Selective Targeting of Proteins within Secretory Pathway for Endoplasmic Reticulum-associated Degradation

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Background: The endoplasmic reticulum-associated degradation (ERAD) is a cellular mechanism to eliminate misfolded proteins. The endoplasmic reticulum is an integral part of the secretory pathway where proteins are folded and transported. ERAD uses different pathways to selectively target and degrade misfolded proteins.

Results: Fusion of a target-binding domain to a fragment of the ERAD-associated protein SEL1L induces specific degradation of secretory and membrane-bound target proteins.

Conclusion: The new recombinant proteins (degradins) efficiently induce degradation of targets within the secretory pathway.

Significance: ERAD can be redirected to induce degradation of defined protein targets.

Proteins synthesized within the secretory pathway initiate their folding while in the lumen of the ER, assisted by ER-resident molecular chaperones. Protein folding is a complex process, and many molecules often do not reach their native conformation required for their proper localization and function. The ER quality control mechanism monitors the final conformational state and, when detected, terminally unfolded or misfolded proteins are selected for ER-associated degradation (ERAD). During ERAD, protein substrates are retrotranslocated to the cytosol (also known as dislocation) for proteasomal degradation, after ubiquitylation by different E3 ubiquitin ligases located on the cytosolic side. Different ER-resident proteins have been described to be involved in retrotranslocation, such as Derlin-1 and Sec61.

In yeast, three different pathways of ERAD have been described with the participation of distinct E3 ligases, depending on the location of the misfolded lesion: cytosolic, luminal, or membrane-spanning domains. An important protein complex in yeast involved in ERAD of luminal proteins is the one formed by the E3 ubiquitin ligase Hrd1p and the ER-resident glycoprotein Hrd3p. The mammalian counterpart of the yeast Hrd1p-Hrd3p complex is the one formed by the E3 ubiquitin ligase HRD1 that associates to the ER-resident protein SEL1L.

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C-terminal portion, including the second luminal domain was described to be involved mainly in retrotranslocation and in the binding to HRD1 (15). SEL1L also interacts with Derlin-1 and Derlin-2 and with the cytosolic ATPase p97, which has been shown to participate during ERAD-extracting proteins from the ER membrane (16). Several interactions have been described for the HRD1-SEL1L complex, including association with the E2 ubiquitin-conjugating enzymes UBC6 and UBC7 (17) and the ER-resident lectins OS-9 and XTP3-B, which also are responsible of targeting ERAD substrates (15, 18). These lectins seem to be involved in coordinating substrate recognition in the ER lumen with ubiquitin conjugation in the cytosol. The C-terminal portion of SEL1L (373-end) was shown to play an essential role in the interaction with HRD1, OS9, XTP3-B, p97 ATPase, and Derlin-1 (15). The luminal N-terminal portion of SEL1L, characterized by the presence of a fibronectin domain (14), is supposed to be important for the interaction with chaperones or for the direct interaction with misfolded proteins, by analogy with Hrd3p (10).

Here, we describe the construction and in vivo activities of fusion proteins, termed degradins, composed of a fragment of SEL1L fused to a target-specific binding moiety constituted by specific ligands or derived from mAbs. Degradins are able to recognize target proteins within the ER and to induce their degradation following retrotranslocation to the cytosol, resulting in an effective technique to induce protein knock-out in cells with high specificity.

