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# Intracellular trafficking of the vacuolar $H^+$-ATPase accessory subunit Ac45

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## SUMMARY

**Ac45 is a type I transmembrane protein associated with vacuolar $H^+$-ATPase, a proton pump mediating the acidification of multiple intracellular organelles. In this study, we examined the intracellular routing of Ac45 in transfected CV-1 fibroblasts. Steady state immunolabeling showed that Ac45 is located on the plasma membrane and in a vacuolar compartment in the juxtanuclear region. Antibody internalization experiments revealed that Ac45 is rapidly retrieved from the cell surface and is targeted to the vacuolar structures. The 26-residue cytoplasmic tail of Ac45 was intrinsically capable of mediating endocytosis of the cell surface protein Tac, indicating that the tail contains an autonomous internalization signal. Immunolocalization studies on cells expressing carboxy-terminally truncated Ac45 mutants showed the presence of essential routing information in the membrane-distal region of the cytoplasmic tail. Further mutational analysis of this region, which lacks the recognized tyrosine- or di-leucine-based sorting motifs, suggested that multiple sites rather than a short linear sequence are responsible for the internalization. Collectively, our results indicate that the cytoplasmic tail of Ac45 contains autonomous targeting information distinct from previously described routing determinants.**

Key words: $H^+$-ATPase, Ac45, Intracellular trafficking, Cytoplasmic tail

## INTRODUCTION

Acidification of organelles in eukaryotic cells is accomplished by a unique class of ATP-driven proton pumps called vacuolar $H^+$-ATPases (V-ATPases) (Forgac, 1989; Harvey and Nelson, 1992). How V-ATPases are directed to and acquire their specialized activities in a large variety of intracellular organelles and membranes is still unknown. One possible mechanism for generating this topological and functional diversity may arise from V-ATPases assembled with organelle-specific membrane proteins (Nelson, 1992). In a search for organelle-specific proteins in purified preparations of V-ATPase from bovine chromaffin granules, a novel type I transmembrane 45 kDa protein was identified and named Ac45 (Supek et al., 1994). Cold-inactivation experiments revealed that this protein is associated with the membrane sector of the pump. Besides, in bovine adrenal medulla, Ac45 was detected in various parts of the brain and in the pituitary gland. Compared with V-ATPase in chromaffin granules, the pump purified from kidney microsomes contained reduced amounts of Ac45, whereas kidney membrane V-ATPase preparations were devoid of the protein (Supek et al., 1994). Using a differential screening strategy designed to identify genes coexpressed with the prohormone proopiomelanocortin (POMC) in the melanotrope cells of *Xenopus* intermediate pituitary, we recently identified the *Xenopus* ortholog of human and bovine Ac45 (Holthuis et al., 1995).

Intracellular trafficking of type I transmembrane proteins along the secretory and endocytic pathways in eukaryotic cells is often directed by information contained within their cytoplasmic domains (Trowbridge et al., 1993; Sandoval and Bakke, 1994). This information primarily consists of short, linear arrays of amino acid residues that function as sorting signals. Three well-characterized types of sorting signals are tyrosine-based, di-leucine-based and di-lysine-based signals. Tyrosine-based signals generally conform to the motifs N*XX*Y or Y*XX*Z (where Z corresponds to any amino acid with a bulky hydrophobic side chain) (Sandoval and Bakke, 1994), although there are also other contexts in which tyrosine residues appear to be active in sorting (Thomas and Roth, 1994). Di-leucine-based signals, on the other hand, consist of critical LL or LI sequences (Letourneur and Klausner, 1992). Both types of signals can mediate endocytosis of transmembrane proteins through clathrin-coated pits. Tyrosine-based and di-leucine-based signals also participate in sorting at the *trans*-Golgi network (TGN) (Sandoval and Bakke, 1994). Di-lysine-based signals (consensus sequence KK*XX* or K*X*K*XX*) are found at the extreme carboxy termini of many type I transmembrane proteins of the endoplasmic reticulum (ER) (Nilsson et al., 1989; Jackson et al., 1990) and proteins bearing such signals are continuously retrieved from post-ER compartments to the ER (Jackson et al., 1993). The cytoplasmic tail of Ac45 does not contain any of the above sorting signals and the intracellular routing of this protein has not been studied. Here

we describe the intracellular trafficking of Ac45 in transfected CV-1 fibroblasts and find that its cytoplasmic tail contains critical information for the endocytosis and steady state localization of the protein. This internalization signal is distinct from the tyrosine-based, di-leucine-based and di-lysine-based signals.

