THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 33, pp. 27537–27546, August 10, 2012

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

Received for publication, February 27, 2012, and in revised form, June 1, 2012 Published, JBC Papers in Press, June 26, 2012, DOI 10.1074/jbc.M112.356105

Eric J. R. Jansen[‡], Nick. H. M. van Bakel[‡], Nikkie F. M. Olde Loohuis[‡], Theo G. M. Hafmans[‡], Tim Arentsen[‡], Anthon J. M. Coenen[‡], Wim J. J. M. Scheenen[§], and Gerard J. M. Martens[‡1]

From the Departments of [‡]Molecular Animal Physiology and [§]Cellular Animal Physiology, Donders Institute for Brain, Cognition, and Behaviour and Nijmegen Centre for Molecular Life Sciences (NCMLS), Faculty of Science, Radboud University Nijmegen, Geert Grooteplein Zuid 28, 6525 GA Nijmegen, The Netherlands

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 β-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.

[1] To whom correspondence should be addressed: Department of Molecular Animal Physiology, RT 282, Donders Institute for Brain, Cognition and Behaviour, Centre for Neuroscience and Nijmegen Centre for Molecular Life Sciences (NCMLS), Faculty of Science, Radboud University, Geert Grooteplein Zuid 28, 6525 GA Nijmegen, The Netherlands. Tel.: 31-24-3610564; Fax: 31-24-3615317; E-mail: g.martens@ncmls.ru.nl.

[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.

In specialized cell types such as osteoclasts (18–20), and neuroendocrine chromaffin and pituitary cells (21, 22) the V_0-sector 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 ~20-kDa Ac45 N-terminal processing product) were generated by stable *Xenopus* transgenesis using the GFP-transgene constructs described below. The generation of transgenic *Xenopus*, F1 offspring and transgenic lines #452 (intact-Ac45) and #533 (cleaved-Ac45: the naturally-occurring C-terminal ~40-kDa Ac45 protein), have been previously described and defined as GFP/intact-Ac45 and GFP/cleaved-Ac45, respectively (24). For the generation of the Ac45ΔCS mutant, SpeI restriction sites were first introduced into wild-type *Xenopus* Ac45 (clone X1311–4, Ref. 21) using the Quickchange Mutagenesis kit (Stratagene) and the primers: 5′-gaccaagcaattggacaaactagtagcacattaaagtcagagggtg-3′ and 5′-caccctctgactttaatgtgctactagtttgtccaattgcttggtc-3′ (mutation 1, nt 569–574 of clone X1311–4) and 5′-gcgccagttacttgccactactagtcctatgccaagctatcctcc-3′ and 5′-ggaggatagcttggcataggactagtagtggcaagtaactggcgc-3′ (mutation 2, nt 695–700 of clone X1311–4). Following a SpeI digestion, the cDNA was re-ligated and amplified by PCR using forward primer 5′-gggggaattccagcaagtgcccgtgctg-3′ and reverse primer 5′-ggggtctagattactctgtctggggcacagc-3′. For obtaining cDNA encoding Ac45ΔC and cleaved-Ac45ΔC fused to GFP, the *Xenopus* Ac45 ORF (without the signal peptide) was amplified by PCR using the forward primer 5′-gggggaattccagcaagtgcccgtgctg-3′ or forward primer 5′-gggggaattccctatgccaagctatcctcc-3′, respectively, with reverse primer 5′-ggggtctagactaaaccatgtgcagtccatagg-3′ introducing a stop codon proceeding Val^{411} of *Xenopus* Ac45. The Ac45ΔCS, Ac45ΔC or cleaved-Ac45ΔC PCR products were cloned into EcoRI/XbaI restriction sites of the pPOMC(A)$^{2+}$-SP-GFP vector (30). For fusing intact-Ac45 and Ac45Nterm C-terminally to GFP, the *Xenopus* Ac45 ORF (including the signal peptide) was amplified by PCR using the forward primer 5′-gggggatccgaattcaacagagatggcagcgatgg-3′ and reverse primer 5′-gcgcggatccctctgtctggggcacagc-3′ or forward primer: 5′-gggggatccaggctgctcagtggcaag-3′ and reverse primer 5′-gcgcggatccctctgtctggggcacagc-3′, respectively. The PCR fragments were cloned into the EcoRI/BamHI sites of N3delAUG-EGFP (Invitrogen). Next, the EcoRI/XbaI-digested inserts were subcloned into the pPOMC^{2+} vector (31) and used for stable *Xenopus* transgenesis.

Antibodies—The anti-*Xenopus* Ac45-C and anti-Ac45-N rabbit antisera (1311N and 1311C, respectively) have been described previously (21). A polyclonal antiserum raised against GFP (32) was kindly provided by Dr. B. Wieringa (Radboud University Nijmegen, The Netherlands). Rabbit antisera against *Xenopus* POMC (ST62, Ref. 33) and *Xenopus* V1A (ST170, Ref. 24) were obtained from Dr. S. Tanaka (Shizuoka University, Japan) and the rabbit antiserum against *Xenopus* calnexin (34) was a generous gift of Dr. K. Geering (University of Lausanne, Switzerland). We generated a rabbit antiserum (ST201) against a synthetic peptide comprising 16 amino acid residues located between transmembrane domains 2 and 3 of the *Xenopus* NaK-ATPase α-subunit with an additional cysteine at the N terminus (CKVDNSSLTGESEPQTR).

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 antisera and with secondary peroxidase-conjugated goat-anti-rabbit antibody (1:5000) followed by chemoluminescence. Signals were detected and quantified using a BioImaging system with Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK).

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

Melanotrope Cell Isolation, Live Cell Imaging, and Immunofluorescence—Melanotrope cells were isolated from freshly dissected *Xenopus* NILs as described previously (21) and plated onto poly-L-lysine coated glass bottom dishes (Willco-

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 NH_4Cl/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 μM 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 (*i.e.* no need for immunoprecipitation).

