Multiple endocytic signals in the C-terminal tail of the cystic fibrosis transmembrane conductance regulator

Wei HU†‡, Marybeth HOWARD‡ and Gergely L. LUKACS*‡†

†Program in Cell and Lung Biology, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, ‡Department of Laboratory Medicine and Pathobiology, University of Toronto, 100 College Street, Toronto, Canada M5G 1L5, and †Department of Surgery, University of California, San Francisco, 1001 Potrero Avenue, Bldg 1, San Francisco, CA 94110, U.S.A.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent protein kinase (PKA)-activated chloride channel that is localized to the plasma membrane and endosomal compartment. Endosomal targeting of CFTR is attributed to the Tyr^{1152}-based internalization signal, identified in the C-terminal tail of the channel. Mutation of the Tyr^{1152} residue could partly inhibit the endocytosis of CFTR and its association with the adapter protein AP-2. To reveal additional endosomal targeting signals, site-directed mutagenesis of both a chimaera, composed of a truncated form of interleukin 2 receptor α chain (TacT) and the C-terminal tail of CFTR (Ct), and the full-length CFTR was performed. Morphological and functional assays revealed the presence of multiple internalization motifs at the C-terminus, consisting of a phenylalanine-based motif (Phe^{1123}) and a bipartite endocytic signal, comprising a tyrosine (Tyr^{1152}) and a di-Leu-based (Leu^{1152}-Leu) motif. Whereas the replacement of any one of the three internalization motifs with alanine prevented the endocytosis of the TacT-Ct chimaera, mutagenesis of Phe^{1123}-Leu impaired the biosynthetic processing of CFTR, indicating that Phe^{1123} is indispensable for the native structure of CFTR. In contrast, replacement of Leu^{1152}-Leu- and Tyr^{1152}-based signals with alanine increased the cell-surface density of both the chimaeras and CFTR in an additive manner. These results suggest that the internalization of CFTR is regulated by multiple endocytic sorting signals.

Key words: clathrin-coated vesicle, internalization, surface density.

INTRODUCTION

Cystic fibrosis is one of the most prevalent lethal genetic disorders in the Caucasian population [1]. Compelling evidence indicates that the cystic fibrosis gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), is a chloride-selective ion channel, regulated by cAMP-dependent protein kinase (PKA)-mediated phosphorylation, ATP binding and ATP hydrolysis [2,3]. CFTR also seems to participate in the regulation of Na^+, K^+, H^+ and ATP transport across the plasma membrane [4].

Morphological, biochemical and functional evidence indicates that CFTR, besides its predominant cell-surface localization, is also confined to the endosomal compartment. Immunocytochemistry at the light-microscopy level suggested the existence of a sub-apical CFTR pool in T84 and CAPAN-1 epithelia, expressing CFTR endogenously [5]. Co-localization of CFTR with rab4, a member of the small GTP-binding protein family and a constituent of the recycling endosomes, was reported in rat salivary gland epithelia [6]. The expression of CFTR in endosomes was also demonstrated functionally in a number of cell lines expressing exogenous or endogenous CFTR. PKA-stimulated anion conductance was detected in isolated as well as in situ endosomes in Chinese hamster ovary and NIH 3T3 cells, in addition to the apical endosomes of T84 epithelia [7,8]. The endosomal CFTR activity was susceptible to inhibition with the monoclonal M3A7 anti-CFTR antibody [8]. Although the PKA-mediated phosphorylation of CFTR evoked a doubling of the anion conductance of the endosomal membrane, neither the steady-state pH nor the acidification rate of the luminal compartment was affected [7,8].

A growing body of evidence suggests that the distribution of CFTR between the plasma membrane and endosomes is at dynamic equilibrium. The rapid internalization of CFTR seems to be mediated by clathrin-coated pits in both polarized and non-polarized cells [9,10]. Accordingly, the expression of CFTR was detected in isolated clathrin-coated vesicles [11]. Endocytosis of CFTR was inhibited by PKA-dependent and protein-kinase-C-dependent phosphorylation and caused the diminution of the internal CFTR pool [10]. As a corollary, the cell-surface density of CFTR was increased in both polarized and non-polarized cells on stimulation with PKA, as detected by immunofluorescence microscopy [12–14].

The dynamic nature of CFTR distribution has been illuminated in an elegant study of primary culture of shark rectal gland, with the use of a quantitative morphological analysis of immunostained cells [15]. Reversible translocation of CFTR was induced by vasoactive intestinal peptide and forskolin from an internal vesicular compartment to the plasma membrane. The translocation coincided with the activation of the plasma-membrane chloride conductance [15]. These observations are consistent with the notion that the cell-surface density of CFTR is determined by the rates of endocytosis and exocytosis.

Internalization of integral membrane proteins relies predominantly on relatively short peptide sequences in their cytoplasmic domains. Tyr- and Leu-based endocytic signals are the
most extensively studied endocytic sorting signals of type I and type II membrane proteins (reviewed in [16–19]). Depending on primary sequence context, these motifs can also be recognized in sorting events in the trans-Golgi network and endosomes [20–22]. The Tyr-based signal conforms to a consensus of Tyr-Xaa-Xaa-ϕaa, in which the Tyr can be replaced with a Phe, Xaa stands for any amino acid residue, and ϕaa represents a bulky hydrophobic amino acid residue [23]. Yeast two-hybrid screens and binding assays in vitro revealed the association of the Tyr-based motif with the \( \mu_1 \) and \( \mu_2 \) subunits of adaptor proteins (AP-1 and AP-2) [24,25]. The Leu-based signal has two successive leucine residues [26], one of which can be replaced with a bulky hydrophobic residue [27].