## MATERIALS AND METHODS

### Recombinant DNA procedures

A 1.7 kb *Eco*RI fragment of *Xenopus* hypothalamus cDNA clone X1311-4 encoding the entire Ac45 protein was subcloned downstream of the cytomegalo virus (CMV) promotor into the *Eco*RI site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). The Ac45/ICAM expression construct, encoding the lumenal domain of *Xenopus* Ac45 followed by the residues TS and then the entire transmembrane and cytoplas mic domains of human intercellular adhesion molecule-1, ICAM-1 (starting NVLSP... at residue 446; Staunton et al., 1988), was obtained by creating *Spe*I sites in the Ac45 and ICAM-1 cDNAs through oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system (Promega, Madison, WI, USA). Oligonucleotides used to create the *Spe*I sites in Ac45 and ICAM-1 were 5′-CAATGTGACTAGTATGGCATTTTCA-3′ and 5′-CACCCGC-GAGGTGACTAGTAATGTGCTCTCCCCC-3′, respectively. The human ICAM-1 cDNA was obtained from Dr C. Figdor (Department of Tumor Immunology, University of Nijmegen, The Netherlands). A pCDM8 expression construct encoding the human interleukin-2 receptor 1α chain (Tac) antigen (Leonard et al., 1984) was obtained from Dr J. S. Bonifacino (National Institute of Health, Bethesda, USA). The Tac45 expression construct, encoding the lumenal- and transmembrane domains of Tac (ending...GLTWQ at residue 262; Leonard et al., 1984) followed by the residues TS and then the entire cytoplasmic domain of *Xenopus* Ac45 was obtained by creating *Spe*I sites in the Tac and Ac45 cDNAs through oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system. Oligonucleotides used to introduce the *Spe*I sites in Ac45 and Tac cDNAs were 5′-CTGACCTATGGACTAGTCACATGGTTATGAG-3′ and 5′-GCTCACCTGGCAGACTAGTCAGAGGAAGAGTAGA-3′, respectively. Carboxy-terminal truncation mutants were constructed by introducing stop codons immediately downstream of the codons for the desired carboxy-terminal amino acid. This was accomplished by oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system. Oligonucleotides used to introduce stop codons behind *Xenopus* Ac45 residues 423, 418 and 411 were 5′-ATCGTTTTGATGATCTCTAGAGCCCAAGCATTGCTG-3′, 5′-TGAAGACTATGGATTGATTTGATGATCCCAA-3′ and 5′-GG-ACTGCACATGGTCTAGAGCCTGAAGACTATG-3′, respectively. Deletion mutant Ac45/deltaC1 ($S^{413}$LKTM$D^{418}$ deleted) and substitution mutant Ac45/A (substitution of the same sequence by six alanine residues) were constructed by oligonucleotide-mutagenesis. Oligonucleotides used were 5′-ATGGACTGCACATGGTTATG-CGTTTTGATGATCCCAAGGG-3′ and 5′-ATGGACTGCACAT-GGTTATGGCTGCTGCTGCTGCTCGTTTTGATGATCCCAA-GGG-3′, respectively. For the construction of the $Thr^{416}$/Ala mutant (Ac45/TA) and the Ac45/FDAR mutant, oligonucleotides 5′-AAGACTATGGATCGTGCTCGAGATCCCAAGGGCCC-3′ and 5′-TGAGCCTGAAGGCTATGGATCGT-3′, respectively, were used. In order to create the Ac45/TFDAAR mutant, the pAlter-1 construct containing Ac45/TA was subjected to a second round of mutagenesis using the same oligonucleotide as used for constructing Ac45/FDAR. The desired mutations were checked by restriction enzyme digestion and double-stranded DNA sequencing. Appropriate fragments of correctly mutagenized cDNAs were subcloned downstream of the CMV promotor into pcDNA3. DNA for transfection studies was isolated using the Qiagen plasmid kit (Qiagen Inc., Chatsworth, CA, USA).

### Cell transfections

Green monkey CV-1 kidney fibroblasts were cultured in Iscoves-modified Eagle's medium (MEM, Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum. For transient expression studies, CV-1 cells were plated on glass coverslips in 20 mm culture dishes, grown until 30% confluency and transfected using the calcium phosphate precipitation method (2.5 μg DNA per construct per dish; Graham and Van der Eb, 1973) or by electroporation. Electroporation of CV-1 cells was performed at 260 V and 1,070 μF in 300 μl of cell suspension at a density of $2.5\times10^6$ cells/ml in Iscoves-MEM containing 10% fetal calf serum, 15 μg plasmid DNA and 200 μg sonicated herring sperm DNA using a 0.4 cm cuvette and the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Following electroporation, cells were gently resuspended in 5 ml Iscoves medium containing 10% fetal calf serum and per cover slip 1 ml of this suspension was used.

### Immunofluorescence microscopy

For steady-state immunofluorescence localization of Ac45 and the Ac45 truncation mutants, transfected CV-1 cells were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.4) for 1 hour at 4°C, incubated in 100 mM glycine/PBS for 30 minutes at 4°C, permeabilized in ice-cold 0.1% Triton X-100/PBS (PBS-TX), and incubated with anti-Ac45 antiserum (1:300) in PBS-TX containing 2% BSA (PBS-TXB) overnight at 4°C. For immunolabeling of cell surface-expressed and internalized protein, cells were incubated with the anti-Ac45 antiserum (1:300) in culture medium for 20 minutes at 4°C, washed and then fixed immediately, or returned to antibody-free culture medium at 37°C for the indicated time periods prior to fixation and permeabilization. Immunolocalization of Tac and the Tac45 fusion protein in transfected CV-1 cells was performed with a mouse monoclonal antibody (7G7) recognizing a lumenal epitope on Tac (Rubin et al., 1985). To visualize bound anti-Ac45 and 7G7 antibodies, permeabilized cells were incubated with fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (1:100; Nordic, Tilburg, The Netherlands) and FITC-conjugated goat anti-mouse antibodies (1:100; Nordic, Tilburg, The Netherlands), respectively, in PBS-TXB for 2 hours at 4°C. For the identification of the intracellular compartment to which Ac45 was targeted, a number of double immunofluorescence studies with CV-1 fibroblasts were performed. First, cells double transfected with the Ac45 expression construct and an expression construct containing myc-tagged sialyltransferase cDNA cloned behind the CMV promoter were used. The expression of the c-myc-tagged *trans*-Golgi sialyltransferase was studied with the anti-myc monoclonal antibody 9E10 (1:50). The monoclonal antibody was raised against a synthetic peptide immunogen derived from the human c-myc proto-oncoprotein (Evan et al., 1985). The second antibodies used were goat anti-rabbit Texas Red and goat anti-mouse FITC. The sialyltransferase cDNA construct and anti-myc antibody were kindly provided by Dr S. Munro (MRC Laboratory of Molecular Biology, Cambridge, UK). Second, for simultaneous immunolocalization of transfected Ac45 and the endogenously expressed V-ATPase subunit Ac116, CV-1 cells were fixed, permeabilized, consecutively incubated with mouse monoclonal antibody OSW2 (directed against the 116 kDa subunit; Sato and Toyama, 1994), FITC-conjugated goat anti-mouse antibodies (1:100; Nordic, Tilburg, The Netherlands), anti-Ac45 antiserum (1:300) and tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-rabbit antibodies (1:100; Nordic, Tilburg, The Netherlands). Third, colocalization studies with Ac45 and the late endosomal/lysosomal markers CD63, Lamp-1 and Lamp-2 were performed. Anti-CD63 monoclonal ascites (1:1,000, Dr D. Roos, Experimental Immunohematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The