**EXPERIMENTAL PROCEDURES**

**Construct Preparation**—Total RNA was extracted from the human cell line HEK293 using the RNeasy mini kit (Qiagen). After cDNA synthesis, a fragment of 1138 bp, containing codons 423–794 of human SEL1L (corresponding to positions 402–773 of the mature protein) was amplified by PCR using primers SEL1L-NheI (AGTAGCTAGCGGAAGTGACATTGGCAGC) and SEL1L-EcoRI (TCAGAATTCTTACTGTTGGGCTGGCTGCTCT) and KOD high-fidelity DNA polymerase (Novagen). The amplified fragment was inserted into a pcDNA3 vector (Invitrogen) expressing the anti-Fc domain (tgg gctcag). The control constructs containing irrelevant scFvs were obtained by substituting the HindIII/BspEI cassette encoding the 9E1 scFv with the corresponding cassettes encoding different scFvs. The cassette encoding the anti-FceRI degradin scFv9E1-SV5-SEL1L was in addition subcloned into the pcDNA3-Hygro vector (Invitrogen) for the generation of double-transfected stable clones. The ligand-degradin expressing vector has been obtained from the previously described plasmid pCDNA3-SEC-SV5-CH3/CH4 (22). From this vector, a fragment encoding a secretion signal (SEC), the SV5 tag and human IgE domains CH3-CH4 was transferred to the plasmid pCDNA3-scFv9E1-SV5-SEL1L, yielding the construct pcDNA-SEC-SV5-CH3/CH4-SEL1L (see Fig. 1a). A truncated form of this construct was generated, lacking the sequence encoding the CH3 domain, which is the domain responsible for interaction with FcεRI (23), to be used as a non-binding degradin control (Fig. 1c).

**Cell Culture and Transfection**—HEK293 cells were grown in DMEM supplemented with 10% FCS. Transient transfections were performed in six-well plates (∼5×10⁴ cells/well) by standard calcium phosphate technique (24) using 2.5 μg of each plasmid. Serum-free medium was added 18 h after transfection and, when required, the proteasome inhibitor MG132 (Sigma) was added at a concentration of 20 μM. Treatment was carried on for 6 h. As a negative control, the same amount of MG132 solvent, dimethyl sulfoxide, was added for the same time. In retrotranslocation experiments by gel retardation assay (25), 18 h after transfection, medium was replaced by serum-free medium supplemented with 0.1 mM biotin and further incubated for at least 8 h. When required, the proteasome inhibitor MG132 was added after 4-h incubation with biotin. Stable transfection with pcDNA3-mdo was achieved by adding 400 μg/ml G-418 (Invitrogen) 24 h after transfection, HEK293-mdo were afterward transfected with pCDNA3-SEC-9E1-SV5-SEL1L-Hygro and clones selected with 400 μg/ml hygromycine (Invitrogen).

**Cell Extract Preparation and Western Blotting**—HEK293-transfected cells were lysed with 100 μl of TNN lysis buffer (100 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitors mixture (Sigma). 10 μl of cell extract or 20 μl of the corresponding supernatants were separated on 10% SDS-PAGE and transferred to PVDF membrane for immunodetection with anti-SV5 (Invitrogen) or 9E1 antibodies. In retrotranslocation experiments, involving gel retardation analysis of cytosolically biotinylated proteins, cells were lysed with 100 μl of reducing SDS buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 175 mM β-mercaptoethanol), to avoid unspecific activity of the cytosolic biotin ligase BirA and subsequently sonicated to disrupt DNA. Denatured samples were then incubated with 1 μg of streptavidin (Sigma) for 30 min before separation on SDS-PAGE. To normalize the amount of extract loaded to the gels, blots were in addition developed with anti-actin antibodies (Sigma).

**Pulse-Chase [³⁵S]Methionine Labeling and Immunoprecipitation**—HEK293 cells were transfected by calcium phosphate technique in six-well plates. 12 h after transfection, medium was discarded, and cells were incubated for 30 min in DMEM without l-methionine and l-cysteine, supplemented with dialyzed FCS, to deplete the intracellular pool of sulfur-containing amino acids. Cells were then pulse-labeled for 15 min with 200 μCi/ml of a mix of [³⁵S]methionine and [³⁵S]cysteine (PerkinElmer Life Sciences Expre³⁵S) in the same methi-
online and cysteine-free medium. After pulse, radioactive aminoaicids were chased by substituting medium with fresh, complete DMEM. At different times, cells were collected and lysed in TNN buffer with protease inhibitors; degradins and targets were then immunoprecipitated for 2 h at 4 °C with anti-SV5 antibody and protein A-agarose. Immunoprecipitated proteins were eluted from agarose beads with reducing SDS buffer, treated with peptide N-glycosidase F (New England Biolabs) to have all the different glycosylation forms migrate as a single band, therefore allowing a better quantification, and separated on 10% SDS-PAGE; gels were afterward fixed in 10% acetic acid, 10% methanol, incubated for 20 min in an Amplify fluorographic enhancer (GE Healthcare), and dried and exposed to autoradiography on films Kodak BioMax XAR. Bands on autoradiographic films were quantified with ImageJ software (version 1.45s, National Institutes of Health).

