Identification of Domains within the V-ATPase Accessory Subunit Ac45 Involved in V-ATPase Transport and Ca\(^{2+}\)-dependent Exocytosis

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Background: Accessory subunit Ac45 is an important regulator of the V-ATPase pump.

Results: Ac45 deletion mutants (involving its proteolytic cleavage site or luminal/cytoplasmic domains) affected Ac45 transport through the secretory pathway, V-ATPase trafficking, and Ca\(^{2+}\)-dependent secretion.

Conclusion: Proper V-ATPase functioning requires Ac45 processing, and N- and C-terminal domains of Ac45.

Significance: Elucidation of structural requirements for Ac45 to act as V-ATPase regulator.

The vacuolar (H\(^+\))-ATPase (V-ATPase) is crucial for maintenance of the acidic microenvironment in intracellular organelles, whereas its membrane-bound V\(_0\)-sector is involved in Ca\(^{2+}\)-dependent membrane fusion. In the secretory pathway, the V-ATPase is regulated by its type I transmembrane and V\(_0\)-associated accessory subunit Ac45. To execute its function, the intact-Ac45 protein is proteolytically processed to cleaved-Ac45 thereby releasing its N-terminal domain. Here, we searched for the functional domains within Ac45 by analyzing a set of deletion mutants close to the in vivo situation, namely in transgenic Xenopus intermediate pituitary melanotrope cells. Intact-Ac45 was poorly processed and accumulated in the endoplasmic reticulum of the transgenic melanotrope cells. In contrast, cleaved-Ac45 was efficiently transported through the secretory pathway, caused an accumulation of the V-ATPase at the plasma membrane and reduced dopaminergic inhibition of Ca\(^{2+}\)-dependent peptide secretion. Surprisingly, removal of the C-tail from intact-Ac45 caused cellular phenotypes also found for cleaved-Ac45, whereas C-tail removal from cleaved-Ac45 still allowed its transport to the plasma membrane, but abolished V-ATPase recruitment into the secretory pathway and left dopaminergic inhibition of the cells unaffected. We conclude that domains located in the N- and C-terminal portions of the Ac45 protein direct its trafficking, V-ATPase recruitment and Ca\(^{2+}\)-dependent-regulated exocytosis.

The vacuolar (H\(^+\))-ATPase (V-ATPase)\(^2\) is a proton pump and its function is crucial for a broad range of biological processes such as membrane trafficking, receptor-mediated endocytosis, lysosomal protein degradation (1), embryonic left-right patterning (2), Wnt signaling during anterior-posterior patterning (3), and maintenance of the acid-base homeostasis (4). In intracellular organelles, such as lysosomes, secretory granules, and the yeast vacuole, the V-ATPase is the major regulator of the pH (2, 5). Moreover, the V-ATPase provides an electrochemical membrane potential that is required for yeast vacuole membrane fusion (6). In neuroendocrine cells, inhibition of the V-ATPase greatly affects neuroendocrine prohormone processing and regulated secretion (7, 8), and in neuronal cells evokes a deceleration in the kinetics of exocytosis and a reduction in the neurotransmitter content of the vesicles (9).

The V-ATPase consists of two sectors, namely the cytoplasmic V\(_1\)-sector that takes care of ATP hydrolysis and the membrane-bound V\(_0\)-sector that harbors the proteolipid by which protons are translocated (1). The V-ATPase complex displays V\(_1\)-V\(_0\) sector dissociation, which most likely represents a universal mechanism for the regulation of its activity (10).

Apart from its function in proton pumping, also a V\(_1\)-independent role for the V\(_0\)-sector in post-SNARE membrane fusion has been found in yeast vacuoles (11), Drosophila neurons (12), mouse pancreatic \(\beta\)-cells (13), and during apical exosome secretion in Caenorhabditis elegans (14). The formation of the pore preceding membrane fusion is induced by the V\(_0\)-sector and involves the small Ca\(^{2+}\)-binding protein calmodulin (11, 15). The recent discovery of a direct interaction between the v-SNARE synaptobrevin and the V\(_0\)-sector of the V-ATPase in rat neurons underscores the important role of V\(_0\) in post-SNARE membrane fusion and Ca\(^{2+}\)-dependent neurotransmitter release (16). Furthermore, a separate function for the V\(_0\)-sector in the biogenesis of dense-core granules in neuroendocrine cells has recently been proposed (17). Together, these studies show that the V\(_0\)-sector is of great importance to the regulated secretory pathway, in particular for the process of Ca\(^{2+}\)-dependent regulated secretion.