Relatively little is known about the endocytic signals in polytopic membrane transporters. The insulin-regulated glucose transporter GLUT4 is constitutively retrieved from the plasma membrane via clathrin-dependent endocytosis to a specialized endosomal compartment (glucosome) in the absence of insulin. Mutagenesis studies of GLUT4 and of a chimera consisting of GLUT4 and the transferrin receptor (TfR) demonstrated a Phe-based and a Leu-based endosomal targeting signal respectively [28,29]. A highly conserved Tyr (Tyr\(^{141}\)) is responsible for the ligand-induced sequestration of the \( \beta_2 \)-adrenergic receptor [30], whereas a Tyr-based internalization motif in the cytoplasmic tail of the \( \beta \) subunit (type II membrane protein) directs the heterodimeric \( \alpha/\beta \) K\(^+\)/H\(^-\)-ATPase to endosome-like intracellular compartments [31].

Recently, a tyrosine (Tyr\(^{141}\))-based motif was identified as a potential endocytic targeting signal in the C-terminal tail of CFTR, with the use of a chimera consisting of TIR, a type II membrane protein and the C-terminal tail of CFTR [32]. Cross-linking and in vitro pull-down assays verified the Tyr\(^{141}\)-dependent association of the C-terminal tail and CFTR with the AP-2 component of the internalization machinery [33]. Intriguingly, replacement of Tyr\(^{141}\) with alanine could only partly inhibit the internalization of the chimera and the full-length CFTR, and the binding of AP-2 to the C-terminal tail [32,33]. One possible explanation for the partial inhibition is that additional endocytic motifs contribute to the internalization of CFTR. Using site-directed mutagenesis in conjunction with morphological and biochemical assays, we show here that multiple sorting signals account for the internalization of a chimera comprising the C-terminal tail of CFTR and part of the interleukin 2 receptor. The co-operative function of the Tyr\(^{141}\)-based motif and the previously unrecognized Leu\(^{148}\)-Leu-based endocytic signal in the C-terminal tail is demonstrated in the full-length CFTR.

**MATERIALS AND METHODS**

**Cells and antibodies**

COS-1 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 10% \( \times \) fetal bovine serum (FBS, complete medium), 100 i.u./ml penicillin and 100 \( \mu \)g/ml streptomycin. BHK-21 cells were cultured in F-12/DMEM with 5% \( \times \) FBS. Cells were grown at 37°C in humidified air/\( \text{CO}_2\) (19:1) atmosphere.

Monoclonal mouse anti-human interleukin 2 receptor \( \alpha \) chain (Tac) antibody (catalogue no. 0119) and its FITC-conjugated derivative (catalogue no. MCA1319F) were purchased from ImmunoTech (Marseille, France) and SeroTech Ltd. (Oxford, U.K.) respectively. FITC-conjugated (catalogue no. MCA350F) and unconjugated (catalogue no. MCA350) rat monoclonal anti-human Tac antibodies were obtained from SeroTech Ltd. Mouse monoclonal anti-FLAG antibody (M2; catalogue no. 200472-21) and lissamine-rhodamine-conjugated donkey anti-rat IgG (catalogue no. 712-083-153; AffiniPure) were purchased from Stratagene (La Jolla, CA, U.S.A.) and Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.) respectively. \( ^{125}\)I-labelled monoclonal goat anti-mouse IgG (catalogue no. NEX159; 126 Ci/ml) was from NEN (Boston, MA, U.S.A.). Texas Red (TR)-conjugated transferrin (TR–Tf; catalogue no. T-2875) was obtained from Molecular Probes (Eugene, OR, U.S.A.). The M3A7 monoclonal mouse anti-human CFTR antibody was a gift from Dr N. Kartner.

**Plasmid construction**

The schematic structure of the TacT–Ct chimaera, consisting of the extracellular and transmembrane domains of Tac and the C-terminal tail of CFTR, is shown in Figure 1. The coding region of Tac (kindly provided by Dr J. S. Bonifacino, NIH) [34] was inserted into the XhoI–XbaI sites of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) as a SalI–XbaI fragment. TacT, terminating at the C-terminus of the predicted transmembrane domain, was constructed by replacing the Bsu36I–XbaI fragment of Tac with a DNA fragment generated by PCR in which the XbaI site encodes a stop codon (pcDNA3-TacT). An XmaI site was introduced upstream of the XhoI site. This procedure led to the incorporation of two extra amino acid residues (Arg and Gly) between the C-terminus of TacT and the CIR and the CFTR tails. The C-terminal tail of CFTR, corresponding to residues from Thr\(^{1897}\) to Leu\(^{1898}\), was amplified with PCR by using BQ4.7 CFTR cDNA (kindly provided by Dr J. Rommens, Hospital for Sick Children, Toronto, Ontario, Canada) as template. The PCR products were inserted as an XhoI–XbaI fragment into pcDNA3-TacT. Alanine substitutions in the following mutants were generated by site-directed mutagenesis: K2, F1413A,L1414A; K3, Y1424A,L1430A,L1431A; K6, F1413A; K7, L1414A; K8, Y1424A; K9, L1430A; K10, L146A,F17A (see also Figure 5), by using either single-stranded (for K2, K3, K8 and K9; Muta Gene in vitro mutagenesis from Bio-Rad) or double-stranded (for K10; QuickChange site-directed mutagenesis from Stratagene [35]) pcDNA3-TacT–Ct as template.

