Syntaxin Isoform Specificity in the Regulation of Renal H\textsuperscript{+}-ATPase Exocytosis*

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Intercalated and inner medullary collecting ducts (IMCD) cells of the kidney mediate the transport of H\textsuperscript{+} by a plasma membrane H\textsuperscript{+}-ATPase. The rate of H\textsuperscript{+} transport in these cells is regulated by exocytic insertion of H\textsuperscript{+}-ATPase-laden vesicles into the apical membrane. We have shown that the exocytic insertion of proton pumps (H\textsuperscript{+}-ATPase) into the apical membrane of rat IMCD cells, in culture, involves SNAP proteins (syntaxin (synt), SNAP-23, and VAMP). The membrane fusion complex observed in IMCD cells with the induction of proton pump exocytosis not only included these SNAREs but also the H\textsuperscript{+}-ATPase. Based on these observations, we suggested that the targeting of these vesicles to the apical membrane is mediated by an interaction between the H\textsuperscript{+}-ATPase and a specific t-SNARE. To evaluate this hypothesis, we utilized a “pull-down” assay in which we identified by Western analysis, the proteins in a rat kidney medullary homogenate that complexed with glutathione S-transferase (GST) fusion syntaxin isoforms attached to Sepharose 4B-glutathione beads. The syntaxin isoforms employed were 1A, 1B, 2, 4, and 5, and also 1A that was truncated to exclude the H3 SNAP binding domain (synt-1A\(\Delta\)H3). All full-length syntaxin isoforms formed complexes with SNAP-23 and VAMP. Neither GST nor synt-1A\(\Delta\)H3 formed complexes with these SNAREs. H\textsuperscript{+}-ATPase (subunits \(\alpha\), \(\beta\), and \(\gamma\)) bound to syntaxin-1A and to a lesser extent to synt-1B but not to synt-1A\(\Delta\)H3 or synt-2, -4, -5. In cultured IMCD cells transfected to express syntaxin truncated for the membrane binding domain (synt-\(\Delta\)C), expression of synt-1A\(\Delta\)C, but not synt-4\(\Delta\)C, inhibited H\textsuperscript{+}-ATPase exocytosis. In conclusion, because all full-length syntaxins examined bound VAMP-2 and SNAP-23, but only non-H3-truncated syntaxin-1 bound H\textsuperscript{+}-ATPase, and synt-1A\(\Delta\)C expression by intact IMCD cells inhibited H\textsuperscript{+}-ATPase exocytosis, it is likely that the H\textsuperscript{+}-ATPase binds directly to the H3 domain of syntaxin-1 and not through VAMP-2 or SNAP-23. Interaction between the synt-1A and H\textsuperscript{+}-ATPase is important in the targeted exocytosis of the proton pump to the apical membrane of intercalated cells.

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‡‡ The abbreviations used are: IMCD, inner medullary collecting duct; H\textsuperscript{+}-ATPase Exocytosis*;

\(H_1\) ATPase and a specific t-SNARE. To evaluate this hypothesis, we first

‡¶ GST, glutathione S-transferase; SNARE, soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor; R\(t\), reverse transscriptase; IPTG, isopropyl-\(\beta\)-thio-galactopyranoside; DTT, dithiothreitol; synt, syntaxin; v-SNARE, vesicular SNARE; t-SNARE, target SNARE.

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syntaxin-1A (deletion of membrane binding domain, synt-1AΔC) had a marked reduction in regulated \( H^+ \)-ATPase exocytosis compared with wild type cells or cells that expressed a soluble form of syntaxin-4 (synt-4ΔC). Finally, we have shown in these cells by fluorescent microscopy that syntaxin-1 is localized to the apical membrane. Thus, it can be demonstrated that there is isoform-specific interaction between \( H^+ \)-ATPase and syntaxin-1 and that the specificity of the interaction and localization of syntaxin-1 can account for both targeting and fusion of \( H^+ \)-ATPase vesicles with the apical plasma membrane.