Netherlands) was used in a 1:1,000 dilution, the anti-human-Lamp-1 monoclonal antibody ascites H4A3 in a 1:200 dilution and the anti-human-Lamp-2 monoclonal antibody ascites H4B4 (Dr J. T. August, Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) in a 1:500 dilution. Texas Red-conjugated goat-anti-rabbit (1:100; Jackson ImmunoResearch Laboratories,Inc, West Grove, USA) and FITC-conjugated goat-anti-mouse (1:100; Nordic, Tilburg, The Netherlands) antibodies detected bound rabbit and mouse IgGs, respectively. In order to visualize the endocytotic pathway in transfected CV-1 cells, cells were grown in the absence of serum for 15 minutes, rinsed three times with Iscoves MEM, cultured in 30 μg/ml FITC-conjugated transferrin (Molecular Probes Inc., Eugene, OR, USA) in Iscoves MEM for 30 minutes, washed three times with ice-cold PBS and fixed with 4% paraformaldehyde for 60 minutes at 4°C. Following internalization of transferrin, cells were processed for immunofluorescense microscopy as described above for the Ac45 steady-state studies. In all immunofluorescence experiments, the immunostained cells were mounted in Citifluor (Agar Scientific Ltd, Stansted, Essex, UK) and viewed under epifluorescence optics with a Leica DMRB/E microscope (Leica, Heerbrugg, Switzerland) equipped with a Vario Orthomat camera system.

## RESULTS AND DISCUSSION

### Cell surface expression and internalization of Ac45

Immunofluorescence studies on primary cultures of *Xenopus* melanotropes have indicated that Ac45 occurs in the secretory granules of these cells and that low but significant amounts of the protein are present on the cell surface (J. C. M. Holthuis, E. J. R. Jansen, V. Th. G. Schoonderwoert and G. J. M. Martens, unpublished observations). To investigate if Ac45 is capable of reaching the plasma membrane in transfected CV-1 fibroblasts, these cells were incubated with anti-Ac45 antibody (Ab) at 4°C, and then fixed and stained with FITC-conjugated secondary Ab. As shown in Fig. 1 (left panel, '0-minutes' micrograph), the transfected CV-1 cells displayed considerable Ac45 immunoreactivity on their surface. Ab-uptake experiments revealed that this cell surface-expressed Ac45 is rapidly internalized. Following a 10 minute chase period in Ab-free medium at 37°C, the Ac45-Ab complexes formed on the surface of live cells appeared in numerous small intracellular vesicles the majority of which had a juxtanuclear position (Fig. 1, '10-minutes' micrograph). By 30 minutes of chase, most of these immune complexes were associated with larger vesicles that had a more wide-spread distribution (Fig. 1, '30-minutes' micrograph). To find out if the binding of Ab to cell surface-expressed Ac45 by itself is sufficient to trigger internalization, we compared the trafficking of Ac45 with that of a chimeric protein (Ac45/ICAM) in which the lumenal domain of Ac45 is fused to the transmembrane and cytoplasmic portions of the plasma membrane protein ICAM-1 (Staunton et al., 1988). Immunostaining of permeabilized transfected CV-1 cells showed that, unlike Ac45, the Ac45/ICAM fusion protein accumulated on the cell surface (Fig. 1, compare 'steady state' micrographs). When live cells were decorated with anti-Ac45 Ab and then chased in Ab-free medium for up to 30 minutes, hardly any internalization of the fusion protein-Ab complexes was observed (Fig. 1b, right panel), indicating that the internalization of Ac45 is not triggered by the antibody. Ac45 retrieved from the surface of transfected fibroblasts was rapidly transported to the Golgi area where it accumulated in vacuolar structures similar to those containing the protein in fixed permeabilized cells. It therefore appears that internalized Ac45 is responsible for much of the steady-state immunofluorescence staining observed in the juxtanuclear region of the cells.

