells were passed through a ball-bearing homogenizer (20-μm clearance) and the membranes from a post-nuclear supernatant (10 min at 500 g_{av}) were pelleted at 100,000 g_{av} for 1 h. The pellet was solubilized for 1 h in 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/10 mM $CaCl_2$/1 mM $MgCl_2$ containing 1% Triton X-100 and protease inhibitors, followed by a centrifugation at 100,000 g_{av} for 1 h and dialysis of the supernatant overnight against binding buffer (10 mM Tris-HCl (pH 7.4)/150 mM NaCl/10 mM $CaCl_2$/1 mM $MgCl_2$, 0.15% Triton X-100. The dialysate was incubated overnight with the mannose column material. The column was packed and washed with 100 volumes of binding buffer. ERGIC-53 was eluted with 5 volumes of binding buffer containing 0.2 M D-mannose, followed by 5 volumes of 10 mM EDTA in 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/0.15% Triton X-100. ERGIC-53 or mutants were immunoprecipitated from the fractions with mAb G1/93 covalently coupled with dimethylpimelidate (Sigma) to protein A-Sepharose CL4B (Pharmacia, Uppsala, Sweden). Precipitates were separated by SDS-PAGE, blotted onto nitrocellulose, and detected with the polyclonal antiserum against ERGIC-53 followed by ^{125}I-protein A and phosphorimaging or autoradiography. Alternatively, the mutants were detected on the blots with mAb 9E10 against the myc-epitope followed by goat anti-mouse–conjugated horseradish peroxidase and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

To determine the hexoses capable of eluting myc/6xHis from the mannose column, a stepwise elution protocol was applied. ERGIC-53 myc/6xHis was bound overnight to the column material as described above. Equal volumes were packed in individual columns and washed with 100 volumes of binding buffer. Two volumes of the lowest concentration of hexose was run on the column followed by 2 volumes of binding buffer. This procedure was repeated with increasing concentrations of hexose for each step, and the remaining ERGIC-53 myc/6xHis was eluted with 5 volumes of 20 mM EDTA in 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/0.15% Triton X-100. The fractions were concentrated by immunoprecipitation and analyzed as described above.

To analyze the cation requirement, ERGIC-53 myc/6xHis was prepared as described above with the exception that the solubilization buffer contained 10 mM EDTA. The solubilized material was then dialyzed against the appropriate binding buffer containing either no divalent cations or various concentrations of Ca^{2+}, Mg^{2+}, or Mn^{2+}. ERGIC-53 myc/6xHis was bound to the mannose column material overnight, packed, and the columns were washed with 50 volumes of binding buffer. Bound ERGIC-53 myc/6xHis was then eluted with 20 mM EDTA in 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/0.15% Triton X-100. The fractions were concentrated by immunoprecipitation and analyzed as described above.

Immunofluorescence Microscopy

Neoglycoproteins were prepared by coupling glycosylphenyl-isothiocyanate to BSA; fluoresceinylated neoglycoproteins were obtained by using fluorescein isothiocyanate as described (Roche *et al.*, 1983; Monsigny *et al.*, 1984). The fluoresceinylated glycoproteins were purified by gel filtration on Trisacryl GF05 using *n*-butanol/water (5:95) and freeze dried. All double-immunofluorescence experiments were carried out in 8-well multichamber glass slides (Miles Labs, Naperville, IL) according to a procedure modified from that of Pimpaneau *et al.* (1991) as follows. Forty-two to forty-eight hours post-transfection, COS cells were cooled on ice, washed twice with phosphate-buffered saline (PBS), and fixed/permeabilized with 2% paraformaldehyde/0.1% saponin in PBS, pH 7.2, for 5 min. Cells were washed twice with PBS containing 20 mM glycine/0.1% saponin, followed by a 30-min incubation with 100 μg/ml fluorescein-labeled neoglycoprotein in Dulbecco's PBS (D-PBS, containing Ca^{2+} and Mg^{2+}), and four washes with D-PBS/0.1% saponin. All further incubations were at room temperature. ERGIC-53 mutants were stained with mAb 9E10 in D-PBS/0.1% saponin for 30 min, washed four times, and incubated with rhodamine-labeled goat anti-mouse immunoglobulin (Cappel, West Chester, PA) for 30 min. After four final washes with D-PBS/0.1% saponin, the cells were mounted in 90% glycerol, 10% D-PBS, 0.1 mg/ml phenylenediamine and analyzed by a Reichert Polyvar immunofluorescence microscope.

Pulse-Chase Experiments and Precipitations

Transfected COS cells were pulse-labeled and chased as described by Itin *et al.* (1995b). The cells were washed on ice with 10 mM Tris-HCl (pH 7.4)/150 mM NaCl and harvested in solubilization buffer (10 mM Tris-HCl, pH 7.4/150 mM NaCl/1% Triton X-100 and protease inhibitors), solubilized for 1 h and centrifuged for 1 h at 100,000 g_{av}. The supernatant was incubated for 3 h at 4°C with mAbs 9E10 or G1/93 prebound to protein A-Sepharose CL4B, or for 1 h at room temperature with Ni^{2+}-NTA-agarose (Qiagen, Hilden, Germany) in the presence of 20 mM imidazole to reduce unspecific background. Beads were washed four times with solubilization buffer, once with 10 mM Tris-HCl (pH 7.4), boiled in sample buffer, separated by SDS-PAGE (Laemmli, 1970), and analyzed by fluorography.

