Functional Characterization of Two Secreted SEL1L Isoforms Capable of Exporting Unassembled Substrate*

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SEL1L-A, a transmembrane glycoprotein residing in the endoplasmic reticulum (ER), is a component of the ER-associated degradation (ERAD) pathway. Alternative splicing generates two smaller SEL1L isoforms, -B and -C, that lack the SEL1L-A membrane-spanning region but retain some sel-1-like repeats, known to be involved in multi-protein interactions and signal transduction. In this study the functional characteristics of SEL1L-B and -C were investigated in human cell models. We show that these two isoforms are induced upon ER stress and activation of the unfolded protein response, together with SEL1L-A. Using transient transfection experiments (based on wild-type and mutant SEL1L constructs) combined with several biochemical tests we show that SEL1L-B and, more prominently, SEL1L-C are secreted glycoproteins. Although SEL1L-C is in monomeric form, SEL1L-B is engaged in intramolecular/intermolecular disulfide bonds. Both isoforms localize in secretory and degradative cellular compartments and in areas of cell-cell contact. However, whereas SEL1L-B is mainly associated with membranes, SEL1L-C shows the typical intraluminal localization of soluble proteins and is present in intercellular spaces. Furthermore, because of its peroxisomal domain, SEL1L-C localizes to peroxisomes. Both SEL1L-B and -C are involved in sorting and exporting unassembled Ig-μ chains, but do not affect two other ERAD substrates, the null Hong Kong variant of α2-antitrypsin, and mutant α1-AT Z. Overall these findings suggest that SEL1L-B and -C participate to novel molecular pathways that, in parallel with ERAD, contribute to the disposal of misfolded/unfolded or orphan proteins through degradation or secretion.

SEL1L (alias SEL1L-A) is a type I transmembrane glycoprotein resident in the endoplasmic reticulum (ER) and involved in the recognition and/or dislocation of misfolded/unfolded proteins for ER-associated degradation (ERAD) (1–3). ERAD is responsible for the recognition, selection, ubiquitination, and retrotranslocation across the ER membrane of unfolded or misfolded proteins for their degradation by cytosolic proteasome (4). It has been recently demonstrated that several well characterized ERAD substrates are cleared by SEL1L-A and its associated E3 ligase, HRD1 (1–3).

Alternative splicing, a major mechanism for expanding, diversifying, and regulating the repertoire of gene functions (5, 6), can generate a wide range of structurally and functionally diverse protein isoforms from transcripts encoded at a single gene locus. Notably, both soluble and membrane-associated protein isoforms can be produced by alternative exclusion/inclusion of membrane-spanning regions (7, 8). Soluble isoforms can acquire new properties and/or act as diffusible signals that agonize or antagonize functions of the membrane-bound variants.

Here we report the cloning and functional characterization of SEL1L-B and -C, two SEL1L isoforms described as alternatively spliced SEL1L variants by Biunno et al. (10) and by AceView (April 2007). SEL1L-C was also identified by Swiss-prot search (Q9UBV2–2). Clark et al. (11) reported that cDNA clone AY358651, alias SEL1L-C, encodes a putative secretory product.

SEL1L-B and -C are respectively encoded by nine and eight of the twenty-one SEL1L-A exons and lack the SEL1L-A C-terminal transmembrane region but retain respectively four and three of the eleven sel-1-like repeats present in SEL1L-A. Alternative splicing events at the 3′ ends generate a tail of 9 amino acid residues (GIYVSPFTF) in SEL1L-B (10) and an SRL sequence, variant of the conserved peroxisomal targeting signal SKL, in SEL1L-C (9, 10). A fibronectin type II domain is conserved in all three SEL1L isoforms (10, 16, 17).

Here we show that SEL1L-B and -C are both responsive to ER stress. Although SEL1L-A is confined to the ER membrane and is involved in the degradation of unassembled Ig-μ chains, both SEL1L-B and -C are secreted and capable of exporting orphan Ig-μ chains outside cells with substrate specificity.
regard to subcellular distribution, both isoforms localize in secretory and degradative compartments and in areas of cell-cell contact. Although SEL1L-B is mainly associated with membrane profiles, SEL1L-C behaves as a typical soluble glycoprotein, localizing intralumenally in secretory compartments and intercellular spaces, and is also present in peroxisomes.

**EXPERIMENTAL PROCEDURES**

**Constructs**—SEL1L-B and -C were generated by PCR from normal peripheral blood lymphocytes and verified by sequencing. The Myc-tagged SEL1L-B and -C constructs were generated by cloning the full-length SEL1L-B and -C coding sequence fused with a Myc tag at the 3' end into the pCDNA3.1Myc-Hys vector (Invitrogen). SEL1L-B harboring a glycine to glutamic acid substitution at position 312 in the fourth sel-1-like repeat was naturally obtained from bacterial transformants. SEL1L-C deleted of the specific C-terminal peroxisomal domain (SEL1L-Cmyc) was derived from the wild-type SEL1L-C isoform. Vectors driving the expression of the N-terminal region of SEL1L-A (20–372 amino acids), with or without KDEL motif (named SEL1L 20–372KDEL and SEL1L 20–372, respectively), were kindly provided by Drs. R. Sitia and J. C. Christianson (2, 3), respectively. Vectors driving the expression of the null Hong Kong variants of 1-antitrypsin (HHK and 1-AT Z) were kindly provided by Dr. R. Sifers.

