

An acidic sequence within the cytoplasmic domain of furin functions as a determinant of *trans*-Golgi network localization and internalization from the cell surface

Peter Voorhees, Eileen Deignan, Elly van Donselaar¹, Jeffrey Humphrey, Michael S.Marks, Peter J.Peters¹ and Juan S.Bonifacino²

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA and ¹Department of Cell Biology and Institute of Biomembranes, Medical School, University of Utrecht, The Netherlands

²Corresponding author

The mammalian endopeptidase, furin, is predominantly localized to the *trans*-Golgi network (TGN) at steady state. The localization of furin to this compartment seems to be the result of a dynamic process in which the protein undergoes cycling between the TGN and the plasma membrane. Both TGN localization and internalization from the plasma membrane are mediated by targeting information contained within the cytoplasmic domain of furin. Here, we report the results of a mutagenesis analysis aimed at identifying the source(s) of targeting information within the furin cytoplasmic domain. Our studies show that there are at least two cytoplasmic determinants that contribute to the steady-state localization and trafficking of furin. The first determinant corresponds to a canonical tyrosine-based motif, YKGL (residues 758–761), that functions mainly as an internalization signal. The second determinant consists of a strongly hydrophilic sequence (residues 766–783) that contains a large cluster of acidic residues (E and D) and is devoid of any tyrosine-based or di-leucine-based motifs. This second determinant is capable of conferring localization to the TGN as well as mediating internalization from the plasma membrane. Thus, these observations establish the existence of a novel, autonomous determinant distinct from sorting signals described previously.

Keywords: cytoplasmic domains/furin/*trans*-Golgi network localization

Introduction

The mammalian endoprotease furin is a member of a superfamily of subtilisin-related serine proteases that cleave proteins preferentially at dibasic residue sites (see reviews by Barr, 1991; Seidah *et al.*, 1993; and references therein). The molecular cloning of furin cDNAs has revealed that the protein is a type I integral membrane protein composed of a 714 residue luminal domain, a 21 residue transmembrane domain and a 58 residue cytoplasmic domain (Hatsuzawa *et al.*, 1990; Misumi *et al.*, 1991). Furin is expressed in most mammalian cells, where it is responsible for the processing of numerous

protein precursors in the lumen of the secretory pathway. In recent years, furin has been the focus of intense interest, not only because of its critical role in protein processing but also because of its distinctive localization and trafficking within cells. Initially, immunofluorescence microscopy analyses localized furin to the general area of the Golgi complex (Bresnahan *et al.*, 1990; Misumi *et al.*, 1991; van Duijnhoven *et al.*, 1992). Recent ultrastructural studies of cells expressing epitope-tagged forms of furin have refined this localization by demonstrating that most of the protein (>80%) is concentrated within a specific compartment of the Golgi complex: the *trans*-Golgi network (TGN; Bosshart *et al.*, 1994; Molloy *et al.*, 1994). Smaller amounts of furin are also found in vesicles of the endosomal/lysosomal system (Bosshart *et al.*, 1994); this is consistent with the observations that a significant amount of furin cycles between the TGN and the plasma membrane (Chapman and Munro, 1994; Molloy *et al.*, 1994) and that the protein is eventually delivered to lysosomes for degradation (Bosshart *et al.*, 1994). A fraction of the furin molecules has also been shown to undergo shedding into the extracellular space (Wise *et al.*, 1990; Rehemtulla *et al.*, 1992; Bosshart *et al.*, 1994; Molloy *et al.*, 1994; Vey *et al.*, 1994). Thus, furin is one of a small number of integral membrane proteins that are known to be predominantly concentrated within the TGN. As such, it has become a useful model protein in the study of the molecular mechanisms that determine localization to the TGN.

Mutational analyses have provided a means of searching for TGN-targeting information within the different topologic domains of furin (Bosshart *et al.*, 1994; Chapman and Munro, 1994; Molloy *et al.*, 1994). Radical truncations of the furin cytoplasmic domain have been shown to result in a loss of TGN localization and the appearance of the truncated molecules in vesicles characteristic of endosomes or lysosomes and at the plasma membrane (Bosshart *et al.*, 1994; Molloy *et al.*, 1994). In addition, replacing the cytoplasmic domains of plasma membrane proteins, such as the Tac antigen, CD4, CD8 or CD2, with that of furin has resulted in chimeric proteins that are targeted to the TGN (Bosshart *et al.*, 1994; Chapman and Munro, 1994). These observations have led to the conclusion that the cytoplasmic domain of furin plays a major role in localization to the TGN.

A role for cytoplasmic domains in TGN localization has also been demonstrated for rat TGN38 (Luzio *et al.*, 1990; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993; Ponnambalam *et al.*, 1994), the mouse hepatitis virus M glycoprotein (Armstrong and Patel, 1991; Locker *et al.*, 1994) and the yeast proteins Kex1p (Cooper and Bussey, 1992), Kex2p (Wilcox *et al.*, 1992) and DPAP-A (Roberts *et al.*, 1992; Nothwehr *et al.*, 1993). Mutational analyses have identified single tyrosine

residues within the cytoplasmic domains of TGN38 and Kex2p and two phenylalanine residues within the DPAP-A cytoplasmic tail that are critical for TGN localization. Interestingly, these critical aromatic amino acid residues are found in contexts that resemble those of signals for internalization from the cell surface (Trowbridge *et al.*, 1993; Thomas and Roth, 1994). Other studies have shown that, in addition to the cytoplasmic domains, the transmembrane domains of proteins such as TGN38 (Ponnambalam *et al.*, 1994) and the mouse hepatitis virus M glycoprotein (Locker *et al.*, 1994) are also important for TGN localization.

To investigate the structural features that specify protein localization to the TGN further, we examined in detail the nature of TGN targeting determinants in furin. The results of our study show that the cytoplasmic but not the transmembrane domain of furin is sufficient for TGN localization. Interestingly, the cytoplasmic domain of furin contains at least two separate determinants that contribute to the localization and trafficking of furin within cells. One of these determinants, located within the N-terminal half of the cytoplasmic domain, is the tyrosine-based motif YKGL (residues 758–761). This motif resembles other tyrosine-based targeting signals, including those present in other TGN-localized proteins. The YKGL motif is active as a signal for internalization from the cell surface but seems insufficient to confer TGN localization by itself. The second determinant, found within the C-terminal half of the furin cytoplasmic domain, consists of a strongly hydrophilic sequence (residues 766–783) containing a cluster of seven acidic amino acid residues (E and D). This determinant alone is capable of conferring localization to the TGN and can also mediate endocytosis from the cell surface, independent of any other signals. Thus, these observations establish the existence of a novel targeting determinant within the cytoplasmic domain of furin.

Results

The cytoplasmic domain but not the transmembrane domain of furin mediates localization to the TGN

In a previous study (Bosshart *et al.*, 1994), we demonstrated by immunogold electron microscopy that the cytoplasmic domain of furin was sufficient to confer steady-state localization of the plasma membrane protein Tac (α chain of the interleukin-2 receptor; Leonard *et al.*, 1984) to the TGN. Because both the cytoplasmic and membrane-bound domains of the TGN-localized proteins, TGN38 (Ponnambalam *et al.*, 1994) and the mouse hepatitis virus M glycoprotein (Locker *et al.*, 1994), have been implicated in localization to the TGN, we decided to test whether this was also the case for furin. To this end, we examined the localization of two chimeric proteins (TTF and TFT) made by exchanging analogous domains between furin and Tac (Figure 1). The TTF chimera (Bosshart *et al.*, 1994) consists of the luminal and transmembrane domains of Tac and the cytoplasmic domain of furin, whereas the TFT chimera contains the luminal domain of Tac, the transmembrane domain of furin and the cytoplasmic domain of Tac (Figure 1).

Pulse-chase analyses of transiently transfected cells expressing Tac, TTF and TFT demonstrated that the three

proteins were synthesized and processed normally (Figure 2). The N-linked oligosaccharide chains of all three proteins became resistant to endo H during the chase (H lanes), indicating that the proteins were transported out of the endoplasmic reticulum (ER) and into the Golgi complex. However, the fraction of endo H-resistant forms after the chase varied for the three proteins, consistent with a different rate of transport for each chimera. The endo H-resistant forms of the three proteins were completely sensitive to neuraminidase (N lanes), indicating that the proteins were capable of reaching the *trans*-Golgi cisternae and/or the TGN.

The subcellular localization of Tac and the chimeric proteins was examined by immunofluorescence microscopy of transiently transfected normal rat kidney (NRK) cells (Figure 1). As expected, Tac displayed a typical pattern of plasma membrane localization (Figure 1a). In contrast, TTF localized to a juxtannuclear structure (Figure 1c) similar to that containing TGN38 (Figure 1d); this structure was identified previously as the TGN by immunogold electron microscopy (Bosshart *et al.*, 1994). TFT was found mainly at the plasma membrane and did not significantly accumulate in juxtannuclear structures relative to the Tac control (Figure 1e). Similar results were obtained in various other cell lines, including HeLa and Madin-Darby canine kidney cells (data not shown). These observations indicated that the cytoplasmic domain but not the transmembrane domain of furin was capable of targeting a reporter protein to the TGN.

C-terminal truncations of the furin cytoplasmic domain identify two segments with targeting activity

Having established that the cytoplasmic domain of furin had sufficient information for TGN localization, we sought to identify the sequences or structural motifs that were responsible for this targeting. Initially, we focused our analysis on the tetrapeptide sequence YKGL (amino acids 758–761). This sequence resembles the sequence YQRL that was shown previously to be important for the TGN localization of TGN38 (Bosshart *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993) and conforms to the YXX \emptyset consensus defined for a subset of tyrosine-based targeting motifs (Trowbridge *et al.*, 1993) (Y corresponds to tyrosine, X to any amino acid and \emptyset to an amino acid with a bulky hydrophobic side chain). Surprisingly, the mutation of Y758 (asterisk in Figure 3) to A had no noticeable effect on the localization of the TTF chimera in transiently transfected NRK cells, as assessed by immunofluorescence microscopy (Figure 3a compare with b). Although this technique may not be sensitive to subtle changes in protein distribution, the observation of a Golgi-like staining pattern for the mutant protein suggested the existence of some other targeting determinant within the furin cytoplasmic domain that was distinct from YXX \emptyset motifs.