RESULTS

ERGIC-53 Mutants Containing an N-Terminal Epitope Tag and a C-Terminal 6xHis Tag Are Stable and Properly Oligomerized

To allow for simplified detection and purification of ERGIC-53 we engineered an ERGIC-53 mutant termed ERGIC-53 myc/6xHis. A c-myc epitope tag (Evan *et al.*, 1985) was introduced at the N-terminus and a 6xHis tag at the C-terminus of ERGIC-53 replacing the C-terminal KKFF-tetrapeptide targeting signal (Itin *et al.*, 1995b). To test the potential relationship of ERGIC-53 to leguminous lectins we generated two mutants in ERGIC-53 myc/6xHis: N156 with N156 changed to A, and AD120/121 was changed to VA (for simplicity called D121 in this study). Figure 1 shows a sequence alignment of the putative carbohydrate binding site of ERGIC-53 with that of two leguminous lectins: Lathyrus ochrus isolectin I (LOL I) and Erythrina corallodendron lectin (ECoRL) (Sharon and Lis, 1990; Fiedler and Simons, 1994). For both lectins the

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EcoRL      85  P L P A D G L . V F F M G P T K S . K P A Q G Y G Y L G I F F N S K Q D N S Y Q T L G V E F D T F S N P . W  135
LOL        77  Y N V A D G F T . F F I A P V D T . K P Q T G G G Y L G V F N S K D Y D K T S Q T V A V E F D T F Y N T A W  128
ERGIC-53  117  R I G A D G L A I W Y A E N Q G L E G P V F G S A D L W N G . . . . . V G I F F D T F D N D G .  158
                      ♦                                                                          ♦
D121                  V A
N156                                                                                             A
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Figure 1. Amino acid sequence alignment of the leguminous lectins Erythrina corallodendron (ECoRL) and Lathyrus ochrus (LOL) with ERGIC-53 according to the methods of Sharon and Lis (1990), and Fiedler and Simons (1994). For the ERGIC-53 mutants D121 (AD 120/121 to VA) and N156 (N156 to A) only the substituted amino acids are indicated. The conserved aspartate (D121 in ERGIC-53) and asparagine (N156 in ERGIC-53) are marked with a diamond. Underlined are conserved amino acids. Amino acids are given in the one-letter code.

three-dimensional structures with their ligands have been determined, LOL I with mannose, and ECoRL with galactose (Bourne *et al.*, 1990; Shaanan *et al.*, 1991). The location of the conserved residues D121 and N156 of ERGIC-53 are indicated in Figure 1 with a diamond symbol. In Figure 2A we show that the mutants exhibit the expected features when expressed in COS cells. All three mutants can be quantitatively precipitated with mAb G1/93 against ERGIC-53 and nonquantitatively with mAb 9E10 against the c-myc epitope tag. In addition, the mutants can also be precipitated with Ni-NTA agarose binding to their C-terminal 6xHis tag (Hochuli *et al.*, 1988). The rate of synthesis of D121 is reduced compared with the other mutants and therefore all fluorograms shown for D121 in Figure 2 were about three times longer exposed. In Figure 2B we tested for the stability of the putative binding site mutants, D121 and N156 in [^{35}S]methionine pulse-chase experiments, and compared it with that of ERGIC-53 myc/6xHis. The three proteins were found to have a similar turnover although the turnover of D121 may be slightly enhanced. To address whether any of the mutations interfered with the overall folding of the protein we compared the kinetics of oligomerization of the mutants with that of overexpressed wild-type ERGIC-53. Figure 2C shows that ERGIC-53 and its mutants oligomerize with identical kinetics. Both stability and oligomerization, therefore,