**Cell Lines, Culture Conditions, and Transfections**—Multiple myeloma KMS11 and breast cancer SKBr3 cells were maintained in RPMI containing 10% fetal bovine serum (Euroclone, FIGURE 1. A, identification of SEL1L-B and -C transcripts and induction by ER stress. RT-PCR was performed on RNA extracted from 293 FT (panel A.1), KMS11 (panel A.2), and SKBr3 (panel A.3) cells, untreated and treated with DTT (2 mM) for 150 min, or with tunicamycin (T) (2 μg/ml) for 24 h. PCR with isoform-specific primers was as described under “Experimental Procedures,” signals shown here were obtained with 23 cycles for SEL1L-A and 32 cycles for SEL1L-B and -C. HPRT was used as a loading control, CHOP expression and spliced XBP-1 as indicators of ER stress. Panel A.4, down-modulation of SEL1L-B and -C transcripts by SEL1L siRNA. SKBr3 cells were treated with scrambled siRNA (lane 1) or siRNA specific to SEL1L (lane 2) for 72 h. Silencing efficiency was verified by RT-PCR. All of the SEL1L isoforms were down-modulated after SEL1L RNA interference. Densitometric quantifications were normalized relative to the housekeeping HPRT signals, as determined through the Scion imaging program. The data, expressed as fold-decrease relative to untreated, are the averages of two independent experiments, as in the top panel, + S.E., *p > 0.05 t test. B, identification of SEL1L-B and -C transcriptional start sites. The sequence of the proximal 5'-flanking region of SEL1L-A (−300 to −1) is shown. The numbers indicate nucleotide positions from the translation initiation site (ATG, bold and double-underlined). GC motifs are in gray boxes, and the CAAT box is in open box. B* and C* indicate the transcription start sites predicted by Aceview, and B and C are the transcription start sites identified by 5'-RACE PCR and sequencing.
Clonal antibodies anti-Myc, anti-actin, anti-vinculin, and anti-SEL1L was kindly provided by Dr. H. L. Ploegh (1). Monoclonal antibody anti-SEL1L was raised against an N-terminal peptide of human SEL1L (28). Rabbit polyclonal antibody was directed against a 180 amino acid-long fragment of the C-terminus of human SEL1L (29). Monoclonal antibody against human 1-antitrypsin was purchased from Sigma-Aldrich. Anti-actin and anti-Myc antibodies were purchased from Zymed Laboratories Inc. (Invitrogen).

RT-PCR—Total RNA was extracted using the SV Total RNA isolation system (Promega, Milan, Italy). RNA was reverse-transcribed with SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR amplifications were performed with 2 µl of RT product/reaction and 0.15 units of Platinum Taq DNA polymerase High Fidelity (Invitrogen) using the following specific primers (and conditions): SEL1L-A, sense: 5'-ctctgctaaagggctcattc-3' and antisense: 5'-gccacctgctacatcttgac-3' (annealing at 60 °C, 23 amplification cycles); SEL1L-B, sense: 5'-ccggccccgagaggaggatgcgggtc-3' and antisense: 5'-ggggaaacatagatcaagagacag-3' (annealing at 58 °C, 32 amplification cycles); SEL1L-C, sense: 5'-ccggccccggagaggaggtggctc-3' and antisense: 5'-ggggcaatatcagccaaactaatca-3' (annealing at 58 °C, 32 amplification cycles); SEL1L-D, sense: 5'-atgcttggtgcagattcacc-3' and antisense: 5'-ggggccatactgcaaaactaatca-3' (annealing at 58 °C, 23 amplification cycles); XBP-1, sense: 5'-ctctgctacatcttgacagcagggaggctcattc-3' and antisense: 5'-ctctgctacatcttgacagcagggaggctcattc-3' (annealing at 60 °C, 38 amplification cycles); and HPRT, sense: 5'-aatatggaagcagactgcaacctc-3' and antisense: 5'-gtggtgtgatatatggtgaaag-3' (annealing at 60 °C, 23 amplification cycles).