To delineate the region(s) of the furin cytoplasmic domain involved in TGN localization, we performed a series of sequential deletions from the C-terminus of TTF (Figure 3). Upon transient expression of the truncated chimeras in NRK cells, we observed a gradual loss of TGN localization as sequences were removed from the C-terminus of the protein. Moreover, there was an increased detection of the truncated molecules in cytoplasmic

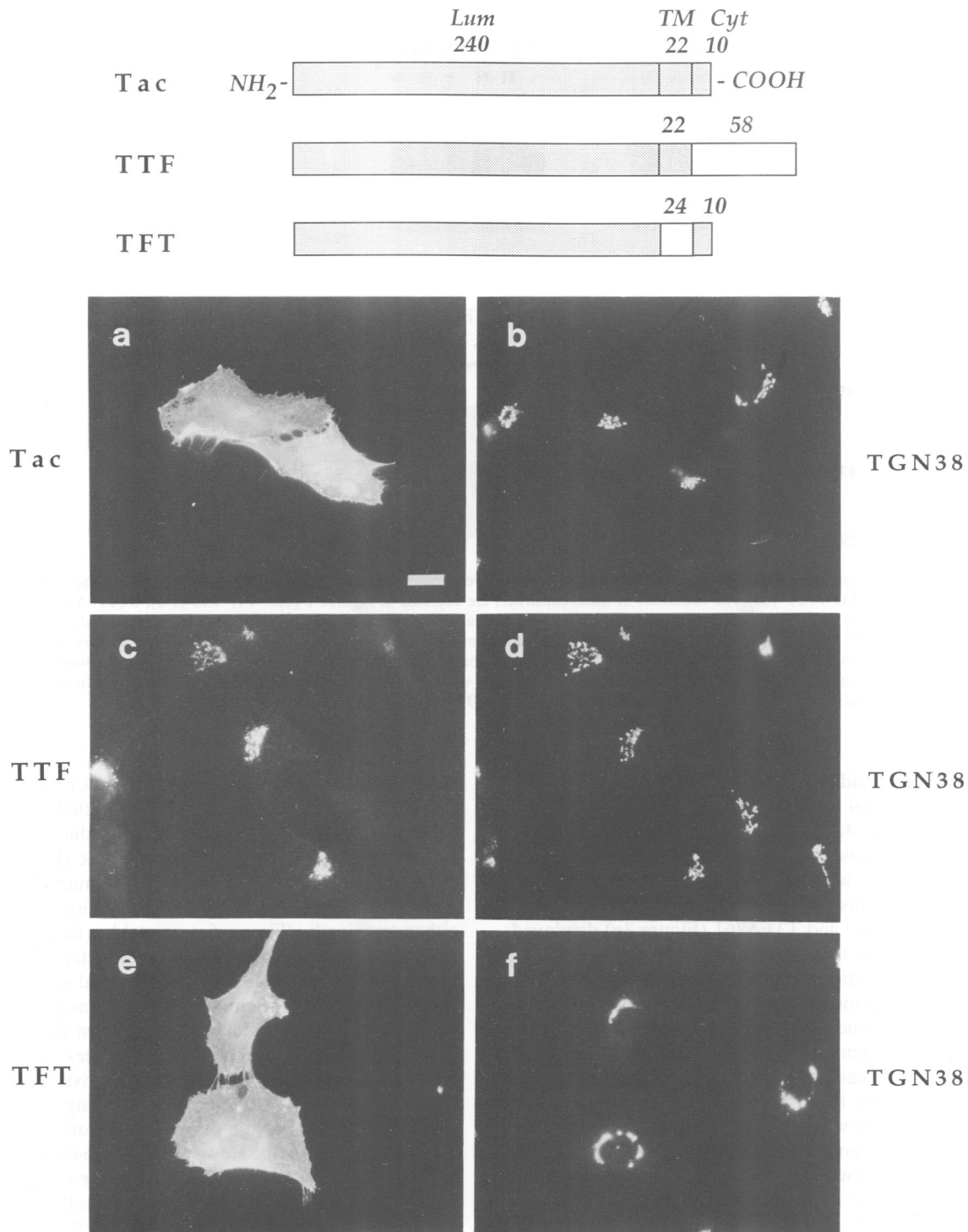


Fig. 1. Comparison of the roles of the cytoplasmic and transmembrane domains of furin in TGN localization. Upper panel: schematic representation of Tac and the Tac-furin chimeric proteins, TTF and TFT. Tac domains are indicated in gray and furin domains in white. The numbers of amino acid residues in the luminal (Lum), transmembrane (TM) and cytoplasmic (Cyt) domains are indicated. Residues in the luminal domain of Tac are counted from the initiating methionine. Lower panel: immunofluorescence microscopy of NRK cells expressing Tac (a and b), TTF (c and d) or TFT (e and f). Transiently transfected NRK cells were fixed, permeabilized and stained simultaneously with a mouse antibody to Tac (7G7) and a rabbit antibody to endogenous TGN38 (JH4), followed by rhodamine-conjugated donkey antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. (a, c and e) Rhodamine channel, Tac staining; (b, d and f) fluorescein channel, TGN38 staining. Notice the presence of normal Tac (a) and TFT (e) at the plasma membrane and the co-localization of TTF (c) with endogenous TGN38 (d). Bar: 10 μ m.

vesicles and at the plasma membrane concomitant with the loss of TGN localization (Figure 3c-i). A particularly noticeable transition, from a tight Golgi-like pattern to a more vesicular pattern, occurred upon the removal of

residues 775-779 (EEDEG; Figure 3e). The construct TTF Δ 774 was found in a diffuse juxtannuclear structure (Figure 4a) that was in the same general area of the cell as TGN38 (Figure 4b), although it had a more vesiculated

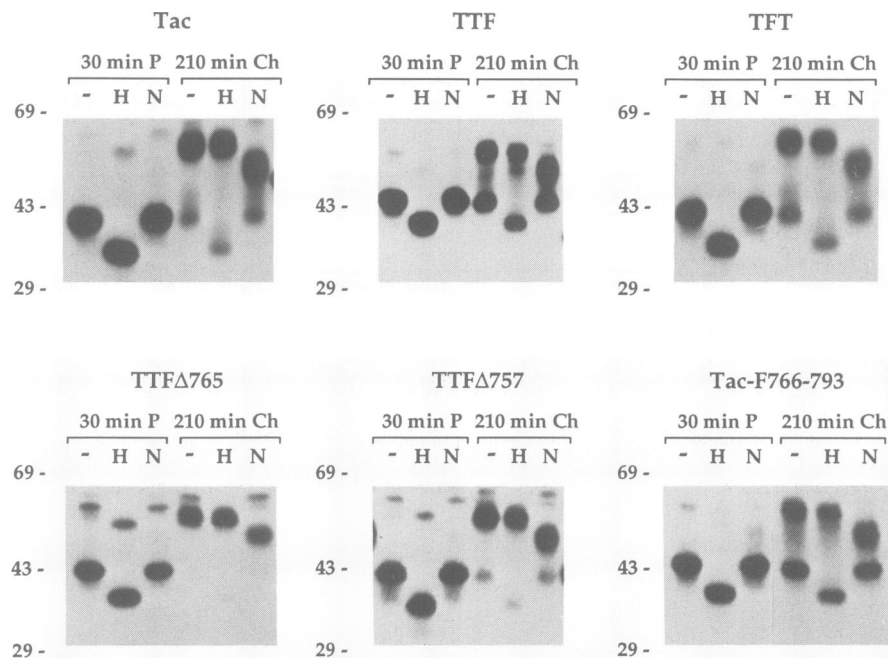


Fig. 2. Pulse-chase analysis of Tac and Tac chimeric proteins. HeLa cells were transiently transfected with plasmids encoding Tac, TTF, TFT, TTF Δ 765, TTF Δ 757 or Tac-F766-793. The structures of these constructs are depicted in Figures 1 (Tac, TTF and TFT), 3 (TTF Δ 765 and TTF Δ 757) and 5 (Tac-F766-793). At 36 h after transfection, cells were metabolically labeled for 30 min with [³⁵S]methionine and collected either immediately after the pulse (30 min P) or after a 210 min chase in regular culture medium (210 min Ch). Tac and the Tac-furin chimeras were isolated from detergent extracts using antibodies to a Tac luminal epitope (7G7). Each immunoprecipitate was divided into three aliquots that were respectively incubated with no additions (–), endo H (H) or neuraminidase (N). Samples were resolved by SDS-PAGE under reducing conditions on 10% acrylamide gels. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown to the left of each panel.

appearance. In addition, TTF Δ 774 was present in numerous peripheral vesicles that contained internalized transferrin (Figure 4c and d). These observations were consistent with at least partial localization of this construct to a compartment with morphologic characteristics of endosomes (Hopkins, 1983; Yamashiro *et al.*, 1984). TTF Δ 765 (Figure 3f) and TTF Δ 761 (Figure 3g) displayed distributions similar to TTF Δ 774.

A second transition, from a juxtannuclear/vesicular pattern to a plasma membrane pattern, was observed upon the removal of residues 758–761 (YKGL; Figure 3h). This latter observation demonstrated that while the integrity of the YKGL motif was not essential for TGN localization in the context of the full-length furin tail (as suggested in Figure 3b), the motif was nonetheless capable of mediating localization to an endosomal compartment. These data thus suggested that two segments of the furin cytoplasmic domain were active in targeting. The more membrane-distal determinant was located within the C-terminal half of the tail and included a group of acidic amino acid residues; the more membrane-proximal determinant was found within the N-terminal half of the tail and included a canonical tyrosine-based motif.

The membrane-distal region of the furin cytoplasmic domain contains an autonomous determinant of TGN localization

The observed loss of TGN localization caused by truncations in the C-terminal half of the furin cytoplasmic domain could have been caused by either a direct involvement of this segment in targeting or an indirect effect on other sequences. To investigate if the C-terminal half of the

furin cytoplasmic domain was capable of independently mediating TGN localization, we constructed an additional chimera (Tac-F766–793) in which residues 766–793 of furin were fused to the C-terminus of Tac (Figure 5); this sequence excluded the YKGL motif. Immunofluorescence microscopy of NRK cells transfected with this construct either transiently (Figure 5a) or stably (data not shown) revealed a typical Golgi-like staining pattern. An analysis of the stable transfectants by immunogold electron microscopy showed that the chimeric protein was predominantly localized to tubulo-vesicular structures in the area of the TGN (Figure 6). Some of these structures were covered with an electron-dense coat characteristic of clathrin (Figure 6, arrow). Little, if any, labeling of the Golgi stack (Figure 6, g) or the plasma membrane (Figure 6, p) was seen in these cells. However, radio-iodinated antibody binding experiments revealed the presence of a small amount of the chimeric protein at the cell surface (data not shown and Figure 9). From these observations, we concluded that the segment comprising residues 766–793 was sufficient to target a reporter protein to the region of the TGN.