In order to identify in the transfected CV-1 cells the vacuolar structures containing Ac45, a number of marker proteins were

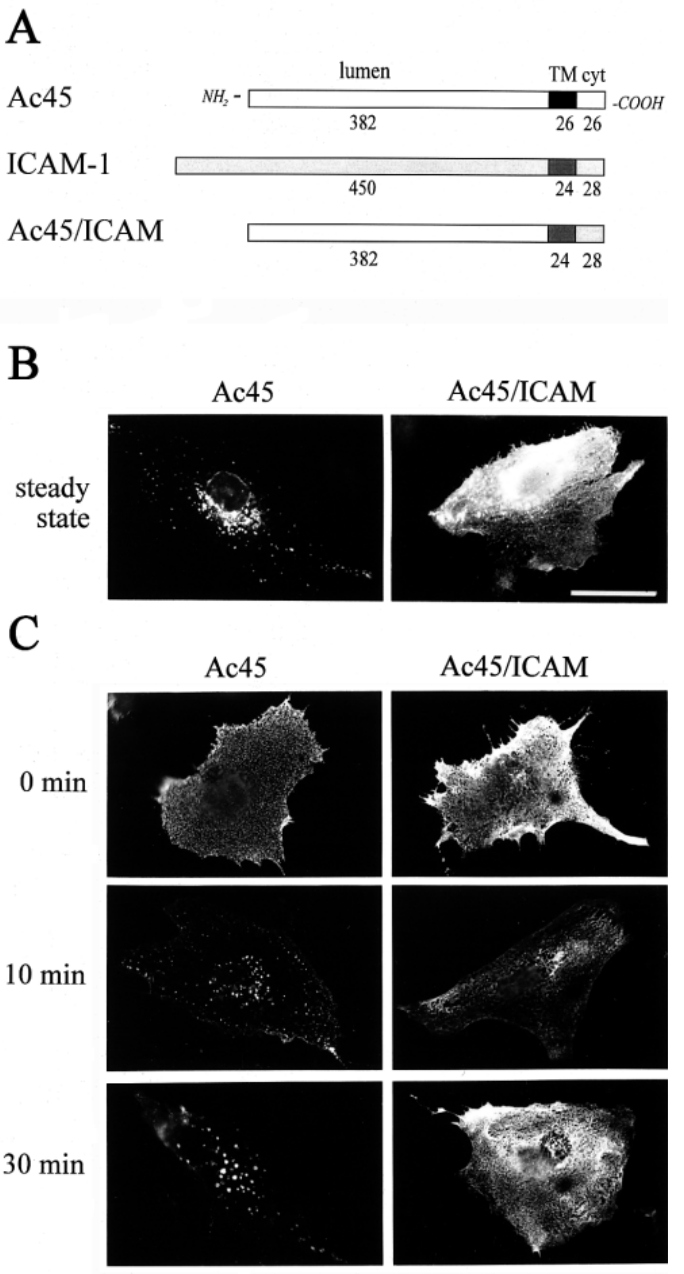


**Fig. 1.** Ac45 contains sorting information recognized by CV-1 fibroblasts. (A) Schematic representation of Ac45, ICAM-1 and the chimaeric protein Ac45/ICAM in which the lumenal domain of Ac45 was fused to the transmembrane (TM) and cytoplasmic (cyt) domains of ICAM-1. Cells were transiently transfected with Ac45 or Ac45/ICAM and either (B) fixed, permeabilized and incubated with anti-Ac45 antiserum or (C) first incubated with the antiserum at 4°C, washed and then returned to 37°C for the indicated time periods before fixation and permeabilization. Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies. Bar, 40 μm.

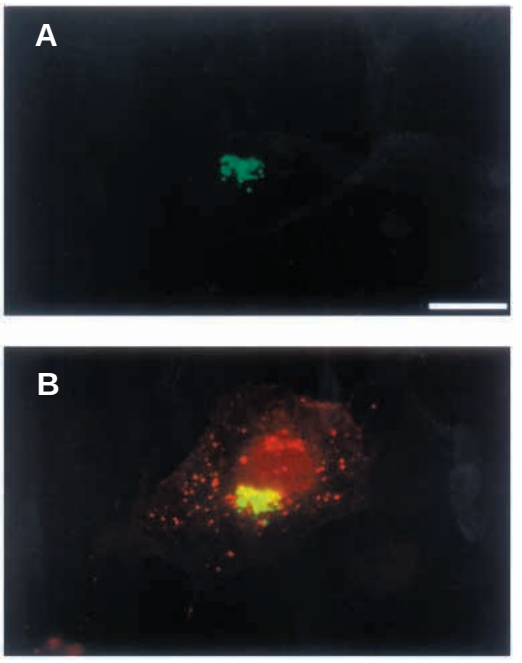


**Fig. 2.** Ac45 does not colocalize with the *trans*-Golgi/TGN marker protein sialyltransferase. CV-1 fibroblasts were transiently transfected with Ac45 and c-myc-tagged sialyltransferase, fixed, permeabilized and simultaneously incubated with rabbit anti-Ac45 antiserum and mouse anti-c-myc monoclonal antibody. (A) sialyltransferase was detected by FITC-conjugated goat anti-mouse secondary antibodies. (B) for double staining, Ac45 was detected using Texas Red-conjugated goat anti-rabbit secondary antibodies and sialyltransferase using FITC-labeling. Bar, 20 μm.