**Induction of Degradin Expression with Tetracycline**—The cassettes encoding the anti-FcRI/89280RI degradins scFv9E1-SV5-SEL1L (scFv-degradin) and SV5-CH3/CH4-SEL1L (ligand-degradin) were subcloned in the vector pcDNA5-FRT-TO (Invitrogen) under the control of a tetracycline-inducible CMV promoter. The resulting plasmids, together with that expressing the mdα/89251(pcDNA3-mdα), were used to transiently co-transfect Flp-In T-REx 293 cells (Invitrogen), stably expressing the tetracycline repressor, cultured in DMEM containing 4.5 g/liter glucose, 10% FCS, 100 μg/ml Zeocin (InvivoGen), and 15 μg/ml blasticidin (InvivoGen). The day after transfection, degradin expression was induced by treatment with 1 μg/ml of tetracycline for 24 h. Cell extracts were prepared at the time of induction (T₀) or at 24 h post-induction, by lysis in TNN and analyzed in Western blot for mdα and degradin expression.

**FACS Analysis**—HEK293 clones either transfected only with mdα or double-transfected with mdα and scFv-degradin were analyzed by FACS: ~10⁶ cells were incubated with the anti-FcRI mAb 9E1 and fluorescein-conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories) and analyzed in a FACSDiabur (Becton Dickinson) with CellQuest software.

**RESULTS**

**Design and Construction of Degradins**—We designed and constructed a genetic version of the fusion molecule that we called degradin (dg), consisting of an N-terminal target-specific binding moiety, such as a scFv from a monoclonal antibody or a ligand of the target, and a C-terminal part derived from the last 372-amino acid-long region of SEL1L. The SEL1L moiety lacks the first N-terminal 401 amino acids, preserving the portion interacting with ERAD active proteins HRD1, OS9, XTP3-B, p97 ATPase, and Derlin-1 (15). Instead, we fused a scFv fragment (scFv-degradin, scFv-dg) or a ligand of the target (ligand-degradin, L-dg) that would be exposed to the luminal side of the ER. We reasoned that a target molecule within the ER engaged by the recognition-specific moiety of degradin would be retrotranslocated to the cytosol and degraded by the activity of the SEL1L moiety and the associated ERAD complex.

A schematic diagram of degradin constructs is shown in Fig. 1a. To facilitate detection, degradins were tagged with the 11-amino acid-long SV5 tag between the recognition and SEL1L moieties or at the N terminus (Fig. 1a).

Degradin activity was tested on the extracellular domains of the α chain of the human high affinity receptor for IgE (FcRIα), as a model target for degradation, in both a secretory and membrane-bound format. The two versions of the FcRIα target contain the two extracellular D1 and D2 domains, which are sufficient to bind human IgE with high affinity, fused to (i) the
γCH3 dimerizing domain of IgG1 H-chain in its secretory form to yield the secretory dimeric α chain construct (sΔα) (20, 26) or (ii) the γCH3 in its membrane-bound form to yield the membrane dimeric α chain construct (mΔα) (Fig. 1b). Two different versions of degradins specific for FcεRIα were then constructed, which contained as recognition-specific moieties either the scFv derived from the highly specific anti-FcεRIα monoclonal antibody 9E1 (scFv-dg) (20) or a truncated version of Fcε (domains eCH3-eCH4 of human IgE heavy chain), which retains full ligand binding activity to FcεRIα (L-dg) (Fig. 1b). Other constructs used are shown in Fig. 1c.

Degradins Induce Target Degradation—The activity of the scFv-dg was first assayed in HEK293 cells by co-transfection with sΔα (secretory version of FcεRIα). Western blot analysis of culture supernatants showed that the scFv-dg had a strong effect on sΔα secretion, whereas the control construct, a degradin with an irrelevant scFv (irr-dg), did not (Fig. 2). Analysis of cellular extracts showed that the target protein was not retained intracelularly as well. In contrast, the scFv fused to the ER retention sequence KDEL (scFv-KDEL) used as a control, which blocked secretion only partially, produced instead large accumulation of the protein within the cell (Fig. 2a). The levels of the specific and irrelevant degradins were similar, whereas the one of the scFv-KDEL was even higher. This molecule was in part also secreted (Fig. 2a).