5'-RACE—5'-RACE was performed using the SMART RACE cDNA amplification kit (Clontech, Celbio, Pero, Italy). RNA was retrotranscribed by PowerScript reverse transcriptase with the 5' CDS and SMART II oligonucleotide. A universal primer A mix and gene-specific primers (SEL1L-B, sense: 5'-ggggaaacatagatcaagagacag-3', and antisense: 5'-ggggcaatatcagccaaactaatca-3'; SEL1L-C, sense: 5'-ctctgctacatcttgacagcagggaggctcattc-3', and antisense: 5'-ctctgctacatcttgacagcagggaggctcattc-3'; SEL1L-D, sense: 5'-atgcttggtgcagattcacc-3', and antisense: 5'-ggggccatactgcaaaactaatca-3') were used for amplification of 5' CDS ends. PCR conditions were: denaturation at 94 °C for 5 min; 28 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; and elongation at 72 °C for 5 min. PCR products were cloned in TOPO TA cloning (Invitrogen) and sequenced.

Western Blotting, Immunoprecipitation, and Analysis of Cell Supernatants—Monoclonal anti-SEL1L was raised against an N-terminal peptide of human SEL1L (28). Rabbit polyclonal anti-SEL1L was kindly provided by Dr. H. L. Ploegh (1). Monoclonal antibodies anti-Myc, anti-actin, anti-vinculin, and anti-α1-antitrypsin were purchased from Sigma-Aldrich. Anti-μ was obtained from Zymed Laboratories Inc. (Invitrogen).

The cells were lysed in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, containing protease inhibitors (Pierce). Protein concentrations were determined by the Bradford assay; the samples were resolved on SDS–polyacrylamide gels, blotted onto polyvinylidene difluoride membranes, decorated with specific antibodies, and developed with ECL (GE Healthcare, Milan, Italy).

For immunoprecipitation studies cell lysates were precleared with antibodies immobilized on protein A-Sepharose. The immunoprecipitates were washed twice with PBS; Sigma-Aldrich) and polyclonal antibodies against markers of cellular compartments, including the ER marker calreticulin.
Secreted SEL1L Isoforms

FIGURE 3. A, stability of exogenous isoforms. 293 FT (4 × 10⁶) transfectants expressing Myc-tagged SEL1L-B or -C were treated for 15 h with cycloheximide (CHX) and incubated for 24 h with Opti-MEM. Aliquots (30 μg) of lysates (left) and of secreted proteins (right) were resolved by 10% SDS-PAGE and probed with anti-SEL1L and anti-actin. The levels of exogenous isoforms in lysates decreased after cycloheximide treatment, concomitantly with increases in the culturing medium. Densitometric quantifications were normalized relative to housekeeping signals by the Scion imaging program. The data are the averages of two independent experiments, as in the top panel, expressed as fold modulation relative to untreated, ± S.E., *p < 0.05 t test. B, SEL1L-B forms dimers. Lysates from 293 FT (4 × 10⁶) transfectants expressing Myc-tagged SEL1L-B or -C were immunoprecipitated with monoclonal anti-Myc and probed with anti-Myc. Under nonreducing condition SEL1L-Bmyc immunoprecipitated both as dimer and monomeric forms, whereas SEL1L-Cmyc immunoprecipitated only as monomeric forms. SEL1L-Bmyc dimers were reduced in the redox running condition. Aliquots of lysates (40 μg) were loaded to verify protein expression levels and immunoprecipitation efficiency. C, intra/intermolecular disulfide bonds in SEL1L-B. 293 FT cells (4 × 10⁶) transiently transfected with Myc-tagged SEL1L-B and -C were maintained for 24 h in Opti-MEM. Secreted protein and aliquots of cell lysates (25 μg) were resolved by SDS-PAGE (7%) under reducing (redox) and nonreducing (non redox) conditions and blotted with monoclonal anti-SEL1L (panels C.1, C.2, and C.4) or anti-Myc (panel C.3). The lower bands correspond to the exogenous SEL1L-Bmyc and -Cmyc isoforms. The upper multiple bands revealed by anti-SEL1L in lysates (panel C.2) and culturing medium (panel C.4) and by anti-Myc in lysates (panel C.3) of SEL1L-Bmyc transfectants under nonreducing conditions may correspond to intra/intermolecularly disulfide-bound SEL1L-B. D, overexpression of SEL1L-B and -C isoforms does not affect the UPR response. RNA was extracted from 293 FT (4 × 10⁶) cells transfected with empty vector (pCDN3.1 myc), SEL1L-Bmyc, and SEL1L-Cmyc, respectively, and analyzed by RT-PCR for the UPR response. The exogenous SEL1L isoforms did not modulate SEL1L-A and CHOP expression or XBP-1 splicing. HPRT serves as internal control. On top, aliquots of lysates (30 μg) were loaded to verify transfection efficiencies.