Treatment with cycloheximide did not change the Golgi-like staining pattern observed by immunofluorescence microscopy of the Tac-F766–793 construct in the stably transfected cells (data not shown), thus ruling out that residence to the TGN was a transient phenomenon. Pulse-chase analysis of Tac-F766–793 gave results that were consistent with the morphologic observations. Although the newly synthesized protein seemed to exit the ER slowly, it became resistant to endo H and sensitive to neuraminidase, in agreement with its final localization to

		740	750	* 760	770	780	790																																				
a) TTF		HRC	SGF	SFR	GVK	VYTM	DRGLI	SYK	GLP	PEAW	QEE	CP	SD	SEE	DE	GR	GERT	AFI	KD	Q	S	A	L																				
b) TTF (Y758→A)		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	A	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
c) TTFΔ787		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
d) TTFΔ779		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
e) TTFΔ774		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
f) TTFΔ765		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
g) TTFΔ761		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
h) TTFΔ757		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L
i) TTFΔ746		HRC	SGF	SFR	GVK	VYTM	DRGLI	S	Y	K	G	L	P	E	A	W	Q	E	E	C	P	S	D	S	E	E	D	E	G	R	G	E	R	T	A	F	I	K	D	Q	S	A	L

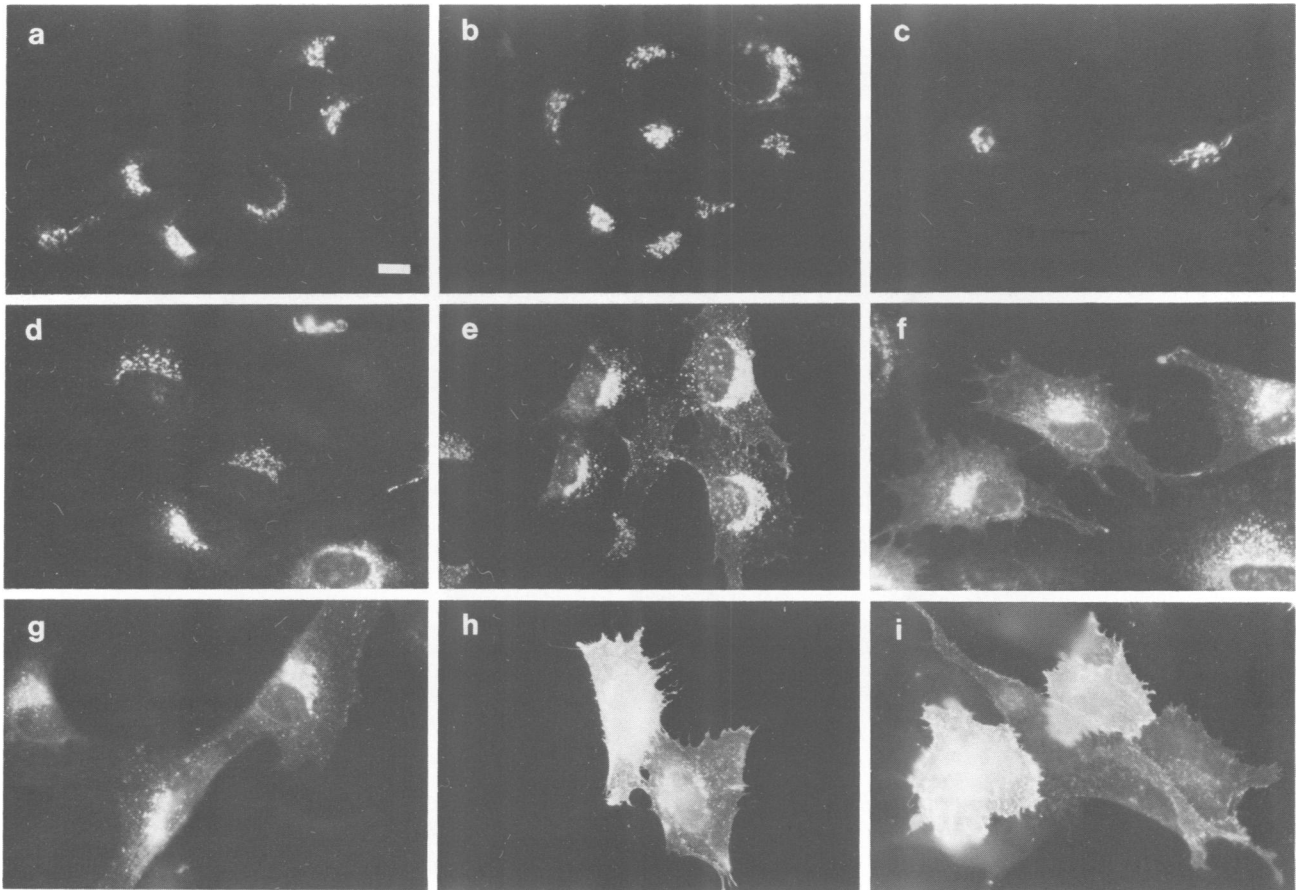


Fig. 3. Immunofluorescence microscopy of cells expressing mutants of the TTF construct. Upper panel: sequences of the cytoplasmic domains of TTF, TTF (Y758 → A) and a series of truncated forms of TTF. The structure of the basic construct TTF is shown in Figure 1. Numbers correspond to residues counted from the initiating methionine of furin. The asterisk indicates the position of Y758. Lower panel: immunofluorescence microscopy of transiently transfected NRK cells expressing the constructs shown in the upper panel. Fixed permeabilized cells were stained with a mouse monoclonal antibody to Tac (7G7) and rhodamine-conjugated antibodies to mouse IgG. Notice the lack of an effect of substituting alanine for tyrosine within the furin cytoplasmic domain (compare a with b). Also notice the gradual loss of TGN localization upon the removal of increasingly longer portions of the cytoplasmic domain (c–i). Bar: 10 μ m.

the TGN (Figure 2). Constructs TTFΔ765 and TTFΔ761 also underwent Golgi and TGN modifications of their carbohydrate chains, consistent with their localization to post-Golgi compartments (Figure 2). All three constructs were quite stable over a 3.5 h chase period (Figure 2), indicating that they were not rapidly delivered to lysosomes for degradation.

The ability of different portions of the furin cytoplasmic domain to maintain the Tac reporter protein at intracellular locations was assessed by flow cytofluorometry; the results obtained for some key constructs are shown in Figure 7. We observed that whereas Tac was expressed at high levels on the cell surface, the surface expression of TTF

was very low. Both the membrane-proximal (736–765) and membrane-distal (766–793) segments of the furin cytoplasmic domain were independently capable of maintaining the Tac reporter protein intracellularly (Figure 7, TTFΔ765 and Tac-F766–793), although each of them separately was less efficient than the full-length furin tail. For TTFΔ765, this capability was mediated by the YKGL motif, as its removal caused surface expression levels to rise to the level of normal Tac (Figure 7, TTFΔ761). These results were consistent with the observations made in the morphologic studies and confirmed that segment 766–793 was as effective as segment 736–765 at causing intracellular localization of the reporter protein.

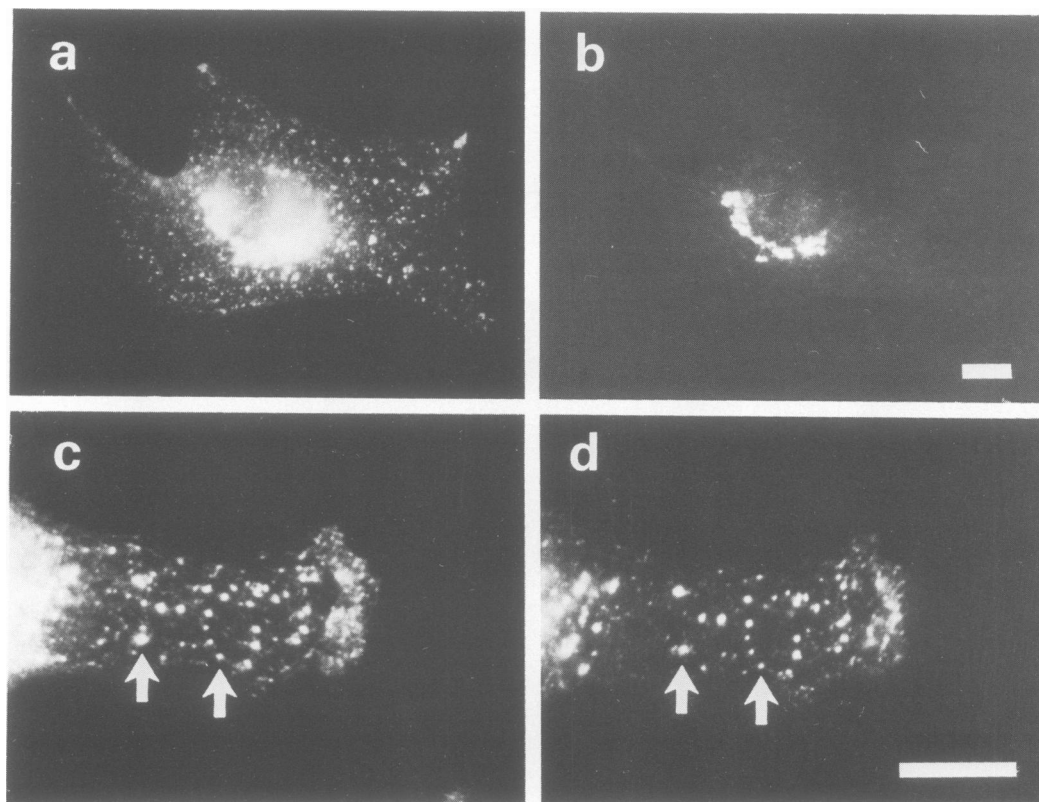


Fig. 4. Analysis of the co-localization of TTF Δ 774 with TGN38 and with endocytosed transferrin. NRK cells were transiently transfected with a plasmid encoding TTF Δ 774. (a and b) Cells were fixed and stained with a mouse antibody to Tac (7G7) and a rabbit antibody to endogenous TGN38 (JH4), followed by rhodamine-conjugated donkey antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. (a) Rhodamine channel, TTF Δ 774 staining; (b) fluorescein channel, TGN38 staining. (c and d) Cells were allowed to endocytose human transferrin for 2 h, after which cells were fixed and stained with a mouse antibody to Tac (7G7) and a rabbit antibody to human transferrin, followed by Cy3-conjugated goat antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. (c) Cy3 channel, TTF Δ 774 staining; (d) fluorescein channel, endocytosed transferrin staining. Arrows in (c) and (d) point to vesicles in which TTF Δ 774 and endocytosed transferrin are co-localized. Bars: 10 μ m.