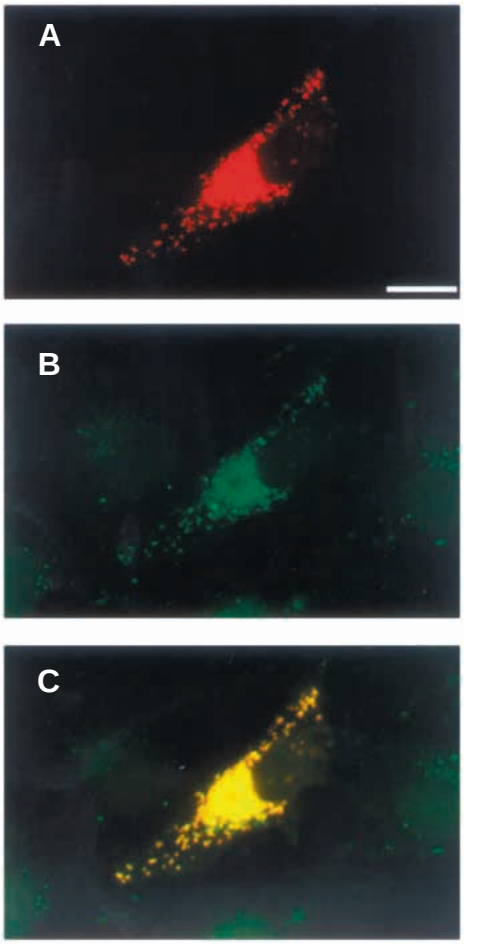


**Fig. 3.** Ac45 colocalizes with the late endosome/lysosomal marker protein CD63. CV-1 fibroblasts were transiently transfected with Ac45, fixed, permeabilized and simultaneously incubated with anti-Ac45 antiserum and anti-CD63 monoclonal antibody. (A) Ac45 was detected using Texas Red-conjugated goat anti-rabbit secondary antibodies, and (B) endogenous CD63 was detected using FITC-labeled goat anti-mouse antibodies. (C) For double staining, the two images were overlayed. Bar, 20 μm.

used in cotransfection studies and double immunofluorescence. We first compared the expression of Ac45 with that of the *trans*-Golgi/TGN enzyme sialyltransferase. The two proteins did not colocalize, suggesting that the vacuolar structures do not represent TGN-derived membranes (Fig. 2). Next, we compared the distribution of Ac45 to that of internalized FITC-labeled transferrin, a marker of the early endosomes. Although some colocalization was detected the overall pattern of distribution was different, indicating that only a minor amount of Ac45 was present in early endosomes. To compare the intracellular distribution of Ac45 and endogenous Ac116, a V-ATPase subunit mainly localized to endosomal and lysosomal structures (Sato and Toyama, 1994), transiently transfected CV-1 cells were double stained and Ac45 was found in the same vacuolar structures as Ac116. Furthermore, the distribution of Ac45 resembled that of the late endosome/lysosomal marker proteins CD63, Lamp-1 and Lamp-2 (data not shown), and a double immunofluorescence study indeed showed colocalization of Ac45 with CD63 (Fig. 3), suggesting that the vacuolar structures are late endosome/lysosome-like.

### Intracellular trafficking of Ac45 is dependent on structural information in its cytoplasmic domain

The above data indicate that Ac45 is actively retrieved from the cell surface to late endosome/lysosome-like structures and that internalization of the protein is dependent upon signals contained within its transmembrane and/or cytoplasmic domain. Since the cytoplasmic tails of many type I transmembrane proteins play a critical role in trafficking (Trowbridge et al., 1993; Sandoval and Bakke, 1994), we analyzed the effects of carboxy-terminal truncations on the intracellular transport of Ac45. For this purpose, three Ac45 carboxy-terminal truncation mutants were constructed, namely Ac45/423, Ac45/418 and Ac45/411 which lack 11, 16 and 23 of the most carboxy-terminal amino acids in the 26-residue cytoplasmic tail of Ac45, respectively (Fig. 4). When these constructs were transiently expressed in CV-1 cells, newly synthesized Ac45 proteins of the expected sizes could be immunoprecipitated from the cell extracts (data not shown). To determine whether the carboxy-terminal truncations altered the trafficking of Ac45, the transfected CV-1 cells were incubated with anti-Ac45 antibodies and processed for immunofluorescence microscopy. Proteins on the cell surface were distinguished from those associated with internal membranes by comparing cells that were first exposed to the

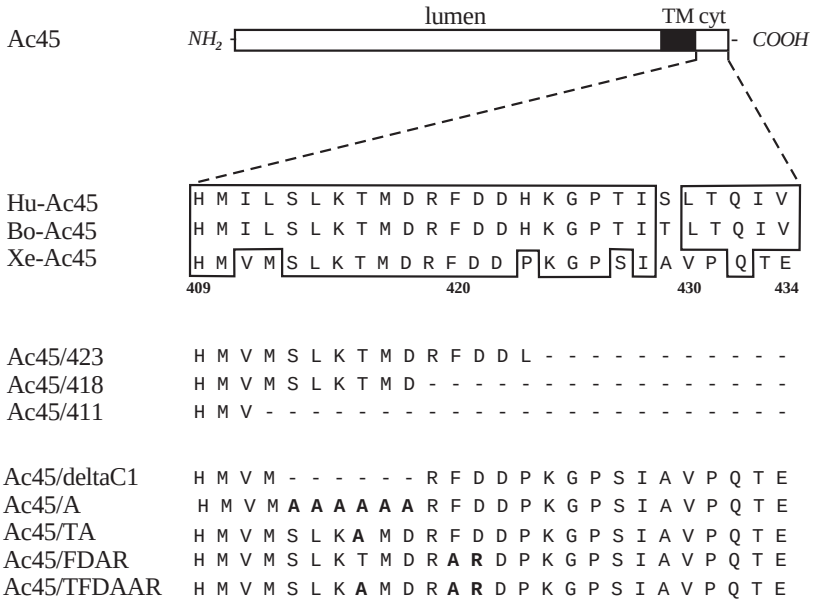


**Fig. 4.** The cytoplasmic tails of Ac45 and Ac45 mutant proteins. Comparison of the cytoplasmic tails of human, bovine and *Xenopus* Ac45. Identical residues are boxed. Also shown are the tails of the mutant proteins used in this study. A dash indicates that the corresponding amino acid residue is not present in the mutant protein. Alanine/arginine residues substituting the corresponding amino acids are in bold. The human (Hu), bovine (Bo) and *Xenopus* (Xe) Ac45 sequences have been taken from Yokoi et al. (1994), Supek et al. (1994) and our unpublished work (EMBL accession number X82421), respectively. TM, transmembrane domain; cyt, cytoplasmic domain.