(1:100 in PBS; Affinity Bioreagents, Breda, The Netherlands), the Golgi marker giantin (1:50 in PBS; Covance, Princeton, NJ), the late endosomal marker CD63/lamp3 (1:20 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA), and the peroxisomal marker catalase (1:100 in PBS; Calbiochem, Gibbstown, NJ). The nuclei were stained using 4',6-diamido-2-phenylindole diluted 1:10,000 in PBS (Sigma-Aldrich). After appropriate washing with PBS, primary antibodies were visualized using goat anti-mouse IgG fluorescein isothiocyanate (1:50 in PBS; Cappel Research Products) or goat anti-rabbit IgG-TxRed (1:200 in PBS; Jackson Immunoresearch Laboratories) for 30 min at 25 °C. The coverslips were finally mounted with 90% glycerol in PBS for observation with a Zeiss Axiosvert 200M microscope fitted with an ApoTome Imaging System. Optical section image analysis was performed on a single central section using a Zeiss KS300 3.0 image processing system (Zeiss). The absence of SEL1L-Bmyc and SEL1L-Cmyc signals in non-transfected 293 FT kidney cells or in cells transfected with empty pCDN3.1Myc-Hys(-) vector confirmed the specificity of the immunofluorescence signals.

Cryoimmunoelectron Microscopy—Cells processed for cryoimmunoelectron microscopy were grown as above and fixed in 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 25 °C. Collected cells were embedded into 10% gelatin (Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.4, solidified on ice, infused in 2.3 M
Sucrose overnight at 4 °C, and frozen in liquid nitrogen. Ultrathin cryosections were cut at 100 °C using an Ultracut EM FC6 (Leica Microsystems, Vienna, Austria), collected with sucrose and methyl cellulose, and indirectly single- or double-labeled. Bound antibodies were visualized by goat anti-mouse conjugated with 10- or 15-nm colloidal gold (British BioCell International, Cardiff, UK) or by protein A conjugated with 10-nm colloidal gold (supplied from G. Posthuma and J. Slot, Utrecht, The Netherlands). Immunolabeling was performed with the following antibodies: monoclonal anti-c-Myc (Sigma-Aldrich), polyclonal anti-calreticulin (Affinity Bioreagents), monoclonal anti-lamp1 (Calbiochem, Gibbstown, NJ), and polyclonal anti-catalase (Calbiochem).

Sections were analyzed with a Philips CM10 transmission electron microscope SEL1L-Bmyc and -Cmyc immunolabeling was quantitated by counting gold particles/unit of surface area (golds/μm², mean ± S.E.) on the ER, Golgi, and endosomes and per unit of linear length (golds/μm, mean ± S.E.) along the plasma membrane. Surface areas and linear plasma membrane lengths were examined and calculated using a digital transmission electron microscope equipped with an image analysis system (Fei-Philips Morgagni 268D, FEI Italia SRL, Milan). For each transfected sample, at least 12 different images of each compartment were used for counting. Background labeling was determined as above on cells transfected with pCDNA3.1Myc-Hys(+)A vector alone, p values were calculated using Student’s t test, with significance at p < 0.05.

RESULTS

Cloning and Characterization of Endogenous SEL1L-B and -C—Using RT-PCR and sequence analysis, we investigated the expression of endogenous SEL1L-B and -C in 293...
FT human embryonic kidney cells and in the human cancer cell lines KMS11 (myeloma) and SKBr-3 (breast carcinoma) (Fig. 1A, panels A.1–A.3). All of these cell lines expressed SEL1L-B and -C transcripts, although at levels lower than SEL1L-A (12). In fact the SEL1L-B and -C signals in Fig. 1A required 32 PCR cycles compared with 23 for SEL1L-A. SEL1L-B and -C mRNA levels were higher in cancer cells relative to 293 FT cells.

As shown in Fig. 1A (panels A.1–A.3), all three SEL1L transcripts were up-modulated by DTT or tunicamycin, reagents that induce ER stress and activation of the unfolded protein response (UPR) by preventing disulfide bond formation and N-glycosylation, respectively. CHOP, a well known ER stress-inducible gene (13), was up-regulated in response to both DTT and tunicamycin, whereas XBP-1 splicing, another UPR indicator, was more evident after DTT treatment. The expression of SEL1L-A, -B, and -C was significantly down-modulated by RNA-mediated interference of SEL1L-A exon 2 (Ambion, Celbio, Pero, Italy) (*, p < 0.05, t test) (Fig. 1A, panel A.4).

5′-RACE PCR analysis of RNA from SKBr3 cells demonstrated that SEL1L-B and -C originate from different transcription initiation sites within the unique TATA-less SEL1L-A promoter (14) (Fig. 1B). Direct sequencing of the RT-PCR products established that the transcription start sites of SEL1L-B and -C are 96 and 79 nucleotides upstream to the canonical ATG, respectively. These results indicate that SEL1L-B and -C, although expressed at lower levels, are induced, along with SEL1L-A, in cells under ER stress.