Identification of a sequence containing a cluster of acidic amino acids as a determinant of TGN localization

Remarkably, segment 766–793 does not have any known targeting signal, such as tyrosine- or di-leucine-based motifs (reviewed by Trowbridge *et al.*, 1993; Sandoval and Bakke, 1994). The truncation analysis shown in Figure 3 suggested that the region comprising residues 775–779 might be part of the more distal determinant. To explore the targeting potential of sequences in this region further, we constructed other chimeras in which various segments between residues 766 and 793 were fused to the C-terminus of Tac (Figure 5). Constructs Tac-F766–787 (Figure 5b) and Tac-F766–783 (Figure 5c) were still mainly localized to the Golgi complex, although the latter displayed increased vesicular and plasma membrane staining. Tac-F766–779, on the other hand, showed a very prominent staining of cytoplasmic vesicles and the plasma membrane (Figure 5d). This established the approximate C-terminal boundary of the targeting determinant at residue 783. Tac-F768–793 (Figure 5e) also gave a typical Golgi-like staining pattern, whereas Tac-F772–793 (Figure 5f), Tac-F774–793 (data not shown) and Tac-F780–793 (Figure 5g) displayed increasingly vesicular and plasma membrane staining. This indicates that residue 768 demarcates the approximate N-terminal boundary of the determin-

ant. Thus, the TGN localization determinant appears to span residues 768–783.

A striking feature of sequence 768–783 is the presence of eight acidic amino acid residues (E and D), seven of which are in a stretch uninterrupted by any basic residues. Our previous analyses of C-terminally truncated constructs have already suggested that segment 775–779 (EEDEG) was important for TGN localization (Figure 3). To investigate the possible role of those acidic residues further, we mutated residues 775–778 (EEDE) to four alanine residues in the context of the Tac-F766–783 construct. As shown in Figure 5h, the mutation of the four acidic residues resulted in a significant loss of TGN localization and an increased expression of the protein at the plasma membrane. Moreover, the introduction of a positively charged residue, R, into the acidic tetrapeptide (EEDE to ERDE) also caused the partial loss of TGN localization and an increased expression at the cell surface (data not shown). Segment 768–783 also contains two serine residues (S772 and S774) that are potential sites for phosphorylation by casein kinase II (see Discussion). The mutation of both serine residues to alanine resulted in an increased staining of the plasma membrane (Figure 5i), although the loss of TGN localization was less pronounced than that caused by mutation of the acidic residues (Figure 5h). Taken together, the above observations suggest that the more

		770	780	790
a)	Tac-F766-793	WQEECP	SDSEED	EGRGERTAFIKDQSAL
b)	Tac-F766-787	WQEECP	SDSEED	EGRGERTAFI
c)	Tac-F766-783	WQEECP	SDSEED	EGRGER
d)	Tac-F766-779	WQEECP	SDSEED	EG
e)	Tac-F768-793		EECP	SDSEED
f)	Tac-F772-793		SDSEED	EGRGERTAFIKDQSAL
g)	Tac-F780-793			RGERTAFIKDQSAL
h)	Tac-F766-783 (AAAA)	WQEECP	<u>SDS</u>	<u>AAAA</u> GRGER
i)	Tac-F766-783 (ADA)	WQEECP	<u>AD</u>	<u>AD</u> EEDEGRGER

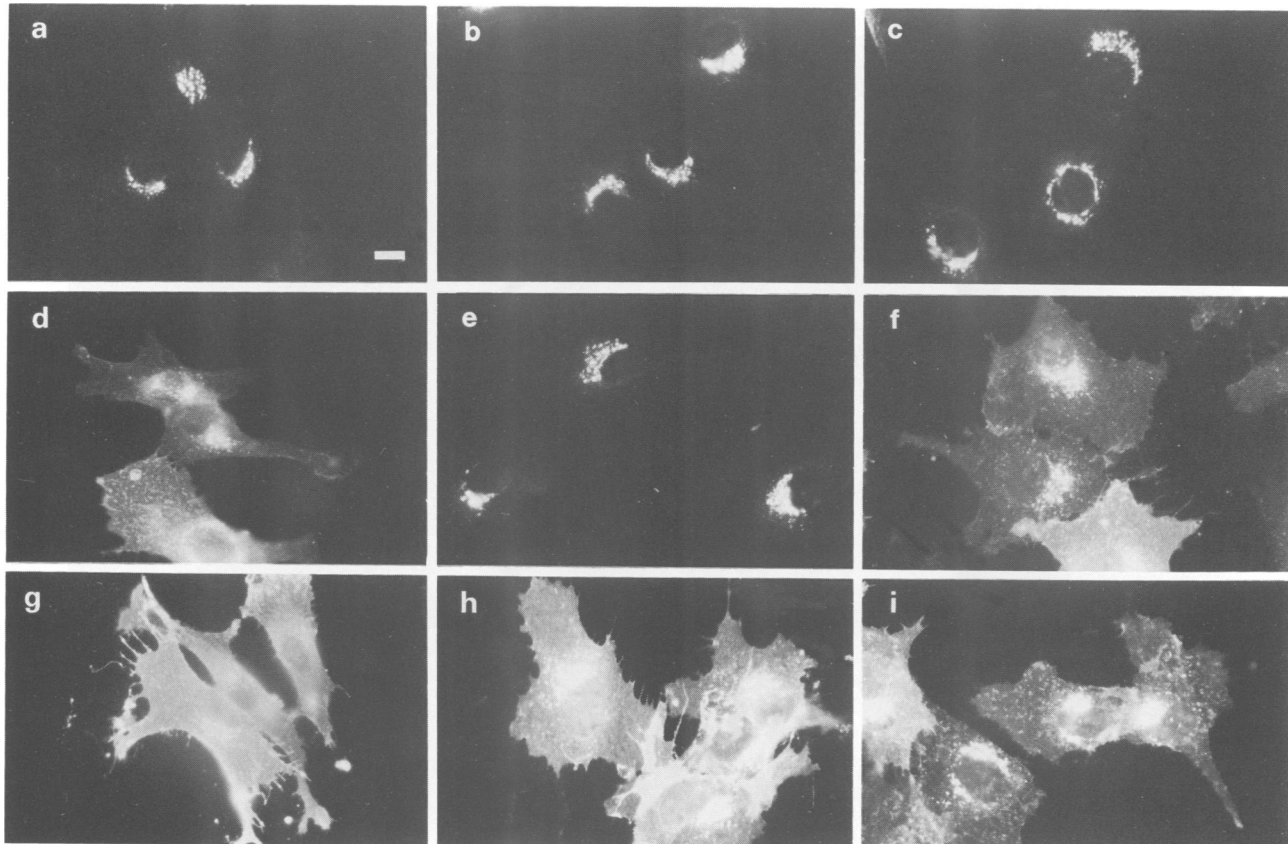


Fig. 5. Effect of adding various portions of the furin cytoplasmic domain to the C-terminus of Tac. Upper panel: sequences from the C-terminal half of the furin cytoplasmic domain were fused to the C-terminal tail of Tac. Numbers correspond to residues counted from the initiating methionine of furin. Four alanine residues inserted in place of the sequence EEDE are underlined in (h). Two alanine residues substituted for two serine residues are underlined in (i). Lower panel: immunofluorescence microscopy of transiently transfected NRK cells expressing the constructs shown in the upper panel. Fixed permeabilized cells were stained with a mouse monoclonal antibody to Tac (7G7) and a rhodamine-conjugated antibody to mouse IgG. Notice that an 18 amino acid sequence (766–783) from the furin cytoplasmic domain is sufficient for localization to a Golgi-like structure (c). Bar: 10 μ m.

distal determinant of TGN localization consists of a relatively long amino acid sequence containing a cluster of acidic residues.

Both the tyrosine-based motif and the acidic determinant can independently mediate internalization from the cell surface

While furin predominantly accumulates within the TGN at steady state, small amounts of the protein are also expressed at the cell surface (Molloy *et al.*, 1994). The surface-expressed furin appears to be part of a highly dynamic cycling pool, as antibodies to epitope-tagged furin constructs added to live, transfected cells are rapidly endocytosed and delivered to the area of the Golgi complex (Molloy *et al.*, 1994). Information leading to the rapid

internalization of furin from the cell surface resides within the cytoplasmic domain of the protein (Chapman and Munro, 1994). These observations prompted us to examine what segments of the furin cytoplasmic domain were responsible for internalization from the cell surface and what the relationship was between the determinants for internalization and TGN localization.