**Figure 1 Schematic representation of the TacT–Ct chimaera**

The arrangement of Tac (open bar) and the C-terminal cytoplasmic tail of CFTR (filled bar) is illustrated. The C-terminal sequences of Tac and TacT (normal type) and CFTR (bold type, underlined) are indicated with numbers representing their original positions in Tac and CFTR respectively. Two inserted amino acid residues (Arg-Gly) are indicated in small capitals. The dippeptide sequence Arg-Gly is encoded by the XmaI site, introduced for insertion of the CFTR fragments and for proper termination of the transmembrane segment (56). TM refers to the transmembrane segment of Tac.

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template. ‘Mega-primer’ PCR [36] was used for the generation of mutants K6 and K7.

To measure the cell-surface expression of the endocytosis-deficient CFTR molecules, first a Asp-Tyr-Lys-Asp-Asp-Asp-Lys (FLAG) epitope was inserted into the fourth extracellular loop of CFTR (CFTRM2) [12]. The alanine substitutions corresponding to the K2, K3, K8 and K9 mutations were introduced into CFTRM2 by replacing the ApaI and PflMI fragment in the wild-type CFTR with the mutant sequences. Stably transfected BHK-21 cells, expressing wild-type and mutant TacT–Ct and CFTRM2 forms, were generated as described previously [37]. All constructs were verified by DNA sequencing.

**Transplant and stable transfection**

COS-1 cells were transiently transfected at 25–30 % confluence on glass coverslips by a modified calcium phosphate precipitation method [38]. Cells were plated at least 4 h before transfection in 35 mm wells. Plasmid DNA (3 μg) was mixed with 6.3 μl of 2.5 M CaCl₂ and 63 μl of 2× Bes solution [50 mM Bes (pH 6.95)/280 mM NaCl/1.5 mM Na₂HPO₄], water was added up to 125 μl. After incubation for 15 min at room temperature, the DNA was mixed with 2.2 ml of complete medium and added to the cells.

The cell-surface density and internalization rates of wild-type and mutant TacT–Ct were measured in COS-1 cells after 40 h of transfection with LIPOFECTAMINE (Gibco BRL Life Technology, Gaithersburg, MD, U.S.A.). Stable transfectants of BHK-21 cells expressing TacT–Ct chimaeras or CFTRM2 forms were obtained as described above. Representative clones, obtained by immunostaining screens, were used to investigate the fate of Tac–CFTR chimaeras. To avoid clonal variations, at least 20 of the wild-type or mutant CFTRM2-expressing clones were combined.

**Immunofluorescence microscopy**

Cells were fixed for 20 min with 4 %, (v/v) paraformaldehyde in PBS at room temperature. When indicated, the plasma membrane was permeabilized by 0.2 %, (v/v) Triton X-100 in PBS for 5 min at room temperature. Samples were stained with primary and secondary antibodies (0.4 μg/ml) in blocking solution (0.5 %, BSA in PBS) for 1 h and mounted with PBS containing 50 %, (v/v) glycerol and 1 %, (v/v) n-propylgallate.

Immunostaining of the extracellular epitope of Tac and the subcellular distribution of Tac–CFTR chimaeras were assessed by direct and indirect immunostaining with monoclonal mouse FITC-conjugated anti-Tac antibody and monoclonal rat or mouse anti-Tac antibody with lissamine-rhodamine-conjugated donkey anti-rat or tetramethylrhodamine-isothiocyanate-conjugated donkey anti-mouse IgG. Early endosomes were detected by TR–Tf accumulation. After the depletion of endogenous transferrin in serum-free medium, cells were left for 40 min at 37 °C to internalize the TR–Tf (5 μg/ml). Fluorescence micrographs were taken as described previously [39]. All micrographs are representative of experiments repeated at least three times. To follow the time course of anti-Tac antibody internalization images were taken with the same exposure time and processed with Adobe PhotoShop (Adobe Systems, San Jose, CA, U.S.A.).

**Monitoring the internalization of TacT–Ct chimaeras**

**Fluorescence microscopy**

Internalization of TacT–Ct chimaeras was followed by incubating COS-1 cells for 15–180 min in complete medium supplemented with FITC-conjugated anti-Tac antibody (0.4 μg/ml) at 37 °C. Cell-surface-associated antibody was removed with acid washes [0.2 M acetic acid/0.2 M NaCl (pH 2.5) for 2 min, followed by 150 mM NaCl/20 mM Hepes/1 mM CaCl₂/5 mM KCl/1 mM MgCl₂ (pH 7.4) for 3 min] [40].