As for the secretory form, expression of the membrane-bound mΔα was also heavily impaired by scFv-dg and intracelularly retained by scFv-KDEL (Fig. 2b). Similarly, the L-dg, which contains the ligand Fcε fragment as target recognition moiety, showed a strong effect on both sΔα and mΔα (Fig. 2, c and d).

For both sΔα and mΔα, the small amount of material retained intracelularly by the degradins, as well as the material retained by scFv-KDEL, corresponded to molecules in the ER, as indicated by their PAGE migration and confirmed by sensitivity to endoglycosidase H (Fig. 2, e and f). The retention of the intracellular secretory and membrane-bound targets within the ER is consistent with the fact that degradins are themselves ER-resident proteins, as expected because of the ER retention signal present in SEL1L. In fact, neither the irr-dg nor the scFv-dg or the L-dg were transported to the cell surface, as shown by cytotoxicity analysis (Fig. 3a) and were totally sensitive to endoglycosidase H (Fig. 3b).

The ability of degradins to degrade newly made target protein was further demonstrated by (i) [35S]methionine pulse-chase labeling of cells expressing mΔα and L-dg and (ii) tetracycline-inducible expression of the degradins in cells expressing the mΔα target. As shown in Fig. 4a, the amount of target recovered after a 90-min chase following the 15-min pulse was reduced to <30% in the presence of degra-
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din, whereas it was practically unchanged in the control. Similarly, mdα levels were compromised strongly following tetracycline-induced transcription of either of the two specific degradins (Fig. 4b).

The specificity of degradins for the target was next tested, co-expressing the scFv-dg and L-dg with a control secretory protein (scFvcontr) not recognized by either of them. As expected, both degradins did not affect expression of the irrelevant protein (Fig. 4c).

Degradins were able to constitutively knock-down the target protein. The scFv-dg stably transfected into a HEK293 transfectant already expressing mdα, as shown by a representative clone (G6), resulted in very reduced levels of total and surface displayed mdα (Fig. 5). The G6 clone was maintained in culture for long periods without changes in the mdα expression profile, demonstrating that degradins can induce strong and sustained target degradation.

Degradin Activity Depends on SEL1L Moiety—The role of the SEL1L moiety on the activity of degradins was assessed with constructs deleted of the complete luminal domain of SEL1L (up to amino acid 716 of the mature protein) (scFv-ΔS, L-ΔS, Fig. 1c), still expressed in the ER. As shown in Fig. 6a, co-expression with scFv-ΔS resulted in a reduced secretion of sdα, yet with a significant increase in the protein retained intracellularly, comparable with the levels obtained with scFv-KDEL. Analogous results were observed for L-ΔS, which also produced strong intracellular retention (Fig. 6b). We tested, in addition, a further relevant control for the L-dg in which the CH3 domain (Δ3) of the Fc ligand recognition moiety, absolutely essential for binding to FcεRIα, was deleted (L-Δ3, Fig. 1c). As expected, this construct behaved as an irrelevant degradin (Fig. 6b).

Similarly to sdα, degradins with SEL1L deletion (scFv-ΔS and L-ΔS) showed mdα impaired degradation and increased ER retention, whereas no effect was observed for L-Δ3 (Fig. 6c).
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FIGURE 6. Degradin activity depends on SEL1L moiety. Western blot analysis of cell culture supernatants (supn.) and cellular extracts (cell extr.) from HEK293 cells co-transfected with vectors expressing the target proteins sΔx (a and b) and mΔx (c) and the indicated constructs. Blots were developed with anti-FcεRI for sΔx and mΔx, anti-SV5 for the degradins and anti-actin.

