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The Prodomain of a Secreted Hydrophobic Mini-protein Facilitates Its Export from the Endoplasmic Reticulum by Hitchhiking on Sorting Receptors*

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Silvestro G. Conticello‡, Noga D. Kowalsman‡, Christian Jacobsen§, Guennady Yudkovsky¶, Kazuki Sato‖, Zvulun Elazar‡, Claus Munck Petersen§, Ami Aronheim¶, and Mike Fainzilber‡**

From the ‡Department of Biological Chemistry, Weizmann Institute of Science, 76100 Rehovot, Israel, the §Department of Medical Biochemistry, University of Aarhus, DK-8000, Denmark, the ¶Department of Molecular Genetics, Rappaport Faculty of Medicine, Technion, 31096 Haifa, Israel, and the ‖Fukuoka Women's University, Kasumigaoka, Higashi-ku, Fukuoka 813-8529, Japan

Misfolded secretory proteins are retained in the endoplasmic reticulum (ER) by quality control mechanisms targeted to exposed hydrophobic surfaces. Paradoxically, certain conotoxins expose extensive hydrophobic surfaces upon folding to their bioactive structures. How then can such secreted mini-proteins traverse the secretory pathway? Here we show that secretion of the hydrophobic conotoxin-TxVI is strongly dependent on its propeptide domain, which enhances TxVI export from the ER. The propeptide domain interacts with sorting receptors from the sortilin Vps10p domain family. The sortilin-TxVI interaction occurs in the ER, and sortilin facilitates export of TxVI from the ER to the Golgi. Thus, the prodomain in a secreted hydrophobic protein acts as a tag that can facilitate its ER export by a hitchhiking mechanism.

Biosynthesis and folding of secreted proteins are regulated by quality control mechanisms targeted to exposed hydrophobic surfaces or free thiols on misfolded proteins, thus retaining them in the ER[1] (1, 2). Misfolded proteins that escape ER quality control can also be directed to vacuolar degradation from the trans-Golgi network (TGN) or returned via retrograde transport to the ER (3). Paradoxically, certain conotoxins expose extensive hydrophobic surfaces upon folding to their stable bioactive structures (Fig. 1*A* and Refs. 4 and 5), suggesting the existence of mechanisms for efficient secretion of hydrophobic mature domains. Conotoxin precursors consist of a signal peptide, a propeptide domain, and the mature toxin domain. An early suggestion that the propeptide domain might act as an intramolecular chaperone (6) was not supported by *in vitro* folding experiments on a hydrophilic conopeptide (7). Nonetheless, the high degree of propeptide conservation in hydrophobic conotoxins (Fig. 1*B*) indicated that this domain might be required *in vivo* for efficient secretion. We used the extremely hydrophobic conotoxin-TxVI (8) to examine this question. Here we show that secretion of TxVI is strongly dependent on its propeptide domain, which enhances TxVI export from the endoplasmic reticulum (ER) by hitchhiking on sorting receptors from the sortilin Vps10p domain family.

EXPERIMENTAL PROCEDURES

Plasmids—An FL-Fc construct comprised of the open reading frame of TxVI (GenBank™ accession number AF193261) fused at its C-terminal to the Fc domain of human IgG was cloned into pCDNA3. A propeptide deletion (ΔP) was prepared by ligating oligonucleotides encoding the signal peptide to a PCR product encoding the mature toxin domain fused with the Fc. A GFP fusion (FL-GFP) was constructed by excision of the Fc domain followed by ligation of EGFP (Clontech, Palo Alto, CA). A GFP construct targeted to the ER (Sig-GFP) was made by replacing the TxVI sequence of FL-GFP with a signal sequence derived from the p75 neurotrophin receptor.

