Maturation of the \textit{trans}-Golgi Network Protease Furin: Compartmentalization of Propeptide Removal, Substrate Cleavage, and COOH-terminal Truncation

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Abstract. We have cloned a bovine cDNA encoding the \textit{trans}-Golgi network (TGN) protease furin and expressed it via recombinant vaccinia viruses to investigate intracellular maturation. Pulse-chase labeling reveals that the 104-kD pro-furin bearing high mannose \textit{N}-glycans is rapidly processed into the 98-kD protease whose \textit{N}-glycans remain sensitive to endoglycosidase \textit{H} for a certain period of time. Furthermore, in the presence of brefeldin A, pro-furin cleavage occurs. From these data we conclude that the ER is the compartment of propeptide removal.

Studies employing the ionophore A23187 and DTT show that autocatalysis is \textit{Ca}^{2+} dependent and that it does not occur under reducing conditions. Pro-furin produced under these conditions never gains endo \textit{H} resistance indicating that it is retained in the ER. Coexpression of furin with the fowl plague virus hemagglutinin in the presence of brefeldin A and monensin reveals that furin has to enter the Golgi region to gain substrate cleaving activity.

\textit{N}-glycans of furin are sialylated proving its transit through the \textit{trans}-Golgi network. A truncated form of furin is found in supernatants of cells. Truncation is inhibited in the absence of \textit{Ca}^{2+} ions and in the presence of acidotropic agents indicating that it takes place in an acidic compartment of cells.

Comparative analysis with furin expressed from cDNA reveals that the truncated form prevails in preparations of biologically active, endogenous furin obtained from MDBK cells. This observation supports the concept that secretion of truncated furin is a physiological event that may have important implications for the processing of extracellular substrates.

Many bioactive peptides and proteins like several peptide hormones, growth factors, coagulation factors, or receptors on the plasma membrane are synthesized as precursors which have to be cleaved by cellular endoproteases to gain their full biological potential (Douglass et al., 1984; Sossin et al., 1987; Furie and Furie, 1988; Yoshimasa et al., 1990; Hosaka et al., 1991). Although they differ with respect to their cell type-specific expression and their mode of secretion, they share some common features. Activation cleavage takes place at the carboxyterminal arginine of a di-, tri-, or tetrasalic recognition peptide and this process is mediated by \textit{Ca}^{2+}-dependent endoproteases in a late compartment of the exocytic pathway of cells (Docherty and Steiner, 1982; Barr, 1991). This concept has been developed early in the evolution of eukaryotes because even the unicellular organism \textit{Saccharomyces cerevisiae} secretes bioactive peptides like the alpha mating factor or the killer toxin by cleavage of their protein precursors at Lys-Arg or Arg-Arg sites (Bostian et al., 1984; Julius et al., 1984a). While maturation and activation of these proproteins have been studied in detail, information on the nature and characteristics of the activating enzymes was rare.