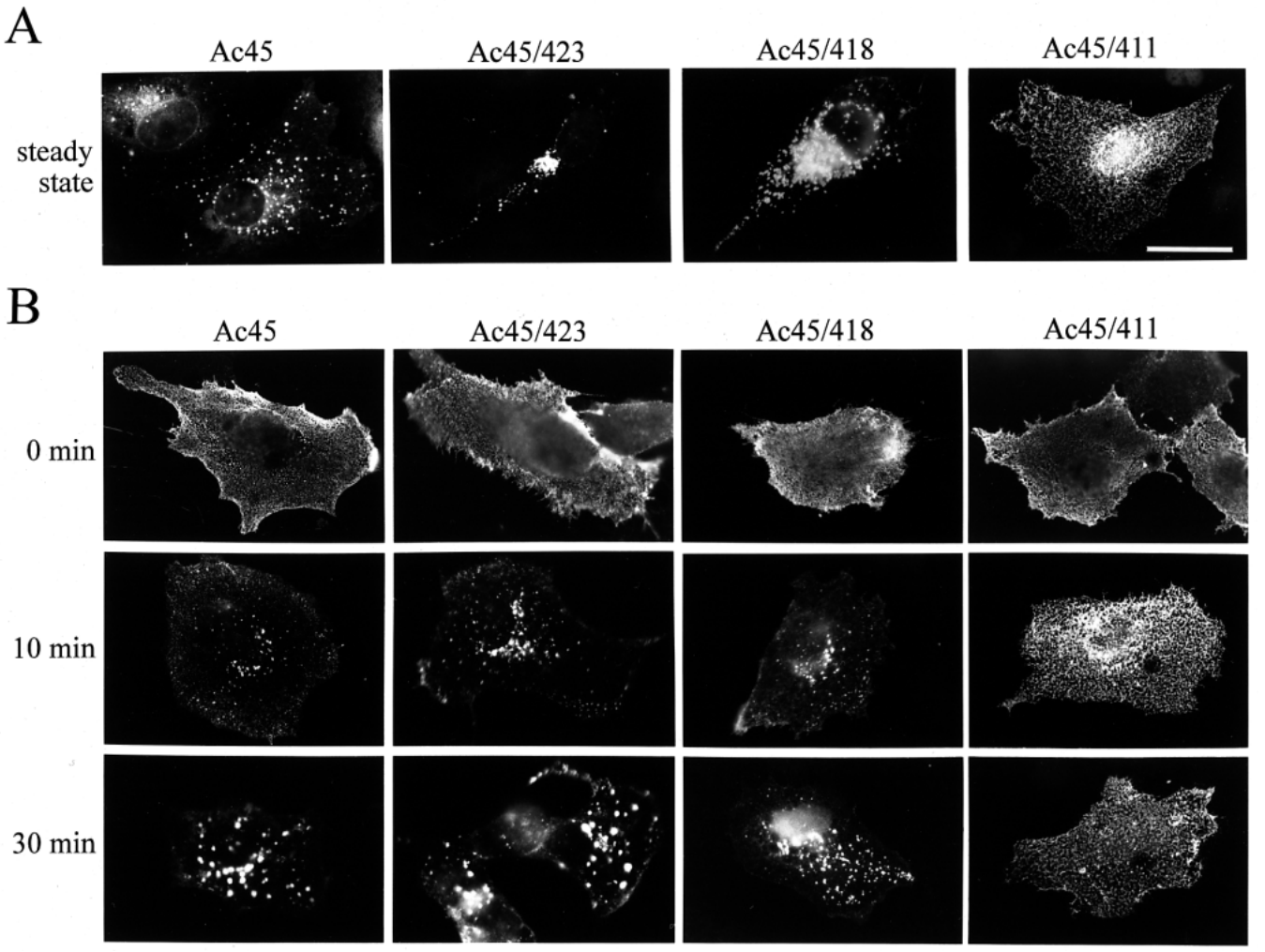


**Fig. 5.** The cytoplasmic domain of Ac45 contains critical targeting information. (A) CV-1 fibroblasts transiently expressing Ac45 or the carboxy-terminal truncation mutants Ac45/423, Ac45/418 and Ac45/411 were fixed, permeabilized and then incubated with anti-Ac45 antiserum or (B) first incubated with anti-Ac45 antibodies at 4°C, washed and then returned to 37°C for the indicated time periods before fixation and permeabilization. Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies. Bar, 40 μm.