FIGURE 7. Degradins induce ER to cytosol retrotranslocation. Western blot of cellular extracts from HEK293 cells co-transfected with vectors expressing the target protein mΔx and the indicated degradins (a and b), or from stable transfecants HEK-mΔx and clone G6 (c) in the presence or absence of the proteasome inhibitor MG132, as indicated. Blots were developed with anti-FcεRI. d and e, Western blot retardation assay performed on extracts derived from cells co-transfected with the BAP-containing targets, sv-BAP (d) and BAP-mΔx (e), the indicated degradins and cytosolic BirA, treated or not with MG132. Samples were run in the presence or absence of streptavidin (StrAv), as indicated. Biotinylated (retrotranslocated) molecules have a retarded PAGE mobility, indicated by arrows. Blots were developed with anti-SV5. Degradins used did not contain the SV5 tag, which was instead present in the targets.

Taken together, these data demonstrate a crucial role of SEL1L moiety for a proper activity of degradins.

Degradins Induce ER-to-cytosol Retrotranslocation—The requirement of the SEL1L luminal domain indicated that degradins perform their activity involving ER-to-cytosol retrotranslocation followed by proteasomal degradation. As shown in Fig. 7, following proteasome inhibition with MG132, an increased amount of intracellular sΔx (Fig. 7a) and mΔx (Fig. 7b) was found in cells co-expressing either of the two degradins (scFv-dg or L-dg). The MG132 effect was also clear in the stable double transfecant G6 clone (Fig. 7c).

These results are consistent with the known ERAD mechanism that involves a retrotranslocation step of the target. We therefore applied a recently developed assay to detect retrotranslocated proteins (25). The assay was based on the specific in vivo monobiotinylation of molecules (tagged with the biotin acceptor peptide BAP in an ER luminal position) retrotranslocated to the cytosol, in cells expressing the cytosolic Escherichia coli biotin ligase BirA. Only molecules retrotranslocated from the ER lumen to the cytosol become substrate of the BirA enzyme and biotinylated and can then be discriminated easily from the non-biotinylated ones by differential migration in PAGE after incubation with streptavidin and detection by Western blotting (retardation assay). Experiments were performed with a C terminus BAP-tagged version of sΔx (sΔ-BAP) and an N terminus BAP-tagged version of mΔx (BAP-mΔx) (Fig. 1d) in the presence and absence of MG132. In cells expressing the two specific degradins, a significant amount of sΔ-BAP was found biotinylated, as reflected by the retarded band observed in the presence of streptavidin (and therefore retrotranslocated) (Fig. 7d). Upon proteasome inhibition, essentially all of the intracellular sΔ-BAP was biotinylated. The biotinylated material consisted not only of deglycosylated molecules because of the cytosolic cellular peptide N-glycosidase, but also of glycosylated ones, as we have reported previously for different proteins that undergo retrotranslocation (25). Similar results were obtained for BAP-mΔx both in the absence and presence of MG132 (Fig. 7e). Taken together, these results demonstrate that the mechanism of action of degradins entails ER-to-cytosol retrotranslocation and proteasomal degradation.

Degradins Decay with Target—To further investigate the mechanism by which degradins induce retrotranslocation of the target, we performed [35S]methionine pulse-chase labeling experiments followed by immunoprecipitation and PAGE analysis.

Cells were co-transfected with the L-dg and either the specific mΔx target or an irrelevant mock target membrane protein (α chain of MHC class-I, MHC-Iα), pulse-labeled with [35S]methionine for 15 min, and then chased for 20, 40, or 60 min before lysis. Cell lysates were used to immunoprecipitate the L-dg, mΔx, and the mock target and analyzed by SDS-PAGE. As shown in Fig. 8, degradin was essentially stable during the chase period in the presence of the mock target, whereas it was reduced strongly when co-expressed with the specific one (mΔx) (Fig. 8a). Similarly, immunoprecipitations of the targets showed stability of the mock target and reduced levels of mΔx (Fig. 8b). These results indicate that the two components of the complex target/dg are retrotranslocated and degraded together. Indeed, as shown in the WB retardation assay of Fig. 8c, most of the BAP-tagged L-dg was found retrotranslocated when co-expressed with mΔx (becoming even more evident...
FIGURE 8. Degradins decay with the target. a, [35S]methionine pulse-chase labeling of cells co-transfected with L-dg and the irrelevant (MHC-I\(\alpha\)) or specific (md\(\alpha\)) targets. L-dg (arrow) was immunoprecipitated at different chase time points, as indicated. The band intensity was determined relative to the 0
min time point. b, a similar experiment as described in a showing immunoprecipitates of mock (MHC-I\(\alpha\), open arrow) or specific (md\(\alpha\), solid arrow) targets. Immunoprecipitates were resolved by PAGE and revealed by autoradiography. c, Western blot retardation assay on extracts derived from cells co-transfected with the BAP-tagged L-dg, cytosolic BirA, and mock (MHC-I\(\alpha\)) or specific (md\(\alpha\)) targets, treated or not treated with MG132. Samples were run in the presence or absence of streptavidin (StrAv), as indicated. Arrowhead indicates biotinylated (retrotranslocated) molecules. Blot was developed with anti-SV5 and anti-actin.