Immunoelectron Microscopy—Immunoelectron microscopy on *Xenopus* NILs using an anti-GFP or an anti-V_1A antibody was described previously (24).

Fluorescence Measurements of Intracellular Ca^{2+}—Dynamic video imaging to measure intracellular Ca^{2+} was essentially performed as described previously (36). Ca^{2+}-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 μM 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 (GFP/intact-Ac45 and intact-Ac45/GFP, respectively). The cDNAs were placed under the control of a POMC gene promoter fragment (31) (Fig. 1*A*) and stable transgenic *Xenopus* lines were established expressing the fusion proteins specifically in the intermediate pituitary melanotrope cells (Fig. 1*B*). To study the steady-state protein expression levels in the transgenic NILs, we performed Western blot analysis with anti-GFP and anti-Ac45-C antibodies. Analysis of the GFP/intact-Ac45 NIL lysate with the anti-GFP antibody showed a product of ~90 kDa, representing the intact-Ac45 protein fused to GFP. The minor ~50 kDa band corresponds to the size of the N-terminal Ac45 cleavage product fused to GFP. The GFP/intact-Ac45 protein was also recognized by the anti-Ac45-C antibody (Fig. 1*C*). In the intact-Ac45/GFP NIL lysate, an ~90 kDa product corresponding to intact-Ac45/GFP and an ~70 kDa representing the C-terminal Ac45 cleavage product fused to GFP (cleaved-Ac45/GFP) were observed. The ~28 kDa product probably corresponds to the stable GFP moiety of the fusion protein, likely resulting from the degradation of the Ac45 portion. The anti-Ac45-C antibody did however not recognize the intact-Ac45/GFP product (Fig. 1*C*). 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, Ac45ΔC, cleaved-Ac45, cleaved-Ac45ΔC, and Ac45Nterm (Fig. 2*A*). 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. 2*B*).

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. 2*C*). In the Ac45ΔC NIL lysate, we detected a major ~90-kDa protein corresponding to the non-cleaved C-terminally truncated Ac45 fusion protein (Fig. 2*C*). 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 72k-Da 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 products, 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. 3*A*). 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-

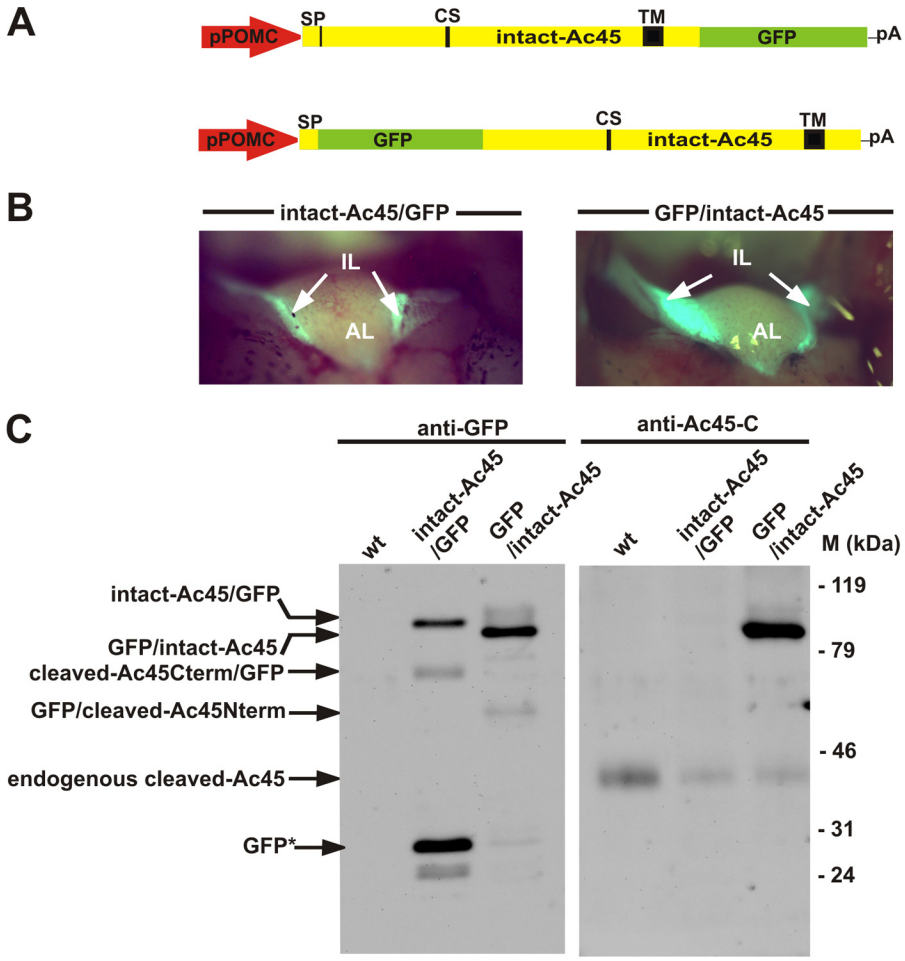

FIGURE 1. **C-terminal fusion of GFP to Ac45 interferes with C-terminal antibody recognition.** *A*, transgenes used to generate transgenic *Xenopus* with expression of intact-Ac45 C- or N-terminally fused to GFP specifically in the intermediate pituitary melanotrope cells. *B*, direct GFP fluorescence was observed only in the IL and not in the AL of the pituitary. *C*, intact-Ac45/GFP fusion protein was recognized by the anti-GFP antibody but not by the anti-Ac45-C antibody. The GFP/intact-Ac45 fusion protein was recognized by both antibodies. *SP*, signal peptide; *CS*, cleavage site; *TM*, transmembrane domain; *pA*, SV40 polyadenylation site.