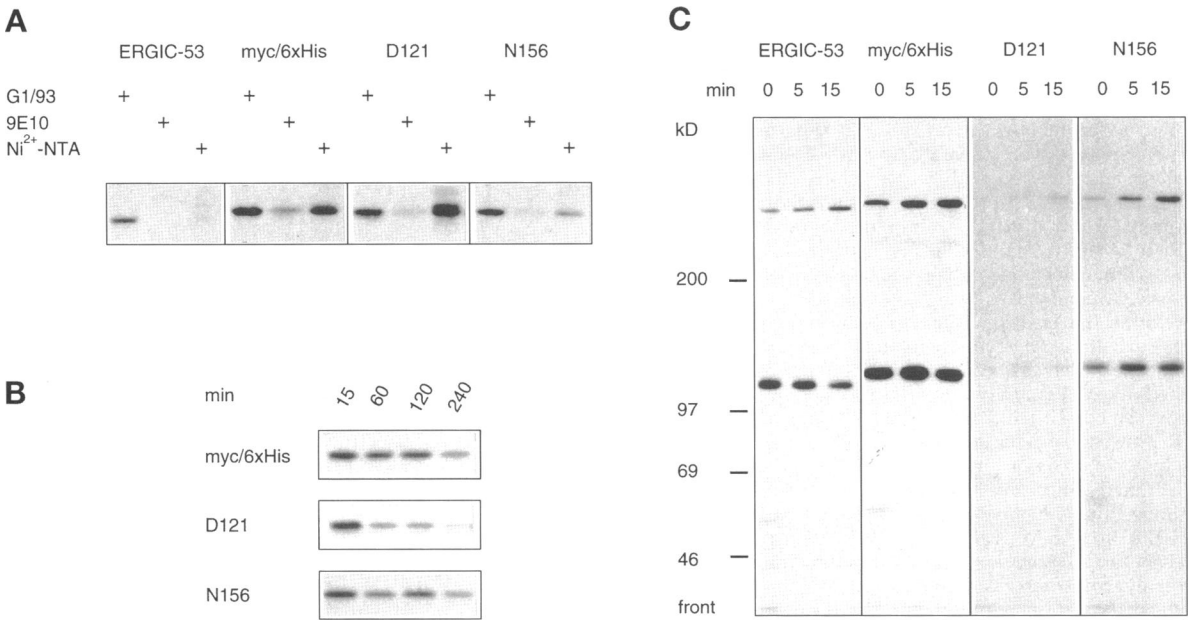

Figure 2. The introduced tags are functional and the mutations do not interfere with the stability and the kinetics of oligomerization of the mutant ERGIC-53 proteins. COS cells were transfected with ERGIC-53 cDNA or with mutant ERGIC-53 DNAs that contained an N-terminal c-myc epitope tag and a C-terminal 6xHis tag. Forty-two hours after transfection the cells were pulsed for 5 min with [^{35}S]methionine followed by a 15-min chase, and the proteins were precipitated with mAb G1/93 against ERGIC-53, mAb 9E10 against the cmyc epitope, or Ni-NTA agarose (A). (B) The cells were chased for the indicated times and the proteins were immunoprecipitated with G1/93. (C) Oligomerization was analyzed at the indicated chase times. *N*-ethylmaleimide (20 mM) was added immediately after the chase to block further formation of di-sulfide linkages. After immunoprecipitation with G1/93 the samples were separated by SDS-PAGE in the absence of dithiothreitol and analyzed by fluorography. Immunoprecipitation with 9E10 in (A) was specific but nonquantitative. The Ni^{2+}-NTA precipitate of the N156 panel in A is weaker than the corresponding G1/93 precipitate due to partial loss of beads.

argue against altered overall folding of the mutant proteins.

Overexpressed ERGIC-53 Binds to a Mannose Column

It was previously suggested that ERGIC-53 was identical to the mannose-binding protein MR60 (Arar *et al.*, 1995). Because not all peptide sequences of MR60 were found in the ERGIC-53 sequence (Roche and Monsigny, unpublished data) it remained uncertain whether ERGIC-53 or an additional, ERGIC-53-associated protein carries the carbohydrate binding activity. In Figure 3A we demonstrate that overexpressed ERGIC-53 indeed binds to the mannose column initially used for the purification of MR60 (Pimpaneau *et al.*, 1991), and that it can be eluted with 0.2 M D-mannose. ERGIC-53 was concentrated from the column fractions by immunoprecipitation with mAb G1/93 against ERGIC-53, subjected to SDS-PAGE, transferred to nitrocellulose, and stained with a polyclonal antibody against ERGIC-53, followed by detection with ^{125}I-protein A (see MATERIAL AND METHODS). In parallel, we tested whether ERGIC-53 myc/6xHis behaves identical to ERGIC-53 (Figure 3A). Binding of ERGIC-53 myc/6xHis to and its elution from the mannose column were virtually identical to those of ERGIC-53, demonstrating that the introduced tags did not interfere with carbohydrate binding.

To test whether binding to the mannose column required divalent cations, we replaced the divalent cations of the binding buffer by 10 mM EDTA. Figure 3B shows that under these conditions all the ERGIC-53 myc/6xHis eluted in the flow-through fraction, consistent with a requirement for divalent cations for carbohydrate binding.

Substitution of N156 with A or Mutation of AD120/121 to VA Prevents Binding to a Mannose Column

Structural analysis by high resolution x-ray crystallography of legume lectins co-crystallized with their appropriate ligands has shown that the two conserved residues corresponding to D121 and N156 in ERGIC-53 (see Figure 1) interact via hydrogen bonds with the calcium ion and the monosaccharide (Derewenda *et al.*, 1989; Bourne *et al.*, 1990; Shaanan *et al.*, 1991). The role of the conserved asparagine in carbohydrate binding was previously tested by its substitution with aspartate in the bean lectin Phaseolus vulgaris leucoagglutinin (PHA-L), and in pea lectin. As predicted from the three-dimensional structure