**Materials and Methods**

**Kidney Inner Medullary Homogenate**

Rat kidney inner medulla was homogenized using Teflon-coated Dounce homogenizer in a buffer consisting of HB, 20 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl\(_2\), 320 mM sucrose. Just before use, 1 mM DTT, 2 \( \mu \)g/ml pepstatin, 10 \( \mu \)g of leupeptin, and 0.3 mM phenylmethylsulfonyl fluoride was added. The homogenate was centrifuged at 10,000 \( \times g \) at 4 °C for 20 min to obtain a post-nuclear supernatant. Triton X-100 was added to the supernatant to a final concentration of 1% and homogenized a second time in a Dounce. This 1% Triton X-100-treated homogenate was centrifuged at 13,000 \( \times g \) for 40 min at 4 °C. The final supernatant was collected and stored at −80 °C for binding assays.

**RT-PCR and Expression Vectors Construction and RNA Purification**

Messenger RNA was purified from IMCD cells, rat renal medulla, and brain tissue using a commercial poly(A) pure kit (Ambion Inc.), and this RNA was analyzed by RT-PCR to identify mRNA expression of syntaxin-1A, -1B, -2, -4, and -5.

**PCR Primer Design**

**Primers for Syntaxin Isoforms to be Cloned into the PGEX Vectors to Express \( \text{GST-Syntaxin Fusion Proteins} \)—To facilitate the subsequent expression vector constructs, some restriction sites were added to the primers so that the PCR products from these primers can be inserted into the corresponding restriction sites of appropriate vectors. The primers used are as follows:**

1. For **Synt-1A**: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-taa ctc gag ATC CAA AGA TGC CCC CGG TGA-3′.

2. For **Synt-2**: upstream, 5′-cac gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-taa ctc gag ATC CAA AGA TGC CCC CGG TGA-3′.

3. For **Synt-4**: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-taa ctc gag ATC CAA AGA TGC CCC CGG TGA-3′.

**An EcoRI and XhoI restriction site was added to the 5′-terminal end of the upstream and downstream primers, respectively. When spliced into the EcoRI and XhoI cloning site of the vector PGEX-4T2, it enables the synt-1A PCR template to fuse with the GST codon beginning at the second codon, including amino acids 2–298 of syntaxin-1A.** 2. **For Synt-1B**: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′.

**Again, EcoRI and XhoI sites were inserted into the 5′-terminal end of the primers. The PCR product was spliced into the EcoRI and XhoI sites of PGE X-4T2 where it fuses with GST in-frame starting at the first codon of S1B, including amino acids 1–289 of syntaxin-1B.** 3. **For Syntaxin Isoforms**

![Diagram](http://www.jbc.org/)

**Fig. 1. Identification of syntaxin isoforms in rat kidney medulla (K) and brain (B) by RT-PCR.** Messenger RNA was isolated from rat kidney and brain and subjected to RT-PCR with primers that would allow for amplification of full-length clones. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The products obtained were the expected size for syntaxin-1A (892 bp), -1B (984 bp), -2 (878 bp), -4 (924 bp), and -5 (925 bp). Identification of these products was confirmed by sequence analysis. RT-PCR analysis of mRNA derived from cultured IMCD cells yielded similar results (data not shown).

**Fig. 2. Expression and purification of GST-synt-1A fusion protein.** Homogenates of \( E. \) coli transformed to express the fusion protein GST-synt-1A were subjected to gel electrophoresis and stained with Ponceau S. Homogenates were obtained prior to induction with IPTG (lane 1), 1 h (lane 2) and 2 h (lanes 3) after addition of IPTG. The expressed protein was purified (lane 4) from the 2-h post-IPTG homogenate by binding the fusion protein to glutathione-Sepharose 4B beads. Lane 5, molecular weight markers.

Synt-2: upstream, 5′-cag gaa ttc acct gtc ggg gac cgg gtc aag-3′; downstream, 5′-gac ctc gag CAT TTG CCA ACC GTC AAG-3′.}

**EcoRI and XhoI sites were also attached to the 5′-terminal end of the primers. The PCR product of these primers was spliced into the cloning sites of EcoRI and XhoI of PGEX 4T2 enabling the fourth codon of the synt-2 template to be fused in-frame with GST, including amino acids 4–291 of syntaxin-2.** 4. **For Synt-4: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-gac ctc gag CAT TTG CCA ACC GTC AAG-3′.**