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

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. 3*B*). 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. 4*A*). Immuno-EM further revealed that the cleaved-Ac45 product was mainly localized to microvillar structures at the plasma membrane (Fig. 4*C* 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. 4*E*). Cleaved-Ac45 co-localized with the endogenous V-ATPase A subunit in the microvillar structures (Fig. 4*G* and Ref. 24), and Ac45ΔC in both the microvillar structures and the non-ruffled plasma membranes (Fig. 4*F*). The localization of the endogenous V-ATPase A subunit in the cytoplasm and its partial association with vesicular structures in the cleaved-Ac45ΔC transgenic

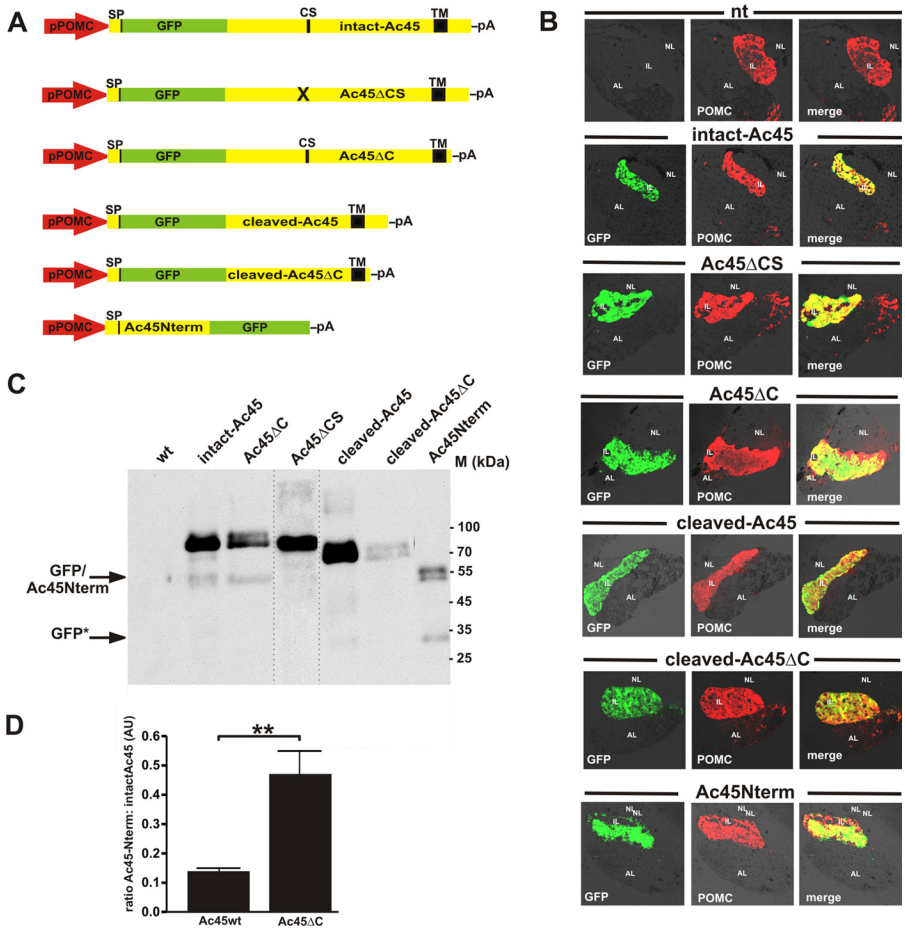

FIGURE 2. **Transgene expression of GFP-Ac45 mutant proteins specifically in the *Xenopus* melanotrope cells.** *A*, overview of transgenes used to express Ac45 mutant proteins fused to GFP in the *Xenopus* melanotrope cells. *B*, sagital cryosections of transgenic *Xenopus* pituitaries. Transgenic Ac45 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 Ac45ΔCS 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 Ac45ΔC 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 Ac45ΔC, respectively. Shown are the means ± S.E. ($n = 4$). Significant difference is indicated by ** ($p < 0.01$).

cells was similar to that in wild-type melanotrope cells (Fig. 4, *E* and *H*, respectively), suggesting that cleaved-Ac45ΔC 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. The radiolabeled proteins are therefore 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. 5*A*). 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. 5*A*). Immunoprecipitations from the NIL lysates with an anti-Ac45-N antibody revealed the specific expression of two protein products of ~50 and ~55 kDa in the transgenic NILs, representing Ac45Nterm transgene products. Since immunoprecipitations from the incubation medium only detected the ~55-kDa Ac45Nterm form, the 50-kDa protein most likely represents its nonglycosylated form and the 55-kDa product the glycosylated form (Fig. 5*B*). 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. 5*A*), showing that the transgene product was secreted in a regulated fashion.