**Radioactive binding assay**

The internalization efficiency of TacT and TacT–Ct was measured by monitoring the cell-surface density of Tac with a mouse monoclonal anti-Tac antibody and 125I-labelled goat anti-mouse secondary antibody as a function of incubation duration. First, cell-surface-resident TacT was labelled for 1 h with the anti-Tac antibody (0.4 μg/ml) in RPMI-H medium [RPMI-1640 medium/5 % (v/v) FBS/20 mM Hepes (pH 7.4)] at 4 °C. Cells were rinsed and internalization was initiated by raising the temperature to 37 °C for 0–15 min. Anti-Tac antibody remaining at the plasma membrane was measured with the specific binding of 125I-labelled anti-mouse IgG (3 μCi/ml) for 1 h at 4 °C. The radioactivity of solubilized cells [in 0.1 M NaOH/0.1 %, (v/v) Triton X-100] was determined with a gamma counter (LKB 1282 Compugamma). Non-specific binding of the primary antibody was measured in mock-transfected cells. The non-specific binding of the secondary antibody was determined in each experiment in the absence of primary antibody and in the presence of an identical concentration of non-specific mouse IgG (Santa Cruz). Specific binding was calculated from the difference of total (0 min internalization) and non-specific binding of the primary and secondary antibodies. The internalization efficiency of the wild-type and mutant TacT–Ct was expressed as a percentage of the decrease in the initial surface binding. All experiments were performed in triplicate and repeated at least three times.

**Determination of the cell-surface density of wild-type and mutant CFTRM2**

The cell-surface density of wild-type and mutant CFTRM2, expressing a FLAG epitope to the extracellular compartment [12], was measured with a monoclonal mouse anti-FLAG (M2) primary antibody and 125I-labelled goat anti-mouse secondary antibody as described for the Tac–CFTR chimaeras. Attempts to follow the internalization of CFTRM2 were compromised by the dissociation of bound M2 antibody at 37 °C, which was conceivably due to the relatively low affinity of the antibody for the FLAG epitope in CFTR (results not shown).

**Inhibition of clathrin-dependent internalization**

Clathrin-coated vesicle formation was inhibited by the depletion of cytosolic K⁺ [41] and hypertonic treatment of the cells, as performed previously [10]. In brief, BHK-21 cells expressing TacT–Ct were incubated in K⁺-free medium [65 mM NaCl/10 mM Hepes (pH 7.4)/0.5 mM MgCl₂/0.05 mM CaCl₂/5 mM glucose] for 10 min at 37 °C in the presence of 1 mM ouabain. Then anti-Tac antibody (0.4 μg/ml) binding was accomplished in K⁺-free medium for 1 h at 4 °C. Unbound antibody was removed and endocytosis was left to proceed for 9 min at 37 °C, then measured as described for the Tac–CFTR chimaera.

**Measuring the plasma-membrane PKA-activated anion conductance**

The macroscopic channel activity of wild-type and mutant CFTRM2 was determined by the iodide efflux assay, essentially as described before [42]. In brief, the chloride content of
BHK-21 cells, stably expressing the wild-type or mutant CFTRM2, was replaced with iodide by incubating the cells for 60 min in loading buffer [136 mM NaI/3 mM KNO$_3$/2 mM Ca(NO$_3$)$_2$/11 mM glucose/20 mM Hepes (pH 7.4)] at room temperature. Iodide efflux was initiated by replacing the loading buffer with efflux medium (composed of 136 mM nitrate in the place of iodide). The efflux medium was subsequently replaced every 1 min. After a steady state had been reached, the intracellular cAMP level was raised by agonists [20 μM forskolin, 0.2 mM CTP-cAMP and 0.2 mM 3-isobutylmethyl-1-xanthine (IBMX)] and collection of the efflux medium resumed for an additional 6–9 min. The amount of iodide in each sample was determined with an iodide-selective electrode [42].

RESULTS

C-terminal tail of CFTR contains endocytic sorting signals

A number of sorting determinants (e.g. internalization, basolateral and apical targeting signals) have been identified in the cytoplasmic tails of polytopic membrane proteins [29–31,43,44]. A sequence alignment of the C-terminal cytoplasmic tail of CFTR from six different species showed the presence of highly conserved consensus Tyr- and Leu-based endocytic sorting signals. The C-terminal tail [45], encompassing residues 1387–1480, contains a putative di-Leu signal (Leu$^{1490}$-Leu), a canonical Tyr-based signal (Tyr$^{1421}$-Asp-Ser-Ile) and a Tyr-like signal (Phe$^{1412}$-Leu-Val-Ile), which might overlap a Leu-based signal. Although the Tyr$^{1421}$-Asp-Ser-Ile targeting motif was experimentally confirmed previously [32], the functional significance of neither the Phe-based signal nor the di-Leu-based signal has been examined. To determine whether these consensus endocytic signals are functional, a fusion protein, TacT–Ct, was first constructed (Figure 1). This approach permitted the fusion of the C-terminus in a physiological orientation to the Tac reporter, a type I membrane protein, which is targeted to plasma membrane by default and has been used extensively to identify sorting determinants [46,47].

The steady-state distribution of the TacT–Ct chimaera was compared with that of TacT, with the use of FITC-conjugated anti-Tac antibody, which recognized the extracellular epitope of Tac. The intense fluorescence immunostaining of non-permeabilized COS-1 cells indicated that a significant amount of TacT accumulated at the plasma membrane (Figure 2A), as reported previously [48,49]. In contrast, negligible immunofluorescence was associated with the plasma membrane of non-permeabilized COS-1 cells expressing TacT–Ct (Figure 2A). The lack of immunostaining cannot be attributed to an impaired biosynthesis of TacT–Ct because a punctate cytosolic distribution pattern with an accentuated juxtanuclear accumulation was revealed after the immunostaining of permeabilized COS-1 transfected (Figure 2A). A similar intracellular distribution of TacT–Ct was observed in BHK-21 cells stably transfected with the chimaera (Figure 2B, and results not shown), which was consistent with the hypothesis that TacT–Ct accumulates predominantly in the endolysosomal compartment.