Characterization of Exogenous SEL1L-B and -C—For in vitro characterization, full-length SEL1L-B and -C cDNAs generated from peripheral blood lymphocytes were cloned in a Myc-tagged vector (Fig. 2A) and used to transiently transfet 293FT cells. To assess Golgi transit, we investigated sensitivity of SEL1L-Bmyc and -Cmyc to PNGase F and endo H. PNGase F and endo H hydrolyzes nearly all types of N-glycan chains added in the Golgi, whereas endo H removes N-linked oligosaccharide side chains of the high-mannose type, found on rough ER proteins that did not transit through the Golgi. As demonstrated by the electrophoretic mobility shifts of their respective 51 and 46-kDa bands, SEL1L-Bmyc and -Cmyc obtained from lysates of transfected cells were sensitive to both PNGase F and endo H (Fig. 2B, panel B.1). Treatment with tunicamycin, which prevents N-glycosylation, determined similar mobility shifts (deglycosylated SEL1L-Bmyc and -Cmyc migrated at about 47 and 41 kDa, respectively; Fig. 2B, panels B.1 and B.2). The culturing medium of transfectants contained SEL1L-Bmyc or -Cmyc that were PNGase F-sensitive, but mostly endo H-resistant, as predicted in case of Golgi transit (Fig. 2B, panel B.3). Taken together, these results indicate that SEL1L-Bmyc and -Cmyc are secreted glycoproteins.

The stability of SEL1L-Bmyc and -Cmyc in transfected cells was analyzed after blockage of protein synthesis with cycloheximide. After a 15-h cycloheximide treatment, 83 and 43% of SEL1L-Bmyc and -Cmyc, respectively, remained in cells (Fig. 3A). Cycloheximide enhanced the secretion of both isoforms, with particular regard to SEL1L-Cmyc: in fact, the respective percentages of SEL1L-Bmyc and -Cmyc over total secreted proteins were 11.4 and 32.00% before versus 21.6 and 83.43% after treatment (Fig. 3A). Therefore the lower SEL1L-Cmyc level maintained in lysates was consistent with the much more pronounced secretion of this isoform relative to SEL1L-Bmyc. Both isoforms resulted more stable than SEL1L-A, which displayed a half-life of 3 h (1).

We next investigated oligomer formation. Lysates of 293 FT cells, transfected with Myc-tagged isoforms, were immunoprecipitated with anti-Myc and analyzed under reducing and non-reducing conditions using anti-Myc. As shown in Fig. 3B, unlike SEL1L-Cmyc, SEL1L-Bmyc immunoprecipitated both as a dimer and as a monomer.
The SEL1L-Cmyc protein migrated as a doublet under both reducing and nonreducing conditions (Fig. 3C, panels C.1 and C.2). Conversely, under nonreducing conditions, SEL1L-Bmyc migrated with multiple high molecular weight bands (panels C.2 and C.3), which disappeared under reducing conditions (panel C.1), suggesting engagement of cysteine residues in inter/intramolecular disulfide bonds. Similar results were obtained using SEL1L-Bmyc recovered from supernatants (panel C.4). Overexpression of SEL1L-Bmyc and -Cmyc in 293FT cells had no effect on UPR activation, as demonstrated by lack of XBP-1 splicing and CHOP up-regulation (Fig. 3D).

In conclusion these results indicate that: (i) SEL1L-Bmyc and -Cmyc are more stable than the endogenous ER-resident SEL1L-A isoform; (ii) secretion is more conspicuous for SEL1L-Bmyc and -Cmyc; (iii) overexpression of exogenous SEL1L-Bmyc and -Cmyc are more stable than the endogenous ER-resident SEL1L-A isoform; (iv) secretion is more conspicuous for SEL1L-Bmyc and SEL1L-20–372KDEL. 293 FT (4×10^6) transfectants expressing Myc-tagged SEL1L-B and SEL1L-20–372KDEL were treated for 4 and 7 h with cycloheximide (CHX 200 μg/μl). Aliquots of lysates (30 μg) were resolved by SDS-PAGE (10%) and probed with anti-Myc (IP myc), resolved by SDS-PAGE (10%) and probed with anti-μ. Lysate aliquots (30 μg) were loaded to verify transfection and immunoprecipitation efficiencies. Abundant μ, co-immunoprecipitated with each of the tested exogenous SEL1L isoforms. B, analysis of μ, stability in presence of SEL1L-Bmyc and SEL1L-20–372KDEL. 293 FT (4×10^6) transfectants expressing Myc-tagged SEL1L-B and SEL1L-20–372KDEL were treated for 4 and 7 h with cycloheximide (CHX 200 μg/μl). Aliquots of lysates (30 μg) were resolved by SDS-PAGE (10%) and probed with anti-μ, anti-SEL1L, and anti-vinculin, as a control for loading. Although SEL1L-B had no effect on μ, stability, SEL1L-20–372KDEL decelerated degradation. WB, Western blot.