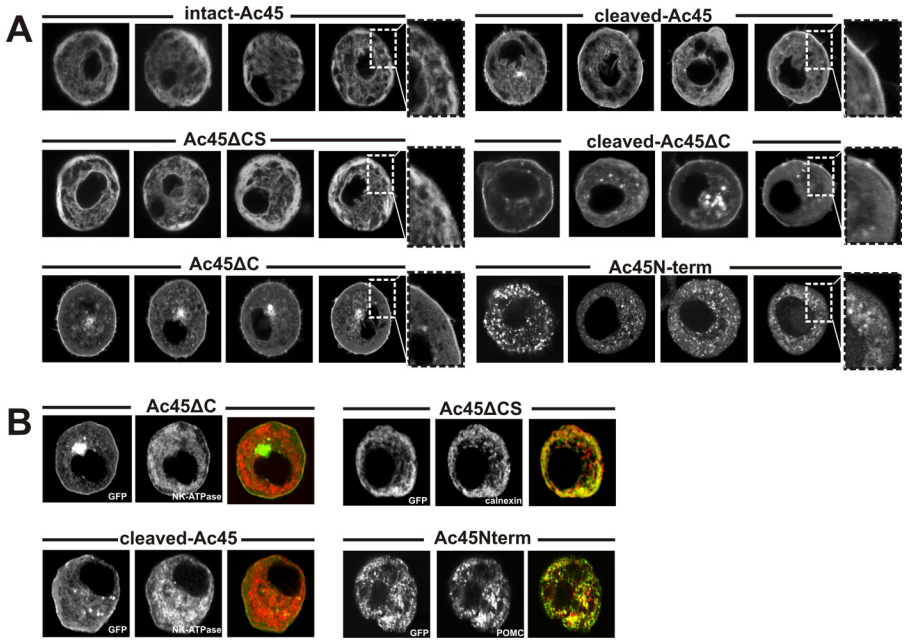

FIGURE 3. **Subcellular localization of GFP-Ac45mutant proteins in transgenic *Xenopus* melanotrope cells.** *A*, *Xenopus* melanotrope cells were isolated from the transgenic pituitaries, cultured and examined with live imaging for GFP fluorescence. Note that in the active melanotrope cells the ER is situated near the plasma membrane (53). *B*, melanotrope cells were fixed and stained with the marker antibodies anti-NaK-ATPase (plasma membrane), anti-calnexin (for the ER) and anti-POMC (for secretory vesicles).

The Dopaminergic Inhibition of Peptide Secretion Is Differentially Affected by the Ac45 Mutant Proteins—Since Ac45 is associated with the V_0-sector of the V-ATPase (18, 26, 39) and given the involvement of V_0 in Ca^{2+}-dependent exocytosis (11, 12), we next wondered how the various Ac45 mutants affect Ca^{2+}-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, Ac45ΔCS, Ac45Nterm or cleaved-Ac45ΔC was inhibited. Surprisingly, in the presence of apomorphine transgenic NILs expressing cleaved-Ac45 or Ac45ΔC 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 Ac45ΔCS), localized to the plasma membrane without affecting V-ATPase localization (cleaved-Ac45ΔC) or secreted by the cells (Ac45Nterm) 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 Ac45ΔC, see above) affect regulated peptide release by the melanotrope cells.

The Dopaminergic Inhibition of Ca^{2+} Oscillations Is Not Affected in the Cleaved Ac45 Transgenic Melanotrope Cells—Since regulated peptide secretion by the cleaved-Ac45 and Ac45ΔC-transgenic melanotrope cells was not effectively inhibited by apomorphine, we wondered whether these transgenic cells would still display normal dopamine D_2-receptor functioning. In wild-type melanotrope cells, Ca^{2+}-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 Ca^{2+}-probe fura-2 and in the absence of apomorphine, both wild-type and the cleaved-Ac45-transgenic melanotrope cells displayed spontaneous intracellular Ca^{2+}-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 Ca^{2+}-oscillations, resulting in minimal, close-to-noise level, Ca^{2+}-oscillations, whereas the remainder of the cells displayed low-amplitude Ca^{2+}-oscillations within the 10-min incubation period (compare Fig. 7, *A* and *B*). Removal of the drug reversed the inhibition and Ca^{2+}-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 Ca^{2+}-oscillations sustained up to 180 min. Comparable with the 10-min measurements, ~25% of the wild-type and transgenic cells gained low-amplitude Ca^{2+}-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 D_2-receptor functioning was unaffected, but the inhibition of the Ca^{2+}-oscillations did no longer lead to the inhibition of peptide secretion.

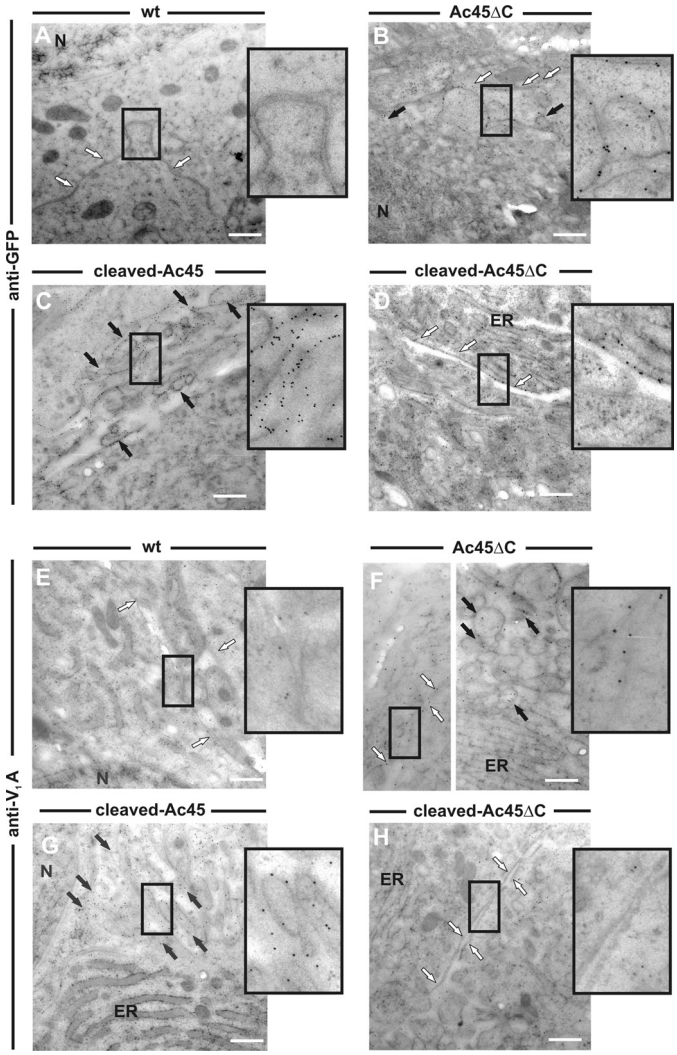

FIGURE 4. **Ac45 mutant proteins differentially affect endogenous V-ATPase localization.** *A–D*, immunogold labeling of ultra-thin pituitary cryosections with anti-GFP antibody confirmed the expression of the GFP/Ac45 mutant transgene products at the plasma membrane of the transgenic *Xenopus* melanotrope cells. No labeling was found in wild-type cells. *E–H*, immunogold labeling with an anti-V_1A antibody showed that in the cleaved-Ac45 and Ac45 ΔC transgenic melanotrope cells the endogenous V-ATPase is recruited to the plasma membrane but not in wild-type cells nor in transgenic cells expressing cleaved-Ac45ΔC. *White arrow*, smooth plasma membrane; *black arrow*, microvillar plasma membrane; *N*, nucleus; *ER*, endoplasmic reticulum .