upon proteasome inhibition with MG132), whereas a reduced amount was observed with the mock target MHC-I\(\alpha\).

Despite the apparent extended half-life of the degradin in the absence of the specific target, its expression did not increase the ER stress response. An increase in the level of spliced XBP1 mRNA, a marker of ER stress (27), was observed upon transfection with an empty plasmid vector; however, transfection with plasmids encoding the degradin or the target alone, or the degradin with either the relevant or the irrelevant target did not show substantial differences between them, indicating that unfolded protein response (UPR) activation was the consequence of transfection and overexpression of proteins in general and not of degradins in particular (supplemental Fig. S1). A very low level of UPR activation also was observed in the two stably transfected cell lines: cells expressing the target alone (HEK-md\(\alpha\)) showed a level of spliced XBP1 comparable with the cell line expressing both target and degradin (clone G6). This lower UPR activation in stable degradin-expressing cells supports the possibility of utilizing our knock-out system constitutively. Furthermore, expression of degradins in transiently or stably transfected cells did not alter the level of other ER proteins, such as calnexin, calreticulin, BAP31, and Derlin-1, and did not alter levels of the cytosolic ERAD-involved ATPase VCP/p97 (supplemental Fig. S2). Therefore, it appears that degradins do not specifically activate the ERAD pathway.

**DISCUSSION**

Here, we report the successful development of a novel procedure to achieve selective degradation of a defined target protein within the secretory pathway. The system is based on the design of a novel fusion protein, termed degradin, consisting of two well defined components: the target-specific recognition moiety and the degradation-inducing moiety.

Our results show that both components are essential to provide, respectively, specificity for the target and induced degradation. Indeed, degradins specifically induce degradation only of recognized proteins: irrelevant degradins (i.e. with recognition moieties unable to interact with the targets) were inactive on the s\(\alpha\)d\(\alpha\) and md\(\alpha\) targets. Similarly, the specific degradins were inactive on the irrelevant targets. In addition, when only the target-specific recognition moiety was maintained, using the KDEl ER retention signal, the target was retained largely within the ER. This characteristic was further demonstrated with constructs with the SEL1L luminal domain deleted, which also showed compromised degradation activity and increased ER retention.

The choice of SEL1L as the degradation moiety was based on the key role of SEL1L in ERAD, linking substrate recognition within the ER lumen and the downstream retrotranslocation and proteasome degradation steps (12). SEL1L was shown to be involved in substrate recruitment to the ubiquitin ligase Hrd1 and the putative retrotranslocation channel protein Derlin-1 (15). We selected the C-terminal portion of SEL1L (from amino acid 402 of the mature protein, to the end), which shares similarities with the yeast (Hrd3p) and Caenorhabditis elegans (SEL1) homologues, shown to interact with the ERAD machinery (10). A fine mapping of the minimal SEL1L moiety in degradins remains to be determined. However, the proline-rich tail and the Hrd3p-like motifs, with essential roles during ERAD, also showed compromised degradation activity and increased ER retention.

