The predominant intracellular localization of the eukaryotic subtilisin-like endoprotease furin is the trans-Golgi network (TGN), but a small fraction is also found on the cell surface. Furin on the cell surface is internalized and delivered to the TGN. The identification of three endocytosis motifs, a tyrosine (YKGL765) motif, a leucine-isoleucine (LI760) motif, and a phenylalanine (Phe790) signal, in the furin cytoplasmic domain suggested that endocytosis of furin occurs via an AP-2/clathrin-dependent pathway. Since little is known about proteins containing multiple sorting components in their cytoplasmic domain, the combination of diverse internalization signals in the furin tail raised the question of their individual role. Here we present data showing that the furin tail interacts with the medium (μ2) subunit of the AP-2 plasma membrane-specific adaptor complex in vitro and that this interaction primarily depends on recognition of the tyrosine-based sorting signal and to less extent on the leucine-isoleucine motif. We further provide evidence that the three endocytosis signals are of different functional importance for furin internalization and retrieval to the TGN in vitro, with the tyrosine-based motif being the major determinant, followed by the phenylalanine signal, whereas the leucine-isoleucine motif is only a minor component. Finally, we report that phosphorylation of the furin tail by casein kinase II is not only important for efficient interaction with μ2 and internalization from the plasma membrane but also determines fast retrieval of the protein from the plasma membrane to the TGN.

The major endocytic trafficking pathways deliver recycling receptors back to the plasma membrane, but endocytosed integral membrane proteins can also have other intracellular destinations. Some of these proteins are selectively targeted to the trans-Golgi network (TGN) after endocytosis, like TGN38 (1), VZV gpI (2), HSV pE (3), and furin (4–7). Localization and movement of such proteins is largely achieved through the recognition of short sequence motifs within the cytoplasmic domains by cellular targeting proteins. One of the most studied processes involving such signal recognition is clathrin-mediated sorting of transmembrane proteins at the plasma membrane and also in the TGN (for review, see Refs. 8 and 9). Clathrin-coated vesicle formation is mediated by the cytosolic adaptor protein complexes AP-2 and AP-1, at the plasma membrane and the TGN, respectively. Both adaptor complexes interact directly with tyrosine- and dileucine-based sorting signals in the cytoplasmic domain of transmembrane proteins and also with clathrin which constitutes the outer layer of the coat. AP-2 complexes consist of four subunits as follows: two 100-kDa large subunits (α-adaptin and β2-adaptin), a 50-kDa medium chain (μ2), and a 17-kDa small chain (σ2). AP-1 complexes consist of four similar subunits (γ-adaptin, β1-adaptin, μ1, and σ1) (for review see Ref. 10). Tyrosine-based motifs conforming to the consensus sequence YXXφ (where φ represents a bulky hydrophobic residue) have been shown to interact directly with the medium chain (μ) subunits of AP-1 and AP-2 (11–13, for review, see Ref. 14), whereas binding of dileucine-based signals to μ-chains is still under discussion. Rapoport and co-workers (15) reported the interaction of leucine-based signals with the β1-subunit of AP-1. On the other hand, Rodionov and Bakke (27) found binding of μ-chains to dileucine motifs.

Furin is a membrane-associated, subtilisin-like eukaryotic endoprotease predominantly localized to the TGN that has been shown to reach the plasma membrane and to become internalized and targeted back to the TGN. The steady state distribution of furin implies that slower exit to the plasma membrane is coupled with rapid internalization and retrieval to the TGN. Furin proteolytically cleaves a large number of proproteins COOH-terminally at the consensus sequence RXK/RR. Among these substrates are endogenous secretory proteins and viral glycoproteins in the exocytotic pathway, as well as bacterial toxins at the cell surface and in endosomes, pointing to an important biological role for this protease in the activation of proproteins in multiple cellular compartments (see Ref. 16 and for review see Refs. 17 and 18). The cytosolic domain of furin contains a tyrosine-based (YKGL765) sorting signal, a leucine-based (LI760) motif, and a monophenylalanine (Phe790) motif, which have been shown to be essential for the internalization of this protein from the cell surface (5–7, 19, 20) and also for interaction with AP-1 Golgi-specific assembly proteins and thus sorting into clathrin-coated vesicles at the TGN (21). The involvement in different sorting processes is an important characteristic of such targeting signals. Furthermore, the furin

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tail contains an acidic cluster CPSDSEEDEG\textsuperscript{783} which is required for TGN localization. Both serine residues (Ser\textsuperscript{776} and Ser\textsuperscript{778}) within this cluster are phosphorylated by casein kinase II (CKII) in vivo and in vitro (5–7, 19). Phosphorylation and dephosphorylation of the furin tail is assumed to regulate transport of the protease in the TGN/endosomal pathway. The cytosolic connector protein PACS-1 (phosphofurin acidic cluster-sorting protein) was identified, which directs transport of phosphorylated furin molecules from endosomes back to the TGN and also from the early endosome to the plasma membrane (23). On the other hand, dephosphorylation of the furin tail by protein phosphatase 2A is assumed to be important for furin transport through the endosomal compartment (22).