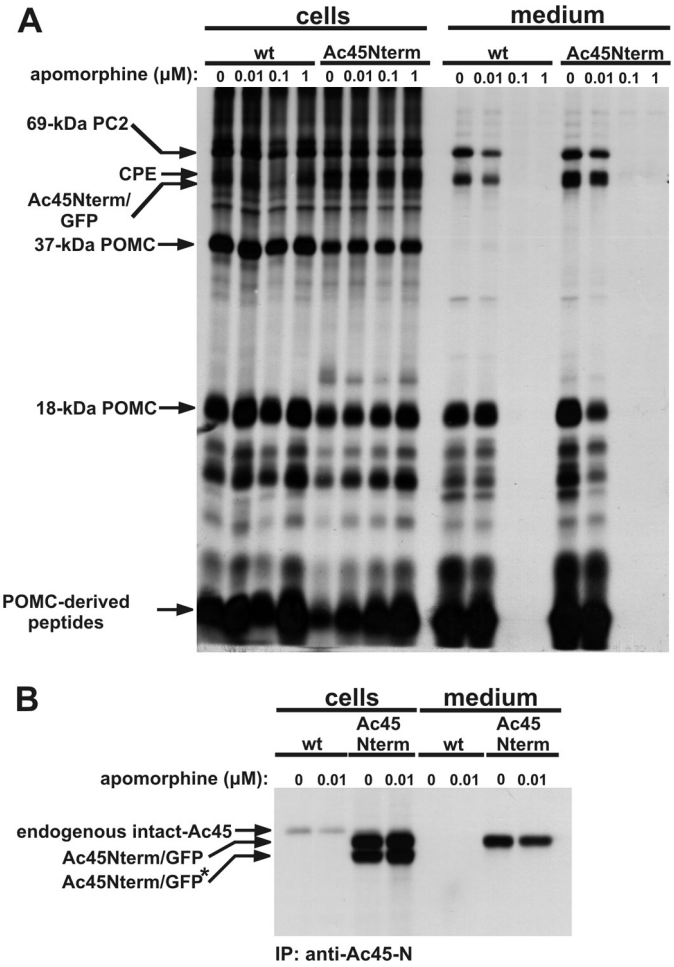

FIGURE 5. **Ac45Nterm is secreted via the regulated secretory pathway.** Wild-type (*wt*) and Ac45Nterm-transgenic NILs were pulse labeled for 30 min and chased for 180 min in the presence of various concentrations of apomorphine, as indicated. *A*, five percent of the total labeled NIL lysates and 20% of the incubation media were directly resolved by SDS-PAGE. *B*, NIL lysates and incubation media were incubated with an anti-Ac45-N antibody, and the immune complexes were immunoprecipititated using protein A-Sepharose and resolved by SDS-PAGE. Radioactive signals were visualized by autoradiography.

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 D_2-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}-Gln^{207} of *Xenopus* Ac45; (21) prevented its processing and caused its accumulation in the ER. This finding indicates that region Val^{164}-Gln^{207}, 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. 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-amidating monooxygenase (PAM), in which cytoplasmic C-tail-binding proteins play a prominent role in trafficking and processing (47–50).

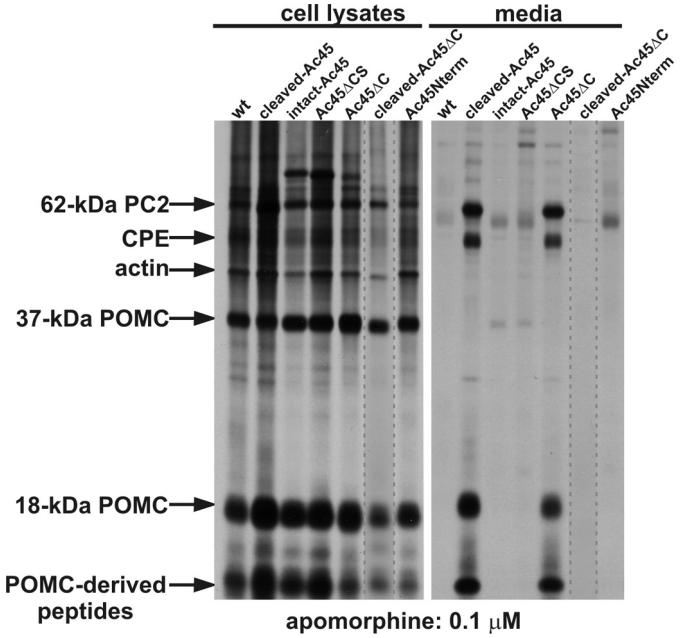

FIGURE 6. **Dopaminergic inhibition of peptide release is affected in cleaved-Ac45- and Ac45 ΔC-transgenic *Xenopus* melanotrope cells.** Wild-type (*wt*) and transgenic NILs were pulse-labeled for 30 min and chased for 180 min in the presence of 0.1 μM apomorphine. NILs were lysed and 5% of the total lysates and 20% of the incubation media was directly analyzed by SDS-PAGE. The lane with the cleaved-Ac45 ΔC NIL lysate was taken from a separate gel. Radioactive signals were visualized by autoradiography.