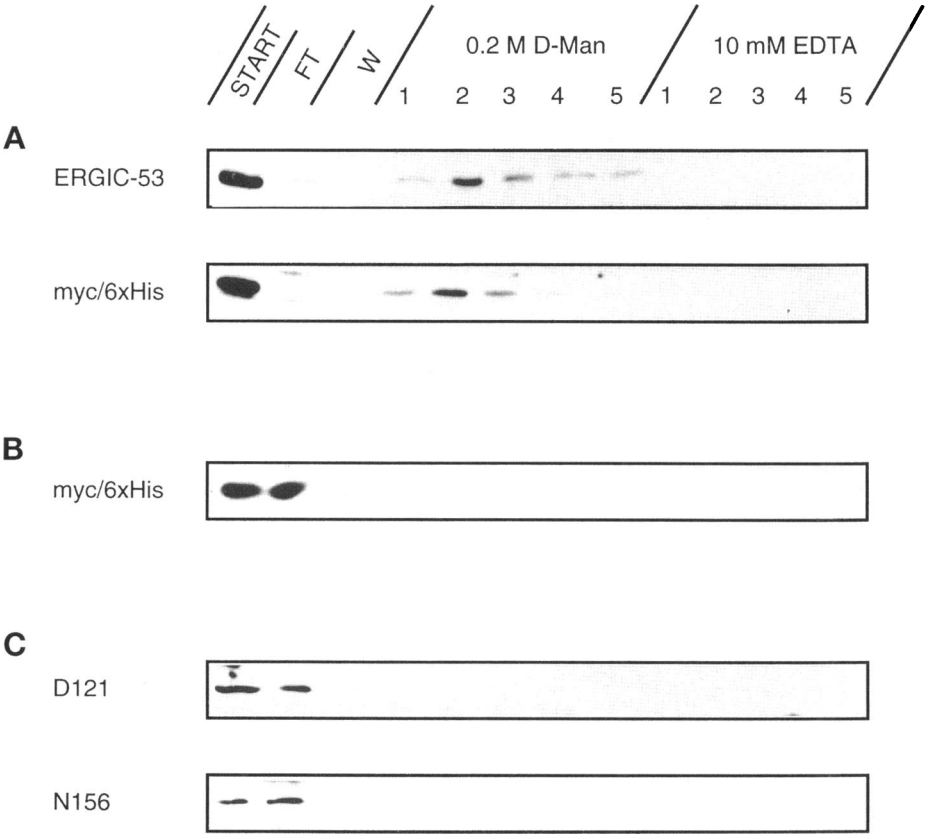

Figure 3. Overexpressed wild-type ERGIC-53 and ERGIC-53 myc/6xHis but not D121 and N156 bind to the mannose column. ERGIC-53 and mutants were overexpressed in COS cells. The cells were harvested 72 h post-transfection, homogenized, and solubilized. The solubilized material was dialyzed overnight against binding buffer that contained 10 mM $CaCl_2$/1 mM $MgCl_2$, and adsorbed to the mannose column material overnight in an end-over-end shaker. The column was packed, washed with 100 volumes binding buffer, and eluted with 5 volumes 0.2 M D-mannose followed by 5 volumes 10 mM EDTA. The antigens were immunoprecipitated from the individual fractions with mAb G1/93, separated by SDS-PAGE, blotted to nitrocellulose, and stained with a polyclonal antiserum followed by ^{125}I-protein A and detected by autoradiography (A), or stained with mAb 9E10 against the c-myc epitope and detected with enhanced chemiluminescence (B and C). (B) EDTA (10 mM) was included in the binding buffer that was devoid of divalent cations. START is 10% of total protein, FT is 10% of flow-through fraction, W is last fraction of washing step.

analysis, and underlining the importance of the conserved asparagine, both mutations eliminated carbohydrate binding (van Eijsden *et al.*, 1992; Mirkov and Chrispeels, 1993).

To test the relationship of ERGIC-53 to leguminous lectins we substituted N156 by alanine. To more severely affect the carbohydrate binding site we also generated mutation AD120/121 to VA (abbreviated D121). In this latter mutation the potential to form hydrogen bonding with D121 is abolished and the change of A120 to V increases the hydrophobicity in the binding site. Although non-neighbouring in primary structure, the homologous asparagine and aspartate are adjacent in the tertiary structure of leguminous lectins. Both mutations should provide a stringent test for functional homology. In case of a functional homology these mutations should abolish binding of the mutant proteins to the mannose column. Figure 3C shows that both mutants N156 and D121 indeed failed to bind to the mannose column and were collected in the flow-through fraction.

Mannosylated Neoglycoprotein Fails to Bind to COS Cells Expressing ERGIC-53 Mutants D121 or N156

To have an independent confirmation of the above result, we tested the binding of fluorescein-labeled neoglycoproteins to detergent permeabilized COS cells expressing the different ERGIC-53 mutants. The cells were incubated with fluorescein-labeled mannose-BSA, and as control for carbohydrate specificity, with fluorescein-labeled GlcNAc-BSA, or with fluorescein-labeled nonglycosylated BSA. It is important to note that equal amounts of each monosaccharide were bound per BSA molecule and that the specific fluorescence was identical for the three probes. Figure 4A (a and b) shows co-staining of ERGIC-53 myc/6xHis with mannosylated-BSA, while GlcNAc-BSA gave weak staining and BSA no staining at all (Figure 4A, c–f). The weak staining with GlcNAc-BSA is limited to a small area of the cell, suggesting that this ligand reacts with a GlcNAc acceptor other than ERGIC-53. The results of Figure 4A were confirmed with overexpressed nontagged ERGIC-53 (our unpublished observations). In contrast, both mutants D121 and N156 failed to co-stain with mannose-BSA (Figure 4B), GlcNAc-BSA, or BSA (our unpublished observations). These results establish in two independent binding assays that the amino acids N156 and AD120/121 are required for carbohydrate binding, which is consistent with ERGIC-53 being a functional animal homologue of leguminous lectins.