Subcellular Localization of Exogenous SEL1L-B and -C—We next analyzed the subcellular localizations of exogenous SEL1L-B and -C in transiently transfected 293FT cells, where the use of Myc-tagged constructs allowed to exclude interference from SEL1L-A (Figs. 4 and 5). Double immunofluorescence analysis using anti-Myc monoclonal antibody (green) and polyclonal antibodies (red) against markers of cellular compartments, including calreticulin (ER), giantin (Golgi), CD63/lamp3 (late endosomes), and catalase (peroxisomes) showed that both SEL1L-Bmyc and -Cmyc were present in stations involved in the secretory and endocytic degradative pathways, such as the ER, the Golgi, and multi-vesicular bodies/late endosomes (Fig. 4, A–P). Furthermore, co-localization with the peroxisomal marker catalase was prominent for SEL1L-Cmyc (Fig. 4, G and H).

In agreement with immunofluorescence, cryoimmunoelectron microscopy clearly showed that SEL1L-Bmyc and -Cmyc codistributed to the ER and to the nuclear envelope with calreticulin (Fig. 5, A and G). However, whereas SEL1L-Bmyc was associated with membrane profiles, SEL1L-Cmyc was mainly intraluminal, as calreticulin. Membranes of electron-lucent structures with the typical morphology of early endosomes were also SEL1L-Bmyc-labeled (Fig. 5, D–E), together with late endolysosomal compartments, identified by Lamp1 (Fig. 5F). These structures were similarly decorated with SEL1L-Cmyc (Fig. 5O). In addition, both isoforms were present near the plasma membrane in small vesicles, mainly in areas of cell-cell interaction and at junctional complexes (Fig. 5, B, C, H, I, L, and M). Finally, SEL1L-Cmyc was also detected in the lumen of Golgi stacks (Fig. 5H), in intercellular spaces (Fig. 5P), and in peroxisomes, with the latter clearly identified by catalase (Fig. 5N). The peroxisomal localization was lost in cells transfected with SEL1L-Cmyc, a construct deleted of the C-terminal peroxisomal signal (supplemental Fig. S1).

To better evaluate the subcellular distribution of SEL1L-Bmyc and -Cmyc, a quantitation of the immunogold labeling in different subcellular compartments was performed. As shown in supplemental Table S1, the absolute labeling densities and their ratios relative to the ER for each isoform point to SEL1L-Bmyc enrichment in endosomal structures.

Taken together, the results indicate that both SEL1L-Bmyc and -Cmyc localize in secretory and degradative compartments and suggest that these isoforms, in addition to exocytosis, could participate in other biological processes, such as endocytosis and cell-cell communication.

Exogenous SEL1L Isoforms Interact with Unassembled Ig-μ,—It was recently reported that SEL1L-A binds unassembled Ig-μ, chains (μ, ) and regulates their turnover in association with HRD1 (2). Here Myc-tagged constructs were used to investigate binding of μ, by SEL1L-Bmyc and -Cmyc, by a SEL1L-Bmyc mutant with glutamic acid to glycine substitution at position 312 in the fourth sel-1-like repeat (SEL1L-Bmutm’myc), and by SEL1L-Cmyc deleted of the peroxisomal domain...

FIGURE 6. A, exogenous SEL1L isoforms interact with μ,. Lysates from 293FT (4×10^6) transfected expressing μ, and combinations of wild-type or mutant Myc-tagged SEL1L isoforms, as indicated, were immunoprecipitated with anti-Myc (IP myc), resolved by SDS-PAGE (10%), and probed with anti-μ,. Lysate aliquots (30 μg) were loaded to verify transfection and immunoprecipitation efficiencies. Abundant μ, co-immunoprecipitated with each of the tested exogenous SEL1L isoforms. B, analysis of μ, stability in presence of SEL1L-Bmyc and SEL1L-20–372KDEL. 293 FT (4×10^6) transfectants expressing Myc-tagged SEL1L-B and SEL1L-20–372KDEL were treated for 4 and 7 h with cycloheximide (CHX 200 μg/μl). Aliquots of lysates (30 μg) were resolved by SDS-PAGE (10%) and probed with anti-μ, anti-SEL1L, and anti-vinculin, as a control for loading. Although SEL1L-B had no effect on μ, stability, SEL1L-20–372KDEL decelerated degradation. WB, Western blot.