To assess whether the TacT–Ct chimaera is targeted via the plasma membrane to endosomes or is delivered directly from the trans-Golgi network, the uptake of FITC-conjugated anti-Tac antibody was monitored. The TacT–Ct chimaera was expressed transiently in COS-1 cells and internalization of the antibody from the extracellular compartment was examined after incubation of the cells at 37°C. Although no fluorescence signal was detected initially (results not shown), a punctate, diffuse cytosolic pattern appeared after 15 min of incubation, reminiscent of the steady-state distribution of TacT–Ct (Figure 3A). The fluorescence signal, which was resistant to stripping with acid, increased progressively with extended incubation and reached saturation between 40 min and 3 h (Figure 3A, and results not shown). No fluorescence signal appeared when the cells were kept at 4°C in the presence of non-specific primary antibody (results not shown) or with the use of mock-transfected cells, suggesting that the staining was specific and reflected the internalization of anti-Tac antibody in a complex with the chimaera.

![Figure 2](image_url)  
Figure 2  Steady-state distribution of TacT–Ct chimaeras in COS-1 and BHK-21 cells

(A) COS-1 cells were transiently transfected with TacT or TacT–Ct construct. After fixation, direct immunostaining was performed on non-permeabilized or permeabilized cells with FITC-conjugated anti-Tac antibody. Whereas a negligible quantity of TacT–Ct chimaeras could be detected in non-permeabilized cells, a punctate cytoplasmic distribution pattern became evident after permeabilization. (B) Subcellular distribution of the TacT–Ct chimaera in BHK-21 stable transfectants was detected by direct immunostaining after permeabilization of the cells with Triton X-100. Mock-transfected cells did not display any immunostaining signal. Scale bar, 5 μm.
The TacT–Ct chimaera was transiently expressed in COS-1 cells. (A) To monitor the internalization of TacT–Ct, cells were incubated for the indicated durations at 37 °C in the presence of FITC-conjugated anti-Tac antibody recognizing the extracellular epitope of Tac. Before fixation, cell-surface-associated anti-Tac antibody was removed with repeated acid washes at 4 °C. Antibody accumulation was detected by fluorescence microscopy. No anti-Tac antibody uptake was observed in mock-transfected cells (results not shown). (B) Co-localization of the TacT–Ct chimaera and recycling endosomes. COS-1 cells transiently expressing TacT–Ct chimaeras were loaded with TR–Tf (5 μg/ml) and FITC-conjugated anti-Tac antibody (0.4 μg/ml) for 40 min at 37 °C. Identical fields are shown for the FITC and the rhodamine channels. The anti-Tac antibody shows extensive co-localization with internalized transferrin (arrowheads). Scale bar, 5 μm.

The endosomal targeting of internalized anti-Tac antibody was confirmed by determining its co-localization with recycling endosomes, labelled with TR–Tf. The significant co-localization of internalized anti-Tac antibody with TR–Tf suggests that most of the internalized chimaeras are sorted for recycling to the plasma membrane (Figure 3B). This conclusion was confirmed by capturing the recycling, FITC-conjugated anti-Tac antibody with a rhodamine-conjugated anti-mouse secondary antibody (results not shown). These observations suggest that endocytic sorting signals are functional C-terminal tails of CFTR and that they account for the internalization of the TacT reporter.

**Internalization efficiency of the TacT–Ct chimaeras**

A radioactive antibody-binding assay was implemented to compare the rate of endocytosis of wild-type and mutant chimaeras quantitatively. The extracellularly exposed epitope of the chimaera was saturated with anti-Tac antibody at 4 °C; internalization was then initiated by shifting the temperature to 37 °C. After 5–15 min, anti-Tac antibody remaining at the cell surface was measured by the specific binding of 125I-labelled anti-mouse IgG at 4 °C. The rapid disappearance of the cell-surface anti-Tac antibody indicates that TacT–Ct was internalized with high efficiency in COS-1 cells (Figure 4). Approximately 50%, and 90%, of the TacT–Ct chimaera was endocytosed during the first 5 and 9 min of incubation respectively. This implies an internalization rate of 11 ± 0.5 %/min (n = 12) for the TacT–Ct chimaera (Figure 4A). In contrast, the internalization rate of TacT was nearly 6-fold slower (1.8 ± 0.3 %/min; n = 6) (Figure 4A). Similar internalization rates were obtained for TacT with fluorophore-conjugated or radioactively labelled Fab fragments, ruling out the possibility that cross-linking with the bivalent anti-Tac antibody triggers the internalization [50].

To preclude any adverse effects of the COS-1 transient overexpression system, the internalization efficiency of the chimaera was also determined in BHK-21 cells stably expressing the chimaera. The results demonstrate that the internalization rates of TacT–Ct were almost identical in BHK-21 and COS-1 cells (Figure 4A), implying that the highly efficient internalization of the chimaera was independent of the expression system used.