The ability of various Tac-furin fusion constructs to mediate the internalization of anti-Tac antibodies from the cell surface was examined initially using an assay in which transiently transfected, intact NRK cells were incubated for 4 h at 37°C in the presence of anti-Tac antibody and subsequently processed for immunofluorescence microscopy to determine the subcellular location of the antibody (Figure 8). We observed that whereas antibodies that had

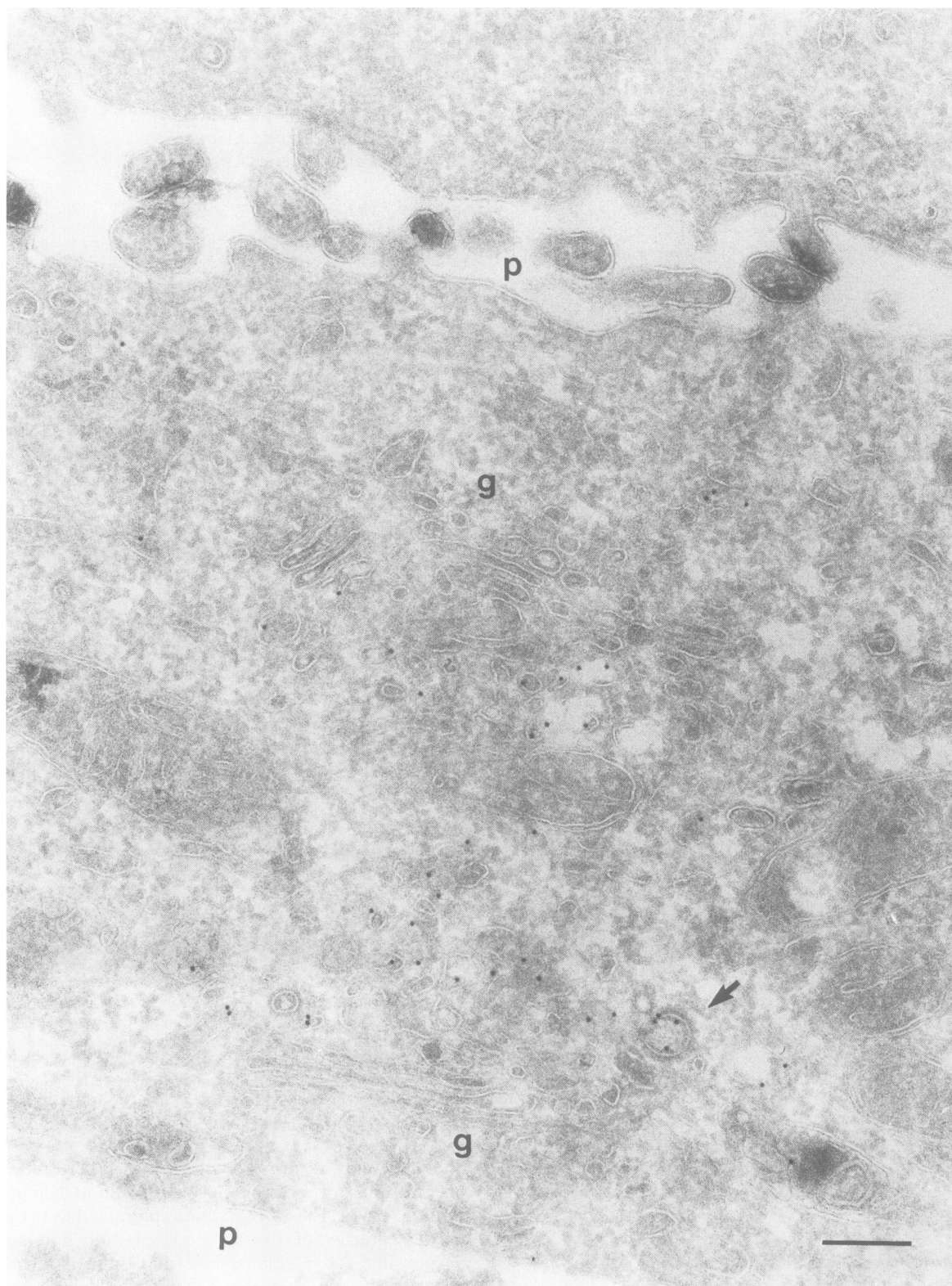


Fig. 6. Immunoelectron microscopic localization of Tac-F766-793 in stably transfected NRK cells. Cells were cultured for 24 h with 1 mM sodium butyrate to enhance expression of the transgene. Thin frozen sections of cells were sequentially immunostained with a rabbit polyclonal antibody to Tac (R3134) and 10 nm gold-conjugated protein A, as described in Materials and methods. g, Golgi complex; p, plasma membrane. The arrow points to a structure with an electron-dense coat. Bar: 0.2 μ m.

bound to the Tac antigen displayed mainly a cell surface staining pattern (Figure 8a), those that had bound to TTF accumulated in numerous intracellular vesicles and in

Golgi-like structures (Figure 8b). Moreover, a large portion of the internalized antibody co-localized with the total pool of TTF (data not shown), suggesting that the endo-

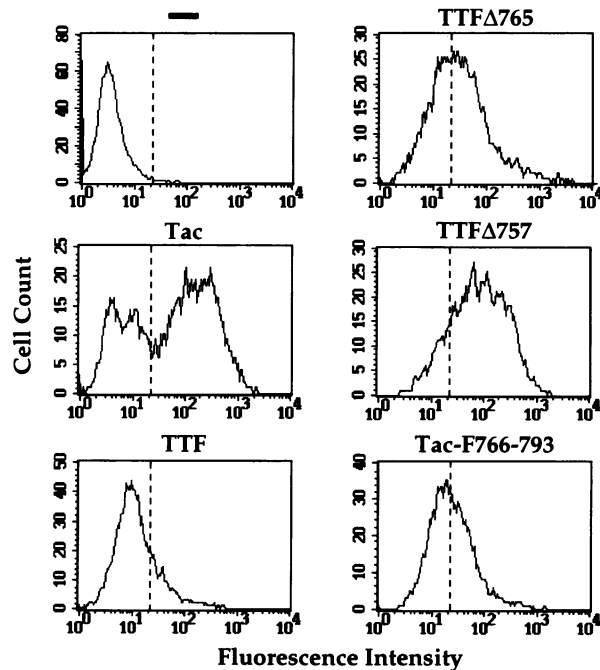


Fig. 7. Surface expression of Tac and chimeric proteins analyzed by flow cytometry. HeLa cells were transfected with plasmids encoding the different Tac constructs indicated in the figure, in combination with a plasmid encoding human CD4 to identify the transfected cells (see Materials and methods). Cells were co-stained with a phycoerythrin-conjugated antibody to Tac and a fluorescein-conjugated antibody to CD4, and analyzed by two-color flow cytometry. The fluorescence profiles represent the surface expression of each Tac construct in transfected ($CD4^+$) cells. The total expression levels of each construct were quantified by pulse labeling and immunoprecipitation. Relative to Tac, the expression levels were as follows: TTF, 10.6-fold; TTF Δ 765, 9.2-fold; TTF Δ 757, 7.7-fold; Tac-F766-793, 17.4-fold. The percentages of $CD4^+$ cells that also expressed the Tac constructs were as follows: Tac, 86%; TTF, 99%; TTF Δ 765, 91%; TTF Δ 757, 97%; Tac-F766-793, 99%. The lower expression levels and transfection efficiency of Tac with respect to the other constructs explain the presence of a population of cells that express CD4 without expressing Tac (left peak in the Tac profile).

cytosed cell surface TTF molecules were at least partially targeted to the TGN. These experiments confirmed the observations of Chapman and Munro (1994) who showed that the cytoplasmic domain of furin was sufficient to mediate the internalization of reporter proteins from the cell surface and subsequently target them to the Golgi complex.

An analysis of constructs TTF Δ 765 (Figure 8c) and Tac-F766-793 (Figure 8d) revealed that both the membrane-proximal (amino acids 736-765) and membrane-distal (amino acids 766-793) segments of the furin cytoplasmic domain had internalization activity. The removal of four residues from the end of segment 736-765 had no effect on antibody uptake (TTF Δ 761, Figure 8e), but the removal of four more amino acids, comprising the sequence YKGL, abolished antibody uptake (TTF Δ 757, Figure 8g). Similar analyses of the C-terminal segment 766-793 revealed that the removal of 10 residues from its end still allowed internalization (Tac-F766-783, Figure 8f), while the removal of four more residues (RGER) resulted in a significant loss of internalization activity (Tac-F766-779, Figure 8h). These observations suggested that the same elements that contribute to TGN localization, the tyrosine-

based motif and the acidic segment, were also capable of mediating internalization from the cell surface.

The immunofluorescence internalization assay was useful as a qualitative screen for the internalization potential of various sequences but did not allow for the quantitation of their activity. In addition, it was not possible to establish the relative contribution of the proximal and distal determinants to the internalization activity of the full-length cytoplasmic domain. To address these issues, we turned to a quantitative internalization assay using ^{125}I -labeled anti-Tac and transiently transfected cells. This assay was implemented previously in the characterization of both tyrosine-based (Letourneur and Klausner, 1992; Humphrey *et al.*, 1993) and di-leucine-based signals (Letourneur and Klausner, 1992). The rates of internalization in these transient transfectants are slower than those observed in stable transfectants, probably because of partial saturation of the internalization machinery by a high expression of the transgenic products (Marks *et al.*, 1995). Normal Tac showed very little internalization (Figure 9a-c), in agreement with previous observations (Weissman *et al.*, 1986; Humphrey *et al.*, 1993). In contrast, TTF was internalized quite rapidly, with >50% internalization achieved after 10 min (Figure 9a). TTF Δ 765 was capable of internalization, but its efficiency was reduced relative to that of a construct having the full-length furin tail (Figure 9a). The deletion of a sequence from the TTF Δ 765 construct containing the YKGL motif (construct TTF Δ 757) resulted in the complete abrogation of internalization activity (Figure 9a), consistent with a role for this signal in internalization. In accordance with this observation, the mutation of Y758 to A in the context of the full-length cytoplasmic domain resulted in a partial decrease of internalization (Figure 9a).

The ability of the TTF(Y758 \rightarrow A) construct to mediate internalization despite the destruction of its YXX Φ motif predicted that the C-terminal half of the furin cytoplasmic tail would also have significant internalization activity. Indeed, Tac-F766-793 mediated endocytosis just as effectively as did TTF Δ 765 (Figure 9b). The rate of internalization of Tac-F766-793 in this system was also similar to that of a construct having a typical tyrosine-based signal (YTPL) from the cytoplasmic domain of the Mb gene product (Marks *et al.*, 1995). Similarly, Tac-F766-783 internalized antibody, although not as well as Tac-F766-793, suggesting that the context in which the sequence functions is critical (Figure 9b). Mutation of the core acidic sequence EEDE to AAAA in the context of the Tac-F766-783 chimera abolished the ability of this construct to internalize (Figure 9c). Moreover, mutating E776 to an R residue substantially reduced the internalization activity of the acidic sequence (data not shown). The substitution of both S772 and S774 by alanine residues decreased the rate of endocytosis by about half (Figure 9c), in agreement with the immunofluorescence microscopy experiments that showed an increased expression of the mutant construct on the cell surface (Figure 5i). Thus, the results demonstrated that the tyrosine-based motif and the acidic segment are equally active in mediating internalization from the cell surface. Furthermore, the internalization activity of the full-length furin cytoplasmic domain seems to be the result of the combined activities of both determinants.