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(SEL1LΔCmyc). Lysates of 293 FT cells, co-transfected with μ, in combination with each of the above-mentioned Myc-tagged constructs, were co-immunoprecipitated with anti-Myc and analyzed by Western blot using anti-μ. As shown in Fig. 6A, significant amounts of orphan substrate were immunoprecipitated by both wild-type and mutated SEL1L-Bmyc and -Cmyc. The absence of signals in single transfec-
tants confirmed the specificity of the observed interactions. We next analyzed the stability of μ, co-transfected with SEL1L-Bmyc or with a construct driving the expression of the SEL1L-A N terminus, including amino acid residues 20–372, elongated with a KDEL retrieval signal (SEL1L-20–372KDEL, kindly provided by Dr. Christianson) (3). These constructs were used because SEL1L-Bmyc exemplifies a physiologically secreted isoform, whereas SEL1L-20–372KDEL, which contains a signal peptide deriving from preprolactin and an intra-repeat region of 48 residues, is retained in the ER. Thus, 293 FT cells expressing μ, alone or in combination with SEL1L-20–372KDEL or SEL1L-Bmyc constructs, were treated with cyclo-
heximide. Although SEL1L-Bmyc did not affect substrate stability, SEL1L-20–372KDEL strongly stabil-
ized μ, with dominant-negative effect (Fig. 6B). In conclusion, SEL1L-Bmyc and -Cmyc are able to bind μ, without altering substrate stability.

Secretion of μ, in the Presence of Exogenous SEL1L Isoforms—Because SEL1L-Bmyc and -Cmyc are secreted and bind μ, we next investigated whether these exogenous iso-
forms were implicated in the secretion of orphan substrate. To assess this point, we compared SEL1L-Bmyc and -Cmyc to other engineered SEL1L variants that are secreted or retained, i.e. SEL1LΔCmyc and SEL1L-20–372 with or without KDEL. As shown in Fig. 7A, μ, accumulated in the culture medium only in the presence of the exogenous SEL1L variants, particularly SEL1L-Bmyc and SEL1L-20–372 (i.e. the anchorless SEL1L-A N terminus) (3). The supernatant of SEL1LΔCmyc transfec-
tants showed the lowest level of μ, accumulation.

Secreted μ, migrated as a doublet at slower rate and were endo H-resistant and PNGase F-sensitive, which points to differ-
ences in glycosylation indicative of Golgi transit (Fig. 7B). Secretion of unassembled substrate had no effect on the UPR response, as monitored by XBP-1 splicing and CHOP activation (Fig. 7C).
We then investigated whether SEL1L-Bmyc, SEL1L-Cmyc, and SEL1L-20–372 could be involved in the secretion of two other well characterized ERAD substrates, the null Hong Kong variant of $\alpha_1$-antitrypsin (HHK), a known SEL1L-A substrate (3), and mutant $\alpha_1$-AT Z (PIZ) (15). Unexpectedly, secretion of HHK and PIZ was not enhanced in cells co-transfected with these secretory SEL1L constructs (Fig. 7D).

Taken together, these results indicate that SEL1L-Cmyc and, more prominently, SEL1L-Bmyc promote secretion of a specific ERAD substrate, i.e. unassembled Ig-$\mu_\alpha$, chains, but that this does not affect UPR. SEL1L-Cmyc and -Bmyc do not affect secretion of two other structurally different ERAD substrates, HHK and PIZ.

**DISCUSSION**

In this study we describe SEL1L-B and -C, splice variants of the ER-resident SEL1L-A protein, a component of the ERAD quality control apparatus that acts as a scaffold coordinating substrate recognition by lectins and ubiquitination via Hrd1 (1–4).

It was previously reported that the SEL1L-A promoter is regulated by the UPR through the ATF-6 pathway (18). Here we show that SEL1L-B and -C are induced, along with SEL1L-A (although at much lower levels), in cells under ER stress with activated UPR, which is consistent with the common transcriptional origin from a unique ER stress-responsive promoter (14). In addition we cannot exclude that during UPR post-transcriptional regulation might stabilize the life of the mRNAs encoding these SEL1L isoforms, affecting their cellular levels. On the other hand, overexpression of the exogenous SEL1L isoforms did not affect UPR.

Lack of the SEL1L-A transmembrane region abrogates insertion into the ER membrane for both the SEL1L-B and -C proteins, which may reflect the secretory features of these isoforms. However SEL1L-B and -C structurally differ in the number of sel-1-like repeats and in the C-terminal region, characterized by a unique nine amino acids tail in SEL1L-B and by a peroxisomal domain in SEL1L-C. Such features may confer new functional properties and localizations to these isoforms.

Using transient transfection experiments based on Myc-tagged constructs combined with several biochemical tests we show that SEL1L-B and -C are highly stable secreted glycoproteins and that secretion is more prominent for the SEL1L-C isoform. On the other hand, biochemical evidence of intra/in-termolecular disulfide bridges suggests that SEL1L-B participates to multi-protein complexes, both within cells and during secretion. The fact that this property is not shared by SEL1L-C indicates that the functionally responsible structural elements reside in the last sel-1-like repeat of the SEL1L-B isoform (that contains an additional cysteine residue relative to SEL1L-C). Further studies, based on competition as well as on mutational approaches, are necessary to better understand the nature of the intra/interdisulfide bonds of SEL1L-B and their biological implications.