**TacT–Ct chimaeras are internalized via clathrin-dependent endocytosis**

To assess whether the TacT–Ct chimaera was retrieved from the cell surface via clathrin-dependent endocytosis, the formation of clathrin-coated vesicles was inhibited with depletion of cytosolic K[+] or exposure of the cells to hypertonic medium. These perturbations inhibit the clathrin-dependent internalization of several plasma membrane proteins [41,51]. Both the depletion of the cytosolic K[+] content and hypertonic treatment almost completely prevented the internalization of TacT–Ct in BHK-21 cells (Figure 4B), suggesting that the retrieval of TacT–Ct to endosomes was clathrin-dependent, as is the full-length CFTR [10].

**Identification of multiple endocytic sorting signals in the C-terminal tail of CFTR**

Sequence alignment of the C-terminal tail of CFTR from six different species predicted the following Tyr- and Leu-based endocytic sorting signals: Phe[1413]-Leu-Val-Ile, Tyr[1414]-Asp-Ser-Ile and Leu[1415]-Leu (Figure 5). To verify the function of these putative internalization signals in the C-terminal tail, alanine-substitution mutagenesis was performed. First, the Phe[1413]-Leu-Val-Ile membrane-proximal signal and the membrane-distal signals containing Tyr[1414]-Asp-Ser-Ile and Leu[1415]-Leu were eliminated separately by replacements with alanine. Considering that the proximal signal might be a combination of an atypical Tyr-based motif (Phe[1412]-Leu-Val-Ile) and a Leu-based motif (Phe[1413]-Leu or Leu[1414]-Val), both Phe[1413] and Leu[1414] were replaced with alanine in the K2 mutant (F1413A,L1414A) (Figure 5). The membrane-distal signals, consisting of a canonical Tyr-based motif and a di-Leu motif, were mutated together and designated as mutant K3 (Y1424A,L1430A,L1431A) (Figure 5).

Deletion of not only the membrane-proximal but also the membrane-distal endocytic signal induced a marked increase in the cell-surface expression of TacT–Ct. This is illustrated by comparing the cell-surface density of K2, K3 and wild-type TacT–Ct with immunofluorescence staining of non-permeabilized cells, with the use of anti-Tac antibody (Figure 6A, left panels). The increased expression of the K2 and K3 mutants at the plasma membrane is conceivably due to their compromised endocytosis rather than to augmented biosynthesis.

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Figure 4  Internalization efficiency of TacT–Ct and TacT

The rate of disappearance of anti-Tac antibody from the cell surface was monitored with 125I-labelled secondary antibody. (A) Transient (COS-1, ◆), or stable (BHK, △) transfectants expressing TacT–Ct were incubated with mouse anti-Tac antibody for 1 h at 4 °C, washed and left to internalize for the indicated durations at 37 °C. Anti-Tac antibody remaining on the cell surface was measured with 125I-labelled anti-mouse secondary antibody. Specific binding was calculated as the difference between total and non-specific binding, as described in the Materials and methods section. Internalization was expressed as a percentage of the specific surface binding detected initially. Results are means ± S.E.M for two to four separate experiments, performed in triplicate. The internalization efficiency of TacT was determined in transiently transfected COS-1 cells (○). (B) Internalization of the TacT–Ct chimaera is clathrin-dependent. BHK-21 cells stably expressing TacT–Ct were incubated in K+
+-depletion medium for 10 min at 37 °C and then in the presence of anti-Tac antibody (0.4 lg/ml) for 1 h on ice. Unbound antibody was removed and endocytosis proceeded in K+-depletion medium for 9 min at 37 °C. The anti-Tac antibody remaining at the cell surface were quantified with 125I-labelled anti-mouse IgG (3 lCi/ml). Alternatively, both anti-Tac antibody binding and internalization were performed in hypertonic medium (0.3 M sucrose in RPMI-H) to inhibit clathrin-dependent endocytosis. The amount of internalized anti-Tac antibody is expressed as a percentage (mean ± S.E.M.) of the radioactivity associated with the cell surface before internalization.

Figure 5 Alanine substitutions of the putative endocytic sorting signals within the C-terminal tail of CFTR

Putative endocytic sorting signals are underlined in the wild-type (wt) sequence; alanine substitutions are indicated. Numbers correspond to the positions of residues in the human sequence of CFTR [45].

or impaired degradation. This assumption was verified qualitatively by detecting the extent of FITC-conjugated anti-Tac antibody uptake by the removal of the cell-surface-bound antibody with acid stripping (Figure 6A, right panels). In contrast with the wild-type TacT–Ct expressors, most of the anti-Tac antibody could be removed from cells expressing the K2 or K3 mutant (Figure 6A, right panels). The total cellular expression levels of the wild-type and mutant chimaeras were comparable, as demonstrated by the comparable immunostaining signals in the permeabilized COS-1 cells expressing the various constructs (Figure 6B). Similar results were also obtained in BHK cells (results not shown).

Direct evidence for the impaired endocytosis of the mutant chimaeras was obtained by determining their internalization rates and relative cell-surface densities with the radioactive antibody binding assay (Figures 7A–7C). The internalization rates of K2 and K3 mutants were indistinguishable from that of TacT, implying that alanine substitution completely abolished the endocytotic activity of the C-terminal tail (Figure 7A). As a consequence the cell-surface density of the K2 and K3 chimaeras increased 7–19-fold in a transient expression system (Figure 7B), and 4–6-fold in a stable expression system (Figure 7C). We therefore concluded that neither the membrane-proximal nor the membrane-distal signal was sufficient to maintain the internalization activity of TacT–Ct.