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_0-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-

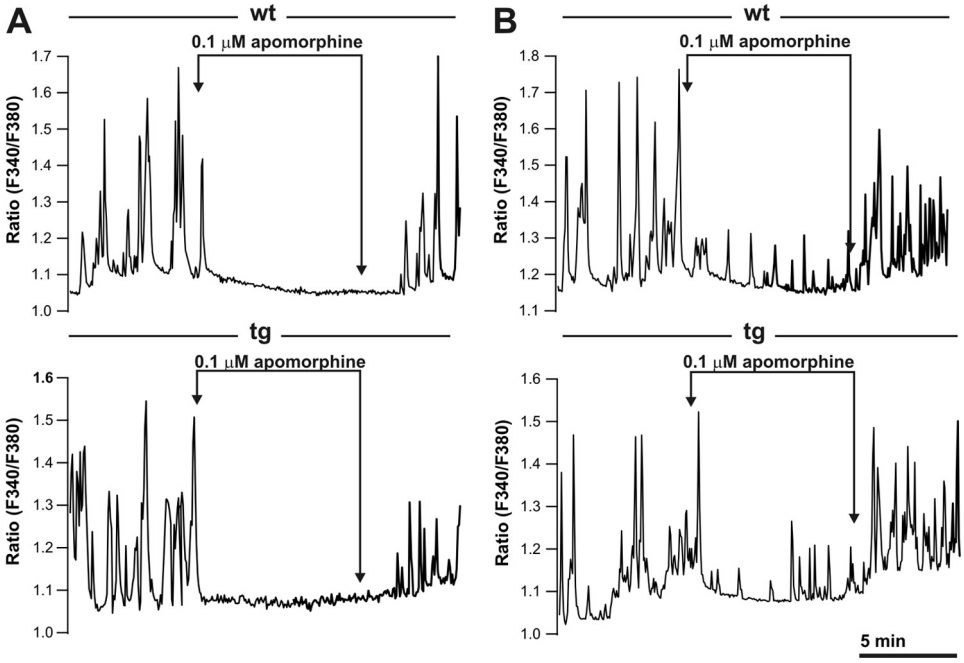

FIGURE 7. **Excess of cleaved-Ac45 does not affect the inhibition of the spontaneous Ca^{2+}-oscillations in cleaved-Ac45-transgenic *Xenopus* melanotrope cells.** *A*, Ca^{2+}-oscillations in wild-type (*wt*) and cleaved-Ac45-transgenic (*tg*) cells loaded with fura-2 are inhibited in the presence of 0.1 μM apomorphine and reappear after removal of the drug. *B*, in the presence of 0.1 μM apomorphine low-frequency Ca^{2+}-oscillations occur in ~25% of the wt and tg cells.

tion (42, 51), were similarly inhibited upon apomorphine treatment, suggesting that D_2-receptor activation and the resulting inhibition of high-voltage activated Ca^{2+}-channels (36, 52) were unaffected. The low-amplitude Ca^{2+}-oscillations observed in the transgenic cells apparently provide sufficient driving force for regulated Ca^{2+}-dependent membrane fusion. We recently showed that in the cleaved-Ac45-transgenic cells the secretion efficiency (the direct link between influx of Ca^{2+} 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 Ca^{2+}-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 V_0 in the vesicular membrane. V_0 recruits via its V_0a subunit the small Ca^{2+}-binding protein calmodulin (15) and interacts with the SNARE fusion machinery via synaptobrevin (16) and therefore may facilitate Ca^{2+}-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 Ca^{2+}-dependent regulated exocytosis.

Acknowledgments—We thank Drs. B. Wieringa (Radboud University, Nijmegen, The Netherlands), K.Geering (University of Lausanne, Switzerland), and S. Tanaka (Shizuoka University, Japan) for antibodies and Ron Engels for animal care. Microscopic imaging was performed at the Microscopic Imaging Centre (MIC) of the NCMLS.