Elution of ERGIC-53 Is Selective for D-Mannose

Based on its ability to bind to the mannose column and to co-stain with mannosylated-BSA, it was clear that ERGIC-53 is a mannose-specific lectin. However, the above experiments did not allow us to conclude whether ERGIC-53 is indeed selective for D-mannose. From its intracellular localization, that is ER, ERGIC, and to a minor extent cis-Golgi (Schweizer *et al.*, 1988, 1990; Chavrier *et al.*, 1990), we expected the putative natural monosaccharide ligands of ERGIC-53 to be either glucose, mannose, or GlcNAc but not galactose that is attached to glycoproteins only in the trans-Golgi (Roth and Berger, 1982; Kornfeld and Kornfeld, 1985). To address the question of monosaccharide selectivity we tested the hexose concentration required for elution of myc/6xHis from the mannose column in a sequential elution protocol (see MATERIAL AND METHODS). Figure 5 shows the elution profile of ERGIC-53 myc/6xHis. D-Mannose started to elute ERGIC-53 myc/6xHis at a concentration of 100 mM with a peak at 150 mM. D-Glucose and D-GlcNAc both eluted ERGIC-53 myc/6xHis starting at about 200 mM with a peak at 400 mM. All three monosaccharides eluted ERGIC-53 myc/6xHis completely, as indicated by the fact that a final wash with 20 mM EDTA did not elute any ERGIC-53 myc/6xHis anymore. In contrast, D-galactose was unable to elute ERGIC-53 myc/6xHis, and ERGIC-53 myc/6xHis was only eluted by removing the divalent cations with 20 mM EDTA. Likewise, D-fucose, a sugar not found in animal glycoproteins used as a negative control, was unable to dissociate bound ERGIC-53 myc/6xHis from the mannose column (note that fucose in glycoproteins has an L configuration).

These results show that ERGIC-53 is selective for mannose. Although the protein also appears to have some affinity for glucose and GlcNAc in the column assay, we consider it less likely that these two sugars are natural ligands in the cell because rather high concentrations were necessary for dissociating ERGIC-53 from the mannose column. Moreover, MR60 can aggregate beads bearing α-D-mannosyl but not α-D-glucosyl residues (Carpentier *et al.*, 1994).

Binding of ERGIC-53 to the Mannose Column Requires Calcium

In Figure 2B we have shown that 10 mM EDTA prevents binding of ERGIC-53 myc/6xHis to the mannose column. However, this experiment did not allow us to decide which divalent cation was required for binding. To analyze this requirement we first removed divalent cations by treatment of ERGIC-53 myc/6xHis with 10 mM EDTA and subsequent dialysis against binding buffer that contained no cations, Ca^{2+}, Mg^{2+}, or Mn^{2+}. The ERGIC-53 myc/6xHis protein was allowed to bind to the mannose column material in the corresponding buffer,