Previous studies demonstrating the dynamin I-dependent internalization of furin at the plasma membrane (22) and the AP-1/clathrin-dependent sorting of furin in the TGN (21) strongly suggested that endocytosis of furin at the plasma membrane also occurs via a clathrin-dependent pathway. Furthermore, the combination of the three internalization signals raised the question of redundancy. In this study we show that the cytoplasmic tail of furin interacts with the \( \mu \)-subunit of the AP-2 complex in vitro, and we give a detailed examination of the furin tail sorting signals required for \( \mu \)-binding. By using internalization kinetics, immunofluorescence analysis, and resialylation assays, we found that the three endocytosis signals are of different functional importance for furin internalization and retrieval to the TGN. For efficient sorting of furin, the motifs have to cooperate; they cannot substitute for each other. Finally, we report that CKII phosphorylation of the furin tail enhances the interaction with the \( \mu \)-subunit and determines fast internalization and retrieval of furin molecules from the plasma membrane to the TGN.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Methods**—The \( \mu \)-subunit of plasma membrane adaptor AP-2 (AP50) was cloned by PCR technique from human placenta Agt10 cDNA library (CLONTECH). The primers were derived from the human sequence (GenBank\textsuperscript{TM} accession number U38189) as follows: forward primer, 5'-GGAGTTGGAGCGGTGCTGATT-3'; and reverse primer, 5'-CTGGGAGGCTCGAGGCTGGGGAGTGGGAGTAG-3'. The PCR product was ligated into the multiple cloning site of pBluescript vector (Stratagene) and was sequenced by the dyelex chain method using the ABI PRISM\textsuperscript{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

For generation of the different furin tail mutants the pSG5:bfur construct, described by Schäfer et al. (5), was used. Substitutions in the cytoplasmic tail were introduced by a PCR-based approach using pSG5:bfur as a cDNA template and synthetic oligonucleotides that contained the desired mutations within their sequence. Recombinants of the plasmid pSG5 containing the CD46-furin chimeras (CDF) were produced by a recombinant PCR technique (24), using the cDNA of membrane cofactor protein (CD46) isoform BC1 (25) cloned in the pSG5 vector and the cDNA of bovine furin, wild type or tail mutants, as template.

**GST Fusion Protein Production**—The cDNA coding for the cytoplasmic domains of wild type furin and furin tail mutants was ligated in-frame to the COOH terminus of GST using the pGEX-5X-1 vector (Amersham Pharmacia Biotech). The fusion proteins were expressed in Escherichia coli strain BL21, and protein purification was carried out according to the manufacturer's instructions (Amersham Pharmacia Biotech).

**Cell Culture and Transfection**—Normal rat kidney (NRK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For stable expression, NRK cells were co-transfected with pSG5 constructs and the neomycin resistance-conferring plasmid, pG-L, at a ratio of 10:1, by electroporation in a 4-mm cuvettes with a GenePulser (Bio-Rad). Geneticin-resistant cell clones were selected by addition of 1 mg of genetin per ml of medium (Calbiochem). The selected cell clones were screened for expression of furin or CD46-furin chimeras by immunofluorescence.

**Indirect Immunofluorescence**—NRK cells stably expressing the furin tail mutants, CDF wt or CD46, were grown on coverslips for 24 h at 50% confluency; 1 h prior to immunolabeling cycloheximide was added to the medium to a final concentration of 100 \( \mu \)g/ml. For surface immunolabeling of CD46, cells were incubated directly at 4 °C with CD46-specific monoclonal antibody J4/48 (Dianova) diluted 1:50 in PBS containing 0.1 mg/ml Ca\textsubscript{2}O\textsubscript{4} and 1 mg/ml Mg\textsubscript{2}O (PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+}) containing 4% formaldehyde in PBS containing with 50 \( \mu \)M NH\textsubscript{4}Cl in PBS.

For co-localization of furin and TGN38, NRK cells were fixed with acetone/methanol and then incubated with a furin-specific antiserum from goat (Jackson ImmunoResearch Laboratories) diluted 1:100 in PBS. For co-localization of CD46-furin chimera and TGN38, after fixation with acetone/methanol cells were incubated with monoclonal antibody J4/48 (diluted 1:100 in PBS) and immunofluorescence purified antibody from guinea pig specific for TGN38 (5) diluted 1:50 in PBS. The primary antibodies were detected by incubation with FITC-conjugated anti-mouse IgG from rabbit (Dako) preadsorbed to guinea pig IgG-agarose and rhodamine-conjugated anti-guinea pig antibody from rabbit (Sigma) preadsorbed to mouse IgG-agarose, both diluted 1:40 in PBS. Finally, the cells were mounted in Moviol (Hoechst) and 10% 1,4-diazabicyclo[2.2.2]octane and visualized by a Zeiss Axioshot microscope equipped with UV optics.

**Sialidase Protection Assay**—NRK cells stably expressing the CD46-furin chimeras were grown on coverslips in 24-well dishes. CD46-specific monoclonal antibody J4/48 (Dianova), diluted 1:50 in DMEM containing 5% FCS and 20 mM HEPES, pH 7.3, was then added. The cells were incubated at 37 °C for 30 min to allow antibody uptake and then washed and processed for immunofluorescence as described above.