Cell Culture, Transfection, and Pulse Chase—COS7 cell lines were maintained in DMEM with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel), and transfected with FuGENE-II (Roche Molecular Biochemicals). Pulse-chase experiments were carried out 24–48 h post-transfection. Cells were transferred to serum-free DMEM deficient in cysteine and methionine (Sigma), and pulsed in medium supplemented with [^{35}S]Cys-Met (0.1 mCi/ml) (Amersham Biosciences). Cells were chased in full medium. Cell lysates were in 10 mM Tris, pH 8, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate and proteinase inhibitors (Merck).

Gel Analysis and Quantitation—^{35}S-Labeled proteins were separated on 10 or 12% SDS-polyacrylamide gels and exposed on a BAS-2500 (Fuji) phosphorimager screen. Results were analyzed and quantified using Image Gauge 3.41 software (Fuji).

Protein Interaction Screens—The RRS system selects for protein interactions that rescue a yeast strain in which the endogenous Ras signaling pathway is repressed at 36 °C. Yeast strains, plasmids, and screening protocols for RRS were as described previously (9). Baits were prepared by cloning the propeptide domains of TxVI, PnIB, or BDNF in pADH-Ras, and proTxVI-ADH-Ras was used to screen a rat pituitary cell line cDNA library.

Enzymatic, Antibody-based, or Protein Interaction Assays— endoglycosidase-H (Endo-H) (Roche Molecular Biochemicals) digests were carried out as described previously (10). Fc constructs were purified over protein A-Agarose (Roche Molecular Biochemicals) according to the manufacturer's instructions. A rabbit anti-sortilin antibody (11) was used at 5 mg/ml for precipitation and at 1:1500 dilution for Western blotting. Surface plasmon resonance measurements were performed on BIAcore 2000 (Biacore) equipped with CM5 sensor chips maintained at 20 °C under a continuous flow of HEPES-buffered saline or MES buffers at the indicated pH (12).

RESULTS AND DISCUSSION

Full-length and deletion constructs of TxVI were tagged C-terminally with the Fc domain of IgG. This relatively large tag was deliberately chosen to avoid the possibility of degradation

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** Incumbent of the Daniel Koshland Sr. Career Development Chair at the Weizmann Institute of Science. To whom correspondence should be addressed. Tel.: 972-8-9344266; Fax: 972-8-9344112; E-mail: mike.fainzilber@weizmann.ac.il.

[1] The abbreviations used are: ER, endoplasmic reticulum; TGN, trans-Golgi network; DMEM, Dulbecco's modified Eagle's medium; Endo-H, endoglycosidase-H; RRS, Ras rescue system; GFP, green fluorescent protein; MES, 4-morpholineethanesulfonic acid.