Morphological analysis revealed that both SEL1L-B and -C localize in secretory and degradative compartments. However, the SEL1L-B isoform preferentially associates with membrane profiles, particularly in the ER and in endosomal compartments. This might be consistent with participation to multi-protein complexes including membrane-bound proteins. On the other hand SEL1L-C is found within the ER and the nuclear envelope, typical intraluminal localizations of soluble proteins. The presence of the two isoforms in the intercellular space, plasma membrane invaginations, peripheral vesicular structures, and intercellular junctions suggests a possible involvement in autocrine/paracrine release/uptake circuits. Furthermore, the localization of both isoforms in early and multivesicular endosomal compartments could be consistent with possible shared roles in sorting and/or accompanying molecules en route toward degradation or secretion. In this regard a peripheral quality control system involved in the recognition and degradation of structurally destabilized proteins that escape ER quality control has been described (19). For SEL1L-C, the specific peroxisomal localization suggests a role in peroxisomal quality control, a process that shares striking similarities with ERAD in the ubiquitin-based protein targeting system (20). In fact components of the peroxisome import system, such as pex2 and pex10, contain an ubiquitin ligase domain homologous to Hrd1; pex 4 contains an E2 ubiquitin-conjugating enzyme domain and is homologous to the ERAD ubiquitin-conjugating enzymes; pex1 and pex6 contain an AAA cassette that evolved from Cdc48/p97; finally pex5 contains TPR repeats homologous to sel-1-like repeats.

The differences in the subcellular distribution of the two isoforms (particularly the association of SEL1L-B with membrane profiles versus the intraluminal location of SEL1L-C) point to the specificity of localization signals, indicating that they are...
not merely due to overexpression of Myc-tagged constructs after transfection.

Finally our data show that, unlike SEL1L-A, SEL1L-B and -C most likely do not participate to the degradation of unassembled \( \mu_c \) being rather involved in their secretion. This secretory ability is enhanced in SEL1L-B and drastically decreased in the SEL1L\( \Delta \)myc mutant with deleted terminal peroxisomai domain, despite probably similar substrate binding affinities. The marked secretory effects exerted by SEL1L-Bmyc on unassembled substrate could be attributed to its ability to participate to multi-protein complexes via intra/intermolecular disulfide bridges. This could facilitate substrate transport.

However, export of \( \mu_c \) is too low to modulate UPR, indicating that these secreted SEL1L isoforms are unable to alleviate the ER stress induced by unassembled substrate. Moreover, export appears to be specific for \( \mu_c \) because two other ERAD substrates, i.e. the null Hong Kong variant of \( \alpha_1 \)-antitrypsin, HHK, and mutant \( \alpha_1 \)-AT Z, are not affected. Such substrate specificity could depend on the sel-1-like repeats. In fact, data concerning the Legionella pneumophila LpEn protein, a secreted virulence determinant possessing sel-1-like repeats, suggest that these repeats might be specifically involved in interactions with proteins characterized by immunoglobulin-like folds (21).

By computational protein design SEL1L-B and -C are predicted to function as hydrolases because of their homology modeling with the Helicobacter pylori cysteine-rich protein C (HcpC), a secreted prokaryotic sel-1-like family member (Biunno personal communication) (22, 23). As their bacterial counterpart, also eukaryotic SEL1L isoforms could elicit their potential hydrolytic activity in response to pathogen attack or overload of misfolded/unfolded or orphan proteins, to alleviate cells from stress. Taken together, the localization in secretory and endocytic degradative pathways, the ability to export unassembled substrate and the potential hydrolytic activity induce us to hypothesize that SEL1L-B and -C could contribute to protect cells against UPR-inducing stresses by disposing aberrant proteins through hydrolitic/autophagic/secretory pathways. Indeed, autophagy, a pathway modulated by the UPR, has been implicated in the clearance of protein aggregates and ubiquitinated proteins (24, 25). The spatial sequestration of SEL1L isoforms and their associated substrates might also facilitate cargo clearance through the autophagic pathway or dilution by asymmetric retention in the dividing cell. This could have important therapeutic implications in a range of diseases. Further studies are needed to shed light on possible enzymatic activities of the various SEL1L isoforms.

In addition to SEL1L-A, another small endogenous protein that shares several features with the exogenous SEL1L isoforms described here, including molecular size and high stability, was detected in most cells analyzed (data not shown). Interestingly, secretion of this putatively new isoform is strongly enhanced by ER stress. Alterations of ER protein expression at several levels, such as post-translational modifications or abnormal secretion, has been correlated with pathological conditions, particularly cancer. Fragments of ER-resident proteins, such as GRP78/Bip, protein disulfide isomerase, GRP94, and calreticulin, are secreted by cancer cells and are found in the serum of cancer patients (26, 27). Further studies are necessary to understand the physiological function of SEL1L-B and -C before attempting to dissect out their role in pathological phenotypes and their possible relevance as disease markers. Nonetheless the presently reported data point our attention to the existence of novel parallel quality control systems acting in cells to manage UPR-inducing stresses.

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