REFERENCES

1. Nishi, T., and Forgac, M. (2002) The vacuolar (H+)-ATPases–nature's most versatile proton pumps. *Nat. Rev. Mol. Cell Biol.* **3,** 94–103
2. Adams, D. S., Robinson, K. R., Fukumoto, T., Yuan, S., Albertson, R. C., Yelick, P., Kuo, L., McSweeney, M., and Levin, M. (2006) Early, H+-V-ATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. *Development* **133,** 1657–1671
3. Cruciat, C. M., Ohkawara, B., Acebron, S. P., Karaulanov, E., Reinhard, C., Ingelfinger, D., Boutros, M., and Niehrs, C. (2010) Requirement of prorenin receptor and vacuolar H+-ATPase-mediated acidification for Wnt signaling. *Science* **327,** 459–463
4. Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D., and Geibel, J. P. (2004) Renal vacuolar H^+-ATPase. *Physiol. Rev.* **84,** 1263–1314
5. Paroutis, P., Touret, N., and Grinstein, S. (2004) The pH of the secretory pathway: measurement, determinants, and regulation. *Physiology* **19,** 207–215
6. Ungermann, C., Wickner, W., and Xu, Z. (1999) Vacuole acidification is required for trans-SNARE pairing, LMA1 release, and homotypic fusion. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11194–11199
7. Schoonderwoert, V. T., Holthuis, J. C., Tanaka, S., Tooze, S. A., and Martens, G. J. (2000) Inhibition of the vacuolar H+-ATPase perturbs the transport, sorting, processing, and release of regulated secretory proteins. *Eur. J. Biochem.* **267,** 5646–5654
8. Tanaka, S., Yora, T., Nakayama, K., Inoue, K., and Kurosumi, K. (1997) Proteolytic processing of pro-opiomelanocortin occurs in acidifying secretory granules of AtT-20 cells. *J. Histochem. Cytochem.* **45,** 425–436
9. Camacho, M., Machado, J. D., Montesinos, M. S., Criado, M., and Borges, R. (2006) Intragranular pH rapidly modulates exocytosis in adrenal chromaffin cells. *J. Neurochem.* **96,** 324–334
10. Beyenbach, K. W., and Wieczorek, H. (2006) The V-type H^+ ATPase: molecular structure and function, physiological roles, and regulation. *J. Exp. Biol.* **209,** 577–589
11. Peters, C., Bayer, M. J., Bühler, S., Andersen, J. S., Mann, M., and Mayer, A. (2001) Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature* **409,** 581–588
12. Hiesinger, P. R., Fayyazuddin, A., Mehta, S. Q., Rosenmund, T., Schulze, K. L., Zhai, R. G., Verstreken, P., Cao, Y., Zhou, Y., Kunz, J., and Bellen, H. J. (2005) The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in *Drosophila*. *Cell* **121,** 607–620
13. Sun-Wada, G. H., Toyomura, T., Murata, Y., Yamamoto, A., Futai, M., and Wada, Y. (2006) The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. *J. Cell Sci.* **119,** 4531–4540
14. Liégeois, S., Benedetto, A., Garnier, J. M., Schwab, Y., and Labouesse, M. (2006) The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in *Caenorhabditis elegans*. *J. Cell Biol.* **173,** 949–961
15. Zhang, W., Wang, D., Volk, E., Bellen, H. J., Hiesinger, P. R., and Quiocho, F. A. (2008) V-ATPase V0 sector subunit a1 in neurons is a target of calmodulin. *J. Biol. Chem.* **283,** 294–300
16. Di Giovanni, J., Boudkkazi, S., Mochida, S., Bialowas, A., Samari, N., Lévêque, C., Youssouf, F., Brechet, A., Iborra, C., Maulet, Y., Moutot, N., Debanne, D., Seagar, M., and El Far, O. (2010) V-ATPase membrane sector associates with synaptobrevin to modulate neurotransmitter release. *Neuron* **67,** 268–279
17. Sobota, J. A., Bäck, N., Eipper, B. A., and Mains, R. E. (2009) Inhibitors of the V0 subunit of the vacuolar H^+-ATPase prevent segregation of lysosomal and secretory pathway proteins. *J. Cell Sci.* **122,** 3542–3553
18. Feng, H., Cheng, T., Pavlos, N. J., Yip, K. H., Carrello, A., Seeber, R., Eidne, K., Zheng, M. H., and Xu, J. (2008) Cytoplasmic terminus of vacuolar type proton pump accessory subunit Ac45 is required for proper interaction with V(0) domain subunits and efficient osteoclastic bone resorption. *J. Biol. Chem.* **283,** 13194–13204
19. Xu, J., Cheng, T., Feng, H. T., Pavlos, N. J., and Zheng, M. H. (2007) Structure and function of V-ATPases in osteoclasts: potential therapeutic targets for the treatment of osteolysis. *Histol. Histopathol.* **22,** 443–454
20. Qin, A., Cheng, T. S., Lin, Z., Pavlos, N. J., Jiang, Q., Xu, J., Dai, K. R., and Zheng, M. H. (2011) Versatile roles of V-ATPases accessory subunit Ac45 in osteoclast formation and function. *PLoS One* **6,** e27155
21. Holthuis, J. C., Jansen, E. J., Schoonderwoert, V. T., Burbach, J. P., and Martens, G. J. (1999) Biosynthesis of the vacuolar H^+-ATPase accessory subunit Ac45 in *Xenopus* pituitary. *Eur. J. Biochem.* **262,** 484–491
22. Holthuis, J. C., Jansen, E. J., van Riel, M. C., and Martens, G. J. (1995) Molecular probing of the secretory pathway in peptide hormone-producing cells. *J. Cell Sci.* **108**, 3295–3305
23. Jansen, E. J., and Martens, G. J. (2012) Novel insights into V-ATPase functioning: distinct roles for its accessory subunits ATP6AP1/Ac45 and ATP6AP2/(pro)renin receptor. *Curr. Protein Pept Sci.* **13,** 124–133
24. Jansen, E. J., Scheenen, W. J., Hafmans, T. G., and Martens, G. J. (2008) Accessory subunit Ac45 controls the V-ATPase in the regulated secretory pathway. *Biochim. Biophys. Acta* **1783,** 2301–2310
25. Jansen, E. J., Hafmans, T. G., and Martens, G. J. (2010) V-ATPase-mediated granular acidification is regulated by the V-ATPase accessory subunit Ac45 in POMC-producing cells. *Mol. Biol. Cell* **21,** 3330–3339
26. Supek, F., Supekova, L., Mandiyan, S., Pan, Y. C., Nelson, H., and Nelson, N. (1994) A novel accessory subunit for vacuolar H(+)-ATPase from chromaffin granules. *J. Biol. Chem.* **269,** 24102–24106
27. Schoonderwoert, V. T., Jansen, E. J., and Martens, G. J. (2002) The fate of newly synthesized V-ATPase accessory subunit Ac45 in the secretory pathway. *Eur. J. Biochem.* **269,** 1844–1853
28. Jansen, E. J., Holthuis, J. C., McGrouther, C., Burbach, J. P., and Martens, G. J. (1998) Intracellular trafficking of the vacuolar H+-ATPase accessory subunit Ac45. *J. Cell Sci.* **111,** 2999–3006
29. Kroll, K. L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122,** 3173–3183
30. Collin, R. W., and Martens, G. J. (2006) The coding sequence of amyloid-β precursor protein APP contains a neural-specific promoter element. *Brain Res.* **1087,** 41–51