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\(^2\)The abbreviations used are: V-ATPase, vacuolar (H\(^+\))-ATPase; POMC, proopiomelanocortin; IL, intermediate lobe; AL, anterior lobe; NIL, neurointermediate lobe; CLSM, confocal laser scanning microscopy; PC, prohormone convertase; CPE, carboxypeptidase E.
Functional Domains within the Ac45 Protein

In specialized cell types such as osteoclasts (18–20), and neuroendocrine chromaffin and pituitary cells (21, 22) the V₀⁻sec-tor of the V-ATPase is equipped with an accessory subunit, namely the glycosylated type I transmembrane protein Ac45 (23). Using a transgenic approach in Xenopus neuroendocrine melanotrope cells, we recently identified the Ac45 protein as a crucial regulator of the V-ATPase in the regulated secretory pathway (24, 25). Extensive biosynthetic labeling studies revealed that in the early secretory pathway the intact 62-kDa Ac45 protein is proteolytically cleaved to a C-terminal Ac45 fragment of ~40 kDa (21), representing the endogenous protein originally isolated from bovine chromaffin granules (26). The ~20-kDa N-terminal cleavage fragment has been suggested to be degraded following its cleavage from the Ac45 precursor protein (27).

In this study, we explored the structural requirements for Ac45 to function as a regulator of the V-ATPase. We examined the importance of a number of domains within the Ac45 protein, including of its short cytoplasmic tail which harbors essential and autonomous routing information (28). Using the technique of stable Xenopus transgenesis (29), we expressed the mutants in their natural environment, namely specifically in Xenopus intermediate pituitary melanotrope cells. We found that the N-terminal as well as the C-terminal portion of the Ac45 protein is crucial for its functioning.

EXPERIMENTAL PROCEDURES

Animals—Xenopus laevis were reared in the Xenopus facility of the Department of Molecular Animal Physiology (Central Animal Facility, Radboud University Nijmegen). For transgenesis experiments, adult female X. laevis were directly obtained from South Africa (Africa Reptile Park, Muizenberg, South Africa). Experimental animals were adapted to a black background for at least 3 weeks with a light/dark cycle of 12 h. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and permit RBD0166(H10) to generate and house transgenic X. laevis.

Generation of Xenopus Stably Transgenic for Ac45 Mutants Fused to GFP—Transgenic lines #530 (Ac45ΔCS: intact-Ac45 from which the proteolytic cleavage site has been deleted), #629 (Ac45ΔC: intact-Ac45 from which the C-tail has been removed), #651 (cleaved-Ac45ΔC: cleaved-Ac45 without its C-terminal tail), and #481 (Ac45Nterm: the naturally-occurring C-terminal tail), and #452 (intact-Ac45) were directly obtained from South Africa (Africa Reptile Park, Muizenberg, South Africa). Experimental animals were adapted to a black background for at least 3 weeks with a light/dark cycle of 12 h. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and permit RBD0166(H10) to generate and house transgenic X. laevis.

Cryosectioning and Immunohistochemistry—Cryosectioning, GFP imaging, and anti-POMC immunostainings on Xenopus brain-pituitary preparations were described previously (25).

Western Blot Analysis—Freshly dissected neurointermediate lobes (NILs) were homogenized in lysis buffer (140 mM NaCl, 0.1% Triton-X100, 1% Tween-20, 50 mM Hepes pH 7.4 supplemented with Complete protease inhibitor mix (Roche Diagnostics)). Lysates were denatured, separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane. Blots were incubated with anti-Xenopus Ac45-C (1:5000) or anti-GFP (1:5000) rabbit antiserum and with secondary peroxidase-conjugated goat-anti-rabbit antibody (1:5000) followed by chemiluminescence. Signals were detected and quantified using a BioImaging system with Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK).
Wells, Amsterdam, The Netherlands). For determining the localization of the GFP fusion protein, cells were subjected to live cell imaging after 24 h of culturing using an Olympus FV1000 confocal laser scanning microscope. For immunostaining with marker antibodies, cells were fixed with 4% paraformaldehyde in Xenopus PBS (XPBS; 67% PBS) for 2 h, washed with 50 mM NH₄Cl/XPBS, permeabilized with 0.05% Tween-20/XPBS and incubated with anti-POMC (ST-62, 1:5000), anti-NaK-ATPase (ST-201 1:1000), or anti-calnexin antibodies (1:1000) in blocking buffer (2% BSA, 0.05% Tween-20 in XPBS). After extensive washing with XPBS, cells were incubated for 1 h with a second antibody, Goat-anti-rabbit-Alexa Fluor 568 (1:100 in blocking buffer). Following additional washing steps, cells were mounted in Mowiol and imaged for GFP and Alexa Fluor 568 using an Olympus FV1000 confocal laser scanning microscope using the Image J software package.