An EcoRI and XhoI restriction site was added to the 5′-terminal end of upstream and downstream primers, respectively, to facilitate the subsequent cloning step. When the PCR product of these primers was spliced into the \( \text{BamHI} \) and \( \text{EcoRI} \) cloning site of the vector PGEX 3X, it made the synt-4 template fuse in-frame with GST, starting at the 2nd codon including amino acids 2–299 of syntaxin-4. 5. **For Synt-5: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-gac ctc gag CAT TTG CCA ACC GTC AAG-3′.**

A \( \text{BamHI} \) and \( \text{EcoRI} \) restriction site was added to the 5′-terminal end of upstream and downstream primers, respectively, to facilitate the subsequent cloning step. When the PCR product of these primers was spliced into the \( \text{BamHI} \) and \( \text{EcoRI} \) cloning site of the vector PGEX 3X, it made the synt-5 template fuse in-frame with GST, starting at the 2nd codon including amino acids 2–299 of syntaxin-5. 6. **For Synt-1A: upstream, 5′-cag gaa ttc aa gac tga gct tgg ag-3′; downstream, 5′-gac ctc gag CAT TTG CCA ACC GTC AAG-3′.**

These primers were designed to splice the synt-1A sequence 2–162 (amino acids) into the cloning site of \( \text{EcoRI} \) and \( \text{XhoI} \) of PGEX 4T2 to generate a truncation of the H3 binding and transmembrane domain of synt-1A.

**Primers for Syntaxin Isoform to be Cloned into \( \text{PcDNA3.1/NT-GFP-TOPO} \) for Expression of GFP-Syntaxin Isoforms—For syntaxin-1A full-
length (amino acids 1–289): synt-1AF, upstream, 5'-atg aag gac cga acc cag gag-3'; downstream, 5'-cta tcc aaa gat gcc ccc gat g-3'. For synt-1A cytosolic form with the C-terminal transmembrane domain truncated (amino acids 1–261): synt-1A/H9004C, upstream, 5'-atg aag gac cga acc cag gag-3'; downstream, 5'-cta tgc ctt gct ctg gta ctt g-3'. For synt-1A with both the transmembrane and H3 snare binding domain truncated (amino acids 1–162): synt-1A/H9004H3, upstream, 5'-atg aag gac cga acc cag gag-3'; downstream, 5'-cta act ggt cgt ggt ccg gcc ag-3'. All the primer DNA-oligo samples were synthesized by Integrated DNA Technologies, Inc.

**Rt-PCR**

Reverse transcription was accomplished by using 0.1 μg of mRNA as template and oligo(dT)16 as reverse transcriptase primer in a total volume of 20 μl; after incubation at room temperature for 10 min, the reaction temperature was raised to 42°C for 15 min, denatured at 99°C for 5 min, and then cooled to 5°C for 5 min. Then 1 nmol of each upstream and downstream PCR primer and other components for PCR were added to a total volume of 50 μl (PerkinElmer Life Sciences, GeneAmp RNA PCR kit). The PCR was performed on a MiniCycler TM with the time and temperature as follows: melting point, 95°C, 60 s; annealing, 60°C, 30 s; extending, 72°C, 120 s for 30 cycles followed by a 7-min extension at 72°C. PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide.

**Construction of Expression Vectors**

GST-Syntaxin Isoform Fusion Protein Expression Vectors—Corresponding bands of the PCR products separated on the agarose gel were purified using a commercial kit (QIAEX II, Gel Extraction kit, Qiagen Inc.) and subjected to enzyme restriction cut as indicated by the attached enzyme restriction site in the primers. Vectors PGEX 4T2 and PGEX 3X were also cut with the same enzyme as the PCR product to be inserted. All the cut DNA were electrophoresed on 1% agarose gels and extracted as indicated above.

PCR products encoding synt-1A, -1B, -2, and -1A/H9004H3 sequence were spliced into the EcoRI and XhoI restriction site of PGEX 4T2 for expression of GST fusion protein. PCR products encoding S4 and S5 sequence were cloned into the BamHI and EcoRI cloning site of PGEX 3X for expression of GST fusion protein.

GFP-Syntaxin Isoform Fusion Protein Expression Vectors—Rt-PCR products from primers for cloning into PcDNA 3.1/NT-GFP-TOPO were ligated directly with PcDNA3.1/NT-GFP-TOPO vector, and the clones with the insertion in the right orientation were picked up for transfection of IMCD cells. All the final clones of recombinant vector were DNA sequenced to confirm the correct insertion.