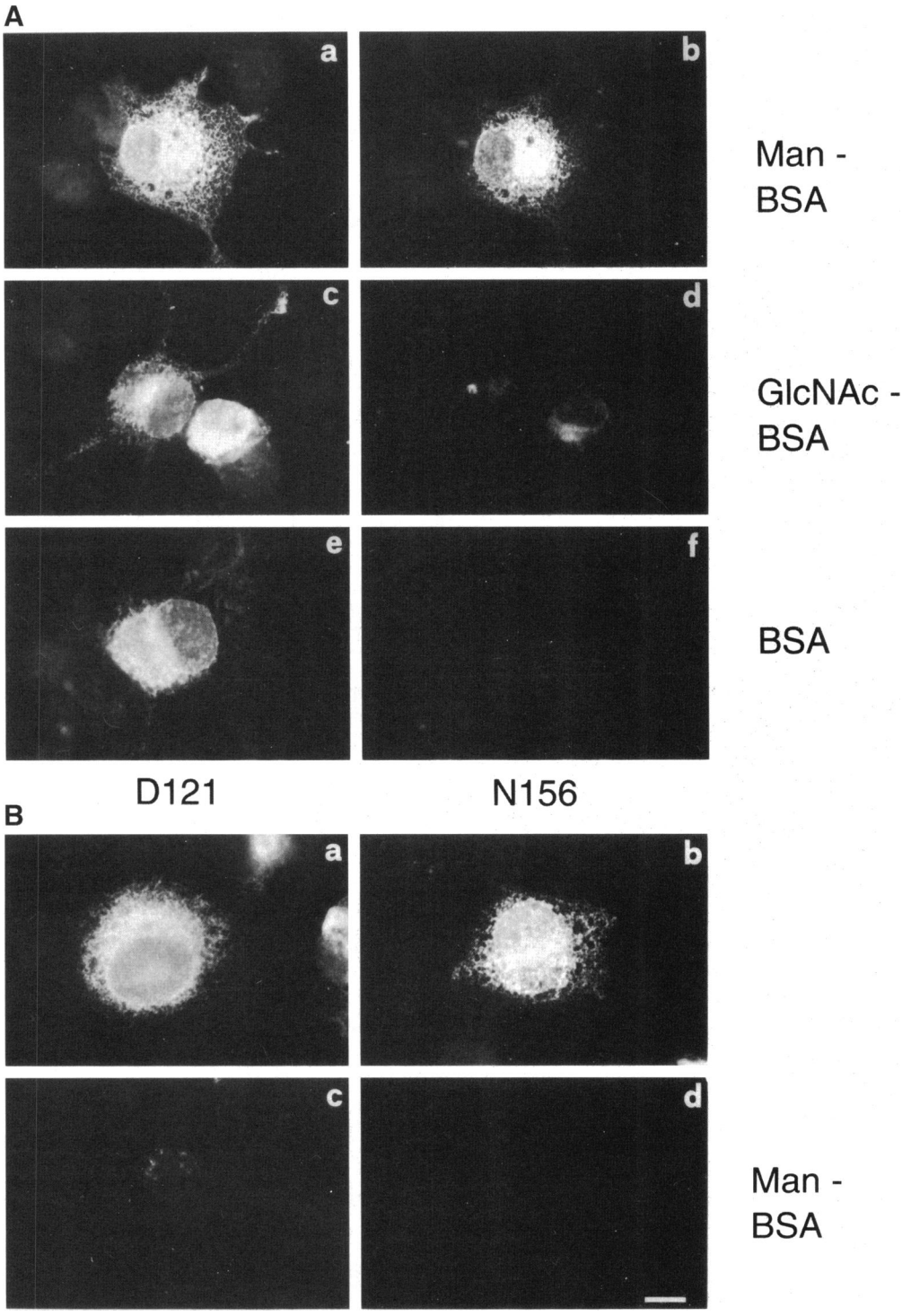

Figure 4. Binding of fluorescein isothiocyanate-labeled neoglycoproteins to transfected COS cells visualized by double immunofluorescence microscopy. (A) COS cells were transfected with ERGIC-53 myc/6xHis cDNA, fixed/permeabilized with para-formaldehyde/saponin, and incubated with fluorescein-labeled Man-BSA (Man-BSA), fluorescein-labeled GlcNAc-BSA (GlcNAc-BSA), or fluorescein-labeled BSA (BSA). Subsequently the cells were incubated with mouse mAb G1/93 against ERGIC-53 followed by a rhodamine-labeled goat anti-mouse secondary antibody. ERGIC-53 myc/6xHis was visualized in the rhodamine channel (a, c, and e) and neoglycoprotein binding in the fluorescein channel (b, d, and f). Note the extensive co-staining of ERGIC-53 myc/6xHis with Man-BSA (a and b) but not with GlcNAc-BSA (c and d) or BSA (e and f). Overexpression of the ERGIC-53 construct leads to a pronounced ER-staining that dominates the intermediate compartment staining. Moreover, there is also slow transport to the cell surface due to the replacement of the KKFF targeting signal by the six histidines (Itin *et al.*, 1995b). (B) COS cells were transfected with the CRD mutants D121 or N156, fixed/permeabilized, and incubated with fluorescein-labeled Man-BSA followed by mAb G1/93 (a) or mAb 9E10 against the c-myc epitope (b) and rhodamine-labeled secondary antibody. D121 (a) and N156 (b) expression was visualized in the rhodamine channel, binding of Man-BSA in the fluorescein channel (c and d). Note that the D121 and N156 mutant proteins are unable to bind Man-BSA. Bar, 10 μm.

the columns were packed, washed with 50 volumes of buffer, and bound ERGIC-53 myc/6xHis was eluted with 20 mM EDTA. Figure 6 shows that in the absence of divalent cations ERGIC-53 myc/6xHis failed to bind to the mannose column, while in the presence of 1 mM Ca^{2+} the protein bound quantitatively. There was no significant difference in binding in a range of 0.1 to 10 mM Ca^{2+} (our unpublished observations). If instead of Ca^{2+}, either 1 mM Mg^{2+} or 1 mM Mn^{2+} was included in the binding buffer,

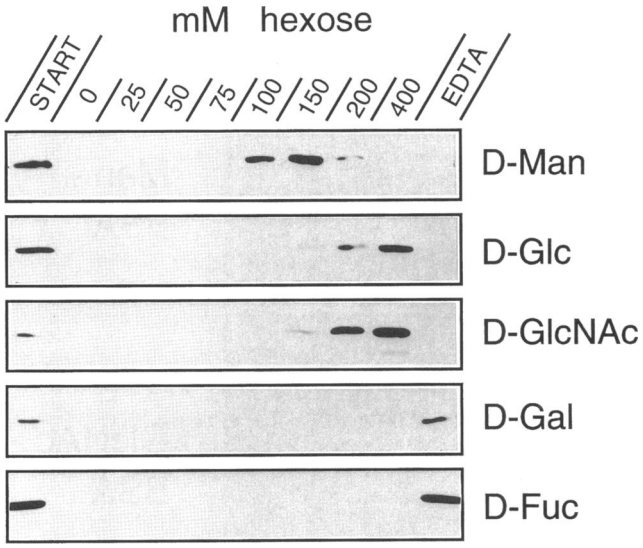

Figure 5. Elution of ERGIC-53 myc/6xHis from the mannose column is selective for D-mannose. ERGIC-53 myc/6xHis bound to the mannose column was eluted by applying 2 volumes of hexose-containing buffer, followed by a wash step with 2 volumes of binding buffer. This procedure was repeated with increasing concentrations of hexose. To remove the remaining ERGIC-53 myc/6xHis from the column, 4 volumes of 20 mM EDTA were applied. All samples were concentrated by immunoprecipitation with mAb G1/93 against ERGIC-53, separated by SDS-PAGE, blotted to nitrocellulose, and detected with mAb 9E10 against the c-myc epitope and enhanced chemiluminescence. Abbreviations are as follows: D-Man, D-mannose; D-Glc, D-glucose; D-GlcNAc, [d]-*N*-acetylglucosamine; D-Gal, D-galactose; D-Fuc, D-fucose.

no ERGIC-53 myc/6xHis bound to the mannose column, demonstrating that neither Mn^{2+} nor Mg^{2+} were sufficient for binding. Higher Mg^{2+} or Mn^{2+} concentrations (i.e., 5 mM) were also ineffective. This result does not entirely rule out a role for either Mn^{2+} or Mg^{2+} as an additional factor required for binding, since we do not know whether the initial treatment with EDTA removed all cations quantitatively. Nevertheless, the data show that Ca^{2+} is required for the lectin function of ERGIC-53.