**Metabolic Cell Labeling and Immunoprecipitations**—Radioactive labeling of newly synthesized proteins from freshly isolated Xenopus NILs was described previously (25). Chase incubations were in the absence or presence of 0.1 mM apomorphine. The gel migration positions of 37-kDa POMC, 18-kDa POMC, CPE, and the various PC2 forms corresponded to those previously observed (22, 24, 35). POMC represents more than 80% of all newly synthesized melanotrope proteins (22) allowing its direct analysis (22, 24).

**Immunoelectron Microscopy**—Immunoelectron microscopy on Xenopus NILs using an anti-GFP or an anti-V₁A antibody was described previously (24).

**Fluorescence Measurements of Intracellular Ca²⁺**—Dynamic video imaging to measure intracellular Ca²⁺ was essentially performed as described previously (36). Ca²⁺-oscillations were measured in a low-speed acquisition mode with a sample interval of 6 s. During the experiment, Ringer’s solution without or with 0.1 mM apomorphine was continuously perfused at a flow rate of 0.7 ml/min; the apomorphine concentration was chosen on the basis of the results of our metabolic cell labeling studies.

**Statistics**—Data are presented as means ± S.E. Statistical evaluation was performed using an unpaired Student’s t test.

**RESULTS**

**Generation of Stable Transgenic Xenopus Expressing (Mutant) Ac45 Tagged with GFP in the Intermediate Pituitary Melanotrope Cells**—To study the functional domains within Ac45, we first expressed in the neuroendocrine Xenopus melanotrope cells intact-Ac45 containing a GFP tag at its N or C terminus (GFPAc45wt) or a variety of Ac45 mutants fused to GFP, namely Ac45ΔC, Ac45ΔΔ, cleaved-Ac45, cleaved-Ac45ΔC, and Ac45Nterm (Fig. 2A). The mutant fusion proteins were colocalized with the main melanotrope cargo protein proopiomelanocortin (POMC) exclusively in the melanotrope cells of the intermediate lobe (IL) and not in the anterior lobe (AL) of the pituitaries (Fig. 2B).

Western blot analysis of a Ac45ΔCS NIL lysate using the anti-GFP antibody revealed only an ~90-kDa product corresponding to the expected size of non-cleaved Ac45 fused to GFP, indicating that this Ac45 mutant indeed prevents Ac45 cleavage (Fig. 2C). In the Ac45ΔC NIL lysate, we detected a major ~90-kDa product corresponding to the non-cleaved C-terminally truncated Ac45 fusion protein (Fig. 2C). The slightly slower migrating diffuse product likely represents a more extensively glycosylated form of Ac45ΔC. Furthermore, we found a significantly higher processing efficiency of Ac45ΔC compared with Ac45wt. The increased processing efficiency of Ac45ΔC was reflected by an increased ratio of Nterm 50-kDa fragment to intact protein (0.463 ± 0.090 for Ac45ΔC and 0.131 ± 0.011, for Ac45wt; p < 0.01, n = 4).

Analysis of the cleaved-Ac45 NIL lysate revealed the expression of an ~70-kDa transgene product corresponding to the cleaved-Ac45 product. The ~70- and 72-kDa cleaved-Ac45ΔC products most likely correspond to two glycosylation states of the transgene product. The steady-state expression level of this transgene was relatively low. Finally, in the Ac45Nterm NIL lysate two ~50-kDa products were detected, presumably also corresponding to differentially glycosylated forms of the N-terminal cleavage product of Ac45 fused to GFP.