31. Jansen, E. J., Holling, T. M., van Herp, F., and Martens, G. J. (2002) Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of *Xenopus laevis*. *FEBS Lett.* **516,** 201–207
32. Cuppen, E., Wijers, M., Schepens, J., Fransen, J., Wieringa, B., and Hendriks, W. (1999) A FERM domain governs apical confinement of PTP-BL in epithelial cells. *J. Cell Sci.* **112,** 3299–3308
33. Berghs, C. A., Tanaka, S., Van Strien, F. J., Kurabuchi, S., and Roubos, E. W. (1997) The secretory granule and pro-opiomelanocortin processing in *Xenopus* melanotrope cells during background adaptation. *J. Histochem. Cytochem.* **45,** 1673–1682
34. Beggah, A. T., and Geering, K. (1997) α and β subunits of Na, K-ATPase interact with BiP and calnexin. *Ann. N.Y. Acad. Sci.* **834,** 537–539
35. Braks, J. A., and Martens, G. J. (1994) 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. *Cell* **78,** 263–273
36. Zhang, H. Y., Langeslag, M., Voncken, M., Roubos, E. W., and Scheenen, W. J. (2005) Melanotrope cells of *Xenopus laevis* express multiple types of high-voltage-activated Ca^{2+} channels. *J. Neuroendocrinol.* **17,** 1–9
37. Martens, G. J. (1986) Expression of two proopiomelanocortin genes in the pituitary gland of *Xenopus laevis*: complete structures of the two prepro-hormones. *Nucleic Acids Res.* **14,** 3791–3798
38. Roubos, E. W. (1997) Background adaptation by *Xenopus laevis*: a model for studying neuronal information processing in the pituitary pars intermedia. *Comp Biochem. Physiol. A. Physiol.* **118,** 533–550
39. Getlawi, F., Laslop, A., Schägger, H., Ludwig, J., Haywood, J., and Apps, D. (1996) Chromaffin granule membrane glycoprotein IV is identical with Ac45, a membrane-integral subunit of the granule's H(+)-ATPase. *Neurosci. Lett.* **219,** 13–16
40. Scheenen, W. J., Jenks, B. G., Roubos, E. W., and Willems, P. H. (1994) Spontaneous calcium oscillations in *Xenopus laevis* melanotrope cells are mediated by omega-conotoxin sensitive calcium channels. *Cell Calcium* **15,** 36–44
41. Shibuya, I., and Douglas, W. W. (1993) Spontaneous cytosolic calcium pulsing detected in *Xenopus* melanotrophs: modulation by secreto-inhibitory and stimulant ligands. *Endocrinology* **132,** 2166–2175
42. Jenks, B. G., Roubos, E. W., and Scheenen, W. J. (2003) Ca2+ oscillations in melanotropes of *Xenopus laevis*: their generation, propagation, and function. *Gen. Comp. Endocrinol.* **131,** 209–219
43. Louagie, E., Taylor, N. A., Flamez, D., Roebroek, A. J., Bright, N. A., Meulemans, S., Quintens, R., Herrera, P. L., Schuit, F., Van de Ven, W. J., and Creemers, J. W. (2008) Role of furin in granular acidification in the endocrine pancreas: identification of the V-ATPase subunit Ac45 as a candidate substrate. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 12319–12324
44. Thomas, G. (2002) Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat. Rev. Mol. Cell Biol.* **3,** 753–766
45. Bass, J., Turck, C., Rouard, M., and Steiner, D. F. (2000) Furin-mediated processing in the early secretory pathway: sequential cleavage and degradation of misfolded insulin receptors. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 11905–11909
46. Roth, J. (2002) Protein *N*-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. *Chem. Rev.* **102,** 285–303
47. Alam, M. R., Caldwell, B. D., Johnson, R. C., Darlington, D. N., Mains, R. E., and Eipper, B. A. (1996) Novel proteins that interact with the COOH-terminal cytosolic routing determinants of an integral membrane peptide-processing enzyme. *J. Biol. Chem.* **271,** 28636–28640
48. Mains, R. E., Alam, M. R., Johnson, R. C., Darlington, D. N., Bäck, N., Hand, T. A., and Eipper, B. A. (1999) Kalirin, a multifunctional PAM COOH-terminal domain interactor protein, affects cytoskeletal organization and ACTH secretion from AtT-20 cells. *J. Biol. Chem.* **274,** 2929–2937
49. Burgos, P. V., Mardones, G. A., Rojas, A. L., daSilva, L. L., Prabhu, Y., Hurley, J. H., and Bonifacino, J. S. (2010) Sorting of the Alzheimer's disease amyloid precursor protein mediated by the AP-4 complex. *Dev. Cell* **18,** 425–436
50. Kuan, Y. H., Gruebl, T., Soba, P., Eggert, S., Nesic, I., Back, S., Kirsch, J., Beyreuther, K., and Kins, S. (2006) PAT1a modulates intracellular transport and processing of amyloid precursor protein (APP), APLP1, and APLP2. *J. Biol. Chem.* **281,** 40114–40123
51. Lieste, J. R., Koopman, W. J., Reynen, V. C., Scheenen, W. J., Jenks, B. G., and Roubos, E. W. (1998) Action currents generate stepwise intracellular Ca^{2+} patterns in a neuroendocrine cell. *J. Biol. Chem.* **273,** 25686–25694
52. Zhang, H., Jenks, B. G., Ciccarelli, A., Roubos, E. W., and Scheenen, W. J. (2004) Dopamine D2-receptor activation differentially inhibits N- and R-type Ca^{2+} channels in *Xenopus* melanotrope cells. *Neuroendocrinology* **80,** 368–378
53. Van Herp, F., Coenen, T., Geurts, H. P., Janssen, G. J., and Martens, G. J. (2005) A fast method to study the secretory activity of neuroendocrine cells at the ultrastructural level. *J. Microsc.* **218,** 79–83