**Expression and Purification of GST-Syntaxin Fusion Protein**

Proteins were expressed in the Escherichia coli BL21 strains. Three hours after induction of the E. coli with IPTG, the bacteria were homogenized in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.3), and the expressed protein was purified from this homogenate by binding the fusion protein to glutathione-Sepharose 4B beads in accordance with the manufacturer's instruction for batch pu-
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In Vitro “Pull-down” Binding Assay

GST-syntaxin isoform fusion protein (25 \(\mu\)g) fixed on beads and 800 \(\mu\)g of kidney homogenate protein were incubated at 4 °C overnight in HB buffer (20 mM Heps, pH 7.2, 100 mM KCl, 2 mM MgCl\(_2\)) containing 1 mM DTT, 2 \(\mu\)g/ml pepstatin, 10 \(\mu\)g/ml leupeptin, and 0.3 mM phenyl-methylsulfonyl fluoride added just before use) with gentle agitation. Proteins bound to beads were recovered by centrifuging at 500 \(\times\) g for 5 min and washed three times with buffer (HB containing 0.5% Triton X-100). The protein attached to the washed beads was solubilized in an equal volume of 2\(\times\) SDS sample buffer (final concentration: 50 mM Tris, pH 6.8, 100 mM DTT, 10% glycerin, 2% SDS, and 0.1 bromphenol blue) and heated for 5 min at 95 °C and then subjected to SDS-PAGE and immunoblot analysis.

Immunoblots

After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes. The membranes were blocked using 5% nonfat dry milk in TBST and incubated overnight at 4 °C with primary antibody. After incubation with appropriate secondary antibodies conjugated to horse-radish peroxidase, the blots were developed using the enhanced chemiluminescence method. The film were scanned and then subjected to band densitometry and quantification (NIH Image).

Antibodies used in this study are as follows: for \(H^+\)-ATPase antibodies: rabbit antibody to the 31-kDa subunit of the \(H^+\)-ATPase (gift of D. Brubaker); rabbit polyclonal antibody to the 116-kDa subunit (Synaptic Systems GmbH); and a rabbit polyclonal antibody to the 16-kDa subunit (Ductin) (Oncogene Research Products). For Snap-23 antibodies: rabbit antibody to Snap-23, from Synaptic Systems GmbH. For synaptobrevin/VAMP: mouse monoclonal antibody to synaptobrevin/VAMP, from Synaptic System GmbH. For syntaxin 1A: mouse monoclonal antibody to syntaxin-1A, clone HPC-1 (Sigma). For syntaxin-4: rabbit antibody to syntaxin-4 Synaptic Systems GmbH.

The secondary antibodies for the immunoblot were peroxidase-conjugated goat anti-rabbit and -mouse IgG and were purchased from Jackson ImmunoResearch.

Transfection and Selection of Stable Transfected Cell Lines

DNA fragments from R\(\text{t-PCR}\) coding the full-length syntaxin-1A-(1-289) (synt-1A/P), syntaxin-1A(1-261), syntaxin 1AΔH3(1-162), and syntaxin-4ΔH3(1-259) were ligated into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen). Cloning with the right ligation was confirmed with PCR using the GFP forward and syntaxin isoform-specific reverse primers. Purified recombinant plasmids were used to transfect the IMCD cell using calcium phosphate precipitation and a selection medium containing 800 \(\mu\)g/ml G418. Survived cell were further selected to yield monoclonal cell lines using the method of limited dilution. Stable cell lines that express GFP-syntaxin mutant protein were confirmed by directly viewing the GFP fluorescence, immunostaining, and immunoblotting with GFP antibody and syntaxin isoform-specific antibodies. Positive clones were used for experiments.