**Localization of the Mutant Ac45 Proteins in the Transgenic Melanotrope Cells**—To examine the subcellular localization of the GFP-tagged mutant Ac45 proteins, we used confocal laser scanning microscopy (CLSM) on cultured live melanotrope cells. The intact-Ac45 and Ac45ΔCS transgene products were mainly found in a reticular network, most likely representing the endoplasmic reticulum (ER) (Fig. 3A). Surprisingly, besides in a perinuclear region, presumably representing the Golgi, Ac45ΔC was mainly localized to the plasma membrane, comparable to the localization of cleaved-Ac45 and cleaved-Ac45/GFP product (Fig. 1C). This finding indicates that the GFP tag prevented detection of the C-terminal Ac45 epitope, presumably due to strong secondary structures being present even under the denaturing SDS-PAGE conditions. Since the C-tail of Ac45 contains important routing determinants (28), we decided to use for our mutational analysis the Ac45 protein fused to GFP at its N terminus.

To gain insight into the significance of the Ac45 cleavage event and the function of the protein domains within Ac45, we generated transgenic Xenopus lines expressing intact-Ac45 (Ac45wt) or a variety of Ac45 mutants fused to GFP, namely Ac45ΔCS, Ac45ΔΔ, cleaved-Ac45, cleaved-Ac45ΔC, and Ac45Nterm (Fig. 2A). The mutant fusion proteins were colocalized with the main melanotrope cargo protein proopiomelanocortin (POMC) exclusively in the melanotrope cells of the intermediate lobe (IL) and not in the anterior lobe (AL) of the pituitaries (Fig. 2B).

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Ac45ΔC (Fig. 3A). The soluble Ac45Nterm product was found in granular structures in the melanotrope cells (Fig. 3A).

To confirm the observed intracellular localizations of the transgene products, isolated melanotrope cells were fixed and probed with specific marker antibodies. The localization of Ac45ΔCS in the ER was confirmed by the analysis using an antibody directed to the ER chaperone calnexin (34). At the plasma membrane, we found co-immunostaining of the plasma membrane marker NaK-ATPase and Ac45ΔC, and of NaK-ATPase and cleaved-Ac45. The localization of the soluble Ac45Nterm transgene product was in granules, as was evident from its colocalization with the main melanotrope prohormone proopiomelanocortin (POMC) (Fig. 3B). Thus, naturally occurring N- and C-terminal Ac45 fragments are transported through the secretory pathway, the cleavage of Ac45 is a prerequisite for its transport and the cytoplasmic tail of Ac45 plays a pivotal role in transporting the Ac45 protein from the ER to the later stages of the secretory pathway.

**Differential Effects of the Ac45 Mutant Proteins on Endogenous V-ATPase Localization**—Previous studies have shown that the cleaved-Ac45 transgene product directs the endogenous V-ATPase through the secretory pathway and to the plasma membrane of *Xenopus* melanotrope cells (24). We now wondered how the expression of the Ac45 mutant proteins would affect endogenous V-ATPase localization. We focused on the cleaved-Ac45, Ac45ΔC, and cleaved-Ac45ΔC transgene products since these were expressed at the plasma membrane. As expected, immuno-EM with an anti-GFP antibody showed no gold label in the wild-type cells, indicative of the specificity of our immunolabeling (Fig. 4A). Immuno-EM further revealed that the cleaved-Ac45 product was mainly localized to microvillar structures at the plasma membrane (Fig. 4C and Ref. 24), Ac45ΔC to both microvillar structures and smooth plasma membrane, and cleaved-Ac45ΔC to smooth, non-ruffled plasma membranes (Fig. 4, B and D, respectively). Next, we studied the intracellular localization of the endogenous V-ATPase A subunit. In wild-type cells, no V-ATPase was found at the plasma membrane of the cells (Fig. 4E). Cleaved-Ac45 co-localized with the endogenous V-ATPase A subunit in the microvillar structures (Fig. 4G and Ref. 24), and Ac45ΔC in both the microvillar structures and the non-ruffled plasma membranes (Fig. 4F). The localization of the endogenous V-ATPase A subunit in the cytoplasm and its partial association with vesicular structures in the cleaved-Ac45ΔC transgenic
cells was similar to that in wild-type melanotrope cells (Fig. 4, E and H, respectively), suggesting that cleaved-Ac45\(|\Delta C|\) did not recruit the endogenous V-ATPase to the plasma membrane.