**Resialylation Assay**—NRK cells stably expressing the CD46-furin chimeras were grown on tissue culture dishes (6 cm in diameter) for 24–48 h at 90% confluency. The cells were chilled on ice, washed with cold PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+} and biotinylated twice for 20 min in 2 ml of PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+} containing 1 mg/ml sulfo-NHS-biotin (Calbiochem). Free sulfo-NHS-Biotin was blocked by washing the cells with 0.1 M glycine in PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+} to 500 milliunits of \textit{Vibrio cholerae} sialidase (Dade Behring) for 1 h and then washed with PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+}. Prewarmed DMEM containing 2% FCS was added, and cells were returned to 37 °C for 2.5 h to allow for endocytosis and recycling to the TGN to occur. Then cells were lysed in radioimmunoprecipitation (RIPA) buffer, and CDF chimeras were immunoprecipitated with CD46-specific monoclonal antibody J4/48 (diluted 1:100) following immunoprecipitation, samples were incubated with 200 milliunits of \textit{V. cholerae} sialidase for 30 min at 37 °C to control resialylation. Finally, the immunoprecipitated material was boiled in sample buffer and separated by SDSPAGE, and proteins were transferred to PVDF membrane by standard procedure (26). After incubation with streptavidin-peroxidase (diluted 1:4000) (Amersham Pharmacia Biotech), biotinylated CDF chimeras were detected using the SuperSignal West Femto chemiluminescence (Pierce), biotinylated CDF chimeras were immunoprecipitated with CD46-specific monoclonal antibody J4/48, and CD46-furin chimeras were incubated at 4 °C with a furin-specific antiserum from goat (Dako) preadsorbed to guinea pig IgG-agarose and rhodamine-conjugated anti-guinea pig antibody from rabbit (Sigma) preadsorbed to mouse IgG-agarose, both diluted 1:40 in PBS. Finally, the cells were mounted in Moviol (Hoechst) and 10% 1,4-diazabicyclo[2.2.2]octane and visualized by a Zeiss Axioshot microscope equipped with UV optics. Quantitation of the luminescence signal of the sialylated and desialylated CDF bands was done by using a FUJI BAS 1000 bioimaging analyzer (Raytest).

**Sialidase Protection Assay**—Stably expressing NRK cells were grown on tissue culture dishes and biotinylated as described above. Following sialylation, prewarmed DMEM containing 2% FCS was added, and cells were returned to 37 °C for different times to allow desialylation to occur. To stop internalization, cells were chilled on ice, and surface proteins that had not been internalized were incubated for 1 h with 500 milliunits of \textit{V. cholerae} sialidase. After some washes with cold PBS-Ca\textsuperscript{2+}/Mg\textsuperscript{2+}, cells were lysed in RIPA buffer, and CDF chimeras were immunoprecipitated. The material was separated by SDSPAGE, proteins were transferred to PVDF membrane, and biotinylated CDF chimeras were detected by streptavidin-peroxidase. Quantitation of the luminescence signal of the sialylated and desialylated CDF bands was done by using a FUJI BAS 1000 bioimaging analyzer (Raytest).

**Sialidase Protection Assay**—The in vitro binding assay was performed as described. Briefly, the \( \mu \)-2-chains was in vitrotranslated in the presence of \textsuperscript{35}S-labeled monomine (Amersham Pharmacia Biotech) using the pBluescript-p2 DNA as template and a coupled in vitro transcription translation kit (Promega). Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) loaded with 30 \( \mu \)g of wild type or mutated GST-furin fusion protein were incubated with or without CKII (25 units/\( \mu \)l) (New England Biolabs) and 2 \( \mu \)M ATP in 30 \( \mu \)l of CKII buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM Mg\textsubscript{2}O, pH 7.5) for 1 h at
30 °C. Then in vitro translated \( \mu \)2 was added, followed by incubation for 2 h at 4 °C in 500 \( \mu \)l of binding buffer (0.05% Nonidet P-40, 50 mM HEPES, pH 7.3, 10% glycerol, 0.1% bovine serum albumin, 200 mM NaCl). After three washes, the bound material was separated by SDS-PAGE, and the radioactively labeled bands were detected by autoradiography. Quantification was done by using a BioImager (Raytest).

RESULTS

Rapid internalization involves recruitment of plasma membrane proteins to clathrin-coated pits, a process that is mediated by interaction of endocytic signals found in the cytoplasmic tail of the proteins with AP-2 clathrin-associated adaptor complexes (10, 14). The medium chain (\( \mu \)2) of AP-2 was identified as a recognition molecule for tyrosine-based motifs and leucine-based sorting signals (11, 27). Subsets of these signals are involved in additional sorting processes such as targeting to lysosomes, to specialized endosomal compartment, to the TGN, or to the basolateral plasma membrane of polarized epithelial lysosomes, to specialized endosomal compartment, to the TGN (21). Phosphorylation of both serine residues within the acidic TGN localization signal CPSDSEEDEG783 by CKII enhances recruitment of AP-1 on Golgi membranes in vivo and is important for high affinity AP-1 and \( \mu \)1 binding to the furin tail in vitro (21, 30).

In this study, we examined first whether endocytosis of furin also occurs via a clathrin-dependent pathway. Therefore we tested interaction of the furin cytoplasmic domain with the \( \mu \)2 adaptor subunit and especially the role of the four furin tail sorting signals for \( \mu \)2 binding.