Effect of Expression of Syntaxin Mutant Proteins on \(H^+\)-ATPase Translocation to the Apical Membrane of IMCD Cells

Wild type IMCD cells and IMCD cells transfected to express various GFP-syntaxin constructs were used to assess the effect of acute cellular acidification on the exocytic insertion of proton pumps into the apical membrane. Apical membranes were isolated from monolayers of wild type IMCD and IMCD cells transfected with GFP-syntaxin constructs by a vesiculation method modified in our laboratory for polarized epithelial cells (7). Control (pH 7.2) or acid-loaded cells, in which the pH falls to 6.5, were incubated for 90 min at 37 °C in a vesiculation medium (CHB or NHB (7)) that contained 50 mM paraformaldehyde, 2 mM DTT, and protease inhibitors. Paraformaldehyde and DTT induced the formation of apical membrane vesicles that are released into the incubation medium. At the end of 90 min, the medium containing the released apical membrane vesicles were collected, filtered through 31-μm nylon mesh to remove whole cells, and centrifuged at 20,000 rpm at 4 °C in a Sorval RC5B centrifuged for 1 h to pellet the vesicles. The pellet was aliquoted for protein determination and dissolved in SDS sample buffer for subjecting to SDS-PAGE and immunoblotting.

RESULTS

Syntaxin Isoforms Expressed in Rat Renal Medulla—Although there are a large number of isoforms of syntaxin, we have restricted our studies to the isoforms of syntaxin that are localized to the apical plasma membrane of renal collecting duct cells and one isoform (syntaxin-5) that is largely restricted to Golgi membranes (10). We were able to demonstrate that rat
renal medulla expresses syntaxin-1A, -1B, -2, -4, and -5 utilizing appropriate primers and RT-PCR of messenger RNA isolated from rat renal medulla (Fig. 1). The products obtained were the appropriate size for syntaxin-1A (882 bp, 2nd and 3rd lanes), -1B (984 bp, 4th and 5th lanes), -2 (878 bp, 6th and 7th lanes), 4 (924 bp, 8th and 9th lanes) and 5 (925 bp, 10th and 11th lanes). No PCR products were identified if the RT step was omitted. Molecular identification of these products was confirmed by sequence analysis, and all products were identical to the published sequences for these rat syntaxins. All of these isoforms were expressed in both kidney and brain with the exception of syntaxin-2, which was only expressed in kidney.

Binding of VAMP, SNAP-23, and H+ ATPase to Syntaxin Fusion Proteins—To test the hypothesis that syntaxin-1A specifically binds H+ ATPase in addition to the other v- and t-SNAREs necessary to form a fusion complex, we compared the capacity of immobilized fusion GST syntaxins to bind SNAP-23, VAMP, and H+ ATPase. The fusion proteins were produced by transformed, IPTG-induced E. coli purified and bound to glutathione-Sepharose G4 beads (Fig. 2). Rat kidney homogenate was incubated with beads coated with either GST alone or equivalent amounts of GST-syntaxin isoforms 1A, 1B, 2, 4, 5, and 1AΔH3, a truncated syntaxin-1A missing the C-terminal and H3 domain (Fig. 3). After extensive washing of the beads with HB + 0.5% Triton X-100, the proteins on the beads were solubilized in SDS sample buffer and subjected to immunoblot analysis as follows: (a) subunits of the H+ ATPase from the V1 domain (31-kDa subunit E) and from the V0 domain (116-kDa subunit a, and 16-kDa subunit c) (Fig. 3A) (14–16); (b) VAMP; and (c) SNAP-23 (Fig. 3B). Compared with control beads coated only with GST, beads coated with syntaxin-1A or -B bound appreciable amounts of H+ ATPase. Syntaxin-1A-coated beads bound 8 times (p < 0.05, n = 6) and syntaxin-1B bound appreciable amounts of H+ ATPase than control beads. The other isoforms, syntaxin-2, -4, -5, and -1AΔH3 bound no more 31-kDa subunit of the H+ ATPase than did control beads. The other isoforms, syntaxin-2, -4, -5, and -1AΔH3 bound no more 31-kDa subunit of the H+ ATPase than background. The binding pattern of the 16- and 116-kDa (V0) subunits of the H+ ATPase to syntaxin isoforms was similar to that of the 31-kDa subunit (Fig. 3A). The binding of both of V1 (31 kD) and V0 (16 and 116 kDa) subunits to syntaxin-1 indicates that intact proton pump units interact with this t-SNARE. In contrast, all of the full-length syntaxin isoforms bound VAMP and SNAP-23 (Fig. 3B). Deletion of the H3 domain of syntaxin-1A (17) resulted in a mutant that, as expected, did not bind VAMP or SNAP-23, but surprisingly, this construct also did not bind H+ ATPase.