DISCUSSION

ERGIC-53 Is a Functional Human Homologue of Leguminous Lectins

Leguminous lectins are a class of proteins that have been extensively studied at the molecular and structural level. They have a highly conserved superimposable overall structure consisting of two antiparallel pleated sheets and a carbohydrate binding site consisting of two interacting cavities (Sharon and Lis, 1990; Sharon, 1993). One cavity contains the cations Mn^{2+} (or Mg^{2+}) and Ca^{2+}, the other contains the monosaccharide. Located in between these cavities are two critical residues, an aspartate and an asparagine, that are conserved in all leguminous lectins. Their side chains bind the Ca^{2+} ion and provide hydrogen bonds to the monosaccharide (Derewenda *et al.*, 1989; Bourne *et al.*, 1990; Sharon and Lis, 1990; Shaanan *et al.*, 1991). In the case of concanvalin A, removal of the cations by EDTA widens the binding site (Reeke *et al.*, 1978), an effect that explains the loss of ligand binding in the absence of cations (Kalb and Levitzki, 1968). Based on these data the Ca^{2+} ion was proposed to orient the side chains of both the conserved aspartate and the conserved asparagine, conferring correct orientation and distance for hydrogen bonding with the monosaccharide. The central role of the conserved asparagine in monosaccharide binding has been tested by substitutional mutagenesis with an aspartate in Pea lectin and in PHA-L (van Eijsden *et al.*, 1992; Mirkov and Chrispeels, 1993). In both cases the mutation of this residue eliminated monosaccharide binding.

We have used a similar strategy to test for the postulated relatedness of ERGIC-53 with leguminous lectins. Replacement of either N156 with A or AD120/121 with VA abolished the binding to the mannose column as well as the mannose-specific co-staining of the mutants with fluorescein-labeled neoglycoproteins in a morphological binding assay. Loss of carbohydrate binding by two independent point mutations that neither affect the overall folding nor the stability of the protein strongly argues against a pure coincidence of the observed effect with the predicted outcome of the experiment. We take this result as strong evidence for a functional homology of the ERGIC-53

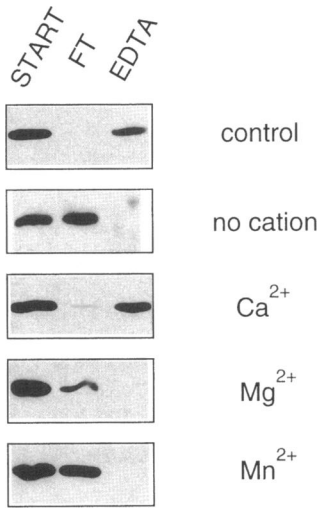

Figure 6. Mannose-column binding of ERGIC-53 myc/6xHis requires calcium. ERGIC-53 myc/6xHis was prepared as described in Figure 3, solubilized in the presence of 10 mM EDTA, and dialyzed against binding buffer that contained either no cation, 1 mM Ca^{2+}, 1 mM Mg^{2+}, or 1 mM Mn^{2+}. The dialyzed material was allowed to adsorb to the mannose-column material overnight and the column was packed and washed with 50 volumes of binding buffer. Bound ERGIC-53 myc/6xHis was eluted with 5 volumes of 20 mM EDTA. START and FT represent one-third of the initial volume of the solubilized material that was applied to the column. EDTA represents the total ERGIC-53 myc/6xHis that could be eluted from the column. All fractions were concentrated by immunoprecipitation with G1/93 against ERGIC-53 and analyzed by SDS-PAGE followed by Western blotting using mAb 9E10 against the c-myc epitope (enhanced chemiluminescence procedure).

carbohydrate recognition domain to that of leguminous lectins. However, formal proof must await crystallization and x-ray defraction analysis of the protein.

In a previous study structural homologies of ERGIC-53 with galectins have been noticed (Arar *et al.*, 1995). However, the biochemical features of ERGIC-53, in particular its mannose selectivity and Ca^{2+} dependence, are different from those of galectins that exhibit galactose selectivity and cation-independent carbohydrate binding. Furthermore, neither D121 nor N156 of ERGIC-53 are conserved in galectins (Arar *et al.*, 1995). In galectins the carbohydrate interaction is mediated by a set of different amino acid residues that are conserved in the galectin family (Barondes *et al.*, 1994). Having established a lectin activity and the functional homology of ERGIC-53 to leguminous lectins, we provide the first direct experimental evidence for the hypothesis of Fiedler and Simons (1994) that ERGIC-53 is a member of a new class of lectins in the secretory pathway.