Association of the Furin Cytoplasmic Domain with the \( \mu \)2-Subunit Is Modulated by CKII Phosphorylation—For our binding studies the cytoplasmic tail of wild type (wt) and mutated furin was expressed as a GST fusion protein (GST-F) (see Fig. 1). Since furin is phosphorylated by CKII both in vivo and in vitro on Ser776 and Ser778, GST-F fusion proteins were incubated either with or without CKII to obtain phosphorylated or non-phosphorylated versions of the furin tail. The medium chain \( \mu \)2 of the clathrin-associated protein complex AP-2 was translated in vitro in a rabbit reticulocyte lysate.

Fig. 2 shows that \( \mu \)2 bound to GST-Fwt but not to GST. Binding to either phosphorylated or non-phosphorylated GST was less than 2% and thus could be considered as background binding. Used as negative control, in vitro translated influenza virus NS1 did not interact with GST-Fwt (data not shown). After phosphorylation by CKII \( \mu \)2 binding to GST-Fwt increased. To ensure that increased \( \mu \)2 binding to the furin tail is really due to phosphorylation, we dephosphorylated CKII-treated GST-Fwt with alkaline phosphatase. After phosphatase treatment the \( \mu \)2 binding was reduced again showing that stronger interaction attributes to CKII phosphorylation (data not shown). This shows that the furin cytoplasmic tail interacts with the \( \mu \)2 adaptor subunit and that CKII phosphorylated furin tail is favored in this interaction.

Since we showed recently that only the number of negative charges in the acidic cluster is crucial for the affinity of AP-1 binding (21), we investigated here whether the \( \mu \)2-subunit of the AP-2 complex behaves in a similar way. The exchange of both serine residues (Ser776 and Ser778) to aspartic acid mimics the dephosphorylated state of furin (19, 30). As expected, such a GST-furin mutant (mutant S776D/S778D) bound \( \mu \)2 as efficiently as CKII-phosphorylated GST-Fwt. Mutant S776A/S778A, where both serine residues were substituted by alanine, behaved like non-phosphorylated wt (Fig. 2). To test whether introduction of negative charges in form of aspartic acid also increases the affinity for \( \mu \)2 binding when they are placed at different positions in the furin tail, the following mutants were used. Mutant D798/799, a GST-furin protein in which two aspartate residues were added to the carboxyl terminus of the furin tail, interacted less efficiently with \( \mu \)2 than the mutant S776D/S778D. On the other hand, the R784D/G785D mutant, in which Arg784 and Gly785 were mutated to aspartic acid, bound the \( \mu \)2-subunit nearly to the same extent as mutant S776D/S778D (Fig. 2). This implies that for efficient \( \mu \)2 binding it makes no difference whether negative charges are introduced by phosphorylation of Ser776 and Ser778 within the acidic sequence or by additional acidic amino acids in this region. It seems that high affinity \( \mu \)2 binding also depends on the number of negative charges in or near the acidic cluster.

The Cytoplasmic Tail Signals YKGL765 and LI760 Are Required for Furin Interaction with the \( \mu \)2 Adaptor Subunit in Vitro—Next we focused on the requirement of the three furin tail endocytosis signals YKGL765, LI760, and Phe790 for interaction with the \( \mu \)2 adaptor subunit. Again GST-F fusion proteins containing the cytoplasmic tail of wt or mutated furin (see Fig. 1) were tested in our in vitro binding assay. As shown in Fig. 3, furin tail substituted in the tyrosine motif (mutant AKGA) bound \( \mu \)2-2-fold less efficiently than wild type, whereas changing LI760 to alanine and asparagine (mutant LI/AN) resulted only in a slight decrease in \( \mu \)2 binding compared with wt furin. Surprisingly, mutation of phenylalanine 790 did not abolish the interaction, although Phe790 had been identified as a furin internalization signal (20). Similar results were found with the double mutants AKGA F/N and LI/AN F/N. They interacted with \( \mu \)2 like the single mutants AKGA and LI/AN, also indicating that substitution of phenylalanine 790 does not
incubated with in vitro material was subjected to SDS-PAGE, and already found for wt furin, we observed that phosphorylation of AKGA F/N, GST-F LI/AN F/N, GST-F LI/AN Y/A, GST-F LI/AN, GST-F F/N, GST-F furin tail mutants (see Fig. 1) were phosphorylated (+) or not (−) with CKII as indicated. Loaded glutathione beads were incubated with in vitro translation and [35S]methionine-labeled material was separated by SDS-PAGE and quantitated by bioimager analysis. The amount of bound μ2-chain is expressed as a percentage of binding to CKII phosphorylated wild type furin tail (GST-Fwt-P). An image of a representative experiment is shown. At least three independent experiments were performed. Error bars represent the standard deviation of the mean.