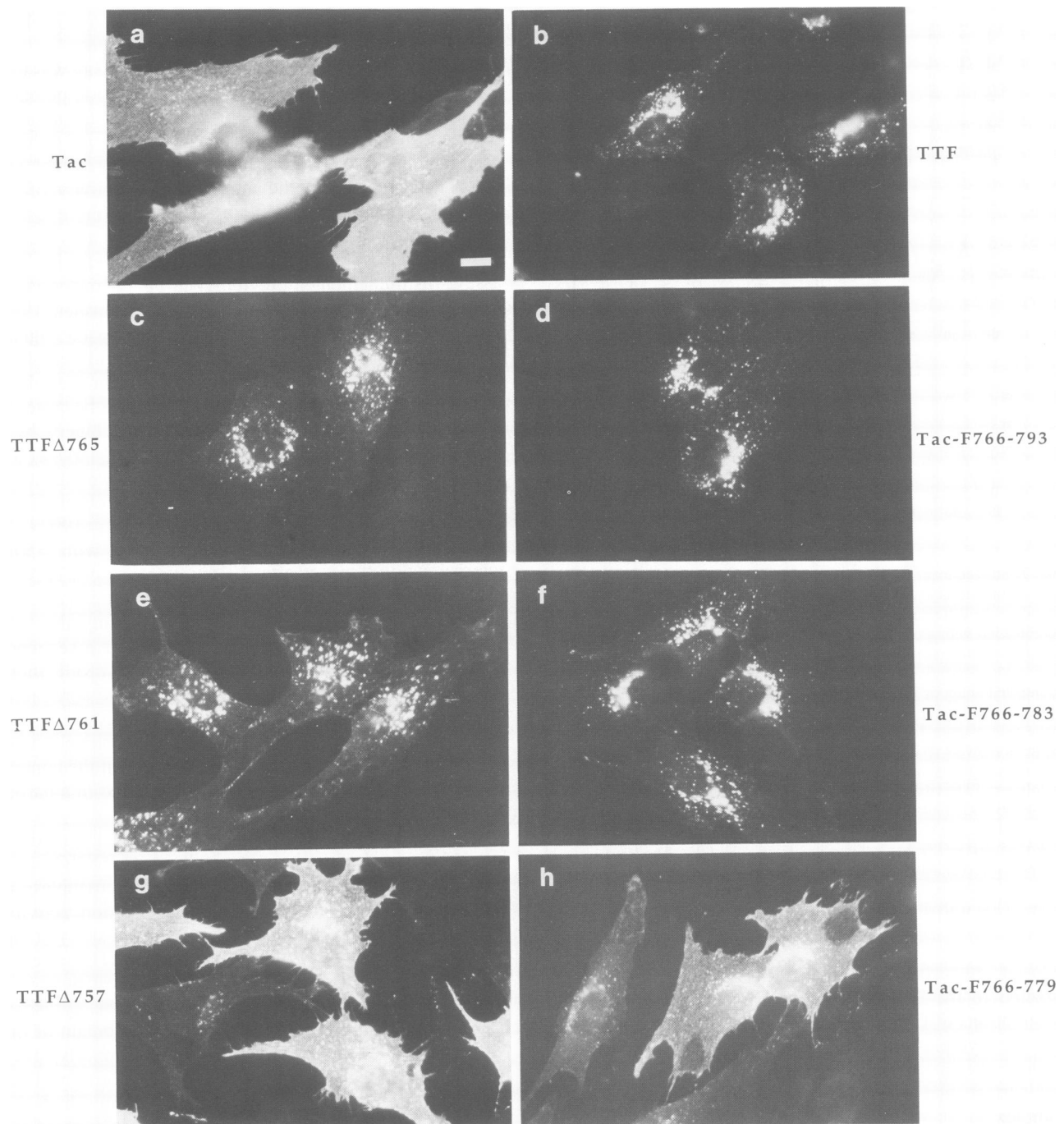


Fig. 8. Immunofluorescence microscopy analysis of the internalization of antibodies to Tac in cells expressing Tac-furin chimeras. Transiently transfected NRK cells expressing Tac or Tac chimeras were incubated for 4 h at 37°C with culture medium containing the monoclonal antibody 7G7. Cells were then fixed-permeabilized and the endocytosed antibody was detected by immunofluorescence microscopy using a rhodamine-conjugated donkey antibody to mouse IgG. No detectable staining or internalization was observed in untransfected cells. Bar, 10 μ m.

Discussion

The sorting of integral membrane proteins within endocytic and exocytic pathways is largely dependent upon information contained within the cytoplasmic domains of these proteins (Trowbridge *et al.*, 1993; Sandoval and Bakke, 1994). This information consists primarily of short, linear arrays of amino acid residues that function as sorting signals. Two types of sorting signal have been identified in the peripheral secretory pathway to date: tyrosine-based and di-leucine-based signals. Tyrosine-based signals are

characterized by the presence of a tyrosine or phenylalanine residue that is essential for function. Most tyrosine-based signals conform to the motifs NPXY (Chen *et al.*, 1990) or YXX Φ (Collawn *et al.*, 1990), 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, have critical LL or LI sequences (Johnson and Kornfeld, 1992b; Letourneur and Klausner, 1992). Both tyrosine-based and di-leucine-based signals participate in various sorting processes, such as internalization from the cell surface, targeting to

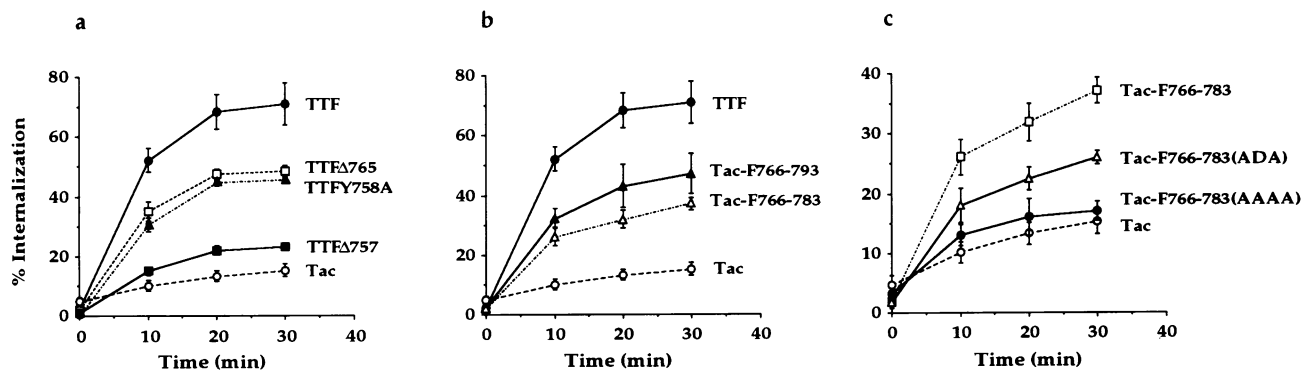


Fig. 9. Internalization of ^{125}I -labeled anti-Tac in HeLa cells transiently expressing: (a) Tac, TTF, TTF Δ 765, TTF Δ 757 or TTF(Y758 \rightarrow A); (b) Tac, TTF, Tac-F766-793 or Tac-F766-783; and (c) Tac, Tac-F766-783, Tac-F766-783(AAAA) or Tac-F766-783(ADA) (structures are shown in Figures 1, 3 and 5). ^{125}I -labeled anti-Tac was pre-bound to transiently transfected HeLa cells at 4°C, and internalization at 37°C was determined as described in Materials and methods. For most constructs, each point represents the average value derived from three to five independent determinations performed in duplicate. Values for TTF(Y758 \rightarrow A) and TTF Δ 757 are the mean of duplicate determinations from one experiment. Error bars indicate the standard deviation. No detectable binding or internalization was observed in mock-transfected HeLa cells.

endosomal and lysosomal compartments, and transport to the basolateral surface of polarized epithelial cells (Trowbridge *et al.*, 1993; Hunziker and Fumey, 1994; Sandoval and Bakke, 1994). Tyrosine-based motifs have also been implicated in protein localization to the TGN (Wilcox *et al.*, 1992; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993).

The 58 residue cytoplasmic domain of furin has been shown to mediate TGN localization and internalization from the cell surface (Bosshart *et al.*, 1994; Chapman and Munro, 1994; Molloy *et al.*, 1994). The analyses presented here demonstrate the existence of targeting information within two regions of the furin cytoplasmic domain. The first region corresponds to the N-terminal half of the cytoplasmic domain. This region contains a typical tyrosine-based motif, YKGL (residues 758–761), which predictably functions as an internalization signal and confers a pattern of steady-state localization that includes numerous endosomal vesicles. The second region with targeting information corresponds to the C-terminal half of the cytoplasmic domain. Strikingly, the targeting information within this region lies within a rather long hydrophilic sequence (residues 766–783) that contains a cluster of acidic residues and is devoid of any tyrosine-based or di-leucine-based motifs. This sequence is intrinsically capable of mediating TGN localization and internalization from the cell surface, thus indicating that it corresponds to a new type of autonomous targeting determinant. These observations suggest that internalization is a function of both the tyrosine-based and the acidic determinant, whereas TGN localization is mainly specified by the acidic determinant. Although the tyrosine-based motif did not seem capable of mediating efficient TGN localization *per se*, we cannot rule out that it might cooperate with the acidic sequence to enhance the efficiency of TGN localization.

The ability of both targeting determinants to mediate internalization from the cell surface has important implications for the trafficking of furin. Our observations as well as those of others (Chapman and Munro, 1994; Molloy *et al.*, 1994) are consistent with some degree of cycling of furin between the plasma membrane and the TGN. The internalization activity of both determinants would thus

be responsible for mediating the efficient retrieval of furin from the cell surface. The delivery of internalized furin to the TGN would necessitate another sorting step in an endosomal compartment, which would most likely be mediated by the acidic segment. Another possible explanation is that the acidic segment causes temporary retention of furin within the TGN. Retrieval from an endosomal compartment or temporary retention in the TGN mediated by the acidic segment could be rendered more efficient by the presence of the tyrosine-based signal.