To evaluate the involvement of single amino acid residues in the membrane-proximal and membrane-distal internalization signals, four additional constructs were prepared that disrupted the three putative endocytic signals individually, as follows: K6, F1413A; K7, L1414A; K8, Y1424A; K9, L1430A (see also Figure 5). Alanine substitutions of each of the four residues decreased the endocytic rate of TacT–Ct by more than 50% (Figures 7A and 7D). The internalization efficiencies of mutants K6 and K7 were decreased to 45 ± 1.7% and 21.7 ± 1.8% of wild-type TacT–Ct respectively. Alanine substitution of Tyr 1424 (K8) and Leu 1430 (K9) decreased the endocytic activity by 66 ± 1.8% and 61.3 ± 1.7% respectively (Figure 7A). Intriguingly, although the cell-surface density of the mutant chimaeras was significantly increased, there was no tight correlation between the internalization rate and surface density. One possible explanation is that alanine substitution influenced not only the internalization efficiency but also the biosynthesis, folding and/or stability of the chimaera. Nevertheless, the results indicate that both the membrane-proximal and membrane-distal motifs are
necessary to maintain the efficient internalization activity of TacT–Ct and suggest that besides the previously recognized Tyr-based signal [32], the Phe-Leu-Val-Ile and Leu-Leu motifs contribute to the internalization activity of CFTR as well.

**Chloride channel function and cell-surface density of CFTR with defective internalization signals**

The physiological significance of the Leu-Leu-Val-Ile and Phe-Leu-Val-Ile internalization motifs identified in the TacT–Ct chimaeras was evaluated in the context of the full-length CFTR harbouring a FLAG epitope in the fourth extracellular loop (CFTRM2) [12,13]. Whereas insertion of the FLAG epitope had no significant impact on the cAMP-stimulated channel activity of CFTRM2 [12,13], it allowed the measurement of the cell-surface density of CFTR by a sensitive antibody-binding assay with anti-FLAG (M2) primary and radioactively labelled secondary IgG.

Alanine substitutions of the endocytic signals delineated in the C-terminal tail were reproduced in CFTRM2. The mutant CFTRM2 molecules were designated as their corresponding chimaeras (K2CFTRM2, K3CFTRM2, K8CFTRM2 and K9CFTRM2) and were expressed in BHK-21 cells. Because a large number of point mutations, located at virtually any position in CFTR, can disrupt biosynthetic processing [52], we first tested the chloride channel function of the mutant CFTRM2 at the plasma membrane of BHK-21 cells. In contrast with the parental BHK-21 cells, which lacked endogenous PKA-stimulated halide conductance of the plasma membrane (results not shown), a significant release of iodide was detectable from all of the mutant CFTRM2 forms except K2CFTRM2 (Figure 8A), implying that the wild-type and mutant CFTRM2 forms were functional. The inability of the K2CFTRM2 to confer PKA-stimulated iodide conductance is conceivable, because of its misprocessing. This notion was confirmed by demonstrating the exclusive expression of the core-glycosylated form of K2CFTRM2 by immunoblot analysis with the monoclonal mouse M3A7 anti-CFTR antibody (results not shown). In contrast, the fully mature complex-glycosylated form was the predominantly expressed form for the other mutants and wild-type CFTRM2 forms (results not shown). Thus the impaired halide conductance of K2CFTRM2 is most
Effect of alanine substitution on the internalization efficiency of TacT–Ct

The internalization efficiency of wild-type (Ct) and mutant (K2, K3, K6, K7, K8 and K9) TacT–Ct chimaeras was measured in COS-1 cells with radioactive antibody-binding assay as described in the Materials and methods section. Results are expressed as percentages of the internalization efficiency of wild-type TacT–Ct, corrected for the slow internalization rate of the TacT. (B, C) The surface density of wild-type and mutant TacT–Ct chimaeras was determined in COS-1 (B) and BHK-21 (C) cells with the radioactive antibody binding assay, normalized for protein and plotted as a fold increase relative to that of the wild-type TacT–Ct (Ct). Results are means ± S.E.M. for two to four separate experiments, each performed in triplicate. (D) Schematic arrangements of the endocytic signals in TacT–Ct and the effect of alanine substitutions on the internalization efficiency of the chimaeras.

Figure 7

probably a consequence of misprocessing, which precluded an assessment of the significance of the Phe113-Leu signal in the internalization of CFTR.

Mutation of the membrane-distal internalization signals (K8, Y1424A; K9, L1430A) did not interfere with the processing of CFTRM2, as demonstrated by the large-amplitude cAMP-dependent iodide release from cells expressing K3CFTRM2, K8CFTRM2 or K9CFTRM2 (Figure 8A). Although we were unable to demonstrate a significant difference in iodide release between these mutants, presumably owing to the insensitivity of the assay at a high level of CFTR expression (M. Sharma and G. Lukacs, unpublished work), a substantial difference in the cell-surface density of the internalization mutants and wild-type CFTRM2 was observed. The K8 and K9 mutations increased the cell-surface expression of CFTRM2 by 4-fold and 3-fold respectively, as determined by the radioactive antibody-binding assay (Figure 8B). Importantly, when both the Tyr1421-based and Leu1430-Leu-based internalization signals were replaced with alanine, the cell-surface expression of CFTRM2 increased 7-fold (Figure 8B). These findings are consistent with those obtained with the TacT–Ct chimaeras and strongly suggest that not only the Tyr1421-Asp-Ser-Ile-based signal but also the Leu1430-Leu-
DISCUSSION