![Fig. 2. Binding of medium chain μ2 to immobilized furin tail and the effect of CKII phosphorylation. GST and GST-furin fusion proteins (GST-Fwt, GST-F S776D/S778D, GST-F S776A/S778A, GST-F D798/799, GST-F R784D/G785D; see Fig. 1) were immobilized on glutathione-Sepharose 4B beads and phosphorylated (+) or not (−) with CKII as indicated. Loaded glutathione beads were incubated with in vitro translated and [35S]methionine-labeled μ2-chain; the bound material was separated by SDS-PAGE and quantitated by bioimager analysis. The amount of bound μ2-chain is expressed as a percentage of binding to CKII phosphorylated wild type furin tail (GST-Fwt-P). An image of a representative experiment is shown. At least three independent experiments were performed. Error bars represent the standard deviation of the mean.](image)

**Fig. 2.** Binding of medium chain μ2 to immobilized furin tail and the effect of CKII phosphorylation. GST and GST-furin fusion proteins (GST-Fwt, GST-F S776D/S778D, GST-F S776A/S778A, GST-F D798/799, GST-F R784D/G785D; see Fig. 1) were immobilized on glutathione-Sepharose 4B beads and phosphorylated (+) or not (−) with CKII as indicated. Loaded glutathione beads were incubated with in vitro translated and [35S]methionine-labeled μ2-chain; the bound material was separated by SDS-PAGE and quantitated by bioimager analysis. The amount of bound μ2-chain is expressed as a percentage of binding to CKII phosphorylated wild type furin tail (GST-Fwt-P). An image of a representative experiment is shown. At least three independent experiments were performed. Error bars represent the standard deviation of the mean.

influence the binding of the μ2-chain. After substitution of both the tyrosine-based motif and the LI motif (mutant LI/AN Y/A) and after removal of all three motifs (mutant LI/AN Y/A F/N) binding of in vitro translated μ2 was at least 5-fold reduced. As already found for wt furin, we observed that phosphorylation of furin tail mutants also resulted in a significant increase in μ2 binding. This observation includes mutants LI/AN Y/A and LI/AN Y/A F/N lacking the critical internalization motifs. To exclude that the loss of μ2 binding to mutated GST-furin is due to less efficient CKII phosphorylation, a kinase assay was performed, showing that mutated GST-F is phosphorylated as efficiently as the wild type (data not shown).

These experiments suggested that the tyrosine-based and to least extent the leucine-based endocytosis signal mediate specific interaction of the furin cytoplasmic domain with the μ2-subunit, whereas CKII phosphorylation modulates the binding affinity by providing additional negative charges (see also Fig. 2).

**Internalization Kinetics of CD46-Furin Chimeras Mutated in One Endocytosis Signal**—With the in vitro binding assay we identified the tyrosine-based motif as the major determinant for μ2 binding, whereas the LI-signal seems to be a minor component. Therefore, it was of interest whether there were also differences in the functional importance of the furin tail endocytosis signals in vivo. To examine this possibility, we analyzed CD46-furin (CDF) chimeras for endocytosis by a sialidase protection assay (31). CD46, a widely distributed complement regulatory protein, is localized to the basolateral surface of polarized epithelial cells but is not endocytosed (31). The CD46 reporter was used instead of furin, because of its increased electrophoretic mobility after removal of sialic acids, its failure of being secreted into the culture medium in contrast to furin and its higher expression level in recombinant cells. The chimeric proteins used in our study are shown in Fig. 1. In the CDF chimeras the cytoplasmic tail of CD46 was replaced with the cytoplasmic tail of wt or mutated furin. The chimeras were stably expressed in NRK cells and first analyzed for endocytosis by antibody uptake as described under “Experimental Procedures.” The pattern of fluorescence in Fig. 4A shows that CDF wt and mutants substituted in one endocytosis signal (mutant LI/AN, F/N, and AKGA) were found to be internalized, whereas surface-expressed CD46 was not subject to endocytosis. To perform the sialidase protection assay, CDF-expressing NRK cells were surface-labeled with biotin and chased for various periods at 37 °C to allow internalization of proteins. The extent of endocytosis was measured by the proportion of biotinylated protein that became inaccessible to extracellular neuraminidase added at 4 °C at the end of the chase period. After digestion with neuraminidase, cells were lysed, and CDF chimeras were immunoprecipitated and separated on an SDS gel. Biotinylated proteins were detected after transfer to PVDF membrane by streptavidin/peroxidase. CDF chimeras on the cell surface were sensitive to neuraminidase treatment. The release of sialic acids resulted in an increased electrophoretic mobility. Internalized protein was resistant to the enzyme treatment and retained its sialic acids. As shown in Fig. 4B, after 5 min 60% of CDF wt was internalized. The amount internalized increased to 80% after an incubation period of 30 min. By using longer chase periods, the percentage of CDF wt internalized did not further increase, suggesting that after 30 min the internalized protein is partly reexpressed at the cell surface (data not shown). This observation is consistent with the view that furin undergoes a local cycling between early endosomes and the cell surface (22). Mutation of the tyrosine-based motif YKGL (mutant AKGA) that led to 50% decrease in association with μ2 also significantly reduced CDF internalization. After 30 min at 37 °C only 45% of surface-labeled CDF AKGA was found to become neuraminidase-resistant. On the other hand, mutation of LI (mutant AKGA) that had little effect on μ2 binding, also showed only a slight decrease in endocytosis compared with CDF wt. In contrast to the μ2 binding behavior of the F/N mutant, substitution of Phe790 resulted in a 20% decrease in CDF internalization compared with wt, confirming previous studies that identified Phe790 as an important factor.
performed, and the S.E. at each time point was