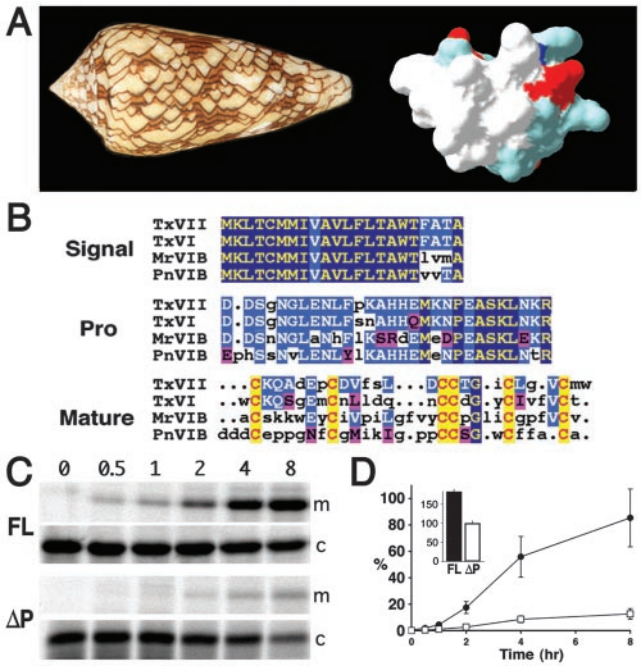

FIG. 1. **Enhancement of TxVI secretion by its propeptide.** *A*: *left*, *Conus textile*, a cone snail with a remarkably high proportion of hydrophobic conopeptides in its venom duct; *Right*, space filling representation of the NMR structure of conotoxin-TxVI. Hydrophobic surfaces are indicated in *white*, acidic in *red*, and others in *blue*. *B*, sequence alignment by domains of hydrophobic conopeptides. *C*, pulse-chase analysis of Fc-tagged constructs of TxVI with and without the propeptide (*FL* and Δ*P*, respectively). Representative gels are shown for TxVI-Fc protein in the medium (*m*) and in the cells (*c*) at given times (hours (*hr*)) after a 30-min pulse with [^{35}S]Cys-Met in transiently transfected COS7 cells. *D*, plot of secretion kinetics for the different constructs, average ± S.D. from four independent experiments (*FL*, *solid circle*; Δ*P*, *hollow square*). Equivalent amounts of transfected cells were used. Secretion levels are expressed as percentage of the total *de novo* synthesized protein measured at the end of the pulse. Relative *de novo* synthesis levels for both constructs at the end of the pulse are shown in the *inset bar graph*, as percentage of the synthesized ΔP protein.