Fig. 5. Immunolocalization of native syntaxin-1A in non-transfected IMCD cells. Cells were stained with a mouse anti-syntaxin-1 monoclonal antibody and examined by confocal microscopy. The photomicrograph in the left panel represents an image obtained at the apex of the cell. The staining indicates that syntaxin-1A is present in the apical membrane. The right panel is an image obtained at the level of the nucleus in the same cells. The staining in a perinuclear distribution indicated that Golgi vesicle may contain syntaxin-1A, but the lack of staining at the periphery of the cell indicates that there is no basolateral localization of this SNARE.

Fig. 6. Western blot analysis showing effect of acute acidification on the 31-kDa subunit of H+ ATPase and on GP-135 in apical plasma membrane of IMCD cells utilizing a vesiculation technique. Parafomaldehyde-isolated apical vesicles from control (C) and acutely acidified (A) wild type IMCD cells and IMCD cells transfected with various syntaxin constructs (synt-1A, 1AΔC, 1AΔCΔH3, and 4C) were subjected to Western analysis for the resident apical protein, GP-135, and 31-kDa subunit of H+ ATPase.
wild type IMCD cells without change in a resident apical membrane protein such as GP-135 (Fig. 6). Transfected cells expressing GFP-synt-1A, GFP-synt-1AΔCΔH3, or GFP-synt-4AC, when subjected to cellular acidification, displayed a similar degree of apical membrane H'-ATPase amplification (Fig. 6) to that of non-transfected cells. However, when cells that express GFP-synt-1AΔC, a form of syntaxin that retains the binding sites for v-SNAREs and H'-ATPase but lacks the membrane binding domain, are subjected to cellular acidification, apical membrane H'-ATPase amplification was abolished (Fig. 6). Thus, this soluble, truncated construct of syntaxin-1A appears to act as a specific inhibitor of regulated exocytosis. To evaluate this hypothesis further, we determined whether any of these constructs of syntaxin could be co-immunoprecipitated with H'-ATPase (Fig. 7). Both GFP-synt-1A and GFP-synt-1AΔC could be co-immunoprecipitated with H'-ATPase, but GFP-synt-4AC could not. These observations indicate that soluble GFP-synt-1AΔC binds to H'-ATPase and by so doing interferes with its targeting to the apical membrane.

**DISCUSSION**

Regulation of H⁺ secretion in the collecting duct of the mammalian kidney involves amplification of the number of apical membrane proton pumps (H⁺-ATPase) by regulated exocytic insertion and reduction by endocytic retrieval of these pumps into a subapical population of recycled vesicles (1, 3). The recycled proton pump containing vesicles is unusual in that their cytoplasmic surface is almost entirely coated with the V₁ portion of the H⁺-ATPase (19). In cultured rat IMCD cells we have demonstrated that the process of regulated exocytic insertion of H⁺-ATPase containing vesicles into the apical membrane involves a process analogous to neurosecretion. The vesicle fusion process in these cells involves SNAREs with the formation of a large, clastroidal, toxin-sensitive complex that includes syntaxin, SNAP-23, VAMP, N-ethylmaleimide-sensitive factor, and α and γ SNAP (4). The complex differs in that it also contains the H⁺-ATPase (4, 5).