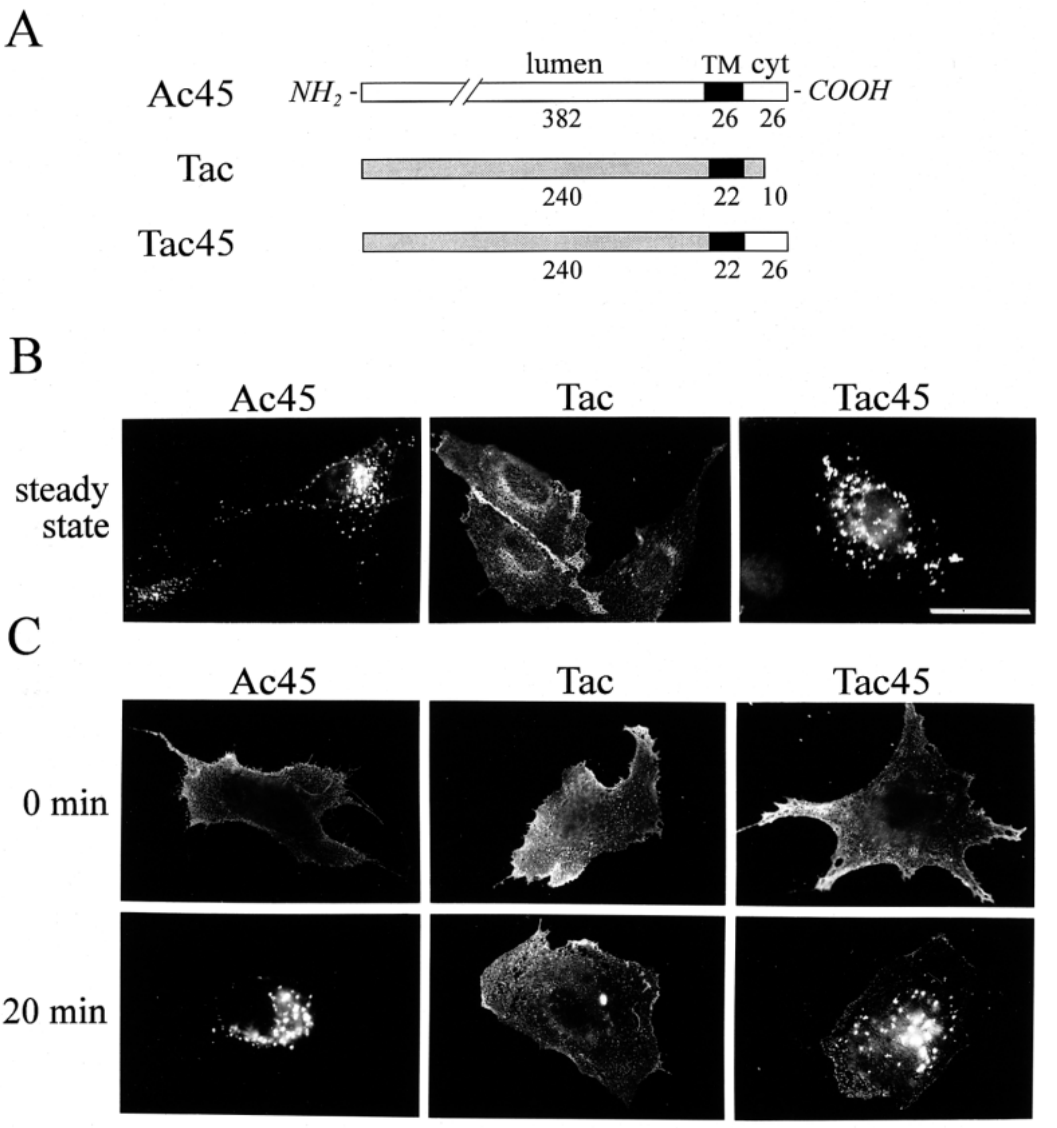


**Fig. 6.** The cytoplasmic domain of Ac45 contains autonomous targeting information. (A) Schematic representation of Ac45, Tac and the chimaeric protein Tac45 which contains the lumenal and transmembrane (TM) regions of Tac and the cytoplasmic (cyt) region of Ac45. (B) CV-1 fibroblasts transiently expressing Ac45, Tac or Tac45 were fixed, permeabilized and then incubated with antibodies or (C) first incubated with antibodies at 4°C, washed and then returned to 37°C for the indicated time periods before fixation and permeabilization. Ac45-expressing cells were incubated with anti-Ac45 antiserum while cells expressing Tac or Tac45 were incubated with a monoclonal antibody recognizing a lumenal epitope on Tac. Bound antibodies were visualized with FITC-conjugated secondary antibodies. Bar, 40 μm.

antibodies at 4°C and then fixed (Fig. 5B, '0-minutes' micrographs) with cells that were fixed and permeabilized before exposure to the antibodies (Fig. 5A, 'steady state' micrographs). To monitor internalization of proteins from the cell surface, cells were incubated with antibodies at 4°C, washed and then returned to 37°C for 10 minutes or 30 minutes before fixation and permeabilization (Fig. 5B, '10-minutes' and '30-minutes' micrographs). The steady state distributions of Ac45, Ac45/423 and Ac45/418 were very similar; in these cases the majority of protein was located in the juxtanuclear region, whereas only a limited amount was present on the cell surface (Fig. 5A). Furthermore, the antibody-uptake experiments revealed that the Ac45/423 and Ac45/418 mutants were retrieved from the cell surface and transported to the juxtanuclear region with kinetics similar to that of the full-length protein (Fig. 5B). In contrast, cells expressing the Ac45/411 mutant displayed a strong immunostaining of the plasma membrane while hardly any labeling was found associated with intracellular compartments (Fig. 5A). Moreover, the Ac45/411-expressing cells failed to take up any appreciable amounts of anti-Ac45 antibodies that had bound to their surface (Fig. 5B). Thus, deletion of the 23 carboxy-terminal residues of Ac45 causes an accumulation of the protein on the plasma membrane. Taken together, the above results indicate that residues 412-418 in the cytoplasmic tail of Ac45 contain at least part of the information necessary for the internalization and intracellular targeting of the protein.