The N-terminal Ac45 Cleavage Product Is Secreted via the Regulated Secretory Pathway—We next performed biosynthetic labeling studies to study the fate of the Ac45Nterm protein in the melanotrope cells. In addition to the biosynthetically active melanotrope cells (in the intermediate lobe), the *Xenopus* NIL consists of nerve terminals of hypothalamic origin (the neural lobe) which are biosynthetically inactive. Therefore, the radiolabeled proteins are synthesized solely by the melanotrope cells. During the 30-min pulse period, in both wild-type and transgenic cells, 37-kDa POMC was clearly the major newly synthesized protein that during the 180-min chase incubation was processed to 18-kDa POMC (Fig. 5A). This protein represents the N-terminal portion of the *Xenopus* POMC molecule, contains the only N-glycosylation site present in the POMC molecule and is the result of the first endoproteolytic cleavage step during POMC processing (37). The biosynthesis of newly synthesized proteins in the Ac45Nterm-transgenic NILs was similar to that in wild-type NILs (Fig. 5A).

Immunoprecipitations from the NIL lysates with an anti-Ac45-N antibody revealed the specific expression of two protein products of \(\sim 50\) and \(\sim 55\) kDa in the transgenic NILs, representing Ac45Nterm transgene products. Since immunoprecipitations from the incubation medium only detected the \(\sim 55\)-kDa Ac45Nterm form, the 50-kDa protein most likely represents its nonglycosylated form and the 55-kDa product the glycosylated form (Fig. 5B). Since the Ac45Nterm protein was secreted by the transgenic melanotrope cells, we wondered whether this secretion occurred in a regulated manner. Under physiological conditions, secretion by the melanotrope cells is under strict control of neurotransmitters of hypothalamic origin, with dopamine, acting through the dopamine D_2-receptor, as one of the main inhibitors of peptide release (38). We applied the dopamine D_2-receptor agonist apomorphine during the 180-min chase incubations and found that in addition to the secretion of the well-defined regulated secretory proteins 18-kDa POMC, prohormone convertase 2 (PC2), and carboxypeptidase E (CPE), also the secretion of Ac45Nterm was blocked (Fig. 5A), showing that the transgene product was secreted in a regulated fashion.

**FIGURE 2.** Transgene expression of GFP-Ac45 mutant proteins specifically in the *Xenopus* melanotrope cells. *A,* overview of transgenes used to express Ac45 mutant proteins fused to GFP in the *Xenopus* melanotrope cells. *B,* sagittal cryosections of transgenic *Xenopus* pituitaries. Transgenic Ac45 mutant/GFP expression was directly viewed under a fluorescence microscope (green). Sections were stained with an anti-POMC antibody (red) showing coexpression of GFP and POMC in the intermediate pituitary melanotrope cells. *C,* Western blot analysis of NIL lysates with an anti-GFP showing the expression levels of the respective transgene products. Ten percent of a total NIL lysate was analyzed. The lane with the Ac45\(|\Delta CS|\) NIL lysate was taken from a separate Western blot. GFP*, stable GFP moiety, probably resulting from Ac45Nterm mutant fusion protein breakdown. *D,* endoproteolytic processing efficiencies of the Ac45wt and Ac45\(|\Delta C|\) proteins are presented as the ratio of the amount of Ac45N-term relative to the amount of intact-Ac45 and the ratio of the amount of Ac45N-term relative to the amount of Ac45\(|\Delta C|\), respectively. Shown are the means \(\pm \) S.E. \((n = 4)\). Significant difference is indicated by \(* * (p < 0.01).\)
The Dopaminergic Inhibition of Peptide Secretion Is Differentially Affected by the Ac45 Mutant Proteins—Since Ac45 is associated with the V₀-sector of the V-ATPase (18, 26, 39) and given the involvement of V₀ in Ca²⁺/H⁻-dependent exocytosis (11, 12), we next wondered how the various Ac45 mutants affect Ca²⁺/H⁻-dependent secretion by the transgenic cells. We therefore performed biosynthetic labeling studies in the presence or absence of apomorphine. During the 30-min pulse and 180-min chase period in the absence of apomorphine, the NILs secreted regulated secretory proteins, including 18-kDa POMC, PC2 and CPE into the incubation medium. In the presence of 0.1 μM apomorphine, peptide secretion from the wild-type NILs was completely blocked. Similarly, peptide secretion from the transgenic NILs expressing intact-Ac45, Ac45/C or cleaved-Ac45/C was inhibited. Surprisingly, in the presence of apomorphine transgenic NILs expressing cleaved-Ac45 or Ac45/C still secreted substantial amounts of the regulated secretory proteins into the incubation medium (Fig. 6). Only ~25% inhibition of secretion was found for the cleaved-Ac45 transgenic melanotrope cells (data not shown). Thus, an Ac45 variant localized to the ER (intact-Ac45 or Ac45/C), localized to the plasma membrane without affecting V-ATPase localization (cleaved-Ac45/C) or secreted by the cells (Ac45/Nterm) does not affect dopaminergic inhibition of peptide release. In contrast, the Ac45 mutants that travel through the secretory pathway and recruit the V-ATPase (namely cleaved-Ac45 and Ac45/C, see above) affect regulated peptide release by the melanotrope cells.