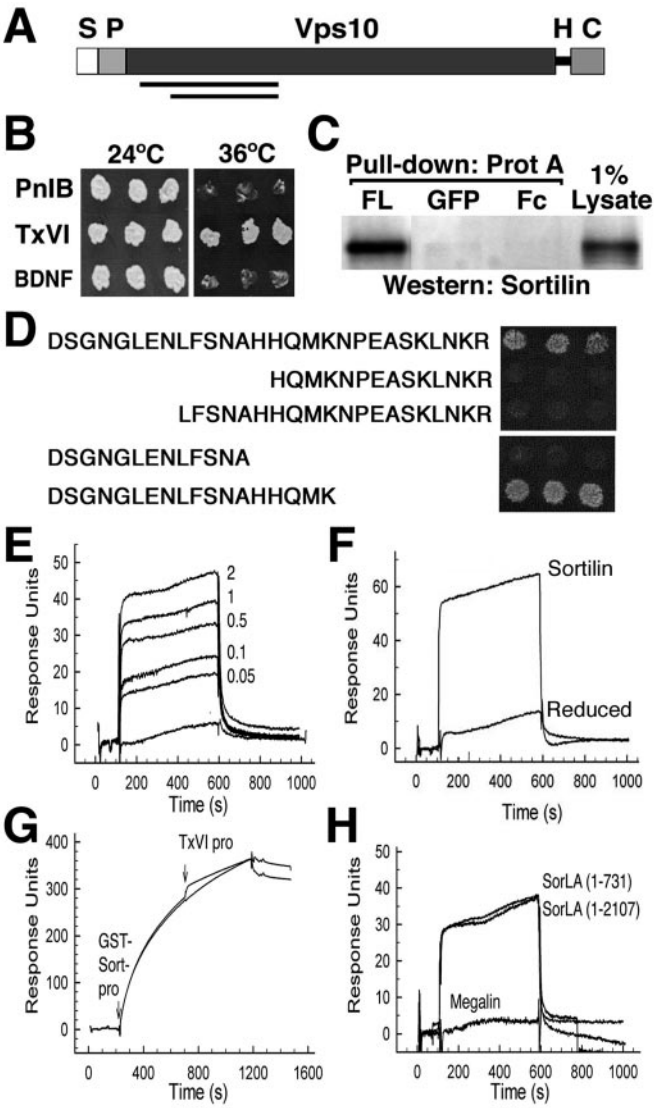

FIG. 2. **The propeptide domain of TxVI interacts with the Vps10p domain of sortilin family receptors.** *A*, schematic showing the position of the two cloned interactors on the full-length sortilin sequence (sortilin amino acid positions 91–353 and 123–353, respectively). *B*, RRS interaction between a full-length sortilin clone and the propeptides of TxVI, α-conotoxin-PnIB, or brain-derived neurotrophic factor (*BDNF*). Triplicate yeast colonies are shown, grown at permissive (24 °C) and non-permissive (36 °C) temperatures. The robust colonies observed at 36 °C for TxVI-transfected yeast reveal the interaction with sortilin. *C*, co-precipitation of sortilin with TxVI-Fc following co-transfection in COS cells; the controls are co-transfections with plasmids encoding GFP or Fc alone. The *rightmost lane* shows total sortilin in 1% of the amount of cell lysate used for the co-precipitation. *D*, interaction between sortilin and the indicated deletion constructs of the TxVI propeptide, as revealed by growth of RRS yeast colonies at 36 °C. *E*, concentration-dependent binding of the TxVI propeptide to sortilin immobilized on a Biacore chip; the *numbers* indicate TxVI propeptide concentration in micromolar. *F*, lack of binding of pro-TxVI to sortilin that was reduced before immobilization. *G*, binding of the TxVI propeptide to sortilin in the presence of excess sortilin propeptide, demonstrating that the propeptide of sortilin does not inhibit binding of the TxVI propeptide. *H*, TxVI propeptide binds to the Vps10p domain (residues 1–731) of sorLA with a response indistinguishable from that observed upon binding to full-length (residues 1–2107) sorLA. Megalin, a sorLA homolog that lacks the Vps10p domain, does not bind the TxVI propeptide.

of extremely short propeptide deletion products (13). Equivalent expression efficacy of the tagged proteins was verified by *in vitro* translation, followed by metabolic labeling and pulse-chase experiments in transfected COS cells. Labeled TxVI-Fc was easily detectable in the medium of cells transfected with the full-length construct and accumulated at a linear rate for 8 h (Fig. 1*C*), thereby establishing that the capacity to efficiently secrete domains with high surface hydrophobicity is not restricted to *Conus* venom ducts. The intracellular expression level of the proprotein deletion was approximately half that of the full-length protein, and it was secreted at a 6–7-fold slower rate than that observed for the full-length product (Fig. 1, *C* and *D*), resulting in an order of magnitude difference in the amount of secreted TxVI. In similar experiments carried out with the extracellular domain of the p75 neurotrophin receptor, which is composed of four hydrophilic cysteine-rich domains, fusion with the TxVI-pro sequence did not significantly enhance secretion of the construct from transfected cells (data not shown).

To clarify the role of the pro domain in enhancing TxVI secretion, we conducted an RRS screen for propeptide-interacting proteins and obtained five independent clones corresponding to two overlapping segments of the lumenal domain of sortilin (Fig. 2*A*). Sortilin is a member of the Vps10p domain family (11, 14), members of which are thought to be involved in TGN-endosome sorting (15, 16). The sortilin-pro-TxVI interaction is specific, as evidenced by a lack of interaction between sortilin and the propeptide domains of two small cysteine-rich hydrophilic proteins (Fig. 2*B*) and by coimmunoprecipitation of sortilin with pro-TxVI from transfected mammalian cells (Fig. 2*C*). Only 1% of the total cellular sortilin was co-precipitated