### The cytoplasmic domain of Ac45 contains autonomous targeting information

The dramatic changes in the subcellular localization induced by the truncations in the cytoplasmic domain of Ac45 can be explained by either a direct involvement of this segment in trafficking or an indirect effect on other sequences. To investigate if the targeting information contained within the cytoplasmic domain of Ac45 can function independently of other regions in the protein, we constructed a fusion protein consisting of the lumenal and transmembrane domains of the cell surface protein Tac (α2 chain of the interleukin-2 receptor; Leonard et al., 1984) and the cytoplasmic domain of Ac45 (Tac45; Fig. 6A). Next, the steady state localization of the fusion protein Tac45 was compared with that of wild type Tac

and Ac45. For this purpose, CV-1 cells were transiently transfected with the appropriate constructs and processed for immunofluorescence microscopy essentially as described above, except that Tac and the Tac45 fusion protein were visualized using a monoclonal antibody (7G7) recognizing an epitope in the lumenal domain of the Tac protein (Rubin et al., 1985). During steady state conditions, Ac45 was predominantly associated with vacuoles in the juxtanuclear region, whereas Tac was nearly exclusively located on the plasma membrane of the transfected cells (Fig. 6B). Cells expressing the Tac45 fusion protein displayed a clear plasma membrane staining accompanied by a strong intracellular vacuolar staining (Fig 6B). Antibody-uptake experiments showed that the Tac45 fusion protein, unlike wild type Tac, was retrieved from the cell surface and accumulated at one side of the nucleus (Fig. 6C). However, this internalization was slower and less efficient than that observed for Ac45; whereas a 30-minutes chase was sufficient for Ac45-expressing cells to take up most of the antibodies that had bound to their surface (Fig. 1C), for the Tac45-expressing cells it took a 60- to 120-minutes chase period (Fig. 6C). From these observations it can be concluded that the cytoplasmic domain of Ac45 harbours an autonomous routing determinant which contributes to both internalization and transport of the protein to the juxtanuclear region.

### Detailed analysis of the targeting signal of Ac45

Having established that the cytoplasmic tail of Ac45 contains autonomous structural targeting information, we decided to dissect in more detail the reponsible region in the tail (residues 412-418). For this purpose, two additional mutants of Ac45 were constructed, namely the deletion of amino acid residues 413-418 (mutant Ac45/deltaC1) and the substitution of amino acids 413-418 to alanine residues (Ac45/A) (Fig. 4). Surprisingly, both of these mutants displayed a steady state localization similar to that of wild-type Ac45 and Ab-internalization experiments showed that the kinetics of internalization were not altered by the mutations (data not shown). The cytoplasmic tail of Ac45 does not contain any of the known signals that mediate localization and internalization from the plasma membrane such as the tyrosine-based and the di-leucine-based motifs nor does it exhibit the di-lysine motif that is often found at the extreme end of type I transmembrane proteins that reside in the ER. Also, an acidic region such as found in the trafficking signal of the proprotein convertase furin (Voorhees et al., 1995; Jones et al., 1995) is absent from the cytoplasmic portion of the Ac45 sequence. Endocytosis and routing of type I transmembrane proteins may also be mediated by phosphorylation of serine or threonine residues in the cytoplasmic tail. For instance, mutation of a serine phosphorylation site in the cytoplasmic tail of peptidylglycine 3α-amidating monooxygenase (PAM) resulted in a redistribution of the protein (Yun et al., 1995). Also for furin (Jones et al., 1995), epidermal growth factor receptor (Lin et al., 1986) and the polymeric immunoglobulin receptor (Hirt et al., 1993), a phosphorylation event in the cytoplasmic region has been implicated as a mediating factor for routing. The carboxy-terminal tail of Ac45 contains a potential phosphorylation site within the sequence $XXK^{415}T^{416}XXR^{419}$ (Kennely and Krebs, 1991) but internalization studies with the mutants Ac45/deltaC1 and Ac45/A, which lack residues $K^{415}T^{416}$, as well as with mutant Ac45/TA in which $T^{416}$ was substituted by an alanine (Fig. 4), revealed no clear effect on the routing of the proteins (data not shown), indicating that threonine residue 416 is not of critical importance. Hence, residues 413-418 of the cytoplasmic tail of Ac45 do not harbour a short linear sequence that on its own is responsible for the internalization of Ac45. However, the fact that this region is identical between human, bovine and *Xenopus* Ac45 (Fig. 4) indicates that it is important for intracellular Ac45 sorting. Interestingly, on the basis of a mutational analysis of the 35-residue cytoplasmic tail of the type I transmembrane leukocyte adhesion receptor P-selectin in transfected CHO cells, it was also concluded that not a short linear sequence but residues throughout the cytoplasmic domain affect the efficiency of internalization (Setiadi et al., 1995). Visual inspection of the amino acid sequences of the cytoplasmic domains of Ac45 and P-selectin revealed a common motif, namely $T^{416}XXXFD^{421}$ in Ac45 (Fig. 4) and $T^{781}XXXFD^{786}$ in human P-selectin. Such a motif is rather unique since a database search on the cytoplasmic tails of type I transmembrane proteins showed a similar motif (SXXXFD) only in the tail of the integral membrane form of PAM. Mutations within the $T^{416}XXXFD^{421}$ motif of Ac45 ($T^{416}$ replaced by A in mutant Ac45/TA, $FD^{412}$ by AR in mutant Ac45/FDAR or $T^{416}XXXFD^{421}$ by AXXXAR in mutant Ac45/TFDAAR, Fig. 4) did not, however, abrogate internalization (data not shown), again suggesting that multiple sites in the Ac45 tail are involved in its intracellular routing.

Taken altogether, our results indicate that the 26-residue cytoplasmic tail of the V-ATPase accessory subunit Ac45 contains autonomous targeting signals that are located in distinct portions of this domain and are different from previously described routing determinants.

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