The Dopaminergic Inhibition of Ca²⁺ Oscillations Is Not Affected in the Cleaved Ac45 Transgenic Melanotrope Cells—Since regulated peptide secretion by the cleaved-Ac45 and Ac45/C-transgenic melanotrope cells was not effectively inhibited by apomorphine, we wondered whether these transgenic cells would still display normal dopamine D₂-receptor functioning. In wild-type melanotrope cells, Ca²⁺-oscillations regulate the secretory activity of the cells, as they are the driving force for regulated secretion and are effectively inhibited by dopamine (40–42). We choose to study receptor functioning in the cleaved-Ac45-transgenic cells, since this protein resembles the naturally occurring Ac45 protein that is found in secretory granules (26). Following loading of the cells with the Ca²⁺-probe fura-2 and in the absence of apomorphine, both wild-type and the cleaved-Ac45-transgenic melanotrope cells displayed spontaneous intracellular Ca²⁺-oscillations. Upon treatment of the cells with 0.1 μM apomorphine for 10 min, in ~75% of both the wild-type cells (25 out of 34 cells, three independent experiments) as well as the transgenic cells (30 out of 41 cells, three independent experiments) showed severe inhibition of the Ca²⁺-oscillations, resulting in minimal, close-to-noise level, Ca²⁺-oscillations, whereas the remainder of the cells displayed low-amplitude Ca²⁺-oscillations within the 10-min incubation period (compare Fig. 7, A and B). Removal of the drug reversed the inhibition and Ca²⁺-oscillations were regained. Since in our peptide release studies we monitored the apomorphine effect following a 180-min chase period, we examined whether the inhibition of the Ca²⁺-oscillations sustained up to 180 min. Comparable with the 10-min measurements, ~25% of the wild-type and transgenic cells gained low-amplitude Ca²⁺-oscillations while other cells remained largely inhibited by the drug for 180 min, and no difference was observed between wild-type and the transgenic cells (data not shown). Thus, in the cleaved-Ac45-transgenic melanotrope cells dopamine D₂-receptor functioning was unaffected, but the inhibition of the Ca²⁺-oscillations did no longer lead to the inhibition of peptide secretion.
DISCUSSION

In this study, we examined the structural domains within the Ac45 protein that are necessary for its function as the V-ATPase regulator in the regulated secretory pathway. We used transgene products representing the naturally occurring N- and C-terminal processing products of Ac45, namely Nterm-Ac45 and cleaved-Ac45, respectively. Microscopic analysis revealed that these transgene products were transported through the secretory pathway of the Xenopus melanotrope cells. The endogenous, soluble N-terminal Ac45 fragment appears to be degraded in vivo (27) however we cannot exclude that this fragment is secreted. In our transgenic melanotrope cells this fragment, apparently stabilized by its fusion to GFP, was released and in a regulated fashion, since its secretion was completely inhibited by the treatment of the cells with the D2-receptor agonist apomorphine.