analysis. Internalization is given as the quotient of sialylated and total biotinylated protein

and desialylated proteins were detected by streptavidin/peroxidase. The amount of biotinylated CDF chimeras was quantitated by bioimager

chimeras were immunoprecipitated; the precipitates were separated by SDS-PAGE and transferred to PVDF membrane. Biotinylated sialylated

stably expressing CD46-furin chimeras, CDFwt ( ), CDF LI/AN ( ), CDF F/N ( ), CDF AKGA ( ), and CD46 ( ) (see Fig. 1) were surface-biotinylated, incubated at 37 °C for 0, 5, 15, and 30 min, and then treated with neuraminidase at 4 °C for 60 min. After cell lysis CD46-furin chimeras were immunoprecipitated; the precipitates were separated by SDS-PAGE and transferred to PVDF membrane. Biotinylated sialylated and desialylated proteins were detected by streptavidin/peroxidase. The amount of biotinylated CDF chimeras was quantitated by bioimager analysis. Internalization is given as the quotient of sialylated and total biotinylated protein × 100%. Three independent experiments were performed, and the S.E. at each time point was <10%.

The results demonstrate that the three furin tail endocytosis signals are of different functional importance, with the tyrosine-based motif being the major determinant, followed by the Phe motif, whereas LI760 is only a minor component.

Involvement of Furin Tail Sorting Signals YKGL765, LI760, and Phe790 in Plasma Membrane to TGN Transport—Little is known about proteins like furin that contain multiple sorting components in their cytoplasmic domain. In the case of furin the combination of diverse internalization signals raises the question of redundancy. From our in vitro binding and internalization studies, we reasoned that the three furin tail endocytosis signals do not equally contribute in clathrin-dependent endocytosis at the plasma membrane. To extend these observations we examined subcellular localization and resialylation of authentic furin or CDF tail mutants, respectively, substituted in LI760, YKGL765, or Phe790 (see Fig. 1). First, NRK cells stably expressing either of the furin tail mutants were analyzed by immunofluorescence whether the expressed foreign protein localizes to the TGN. To indicate the TGN localization in each cell, double immunofluorescence analysis with antibodies against furin and TGN38, a well established endogenous marker protein for the TGN, were performed. As shown in Fig. 5, the furin mutants substituted in one endocytosis signal (mutant LI/AN, F/N, and AKGA) as well as the double mutant LI/AN Y/A, lacking the LI and the tyrosine motif, were found in the TGN of recombinant NRK cells as was the parental furin protein. After mutation of LI760 and Phe790 (mutant LI/AN F/N) significant surface expression was observed, and the strict Golgi staining pattern was lost. The pattern of fluorescence changed further when YKGL765 and Phe790 (mutant AKGA F/N) and also when all three internalization motifs were destroyed (mutant LI/AN Y/A F/N). These mutants were predominantly expressed on the cell surface of recombinant NRK cells.

To confirm the localization studies and especially to learn more about the time course required by wt and furin tail mutants for retrieval from the plasma membrane to the TGN, we used a resialylation assay (32). This study relies on the glycoprotein-modifying enzyme sialyltransferase, which has been localized to the trans-Golgi cisternae and the TGN in a

number of cell types (33, 34). Cells were treated with neuraminidase to remove sialic acid residues from surface proteins so that they are substrates of sialyltransferase when transported to the TGN. As described for the sialidase protection assay, we took advantage of NRK cells stably expressing CDF chimeras (see Fig. 1). CDF wt showed the same pattern of fluorescence as observed with furin wt and co-localized with TGN38 (Fig. 6). Immunofluorescence analysis of CDF tail mutants revealed a
similar distribution as described for the furin tail mutants (data not shown). Only in high expressing cells the CDF chimera were found in additional cytoplasmic, endosomal-like structures, a phenomenon also being seen with overexpressed furin and TGN38 (35), suggesting that transport pathways become saturated by high levels of protein expression. For resialylation, recombinant NRK cells were surface-labeled with biotin and then treated with neuraminidase to remove sialic acids from surface glycoproteins. After culture in growth medium at 37 °C for further 2.5 h, cells were lysed. CDF chimeras were then immunoprecipitated, transferred to nitrocellulose, and biotinylated proteins detected by streptavidin/ peroxidase. As shown in Fig. 7A, sialic acid residues on surface CDF wt were removed by neuraminidase treatment, as indicated by the increased electrophoretic mobility. After 2.5 h of reculture the high molecular weight species reappeared, demonstrating that surface-labeled CDF wt was resialylated and thus retrieved to the TGN. To prove that the reappearance of high molecular weight CDF species in recultured neuraminidase-treated NRK cells was really due to the addition of sialic acid, a second neuraminidase treatment was performed after cell lysis. As seen in Fig. 7, the bands shifted to the desialylated form. The mutant lacking the LI motif (mutant LI/AN) was subject to resialylation as was the parental CDF protein (Fig. 7b), which is consistent with the previous results that LI760 is a minor sorting component. Although mutants substituted in the tyrosine or the phenylalanine motif (mutant AKGA and F/N) as well as double mutant LI/AN Y/A were found to be localized to the TGN at steady state (Fig. 5), these mutants were significantly delayed in retrieval from the plasma membrane to the TGN (Fig. 7, c–e). After 2.5 h of reculture approximately 50% of surface-labeled CDF F/N became resialylated, whereas with mutant AKGA and LI/AN Y/A the amount of resialylated protein was less than 40%. Mutant LI/AN F/N, which localized to the Golgi and the cell surface, and mutants AKGA F/N and LI/AN Y/A F/N that were predominantly expressed on the cell surface were not resialylated within 2.5 h of reculture as was control CD46 (Fig. 7, f–i).