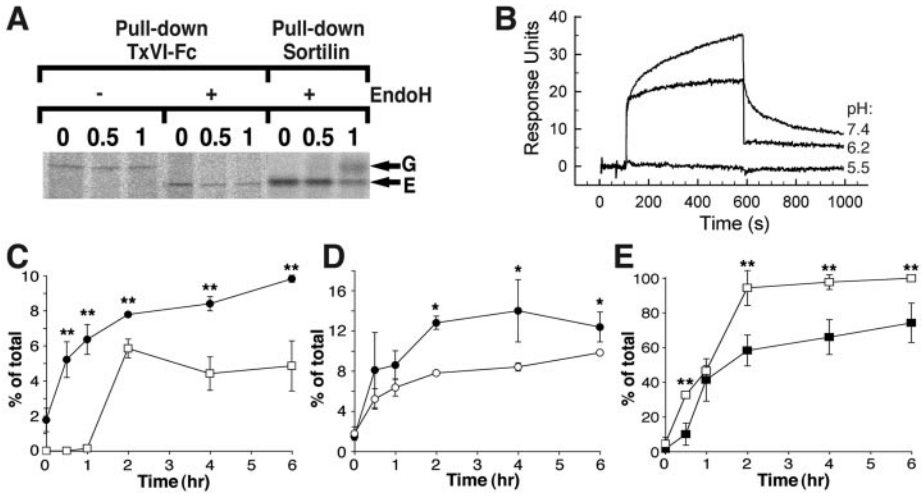

FIG. 3. **Sortilin interaction with the TxVI propeptide enhances ER export.** *A*, Endo-H digest of sortilin from [^{35}S]Cys-Met pulse-chased transfected COS7 cells (10-min pulse-chase times indicated in hours (*hr*)). The *left panels* show sortilin co-precipitated in pull-downs of TxVI-Fc, while the *right panel* shows direct immunopreciptation of sortilin. Since all the sortilin co-precipitated with TxVI is Endo-H-sensitive, the interaction occurs in the ER. *B*, Biacore analyses of TxVI propeptide binding to sortilin at three different pH values, approximating ER (pH 7.4), Golgi (pH 6.2), and secretory granules (pH 5.5). *C*, plot showing ER export of TxVI-Fc with and without the propeptide (FL, *solid circle*; ΔP, *hollow square*). Transfected cells were pulsed for 10 min with [^{35}S]Cys-Met and chased for the indicated times. *D*, ER export of TxVI-Fc in the presence (*solid circles*) or in the absence (*hollow circles*) of co-transfected sortilin. Values in *C* and *D* are expressed as percentage of Endo-H-resistant TxVI-Fc from the total and represent average ± S.D. from three independent experiments. *E*, ER export of sortilin in the presence of FL-TxVI-GFP (*solid squares*) or GFP with the signal peptide of TxVI (*hollow squares*). Data are shown as percentage of Endo-H-resistant sortilin from the total and represent average ± S.D. from three independent experiments. Statistical significance: * indicates $p < 0.05$; ** indicates $p < 0.01$.

with TxVI (Fig. 2*C*), suggesting that sortilin-TxVI interaction in the cell is spatially or temporally restricted. Testing of a number of propeptide deletion constructs localized the sortilin-binding domain to the N-terminal region of the propeptide (Fig. 2*D*). Further verification of the sortilin-pro-TxVI interaction was obtained by surface plasmon resonance, demonstrating concentration dependent binding with an apparent K_d of 1 μM and stoichiometry of 0.2 mol of peptide bound per mol of sortilin immobilized (Fig. 2*E*). Since less than half of the sortilin is likely to be immobilized on the chip in an orientation favorable for peptide binding, the latter value suggests binding of the propeptide to a single site on sortilin. Binding is lost upon reduction of sortilin (Fig. 2*F*). The endogenous sortilin propeptide is known to bind to the lumenal domain of mature sortilin, and its cleavage and dissociation activates certain functions of sortilin (12). We therefore tested for binding of TxVI propeptide in the presence of sortilin propeptide and observed binding of the TxVI propeptide superimposed onto the binding curve for sortilin propeptide, indicating that the binding sites for these two ligands are distinct (Fig. 2*G*). Finally, we tested the TxVI propeptide for binding to SorLA, another member of the Vps10p family (17). Both full-length SorLA and its isolated Vps10p domain bind TxVI propeptide, whereas megalin (an low density lipoprotein domain-containing receptor that lacks the Vps10p domain) does not (Fig. 2*H*).