A tyrosine-based motif (SDYQRL) has been implicated in the localization of glycoprotein TGN38 to the TGN (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993; Ponnambalam *et al.*, 1994). This motif mediates the internalization of TGN38 from the cell surface and also seems capable of directing the internalized TGN38 to the TGN. In comparison, the YKGL motif of the furin tail appears to be less effective at mediating retrieval to the TGN; the retrieval function is in fact more effectively carried out by the acidic sequence. The cytoplasmic tail of TGN38 has no large acidic clusters such as that of furin, although we cannot exclude the possibility that other sequences or residues might fulfill an equivalent role. TGN38 and furin also differ in the targeting potential of their transmembrane domains. Whereas the transmembrane domain of TGN38 contributes to TGN localization (Ponnambalam *et al.*, 1994), the transmembrane domain of furin has no such activity (this study). These observations suggest that similar intracellular distributions can be effected by different sets of targeting determinants.

The minimum segment in the distal region of the furin tail that was shown to confer localization to a Golgi-like compartment comprises residues 766–783 and has the sequence WQEECPDSEEDRGER (Figure 5c). Residues 766 and 767 (W and Q) are dispensable because their deletion in the context of the sequence 766–793 had no effect on the localization of a Tac chimera (Figure 5e). A critical element of the determinant is contained within the acidic tetrapeptide EEDE (residues 775–778), as demonstrated by the loss of TGN localization and internalization activity when EEDE was mutated to AAAA or when a basic R residue was inserted in place of E776. However, other observations argue that the sequence

<u>Protein</u>	<u>Cytoplasmic Sequence</u>
Furin (Rat)	TM-28aa-EAWQE ^{8/15} ECPDSE ^{8/9} EDE-15aa-COOH ^{10/13}
PC6B (Mouse)	TM-42aa-DYDE ^{7/10} DEDD-19aa-DE ^{6/8} TEDDE ^{5/6} LEYDDE-5aa-COOH
CI-M6PR (Human)	TM-31aa-EEETDENETE-36aa-DDQ ^{6/10} DSEDE-70aa-DDSDED-4aa-COOH
CD-M6PR (Human)	TM-50aa-DDQLGE ^{7/10} ESEE-8aa-COOH
PAM (Human)	TM-64aa-EDDGE ^{10/20} SE ^{5/10} EEEE-13aa-COOH
Kex1p (<i>S. cerevisiae</i>)	TM-31aa-DDLESGLGA ^{10/16} EDDLEQDEQLE-30aa-EIDESFEMTD-COOH
Kex2p (<i>S. cerevisiae</i>)	TM-38aa-EP ^{5/9} EEV ^{8/13} EDFDFDLSDED-62aa-COOH
CPY Receptor (<i>S. cerevisiae</i>)	TM-82aa-DQFLDEADD-22aa-EIE ^{5/9} EDDVPTLEEE-38aa-COOH ^{8/13}

Fig. 10. Proteins having clusters of acidic amino acids within their cytoplasmic domains. Acidic clusters were arbitrarily defined as continuous sequences in which at least 50% of the residues are aspartic or glutamic acid, contain no less than five acidic residues and have no basic residues. Numbers on top of each acidic cluster indicate the number of acidic residues over the total number of residues in the sequence. Smaller clusters are found in these as well as other proteins. The proteins in the list were chosen because they either reside within the TGN or are sorted in this organelle. Only sequences from one species are shown in the figure; sequences from other species also have acidic clusters. The sequence of rat furin was taken from Misumi *et al.* (1991), of mouse PC6B from Nakagawa *et al.* (1993), of the human cation-independent mannose 6-phosphate receptor (CI-M6PR) from Morgan *et al.* (1987) and Oshima *et al.* (1988), of the human cation-dependent mannose 6-phosphate receptor (CD-M6PR) from Pohlmann *et al.* (1987), of human peptidyl-glycine α -amidating mono-oxygenase (PAM) from Glauder *et al.* (1990), of *S.cerevisiae* Kex1p from Dmochowska *et al.* (1987), of *S.cerevisiae* Kex2p from Mizuno *et al.* (1988) and Fuller *et al.* (1989), and of the carboxypeptidase Y (CPY) receptor (also known as Pep1p and Vps10p) from Van Dyck *et al.* (1992) and Marcusson *et al.* (1994).

EEDE is not the only important element of the targeting determinant. For instance, the mutation of S772 and S774 to alanines decreased internalization from the surface (Figure 9c) and, as a consequence, increased surface expression (Figure 5i). In addition, the deletion of residues 766–771 (WQE^{8/9}ECP, Figure 5f) or 780–783 (RGER, Figure 5d) decreased TGN localization and internalization. Moreover, the Tac–F774–793 construct, which contains the critical acidic and serine residues, could not mediate endocytosis or localization to the TGN. Finally, the construct Tac–F766–793 displayed a tighter Golgi-like staining pattern than Tac–F766–783. These findings suggest that sequences flanking the critical EEDE tetrapeptide are also essential for efficient targeting. Further analyses will be needed to establish whether the flanking sequences contain other residues that are directly involved in targeting or whether their role is to provide an appropriate context for the function of the central acidic sequence.

While there is no precedent for an autonomous targeting determinant composed predominantly of acidic residues and devoid of any tyrosine-based or di-leucine-based motifs, three studies have suggested that acidic residues might influence targeting events mediated by tyrosine- or di-leucine-based signals. In one of these studies, Prill *et al.* (1993) showed that the mutation of either of two acidic residues located downstream from a tyrosine-based signal in the cytoplasmic domain of lysosomal acid phosphatase partially decreased the rate of internalization of this protein from the cell surface. A second study by Matter *et al.* (1994) demonstrated that the mutation of clusters of three acidic residues downstream from each of two tyrosine-based signals in the cytoplasmic domain of the LDL receptor decreased sorting to the basolateral surface of polarized epithelial cells but had no effect on internalization. A third study, by Chen *et al.* (1993), demonstrated that the mutation of five acidic residues directly upstream of a di-leucine-based motif at the C-terminus of the cation-independent mannose 6-phosphate receptor led to some

missorting and the secretion of lysosomal enzymes. While these observations suggest that acidic residues are important for sorting, they differ from our observations in that the acidic clusters involved seem to act in conjunction with tyrosine- or di-leucine-based signals rather than independently.

The finding that an acidic segment can mediate TGN localization and internalization raises the question whether similar structural motifs might be present in other proteins. To address this question, we surveyed the sequences of various type I integral membrane proteins for the presence of cytoplasmic acidic clusters. We arbitrarily searched for continuous sequences that were composed of $\geq 50\%$ acidic residues, had no less than five acidic residues and contained no basic residues. This definition distinguishes the type of large acidic clusters found in furin from smaller groups of acidic residues that are much more common in proteins. The survey revealed that many proteins that are either localized to the TGN or sorted within this compartment have large acidic clusters (Figure 10). Among mammalian proteins that have this putative motif are PC6B (another membrane-bound member of the furin family; Nakagawa *et al.*, 1993), both the cation-independent (Lobel *et al.*, 1987, 1988; Oshima *et al.*, 1988) and cation-dependent mannose 6-phosphate receptors (Dahms *et al.*, 1987; Pohlmann *et al.*, 1987) and PAM (Glauder *et al.*, 1990). Three yeast TGN proteins, Kex1p (Dmochowska *et al.*, 1987), Kex2p (Mizuno *et al.*, 1988) and the carboxypeptidase Y receptor (Van Dyck *et al.*, 1992; Marcusson *et al.*, 1994), also have acidic clusters. The stretches of acidic amino acids were found to be conserved for the same protein in different species. For instance, furin homologs in *Saccharomyces cerevisiae* (Kex2p; Mizuno *et al.*, 1988), *Kluyveromyces lactis* (Tanguy-Rougeau *et al.*, 1988), *Drosophila melanogaster* (Roebroek *et al.*, 1992), mouse (Hatsuzawa *et al.*, 1990), rat (Misumi *et al.*, 1991) and human (van den Ouweland *et al.*, 1990) all have acidic clusters that vary in sequence but share the same general

properties outlined above. Large acidic clusters seem to be less prevalent among plasma membrane proteins, although they are also present in the cytoplasmic domains of the receptors for epidermal growth factor (Ullrich *et al.*, 1984), prolactin (Edery *et al.*, 1989) and transferrin (McClelland *et al.*, 1984). While the presence of acidic clusters in these proteins could be coincidental, it is tempting to speculate that these sequences might play some role in intracellular trafficking.

The acidic clusters in the cytoplasmic domain of furin and of some of the proteins listed in Figure 10 resemble consensus sites for phosphorylation by the serine/threonine kinase casein kinase II (Meggio *et al.*, 1984; Hagiwara *et al.*, 1992; Boldyreff *et al.*, 1994). The acidic cluster in furin, for instance, has two serine residues (S772 and S774) that are potential substrates for casein kinase II. Preliminary data from our laboratory suggest that one or both of these residues are phosphorylated *in vivo* (P.Voorhees and J.Bonifacino, unpublished observations). Serine 833 in the cytoplasmic domain of the LDL receptor, which is upstream of a cluster of three acidic residues, has also been shown to be phosphorylated *in vivo* (Kishimoto *et al.*, 1987). Similarly, experiments with both types of mannose 6-phosphate receptor have demonstrated the phosphorylation of serine residues contained within their respective acidic clusters (Corvera and Czech, 1985; Corvera *et al.*, 1988; Meresse *et al.*, 1990; Hemer *et al.*, 1993; Körner *et al.*, 1994). The functional significance of these phosphorylation events is still unclear, although two studies on mannose 6-phosphate receptors have suggested that phosphorylation may allow the recognition of the cytoplasmic domain by cytosolic components of the sorting machinery (Le Borgne *et al.*, 1993; Rosorius *et al.*, 1993). In contrast, a study by Johnson and Kornfeld (1992a) showed that the mutation of several serine and threonine residues within the cation-independent mannose 6-phosphate receptor had no effect on sorting. In the case of furin, the mutation of S772 and S774 causes a partial decrease in internalization (Figure 9c) and TGN localization (Figure 5i), although it does not completely abrogate either process. This suggests that the phosphorylation of the serine residues is not strictly required for internalization or TGN localization but may play a regulatory role. In the light of these observations, it will now be of interest to investigate the role of phosphorylation in TGN localization or internalization mediated by the acidic clusters in the furin tail.