ERGIC-53 Has All of the Main Features of a Sorting Receptor

Sorting receptors recognizing carbohydrates have a general domain organization consisting of an exoplasmic domain with one or multiple CRDs, a single transmembrane domain, and a cytoplasmic domain, containing sorting signals (Drickamer and Taylor, 1993). Ligands bind on the extracytoplasmic side to the CRDs and the ligand-receptor complex is then sorted into budding vesicles via the cytoplasmic sorting signals, a process that is likely mediated through the interaction with coat structures (Kornfeld, 1992; Schmid, 1992; Pelham, 1994). ERGIC-53 with its type I transmembrane topology (Schweizer *et al.*, 1988; Schindler *et al.*, 1993), a lumenal CRD (this study), and its cytoplasmic sorting signals (Itin *et al.*, 1995b) fits the domain organization of a sorting receptor. The oligomeric structure of ERGIC-53 (disulfide-linked dimers and hexamers) also provides multiple CRDs per ERGIC-53 complex. Consistent with a role as a sorting receptor, ERGIC-53 is continuously recycling between the different organelles in the early secretory pathway (Lippincott-Schwartz *et al.*, 1990; Foguet, Clausen, Tang, Hong, and Hauri, unpublished data), and the selectivity for mannose fits the modifications expected to be found on potential ligands. Finally, the calcium dependence of ligand binding, and the homology to leguminous lectins suggest that luminal Ca^{2+} levels may regulate the ERGIC-53-ligand interaction.

Hypothesis of ERGIC-53 Function

We propose that ERGIC-53 recognizes fully folded high-mannose glycoproteins in the ER after they have been released from the proposed quality control machinery for protein folding and N-glycan trimming consisting of the UDP-Glc:glycoprotein glucosyltransferase/calnexin/ER glucosidase cycle (Sousa *et al.*, 1992; Sousa and Parodi, 1995; Hammond *et al.*, 1994; Hebert *et al.*, 1995; Peterson *et al.*, 1995). COP I or COP II coats then bind to the cytoplasmic sorting signals of ERGIC-53 and drive the lectin-glycoprotein complex into budding vesicles. During transport or upon arrival in the ERGIC, the ligand is released from ERGIC-53 and is free to be transported to the cis-Golgi, while dissociated ERGIC-53 would return to the ER for the next round of transport. Such a receptor function is in line with the ERGIC-53 trafficking pathway. ERGIC-53 cycles mostly between ER and ERGIC and only to a minor extent via the cis-Golgi (Foguet, Clausen, Tang, Hong, and Hauri, unpublished data; Kappeler, Itin, Foguet, and Hauri, unpublished data). It might also explain the observed concentration of an itinerant membrane glycoprotein, vesicular stomatitis virus G protein, at the exit site of the ER (Balch *et al.*, 1994). Based on ERGIC-53's dependence on calcium for carbohydrate binding we would further suggest that the high calcium concentration in the lumen of the ER (Sambrook, 1990) favors ligand binding whereas reduced calcium concentration in post-ER compartments would trigger release of the ligand.

Alternatively, ERGIC-53 may recycle incorrectly trimmed glycoproteins from ERGIC or cis-Golgi back to the ER and thereby constitute a post-ER quality control mechanism of glycoprotein maturation. According to this notion ERGIC-53 should recognize N-glycans that have escaped trimming by glucosidases, or α-mannosidases in the ER, and as a consequence, their carbohydrate structure would be $Glc_{0-3}Man_{8-9}GlcNAc_2$ (Kornfeld and Kornfeld, 1985). The major difference between correct trimming resulting in $Man_{6-9}GlcNAc_2$, and incorrect trimming would consist in the terminal glucoses. Since we have shown that ERGIC-53 has selectivity for mannose such an interaction based mostly on the recognition of terminal glucoses appears less likely. Furthermore, a fail-safe mechanism for incorrect glucose trimming has been described for the Golgi apparatus. It consists of a Golgi α-D-endomannosidase that removes the outermost $Glc_{1-3}Man$ (Lubas and Spiro, 1987, 1988; Moore and Spiro, 1990; Fujimoto and Kornfeld, 1991). Differently trimmed mannose glycans are unlikely candidates for recognition because of the overlapping substrate specificities of α-mannosidases in the ER (Man_9 to Man_{7-8}) and the Golgi (Man_9 to Man_5), which render a control step for mannose-trimming obsolete (Tabas and Kornfeld, 1979; Tulsiani *et al.*, 1982; Bischoff and Kornfeld, 1983, 1986; Bischoff *et al.*, 1986; Tulsiani and Touster, 1988; Bause *et al.*, 1992).

Because of the transient nature of the proposed binding of glycoproteins to ERGIC-53 via their carbohydrate moieties, we would predict that this interac-

tion is of low affinity very much like the initial binding of the chaperone calnexin to monoglucosylated glycoproteins in the ER (Ware *et al.*, 1995). However, with calnexin, an additional polypeptide-polypeptide interaction appears to stabilize the initial carbohydrate binding which allows co-immunoprecipitation of the ligands (Degen *et al.*, 1992; Ou *et al.*, 1993; Hammond *et al.*, 1994, Hebert *et al.*, 1995; Ware *et al.*, 1995; Zhang *et al.*, 1995). In contrast, our attempts to co-isolate ERGIC-53 and ligands by immunoprecipitation have not been successful (Itin, Bucher, and Hauri, unpublished results). ERGIC-53 is therefore unlikely a chaperone, arguing against a potential role of ERGIC-53 as an additional folding sensor down-stream of the reglucosylation-calnexin system in the ER (Helenius, 1994).

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