Since sortilin is thought to be involved in TGN-endosome trafficking, how might it influence TxVI evasion of quality control mechanisms in the ER? One possibility would be an interaction between sortilin and TxVI commencing in the ER, followed by joint transport to the Golgi. Indeed localization of sortilin binding to the N-terminal region of the TxVI prodomain, and the ability of the TxVI prodomain to bind sortilin before cleavage of the sortilin propeptide, both support such a scenario. Co-precipitates of TxVI-Fc and sortilin from pulse-labeled transfected COS cells were therefore subjected to Endo-H digest to discriminate between ER *versus* Golgi forms of the proteins. The labeled sortilin band that co-precipitated with the TxVI-Fc was clearly Endo-H-sensitive (Fig. 3*A*), confirming that the interaction between the two proteins occurs in the ER. Biacore experiments (Fig. 3*B*) then revealed that the prodomain-sortilin interaction was pH-sensitive, with affinity decreasing from pH 7.4 (ER) to pH 6.2 (Golgi), with a complete loss of the interaction at pH 5.5 (secretory granules). Endo-H treatment of cell lysates from pulse-chase experiments showed a higher efficiency of ER export for full-length TxVI-Fc, with ~6% of labeled full-length TxVI in the Golgi after 1-h chase, compared with less than 1% for the prodeletion construct (Fig. 3*C*). We then proceeded to examine the rate of ER to Golgi trafficking of the TxVI-Fc protein with and without co-transfected sortilin. Co-transfection of sortilin enhanced ER export of TxVI, and the fraction of the protein in the Golgi at later time points in the chase was increased from 6 to 8% at steady state in control to 12% with additional sortilin (Fig. 3*D*). In complementary experiments sortilin was co-transfected with TxVI-GFP or with signal peptide-GFP, and the complex was immunoprecipitated with anti-sortilin. This allowed direct examination of ER-Golgi transport of sortilin by Endo-H digestion, revealing that presence of the toxin markedly slows ER export of sortilin (Fig. 3*E*). Taken together, these data show that the sortilin-pro-TxVI interaction occurs in the ER and that sortilin enhances ER export of the TxVI protein to the Golgi.

Recently it has become apparent that ER export is a process regulated by different signals and facilitating molecules (18, 19). We have shown that the prodomain of conotoxin-TxVI acts as a facilitator of ER export and enhances secretion of this hydrophobic mini-protein. Intriguingly, the sortilin-binding site in the prodomain is close to its N terminus, *i.e.* within precisely those residues that are the first to enter the ER lumen during translation of TxVI. The prodomain-sortilin interaction occurs in the ER; and the TxVI propeptide binds a site on sortilin that is not masked by the sortilin propeptide; thus, the interaction occurs before sortilin is activated in the Golgi by truncation of its own propeptide. These data and the effect of the proteins on each other's ER export kinetics fit a scenario whereby the interaction essentially occurs while both proteins are co-maturing. The slowing of sortilin export in the presence of the toxin may reflect the imposition of a rate limit due to

folding or maturation of TxVI. Thus, the prodomain of TxVI can facilitate ER export by a "hitchhiking mechanism," linking its hydrophobic mature domain to a protein that traverses the ER efficiently and rapidly. Vps10p family members have been found across a very broad evolutionary range in multicellular organisms (20), thus the proposed mechanism may be evolutionarily ancient and could have been co-opted by *Conus* species to allow high expression of hydrophobic conopeptides. It should be noted that our findings do not rule out prodomain interactions with additional proteins outside the sortilin family. Screening for interactors of prodomains from different hydrophobic conotoxins and structure-function analyses of the TxVI prodomain interaction with Vps10p family receptors will help define the prevalence of this mechanism in the secretory pathway. It will also be interesting to see if other eukaryotes utilize prodomain "tags" to mark secreted proteins with hydrophobic surfaces as *bona fide* products that should pass quality control in the ER.

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