Materials and methods

Recombinant DNA procedures

Chimeric proteins consisting of portions of the human Tac antigen (interleukin-2 receptor α chain; Leonard *et al.*, 1984) and rat furin (Misumi *et al.*, 1991) (TTF and TFT, Figure 1) were constructed by fusion of the corresponding cDNA sequences using a double PCR protocol (Higuchi *et al.*, 1988; Saiki *et al.*, 1988). Tac-F766-793, Tac-F768-793, Tac-F772-793, Tac-F774-793 and Tac-F780-793 (Figure 5) were constructed in a similar fashion. To construct various C-terminal truncation mutants of TTF (Figure 3), reverse-strand primers were made to introduce stop codons after the codons for the desired C-terminal amino acid in a single PCR. Tac-F766-787, Tac-F766-783 and Tac-F766-779 (Figure 5) were made using Tac-F766-793 as a template in a single PCR. Point mutants [Tac-F766-783(AAAA) (Figure 5), Tac-F766-783(ADA) (Figure 5) and Tac-F766-783(R776)] were made by double PCR using Tac-F766-783 as a template and primers introducing

the appropriate mutations. All constructs used in this study were cloned in pCDM8.1, a modified version of the mammalian expression vector pCDM8 (Seed, 1987). The DNA sequence of all the recombinant constructs was confirmed by the dideoxy chain-termination method.

Cells

NRK and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) supplemented with 5% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (complete medium). The stable transfection of NRK cells was performed essentially as described by Humphrey *et al.* (1993). Single-cell clones of stably transfected NRK cells were isolated and expanded, and positive clones were identified by immunofluorescence microscopy (see below).

For transient transfections, cells were plated out at 20–30% confluency. The cells were then incubated for 2 h in DMEM containing 2% (v/v) FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin (transfection medium). Cells were transfected using the calcium phosphate precipitation method (Graham and van der Eb, 1973). The transfection medium was replaced 12–14 h after transfection with complete medium (see above). Routinely, transfection efficiencies for NRK cells ranged from 10 to 40%, whereas efficiencies for HeLa cells ranged from 30 to 90%.

Antibodies

The mouse monoclonal antibody 7G7.B6 (referred to as 7G7; Rubin *et al.*, 1985), directed to a luminal epitope of the human Tac antigen, was produced from a hybridoma clone obtained from the American Type Culture Collection. The mouse monoclonal antibody anti-Tac (Uchiyama *et al.*, 1981) was a gift from Dr Thomas Waldmann (National Institutes of Health, Bethesda, MD). The rabbit antiserum R3134 to purified human Tac (Sharon *et al.*, 1986) was a gift from Dr Warren Leonard (National Institutes of Health). The rabbit antiserum JH4 to rat TGN38 was produced in our laboratory (Bosschart *et al.*, 1994). Secondary antibodies conjugated to fluorescein, rhodamine or Cy3, suitable for multiple labeling experiments, were obtained from Jackson Immuno-Research (West Grove, PA).

Immunofluorescence microscopy

Immunofluorescence microscopy analyses were performed in NRK cells because these cells expressed moderate levels of the transfected gene products, thus avoiding saturation of the sorting machinery. Under the conditions described below, only 5–20% of the positive cells displayed overexpression of the different constructs. Transiently transfected NRK cells were grown to 50–75% confluence on glass coverslips (40–48 h after transfection). Cells were fixed for 15 min at room temperature with 2% formaldehyde in PBS. After washing in PBS, the cells were incubated for 1 h at room temperature with primary antibodies (7G7 for Tac; JH4 for TGN38) as supernatant or diluted in PBS containing 0.1% saponin. Unbound antibodies were removed by rinsing in PBS, after which cells were incubated for 30 min at room temperature with fluorescently labeled secondary antibodies in 0.1% saponin/PBS. After rinsing once more in PBS, the coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). For internalization assays, cells were incubated in sterile 7G7 culture supernatant (without NaN_3) for 4 h at 37°C, after which cells were fixed, permeabilized and stained with secondary antibodies as described above. Samples were examined and photographed with a Zeiss photomicroscope equipped with a 63 \times Planapo lens (Carl Zeiss, Oberkochen, Germany).

Electron microscopy

Stably transfected NRK cells expressing Tac-F766-793 were cultured for 24 h with 1 mM sodium butyrate to enhance the expression of the transgene. Electron microscopy of ultrathin frozen sections was performed essentially as described previously (Peters *et al.*, 1991; Humphrey *et al.*, 1993). Briefly, cells were fixed for 2 h at room temperature in 2% formaldehyde/0.2% glutaraldehyde/50 mM sodium phosphate buffer, pH 7.4. Cells were then rinsed in PBS containing 0.15 M glycine and embedded in 10% gelatin. Gelatin blocks were incubated for 16 h in 2.3 M sucrose at 4°C and frozen in liquid nitrogen. 50 nm-thin cryosections were made with a cryo-ultramicrotome (Reichert Ultracut S). Sections were incubated sequentially at room temperature with a polyclonal antibody to Tac (R3134) and protein A conjugated to 10 nm gold particles. Sections were examined with a Phillips CM 10 transmission electron microscope.

Metabolic labeling

At 36 h after transient transfection, HeLa cells were detached from plates by brief treatment with trypsin/EDTA, rinsed twice with PBS and labeled for 30 min at 37°C with 0.5 mCi/ml [³⁵S]methionine (Tran ³⁵S-Label, ICN Radiochemicals, Irvine, CA) in labeling medium (methionine-free DMEM supplemented with 3% dialyzed FBS, 100 U/ml penicillin and 100 µg/ml streptomycin). Labeled cells were either collected immediately after the pulse or chased for 210 min at 37°C in regular culture medium. Cells were collected by centrifugation at 1500 g for 5 min and frozen at -70°C.

Immunoprecipitation and electrophoresis

Labeled cells were thawed at 4°C and lysed by treatment for 15 min at 4°C in 1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4 (regular lysis buffer) containing 0.25 mM 4-(aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml leupeptin and 1.8 mg/ml iodoacetamide. Lysates were cleared by centrifugation for 15 min at 15 000 g at 4°C. The cleared lysates were incubated for 2 h at 4°C with 7G7 bound to protein A-Sepharose. The Sepharose beads were washed five times with 0.1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4 (wash buffer), and once with PBS. Immunoprecipitates were resuspended in 0.1 M sodium citrate buffer (pH 6) and divided into three aliquots. The three aliquots were incubated for 1 h at 37°C with (i) no additions, (ii) 1000 NEB Units of endo H_f (New England Biolabs, Boston, MA) or (iii) 6 mU neuraminidase from *Arthrobacter urefaciens* (Calbiochem, San Diego, CA). Samples were then incubated for 5 min at 95°C in reducing SDS-PAGE sample buffer (Laemmli, 1970). Immunoprecipitated proteins were separated by SDS-PAGE on 10% acrylamide gels, which were processed for fluorography with 1 M sodium salicylate/2% glycerol.

Analysis of surface expression by flow cytofluorometry

Flow cytofluorometry analyses were performed as described by Marks et al. (1995). Briefly, HeLa cells grown on 100 mm culture dishes were co-transfected with 2 µg of plasmids encoding different Tac constructs, 2 µg of a plasmid encoding human CD4 and 6 µg of pCDM8.1. The expression of surface CD4 was used to identify the transfected cells, independent of the surface expression of the Tac constructs. After 36–40 h, the transfected cells were detached from the dishes by incubation in PBS containing 10 mM EDTA. Cells were then co-stained in suspension with a phycoerythrin-conjugated antibody to Tac (B1.49.9, Immunotech, Westbrook, MA) and a fluorescein-conjugated antibody to CD4 (IOT4a, Immunotech), washed with PBS and fixed with 2% formaldehyde in PBS. Two-color fluorescence-activated cell sorting analysis was performed on a Becton-Dickinson FACScan machine using the CyQuest software. In parallel with the flow cytofluorometry analyses, the same cells were analyzed by pulse labeling/immunoprecipitation and by immunofluorescence microscopy to determine the expression levels and transfection efficiency, respectively.

Quantitative internalization assays

The internalization of antibodies to Tac was assayed as described previously (Weissman et al., 1986; Letourneur and Klausner, 1992; Humphrey et al., 1993). The mouse monoclonal antibody anti-Tac (Uchiyama et al., 1981) was labeled with ¹²⁵I (Amersham, Arlington Heights, IL) using chloramine T (Jensenius and Williams, 1974). At 36 h post-transfection, mock-transfected or transiently transfected HeLa cells expressing the appropriate constructs were detached from culture plates by incubation with 10 mM EDTA in PBS. The suspended cells were then washed with DMEM/10 mM HEPES, pH 7.4, and incubated with 1 × 10⁷ c.p.m./ml of ¹²⁵I-labeled anti-Tac for 30 min at 4°C in DMEM/10 mM HEPES, pH 7.4. Cells were washed extensively at 4°C twice with DMEM/10 mM HEPES and once with complete medium to remove unbound radio-iodinated antibody, after which the cells were resuspended in complete medium. The bound antibody was allowed to internalize for various periods of time at 37°C. At each time point, two 50 µl aliquots were removed and added to 200 µl of ice-cold DMEM/10 mM HEPES. At the end of all the incubation periods, the cells were pelleted. One aliquot was resuspended in DMEM/10 mM HEPES with 1 mg/ml proteinase K (Gibco-BRL, Gaithersburg, MD); the other aliquot was resuspended in the same medium but without proteinase K. The resuspended cells were then incubated for 30 min at 4°C and finally pelleted by centrifugation through a cushion of FBS. Cell pellets were cut from the bottom of frozen tubes and counted in a gamma counter. The activity of each sample was corrected by the subtraction of activity values obtained for mock-transfected cells at the appropriate time points. The percent internalization at each time point was calculated according

to the formula: Percent internalization = (Activity)₊ / (Activity)₊ + (Activity)₋ × 100.

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Note added in proof

After submission of our manuscript, an article by Schäfer *et al.* was published reporting that the sequence CPSDSEEDG functions to localize furin to the TGN, consistent with our results [Schäfer *et al.* (1995) *EMBO J.*, **14**, 2424–2435].