An important step in the identification of the proteases was the discovery of the yeast endoprotease kexin which mediates the cleavage of pro alpha-factor and pro killer toxin (Julius et al., 1984a; Fuller et al., 1988, 1989). Homologues of kexin in higher eukaryotes could then be found because all of these enzymes share a subtilisin-like catalytic domain. Furin, encoded by the fur gene, the fes/fps upstream region gene (also called PACE for paired basic amino acids cleaving enzyme), and PACE4 (Kiefer et al., 1991) seem to be ubiquitously expressed in organisms and cell lines. Prohormone convertases (PC) on the other hand can only be detected in specialized cells: PCI/3 and PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990; Creemers et al., 1992)
are confined to endocrine and neuroendocrine tissues (Seidah et al., 1990; Smeekens et al., 1991) where they cleave prohormones like proinsulin or proopiomelanocortin (Benjamin et al., 1991; Thomas et al., 1991); PC4 mRNA has only been detected in testis (Nakayama et al., 1992), and PC6 (also called PC5) expression is mainly found in intestinal cells (Nakagawa et al., 1993). Besides the subtilisin-like catalytic domain, these enzymes share an NH2-terminal signal sequence and a prodomain which for some of these proteases has been shown to be cleaved as is the case with subtilisin (Power et al., 1986) and kexin (Brenner and Fuller, 1992). On the other hand, there are important differences between the eukaryotic subtilisins. While most of them lack a hydrophobic anchor domain, furin and kexin are integral membrane proteins. Furthermore, the prohormone convertases PCI/3 and PC2 are sorted into and act in the regulated secretory pathway of endocrine cells, whereas furin acts in the constitutive secretory pathway present in all cells. According to the intracellular targeting and tissue distribution of furin and the prohormone convertases have different substrates and cleavage site specificities: substrates of furin are activated at the recognition motifs Arg-X-Lys/Arg-Arg or Arg-X-X-Arg (Molloy et al., 1992) in contrast to PCI/3, PC2, and kexin cleaving at Lys-Arg- or Arg-Arg-sites (Fuller et al., 1988; Thomas et al., 1991). Endogenous furin substrates can either be soluble proteins like pro-nerve growth factor (Bresnahan et al., 1990), pro-von Willebrand factor (Van de Ven et al., 1990; Wise et al., 1990) or membrane bound glycoproteins like pro-insulin receptor (Robertson et al., 1993) which are transported via the constitutive secretory pathway.

Moreover furin plays an important role in the pathogenicity of certain bacteria and viruses. *Pseudomonas* exotoxin A and *Bacillus anthracis* protective antigen can be activated by furin although the cellular compartment of cleavage of these exogenous substrates has not been defined yet (Molloy et al., 1992; Moehring et al., 1993). Viruses, on the other hand are intracellular pathogens using the cellular machinery for production of their progenies. Some of the enveloped viruses, for example paramyxoviruses like the pathogenic Newcastle disease viruses (Gotoh et al., 1992; Moehring et al., 1993) and human immunodeficiency virus type III (Ottmann et al., 1994), human immunodeficiency virus (HIV) (Hallenberger et al., 1992), or fowl plague virus (FPV) (Stieneke-Gröber et al., 1992) bear integral glycoproteins that are activated by furin before virions leave their host cells in a budding process (for review see Klenk et al., 1993). For the hemagglutinin (HA) of FPV it has been shown that the endogenous cleaving activity of the permissive Madin Darby Bovine Kidney (MDBK) cell line has the same properties as furin, and in enriched Golgi fractions furin could be detected by immunoblotting (Stieneke-Gröber et al., 1992). Furin is synthesized as a prepro-enzyme at the rough endoplasmic reticulum and later on it is concentrated in the trans-Golgi network (TGN) (Bresnahan et al., 1990; Misumi et al., 1991; Molloy et al., 1994; Schäfer, W., M.-L. Kruse, M. Vey, S. Bergbäuer, J. Seiler, H. F. Kern, H.-D. Klenk, and W. Garten, manuscript submitted for publication). Therefore, maturation and transport processes that happen in between are of special interest, but knowledge on temporal and spatial relationships between these stages of furin maturation is still incomplete. Previous studies have shown that proteolytic removal is an autocatalytic process (Leduc et al., 1992) but different opinions on the cellular compartment in which this reaction takes place, based on soluble furin mutants with attached KDEL sequences, exist (Rehemtulla et al., 1992; Molloy et al., 1994). Pulse-chase studies favor the ER as the compartment of propeptide removal (Molloy et al., 1994).

After activation, wild-type furin accumulates in the TGN (Molloy et al., 1994; Schäfer, W., M.-L. Kruse, M. Vey, S. Bergbäuer, J. Seiler, H. F. Kern, H.-D. Klenk, and W. Garten, manuscript submitted for publication), while truncated furin mutants are secreted from cells (Hatsuzawa et al., 1992; Molloy et al., 1992). Finally, the fate of furin has not been clarified yet, but there are indications that the membrane-bound enzyme is converted into a soluble form that can be found in supernatants of overexpressing cells (Rehemtulla et al., 1992; Vidricaire et al., 1993; Molloy et al., 1994). It should be emphasized that all of our knowledge on furin biosynthesis has been derived from studies on the overexpressed protease.