Degradins were validated using the FcERI-\(\alpha\) chain model, which gave us the opportunity to test both a soluble secretory and a membrane-bound version of the same target. We also took advantage of two different target-specific recognition moieties available: an scFv derived from the anti-Fc \(\alpha\) chain model, which gave us the opportunity to test both a soluble secretory and a membrane-bound version of the same target. We also took advantage of two different target-specific recognition moieties available: an scFv derived from the anti-\(\alpha\) chain model, RI-
\(\alpha\) mAb 9E1 (20) and the ligand (the eCH3-eCH4 fragment of IgE). The two degradin versions worked very efficiently on both the secretory and the membrane-bound target proteins.

Our results provide clear indication that degradins operating mechanism involves several key steps associated to ERAD. First, localization of the degradins within the ER, due to ER localization signals in the transmembrane and cytosolic portions (residues 717–773 of the mature protein) of SEL1L. Degradins are ER-resident proteins, independent of target presence. Indeed, most of the material found intracellularly in cells expressing the target-specific degradin corresponded to molecules that have not trafficked to the Golgi. Second, as expected for a target forced to enter the ERAD pathway, degra-
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dins induced retrotranslocation of the target to the cytosol, as shown using our novel method of retrotranslocation detection (25). Third, as a consequence, the target was degraded by the proteasome, as shown by the significant increase of the intracellular levels observed upon proteasome inhibition. We also showed that the retrotranslocation step concerns the degradins themselves, which are then degraded. This indicates that the level of expression of a degradin needs to be similar to the target to be effective. Despite this, stable expression of a degradin was able to significantly block the constitutive expression of the target.

In contrast to degradins, the N-terminal part of SEL1L contains a PEST motif (rich in proline, glutamate, serine, and threonine) that facilitates rapid degradation (14). The half-life of endogenous SEL1L has been reported to be ~3 h (12), whereas transiently overexpressed SEL1L displays a half-life of ~1 h (28). Instead, probably due to the lack of the N-terminal region, the half-life of the degradin in the absence of the target appeared to be significantly longer than in the presence of it. Yet, this did not imply an increase in the ER stress response.

Degradins thus represent a novel tool for the induction of specific degradation of target proteins with some interesting features and several possible applications: (i) degradins are very flexible in the design offering the possibility to use as recognition units, scFvs, or other recombinant binders derived from mAbs or selected from different type of libraries, in addition to the use of known ligands or interaction partners, including peptides; (ii) the recognition moiety also can be more complex to include more than one specificity for multiple complex targets, or alternatively different degradins can be co-expressed, each with a different specificity; (iii) depending on the availability of a specific binder, degradins could be useful in targeting protein isoforms with differences in their folding or containing alternative sequences, for instance as a consequence of alternative splicing; (iv) degradins could also be of relevance to degrade disease causing proteins such as the amyloid precursor protein in Alzheimer disease avoiding its appearance on the cell surface (29); the prion protein PrP, which needs to be transported to the cell surface to be converted to the PrP^sc aberrant conformation (30) in different transmissible spongiform encephalopathies like Bovine Spongiform Encephalopathy, Creutzfeld-Jacob disease, and Scrapie (31); or the mutated huntingtin protein in Huntington disease, by reducing the formation of aggregates (32); (v) degradins could be used to target exogenous proteins entering the secretory pathway from pathogens able to persist within the cell in intracellular vacuoles (33, 34) such as SlrP from Salmonella typhimurium, major outer membrane protein (MOMP), or IncA from Chlamidia trachomatis, that traffic through the ER and induce ER stress (35, 36); (vi) degradins could selectively redirect antigens to proteasome degradation in antigen presenting cells, to increase presentation of antigen-derived peptides for activation of antigen-specific cytotoxic CD8^+ T-lymphocytes.

Other protein knock-out systems that target proteins to the proteasome have been described previously. In one case, a chemokine was fused to the cytosolic fragment of the HIV protein Vpu. In this way, degradation of the chemokine receptor through a dubious mechanism was reported (37).

To our knowledge, our method is the only one described so far that specifically takes advantage of the ERAD pathway through the involvement of SEL1L, to specifically redirect proteins within the ER to degradation. In contrast to ERAD, however, the target protein does not need to be misfolded because practically all target molecules that enter the ER are recognized, retrotranslocated to the cytosol, and degraded.

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