Thus the results of the resialylation analysis confirmed those obtained by the in vitro binding studies and internalization kinetics. They indicate that both the tyrosine-based motif and the monophenylalanine signal act as efficient targeting signals and are not able to substitute for each other, whereas LI760 is only a minor sorting component. For efficient recycling the interplay of the different motifs is important rather than one distinct signal. It should be pointed out that substitution of one internalization signal had no visible effect on the steady state localization of furin but that furin recycling is significantly slowed down after mutation of YKGL765 or of Phe790 as indicated by resialylation analysis.

Importance of the CKII-phosphorylated Acidic Signal for Internalization and Cell Surface to TGN Transport of Furin—Previous work was engaged in analyzing the effect of furin tail phosphorylation on Ser776 and Ser778 (19, 30). Molloy and co-workers (22) propose that dephosphorylation of the furin tail is necessary for furin transport through the endosomal compartment, whereas the phosphorylated version is required for transport from early endosomes to the plasma membrane and from endosomes to the TGN, in either case via interaction with PACS-1. We reported recently (21) that CKII phosphorylation enhances AP-1 recruitment on Golgi membranes and AP-1 and μ1 binding to GST-F fusion protein. In this study we further demonstrated that phosphorylated furin tail is also favored in μ2 interaction, pointing to a role of CKII phosphorylation in furin internalization. Therefore, we finally examined the requirement of phosphorylated Ser776 and Ser778 for endocytosis and plasma membrane to TGN transport of furin.

Fig. 8A shows the steady state distribution of furin tail mutants S776D/S778D and S776A/S778A in stably transfected NRK cells. Mutant S776D/S778D, imitating the diphasphorylated state of furin by mutation of both serine residues to aspartic acid (19, 30), and also mutant S776A/S778A, containing a non-phosphorylatable acidic signal, were localized to the TGN as was furin wt.

The internalization kinetics were performed as described before, using CD46-furin chimeras (CDF) stably expressed in NRK cells. As shown in Fig. 8B mutant CDF S776D/S778D, imitating the diphasphorylated state of furin, was internalized with high efficiency. After 5 min and also after 15 min at 37 °C, the proportion of internalized protein was higher than the corresponding values for wt CDF. This observation could be due to the fact that in vivo furin exists as di-, mono-, and non-phosphorylated forms (19), whereas mutant CDF S776D/S778D only represents the diphasphorylated and maybe the most efficiently internalized form. With mutant CDF S776A/S778A containing a non-phosphorylatable acidic cluster, we observed a 20% decrease in endocytosis. After 30 min at 37 °C
Fig. 8. Importance of phosphorylated acidic cluster for furin routing. A, NRK cells stably expressing furin wt (a and b) or furin tail mutants S776D/S778D (c and d) and S776A/S778A (e and f) were treated with cycloheximide for 1 h prior to acetone/methanol fixation. Cells were double-labeled with an antisera directed against the cytoplasmic tail of furin (a, c, and e) and a monoclonal anti-TGN38 antibody (b, d, and f) and immunostained. B, analysis of endocytosis by neuraminidase-protection assay. NRK cells stably expressing CD46-furin chimeras, CDF wt (●), CDF S776A/S778A (○), CDF S776/778A (▲), and CDF LI/AN Y/A F/N (△) (see Fig. 1) were surface-biotinylated, incubated at 37 °C for 0, 5, 15, and 30 min, and then treated with neuraminidase for 3 h. Thus we demonstrated here that phosphorylation determines fast recycling of CDF chimeric proteins from the cell surface to the TGN.

60% of surface-labeled CDF S776A/S778A became neuraminidase-resistant compared with 80% for wt CDF and mutant S776D/S778D. On the other hand, mutant LI/AN Y/A F/N, lacking all three internalization motifs and containing only the intact acidic signal was not subjected to endocytosis. The data show, consistent with the result that CKII phosphorylation of Ser776 and Ser778 is important for efficient interaction of the furin tail with μ2, that phosphorylation also modulates internalization of CDF chimeric proteins in vivo. The intact acidic cluster alone is not able to mediate endocytosis.

For resialylation kinetics (Fig. 8C) NRK cells stably expressing either CDF wt, CDF S776D/S778D, or CDF S776A/S778A were surface-labeled with biotin, treated with neuraminidase to remove sialic acids, and then recultured in growth medium at 37 °C for various times. As with the CDF wt, after 30 min of reculture high molecular weight species of surface-labeled CDF S776/778D began to reappear, with a corresponding decrease in desialylated species. After 2 h of reculture desialylated species completely disappeared, indicating that surface-labeled protein was completely resialylated. In contrast to the S776D/S778D mutant, non-phosphorylatable CDF S776A/S778A exhibited a significant decrease in resialylation kinetics. Only after 2 h of reculture resialylated species began to reappear and only 50% of surface-labeled CDF S776A/S778A became resialylated after 3 h. Thus we demonstrated here that phosphorylation determines fast recycling of CDF chimeric proteins from the cell surface to the TGN.