An examination of the internalization efficiency of TIR–CFTR chimaeras led to the conclusion that Tyr1124 has a central role in the formation of an endocytic signal. The physiological importance of this signal was verified in full-length CFTR [32]. On the basis of the observations that alanine substitution in Tyr1124 only partly inhibits both the internalization of CFTR and the association of the C-terminal tail with AP-2 protein [32,33], we hypothesized that one or more additional signals contribute to the endocytic activity of CFTR. To avoid misfolding of the conformationally unstable CFTR by the introduction of point mutations [3], endocytic sorting signals were identified first with a chimaeric approach. This strategy relies on the transposable nature of the endocytic signal and has proved to be instrumental in elucidating targeting determinants to the trans-Golgi network, lysosome and endosome [18,26,53]. Three internalization signals were highlighted in the TacT–Ct chimaera; two of them were verified in the context of full-length CFTR.

Compelling evidence suggests that the C-terminal tail of CFTR contains multiple sorting information to target the TacT reporter (type I membrane protein) from the cell surface to the endocytic

Figure 8 Channel function and cell surface expression of the endocytosis-deficient CFTRM2

Wild-type (wt) and mutant (K2, K3, K8 and K9) CFTRM2 forms were expressed in BHK-21 cells. (A) cAMP-stimulated channel activity of CFTRM2 was measured with the iodide-efflux assay. Each data point represents the amount of iodide released from the indicated cells during 1 min of incubation. PKA-agonist cocktail (20 μM forskolin/0.2 mM CTP-cAMP/0.2 mM IBMX) was added at 0 min. Symbols: ○, control; ■, cAMP added. (B) The cell-surface densities of the wild-type (wt) and mutant (K3, K8 and K9) CFTRM2 forms were measured in BHK-21 cells with the monoclonal M2 anti-FLAG primary and 125I-labelled secondary antibody, essentially as described for the TacT–Ct chimaeras in the legend to Figure 7. The expression level was normalized for total protein and expressed relative to that of wild-type CFTRM2. Data are means ± S.E.M. for two or three separate experiments, each performed in triplicate.
compartment. First, fluorophore-conjugated anti-Tac antibody was targeted to endosomes and co-localized with internalized transferrin in cells expressing TacT–Ct (Figure 3). Secondly, the internalization efficiency of TacT–Ct (11 %/min) was comparable with that of TIR (11 %/min) [40], GLUT4 (14 %/min) and other chimaeras such as TacT–human leucocyte antigen-DM β-chain (8 %/min) [49], GLUT4–TIR (14 %/min) [29] and TacT–TGN38 (15.4 %/min) [50]. The internalization rate of the TacT–Ct chimaera was similar in stable and in transient expression systems (Figures 4A and 4B). Finally, the internalization of the TacT–Ct was arrested by the depletion of cellular K+ and by hypertonic treatment, which are inhibitors of the clathrin-dependent endocytic pathway [41] (Figure 4C), which also compromised the retrieval of CFTR into newly formed endosomes [8]. Taken together, these results show that the C-terminal tails of CFTR harbour endocytic sorting motifs that ensure the efficient clathrin-dependent endocytosis of the chimaera.

The steady-state distribution and internalization efficiency of mutant TacT–Ct demonstrated that the C-terminus contains three (Phe1412-Leu, Tyr1415 Asp-Ser-Ile and Leu1420-Leu) endocytic determinants. Disruption of these signals with alanine substitution largely or completely inhibited endocytosis and induced the accumulation of the chimaera at the cell surface (Figures 6 and 7). Two clusters of endocytic sorting signals were recognized in the C-terminal tail. The membrane-proximal signal (Phe1412-Leu-Val-Ile) starts with a phenylalanine residue, a conserved substitution for tyrosine, found only in a few endocytic chimaeras such as TacT–human leucocyte antigen-DM β-chain [8]. Taken together, these results show that the internalization activity (Figure 7D). Our results therefore confirmed the role of the Tyr-based signal (Tyr1415-Aser-Ile), as proposed by Prince et al. [32]. In contrast, alanine substitution of the membrane-proximal signal (Phe1412-Leu) led to misprocessing of CFTRM2, precluding the evaluation of its targeting function and leaving open the possibility that the Phe1412-Leu residues are critical for both the folding and endocytosis of CFTR. In contrast, the disruption of individual components of the bipartite membrane-distal signal caused a 4-fold (K8, Y1424A) and a 3-fold (K9, L1430A) increase in the cell-surface density of the CFTRM2 (Figure 8B; see also Figure 7D). Because deletion of the two signals simultaneously increased the channel density in an additive manner, we propose that both the Tyr-based and the previously unrecognized Leu1420-Leu-based signal have a determinant role in the regulation of CFTR cell surface density. Although iodide efflux measurements suggested that the endocytosis-deficient CFTRs were functional, further studies are needed to demonstrate that the gating and activation characteristics of these CFTR variants remain unaltered.

In summary, a tripartite endocytic sorting signal has been identified in the C-terminal cytoplasmic tails of CFTR. Although a unique interplay was revealed between three endocytic signals in the TacT–Ct chimera, the co-operative function of two of them was confirmed in the full-length CFTR. These results suggest that the internalization process of CFTR is regulated by the co-ordinated action of multiple endocytic signals.

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