We further observed that in the melanotrope cells the wild-type intact-Ac45 protein was proteolytically processed. Removal of the region harboring the putative cleavage site (Val-164-Asp-207 of Xenopus Ac45; (21)) prevented its processing and caused its accumulation in the ER. This finding indicates that region Val164-Asp207, which includes a potential cleavage site for the endoprotease furin, contains the endoproteolytic processing site within the Xenopus Ac45 protein. In mouse pancreatic β-cells, furin indeed cleaves mouse Ac45 (43). However, the furin cleavage site of mouse Ac45 is not conserved in Xenopus Ac45 and the adjacent, conserved putative furin cleavage site in mouse Ac45 was not recognized by furin (43). Furthermore, since the treatment of Xenopus melanotrope cells with the Golgi-disrupting drug Brefeldin A did not interfere with the proteolytic processing of Xenopus Ac45 (27), Ac45 processing likely takes place in the early secretory pathway, i.e. before the site of furin action (44) has been reached. Thus, furin might not represent the Ac45 cleaving enzyme in Xenopus melanotrope cells.
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cells. Alternatively, in these cells furin could already be activated in the early secretory pathway, as previously found in HEK293 cells (45).

Interestingly, our C-terminally truncated Ac45 mutant was more extensively glycosylated and processed in the secretory pathway than the wild-type Ac45 protein. Removal of the C-tail apparently allowed efficient transport of the Ac45 mutant through the secretory pathway, illustrated by its extensive glycosylation, a Golgi event (46), and its extensive endoproteolytic cleavage. We conclude that, in addition to its role in Ac45 internalization (28), the C-tail also affects Ac45 trafficking efficiency through the secretory pathway. Possibly, the transport of Ac45 resembles that of other type I transmembrane proteins, such as amyloid precursor protein (APP) and peptidylglycine α-aminating monooxygenase (PAM), in which cytoplasmic C-tail-binding proteins play a prominent role in trafficking and processing (47–50).

Besides being efficiently transported through the secretory pathway, our immuno-EM analysis showed that cleaved-Ac45 and the C-tail Ac45 mutant caused recruitment of endogenous V-ATPase to the plasma membrane. In osteoclasts, removal of the Ac45 C-tail still allowed, albeit less tightly, its interaction with the V_{o}-sector of the pump (18). However, we found that removal of the C-tail from the N-terminally cleaved form of Ac45 abolished V-ATPase recruitment, despite of its transport to the plasma membrane. We conclude that domains within both the luminal N-terminal portion and the cytoplasmic C-tail of the Ac45 protein are necessary for interaction with and recruitment of the V-ATPase.

Cleaved-Ac45 and the C-tail Ac45 mutant also affected the dopaminergic inhibition of regulated peptide secretion. Intriguingly, despite of its localization at the plasma membrane the cleaved-Ac45 form lacking its C-tail did not influence the inhibition of regulated exocytosis, although one has to realize that this mutant was expressed at relatively low levels. Nevertheless, affecting dopaminergic inhibition of peptide release apparently necessitates the recruitment of the V-ATPase into the regulated secretory pathway.

In wild-type and the cleaved-Ac45-transgenic melanotrope cells, cytoplasmic Ca^{2+}-oscillations, the driving force for secre-
tion (42, 51), were similarly inhibited upon apomorphine treatment, suggesting that D2-receptor activation and the resulting inhibition of high-voltage activated Ca2+-channels (36, 52) were unaffected. The low-amplitude Ca2+-oscillations observed in the transgenic cells apparently provide sufficient driving force for regulated Ca2+-dependent membrane fusion. We recently showed that in the cleaved-Ac45-transgenic cells the secretion efficiency (the direct link between influx of Ca2+ and exocytosis) was substantially increased (24). Furthermore, these transgenic cells displayed elevated levels of basal peptide release (25), showing that also under non-inhibitory conditions Ca2+-dependent secretion is enhanced. In addition, in the cleaved-Ac45-transgenic melanotrope cells the regulated secretory vesicles were more acidified (25). We hypothesize that an increased V-ATPase recruitment to the regulated vesicles provides a higher abundance of V0 in the vesicular membrane. V0 recruits via its V0a subunit the small Ca2+-binding protein calmodulin (15) and interacts with the SNARE fusion machinery via synaptobrevin (16) and therefore may facilitate Ca2+-dependent secretion.

Taking our results together, we conclude that domains within the N-terminal portion and in the C-tail of Ac45 play a key role in Ac45 transport, V-ATPase recruitment and the process of Ca2+-dependent regulated exocytosis.

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