We report here on the spatial and temporal relationship between synthesis, autocatalytic propeptide removal, transport, substrate cleavage, and secretion of furin. Our studies have been carried out on bovine and human furin overexpressed from cDNA and on endogenous bovine furin enriched from Golgi fractions of MDBK cells.

**Materials and Methods**

**Cell Culture**

African green monkey kidney (CV-1) cells and MDBK cells were grown in Dulbecco's medium supplemented with 10% fetal calf serum. For production of recombinant vaccinia viruses and all labeling procedures, cells were grown on 60-mm-diam culture dishes (GIBCO, Eggenstein, Germany). Human TK-143 cells were grown in Dulbecco's medium supplemented with 5% fetal calf serum and 25 µg/ml of 5-bromodeoxyuridine (Sigma Chem. Co., Deisenhofen, Germany). The Western Reserve strain of vaccinia virus was propagated in CV-1 cells.

**Isolation of Endogenous MDBK Furin and Detection by Immunoblotting**

Golgi fractions were prepared from MDBK cells and furin enriched by chromatography on FPLC as described previously (Stieneke-Gröber et al., 1994). For immunodetection an aliquot of the Superose fractions and lysates of CV-1 cells harvested 5 h after infection with recombinant vaccinia virus vV:hfur (multiplicity of infection [ moi] = 10 pfu/cell) were separated on 12% gels under reducing conditions. Proteins were transferred to nitrocellulose and detected with a furin specific antiserum from rabbits.

**Amplification of a Human Fur Gene Fragment**

DNA of a recombinant vaccinia virus encoding the human furin cDNA (Bresnahan et al., 1990) was isolated by standard procedures (Macket et al., 1985) and used as template for PCR amplification of a part of the region coding for the subtilisin-like domain. The following primers were used: 5'-CACGGCACACGGTGTC~AAGT-3' corresponding to nucleotides 1163 to 1188 of the coding region of the human furin cDNA. For amplification a commercially available kit (Perkin-Elmer Cetus Instruments) was used and reactions were performed in a thermal cycler (Perkin-Elmer Cetus Instruments) for 30 cycles (72°C, 1.5 min). After electrophoresis the DNA fragment was extracted by using tagged probes employing a random primed DNA labeling kit (Boehringer, Mannheim, Germany).
Cloning of Bovine Fur cDNA from an MDBK cDNA Library

About 2 × 10⁶ clones of a XZAPIIIsDNA library of MDBK cells (Stratagene, Heidelberg, Germany) were screened with the human fur probe using standard protocols for blotting and hybridization techniques. Bovine fur-positive clones were visualized with a nonradioactive detection kit employing chemiluminescent reagents (Boehringer). Five fur-recombinant clones were plaque purified and their cDNA sequences were determined (EMBO Data Bank accession number X75956). The deduced amino acid sequence revealed high homology with human furin except for a proline-rich region upstream of the membrane anchor comprising amino acids 686 and 696 of human furin (Table I). In bovine furin this region is extended by three additional amino acid residues, and two of these are prolines. MDBK cells were infected to study the expression of furin (Fig. 1 B). Cells were either infected with human (lanes *VV*hfur) or bovine furin recombinant viruses (lanes *VV*hfur), and proteins were pulse labeled for 45 min (Fig. 1 B, lanes C) or labeled for 45 min and then chased for 4 h with cold methionine (Fig. 1 B, lanes S). Furin proteins were precipitated using a furin specific antiserum and resolved by SDS-PAGE on 10% gels under reducing conditions and visualized by fluorography.