In addition to mediating fusion, the SNARE proteins may be involved in the targeting of the H⁺-ATPase vesicles to the apical membrane. This latter role of SNAREs in targeting is controversial because SNARE pairing can be promiscuous (12). Thus, factors other than the specificity of SNARE pairing may allow these proteins to participate in the targeting process. Important factors that could confer a specific role of a SNARE protein in targeting may include selective affinity of this t-SNARE for a specific non-SNARE in the vesicle and segregation of the target SNARE to a single membrane domain (apical). Consistent with this kind of targeting scheme is the hypothesis that the renal acid secretory vesicles are targeted, in part, by an interaction between an apical membrane syntaxin and the vesicular coating protein, the H⁺-ATPase. To test this hypothesis we initially examined the ability of fusion GST-syntaxin isoforms to form complexes with v-SNAREs and H⁺-ATPase derived from detergent (Triton-X-100)-solubilized extracts from the renal medulla of rats. The isoforms of syntaxin evaluated (syntaxin-1A, -1B, -2, -4, and -5) included only the syntaxins that are expressed by rat kidney and present in the apical membrane of intact tubular epithelial cells with the exception of syntaxin-5, a non-plasma membrane syntaxin that served as a control for these assays (10). Syntaxin 3 which is also expressed in the plasma membrane of renal epithelial cells was not evaluated because it has been shown to be localized to only the basolateral membrane of renal tubular epithelial cells of the collecting duct (20). In the pull-down assay, utilizing these GST syntaxins bound to Sepharose beads, we demonstrated isoform specificity in that only syntaxin-1 (1A ≫ 1B) bound both V₁ and Vₐ H⁺-ATPase subunits, whereas all the
other isoforms (syntaxin-2, -4, and -5) did not. All syntaxin isoforms examined bound VAMP and SNAP-23. Thus, the interaction between the proton pump and syntaxin appears not to be mediated by the binding of the proton pump to the other SNAREs that form the fusion complex. Paring of syntaxin-1 with H⁺-ATPase is direct and a likely process for targeting these vesicles to the apical membrane.

To identify the region of syntaxin involved in binding to H⁺-ATPase, we determined whether the proton pump could be bound by truncated forms of syntaxin-1A. Truncation of the C-terminal membrane-binding region did not alter the binding characteristics for H⁺-ATPase or other SNAREs. On the other hand, deletion of the H3-SNARE binding domain abolished not only VAMP and SNAP-23 binding but also H⁺-ATPase binding. It would appear that the proton pump interacts with the same general region of syntaxin-1A as do other SNAREs but does not attach indirectly to syntaxin through VAMP or SNAP-23.

These in vitro studies also are consistent with the view that there is syntaxin isoform specificity in the process of exocytic insertion of proton pumps into the apical membrane. To evaluate this proposal in an in vivo system, we examined the effect of expressing constructs of syntaxin in cultured IMCD cells on apical membrane H⁺-ATPase amplification. We developed cell lines that express truncated forms of syntaxin-1A and syntaxin-4 that had deletions of the membrane binding domain but not the SNARE binding domain. These cells expressed a soluble form of syntaxin (Fig. 4). We also generated a line that overexpressed full-length syntaxin-1A, a form that was primarily membrane-bound. Finally, we generated a cell line that expressed a form of syntaxin-1A that had truncations for both the membrane binding and SNARE binding domains. Based on the results of the pull-down assay, we predicted that the only truncation form of syntaxin that could act as an inhibitor of exocytosis of the proton pump would be the form that could bind to H⁺-ATPase but would not be localized to the plasma membrane (synt-1AΔC). Our results confirmed this prediction because only the expression of synt-1AΔC but not other forms (synt-1AΔCΔH3 or synt-4ΔC) resulted in an appreciable inhibition of H⁺-ATPase exocytosis. We also have demonstrated that synt-1AΔC but not synt-4ΔC co-immunoprecipitates with H⁺-ATPase. This observation indicates that these two molecules also interact in vivo. Finally, we have shown that syntaxin-1A is primarily distributed to the apical membrane of cultured IMCD cells. This series of observations is consistent with the hypothesis that interaction between the isoform of syntaxin, syntaxin-1A, and the H⁺-ATPase is specifically required for the targeting and fusion of proton pump laden vesicles with the apical membrane.

Although there is compelling evidence in our cultured IMCD cell line (4) and in non-renal tissue (21) for a role for SNAREs in fusion targeting of H⁺-ATPases to the plasma membrane, to date there has been little evidence that indicates that SNAREs regulate exocytic insertion of the proton pump in the intact nephron. In the present study we demonstrate that the rat renal medulla expresses all of the constituents necessary to form fusion complexes. In addition the proteins derived from renal medullary homogenates interact with GST-syntaxin-1A and form a complex that has the same constituents that are observed in cultured IMCD cells when H⁺-ATPase exocytosis is stimulated. Therefore, we predict that as in the cultured cell model system, in the intact nephron, targeting of proton pumps involves the same set of SNAREs and has the same isoform specificity for syntaxin-1A as in the IMCD cell in culture.

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