DISCUSSION

Although the predominant intracellular localization of furin is the TGN, a small fraction of furin is found on the cell surface (5). Furin on the cell surface is internalized and delivered to the TGN. Efficient recycling depends on sequence motifs in its cytoplasmic domain (4, 5, 35, 36). A tyrosine-based motif, a leucine-isoleucine motif, and a monophenylalanine signal mediate internalization at the plasma membrane (5–7, 19, 20) and also interaction with AP-1 Golgi-specific assembly proteins and thus sorting into clathrin-coated vesicles at the TGN (21). On the other hand, an acidic cluster is required for retrieval from endosomes to the TGN (5–7, 19). The presence of the different internalization signals within the furin tail suggested that endocytosis also occurs via a clathrin-dependent pathway. This prompted us to see whether there was evidence for interaction of the furin tail with the AP-2 adaptor medium subunit (μ2) and to analyze in detail the binding sites in the cytoplasmic domain required for this interaction. Since the furin tail contains three internalization signals, a major goal of this study was to determine whether the endocytosis signals of the furin tail are of different functional importance for furin internalization and recycling to the TGN.

In this study we report that furin associates with the μ2 subunit of the AP-2 complex and that furin/μ2 association strongly depends on the tyrosine motif YKG1765 and only to less extent on the leucine-isoleucine signal LI760. Tyrosine-based motifs have been reported to interact with the medium
YKGL765/LI760 sequence to the TGN. At steady state furin wt and mutant S776D/S778D, resembling the diphosphorylated state of furin, and S776A/S778A, containing a non-phosphorylatable acidic signal. We found that resialylation of surface-labeled, desialylated wt and mutant S776D/S778D occurs with the same half-time of approximately 1 h. This compares well with the half-time determined for TGN38 and CI-MPR transport from the plasma membrane to the TGN. Both proteins are concentrated in the TGN at steady state like furin. An epitope-tagged version of TGN38 was found to be transported with a half-time of 45 min from the cell surface to the TGN (37), while resialylation in the TGN of CI-MPR occurs with a half-time of 1–2 h (32). In contrast to wt and the S776D/S777D mutant, non-phosphorylatable CDF S776A/S778A was resialylated three times more slowly, with a half-time of 3 h demonstrating that phosphorylation also determines fast retrieval of CDF chimeric proteins from the plasma membrane to the TGN. At steady state furin wt and furin tail mutants S776D/S778D and S776A/S778A localize to the TGN of stably transfected NRK cells.

There is a discrepancy in the requirement of furin tail phosphorylation for steady state localization and plasma membrane to TGN transport of furin in our study and in that recently published by Molloy and co-workers (22). They found that a furin tail mutant imitating the diphosphorylated state does not predominantly localize to the TGN and that after endocytosis such a mutant only reaches the early endosome. Thus, the delay in cell surface to TGN transport of CDF chimeras lacking one or more internalization signal suggests that a decrease in AP-2/μ2 interaction and thus reduction of clathrin-mediated endocytosis from the cell surface is responsible for this effect. Since all mutants contain an intact, phosphorylatable acidic TGN localization signal, later sorting steps in the endosomal compartment should not be affected. On the other hand, it cannot be excluded that the furin tail sorting signals YKGL765, LI760, and Phε790 might play a role in transport from endosome to TGN. For transport of the shiga toxin B fragment from the early endosome to the Golgi apparatus, Mallard and co-workers (39) suggested that sorting at the level of the early/recycling endosome involves AP-1 clathrin coats.

Furthermore, it is worth mentioning that this study and our previous work revealed that the furin tail sorting signals YKGL765, LI760, Phε790, and the phosphorylated acidic cluster, are involved in the same way in two different sorting processes. The same sorting signals are recognized at two intracellular sites, the plasma membrane and the TGN (21), by adaptor complexes AP-2 and AP-1, respectively. These findings correlate with the model proposed by Molloy and co-workers (22) that the cycling loop between the cell surface and the early endosome represents a mirror image of a TGN/endosome cycling pathway.
In summary, our studies have shown that the furin cytoplasmic domain interacts with the \( \mu_2 \)-subunit of the AP-2 complex and that the \( \mu_2 \)-chain is predominantly recognized by the tyrosine-based sorting signal and to less extent the leucine/isoleucine signal. Furthermore, we were able to demonstrate that the furin tail sorting signals are of different functional importance for furin endocytosis and retrieval to the TGN, with the tyrosine motif being the major determinant, followed by the phenylalanine signal, whereas the leucine-isoleucine motif is only a minor component or part of the tyrosine motif. Phosphorylation of the furin tail by CKII enhances the interaction with the \( \mu_2 \)-subunit and determines efficient internalization and fast retrieval of furin molecules from the plasma membrane to the TGN. Since it has been shown that phosphorylation of the furin tail also determines high affinity AP-1 binding (21), we hypothesize that phosphorylation of the furin tail modulates the interaction with the adaptor complexes AP-1 and AP-2 and thus balances export at the TGN and retrieval from the plasma membrane, respectively.

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