Results

Comparison of Bovine and Human Furin

We have isolated the endogenous furin protease from Golgi fractions of MDBK cells as described previously (Stieneke-Gröber et al., 1992) and compared it with overexpressed human and bovine furin (Fig. 1). The 85-kDa protein band of the endogenous furin (Fig. 1 A, lane *MDBK*) differs significantly from the 94- and 100-kDa doublet of the human protease (Fig. 1 A, *VV*hfur). To find out if this difference is caused by mutations in the coding region of the bovine furin gene we have cloned its cDNA from an MDBK library and determined the nucleotide sequence (EMBO Data Bank accession number X75956). The deduced amino acid sequence revealed high homology with human furin except for a proline-rich region upstream of the membrane anchor comprising amino acids 686 and 696 of human furin (Table I). In bovine furin this region is extended by three additional amino acid residues, and two of these are prolines. MDBK cells were infected to study the expression of furin (Fig. 1 B). Cells were either infected with human (lanes *VV*hfur) or bovine furin recombinant viruses (lanes *VV*hfur), and proteins were pulse labeled for 45 min (Fig. 1 B, lanes C) or labeled for 45 min and then chased for 4 h with cold methionine (Fig. 1 B, lanes S). Furin proteins were precipitated using a furin specific antiserum and resolved by SDS-PAGE on 10% gels under reducing conditions and visualized by fluorography.

Figure 1. Endogenous bovine furin is smaller than overexpressed, membrane-bound forms of human and bovine furin but similar to their secreted forms. (A) Immunodetection of endogenous bovine and overexpressed human furin. Golgi fractions of MDBK cells were enriched and, after solubilization, subjected to FPLC on a Superose 12 column as previously described (Stieneke-Gröber et al., 1992). An aliquot of a furin containing fraction was prepared for SDS-PAGE by boiling in sample buffer containing β-mercaptoethanol (lane *MDBK*). For comparison with overexpressed human furin CV-1 cells infected with recombinant vaccinia viruses VV; hfur (moi. = 10) were lysed in sample buffer (lane *VV*hfur) 5 h p.i. Proteins were separated on a 12% gel and blotted onto nitrocellulose. Furin proteins were detected with a furin specific antiserum from rabbits. (B) Fluorogram of radiolabeled bovine and human furin immunoprecipitated from cell lysates and supernatants of overexpressing cells. MDBK cells were infected with recombinant vaccinia virus encoding human (VV;hfur) or bovine (VV;hfur) furin (moi. = 10). At 3.5 h after infection cells were either pulse labeled for 45 min (lanes C) with 35S-methionine or labeled for 45 min and chased for 4 h with cold methionine (lanes S). Furin was then immunoprecipitated from pulse labeled cell lysates (lanes C) and from supernatants of the pulse-chase-labeled cells with a furin specific antiserum and resolved by SDS-PAGE on 10% gels. The arrows indicate positions of the molecular mass.
from lysates of pulse-labeled cells (Fig. 1 B, lanes C) and from supernatants of the pulse-chase-labeled cells (Fig. 1 B, lanes S) with an antiserum raised against the catalytic domain of human furin. Again bovine furin showed reactivity with this antiserum proving that human and bovine furin are very similar. Bovine furin appeared as a doublet of which the upper band had a relative molecular mass of 104 kD and the very similar. Bovine furin appeared as a doublet of which the lower band migrated at 98 kD, whereas the doublet bands observed with human furin had higher electrophoretic mobilities migrating at 100 and 94 kD. Previous studies on human furin (Bresnahan et al., 1990; Van de Ven et al., 1990; Wise et al., 1990; Leduc et al., 1992; Creemers et al., 1993) had shown that both of these proteins are encoded by the fur open reading frame: the larger protein represents the proenzyme, pro-furin, that is converted into the mature furin protease furin (Bresnahan et al., 1990; Van de Ven et al., 1990; Wise et al., 1990). Earlier reports postulated a post ER compartment for autoprocessing and substrate cleavage (Rehemtulla et al., 1992). Studies on rat furin, on the other hand, showed that the N-linked carbohydrates of pro-furin and cleaved furin were endo H sensitive and were converted only after several hours into complex glycans (Misumi et al., 1991). Additionally, contradictory conclusions were drawn from studies on soluble furin mutants with attached KDEL sequences (Rehemtulla et al., 1992; Molloy et al., 1994). Thus, the compartment of autoprocessing has not been clearly defined.

**Protease Removal Occurs Rapidly and Precedes Transport to Golgi Apparatus and COOH-Terminal Truncation**

Little is known about the temporal and spatial relationship of maturation and transport of furin. Earlier reports postulated a post ER compartment for autoprocessing and substrate cleavage (Rehemtulla et al., 1992). Studies on rat furin, on the other hand, showed that the N-linked carbohydrates of pro-furin and cleaved furin were endo H sensitive and were converted only after several hours into complex glycans (Misumi et al., 1991). Additionally, contradictory conclusions were drawn from studies on soluble furin mutants with attached KDEL sequences (Rehemtulla et al., 1992; Molloy et al., 1994). Thus, the compartment of autoprocessing has not been clearly defined.

**Pulse-chase labeling studies have been performed before on epitope-tagged furin (Molloy et al., 1994) and wild-type human furin (Creemers et al., 1993), and we have used a similar approach to study kinetics of propeptide removal of native bovine and human furin. In addition, we analyzed the status of the N-linked glycans of furin during the maturation processes to link the kinetics of protease removal with data on transport through the compartments of the secretory pathway (Fig. 2A). Only the data observed with bovine furin are shown, but similar results were obtained on human furin. Autocatalysis is a posttranslational event as indicated by the observation that most of the immunoprecipitated furin was labeled in its pro-form during a 10-min pulse (Fig. 2A, panel 0). In addition to the proenzyme, cleaved furin could be detected indicating that propeptide removal had already begun. Both of the precipitated furin bands shifted after treatment with endoglycosidase H to the molecular mass of the deglycosylated forms which had been generated by digestion with N-glycanase F. This means that all N-linked sugars were of the high mannose type and, thus, autoprocessing probably took place in the ER. During the following chase periods (Fig. 2A, 10', 20', and 30') pro-furin was completely converted into cleaved furin. The half time of the autocatalytic reaction could not be measured precisely, but between 0 and

Table I. Differences in the Amino Acid Sequences of Bovine and Human Furin

| Position | Bovine | Human |
|----------|--------|-------|
| 8        | 14     | 656   |
| 14       | 16     | 686   |
| 21       | 24     | 705   |
| 27       | 27     | 710   |
| 37       | 46     | 702   |
| 47       | 47     | 707   |
| 71       | 71     |
| 82       | 82     |
| 83       | 83     |
| 85       | 85     |
| 94       | 94     |

Single letter code has been used for abbreviation of amino acid residues. Amino acid sequence of human furin was taken from Van den Ouweland et al. (1990). Numbers indicate the positions of residues in the respective furin protein, the region between amino acids 686 and 702 of human furin are shown completely.
10 min of chase half of the labeled pro-furin was converted into the cleaved form. Endo H treatment clearly showed that furin still had high mannose glycans indicating that after propeptide removal the cleaved form only slowly left the ER. After 40 and 60 min of chase, again a high molecular mass furin band appeared, which did not shift after endo H treatment demonstrating that N-linked carbohydrates of this protein had been converted into complex types. This larger band now represented cleaved furin because it shifted to the position of the deglycosylated, cleaved furin after digestion with N-glycanase F. These results show that autocatalysis of pro-furin is an early event starting a few minutes after synthesis. Only the cleaved form slowly reaches the medial and late Golgi compartment, where its N-linked glycans are converted into complex types.

Fig. 2 B monitors furin maturation during longer chase periods. In this experiment supernatants of furin expressing cells were incubated with the anti-furin antibody to check if a soluble form of furin had been secreted into the medium as has been reported in earlier studies (Wise et al., 1990; Rehemtulla et al., 1992). After a 45-min pulse without chase, the cleaved furin band dominated over pro-furin, because the labeling period had exceeded the half time of autocatalysis about three times (Fig. 2 B, bottom). After 1 h of chase, pro-furin totally disappeared, and the doublet now consisted of high and low molecular mass cleaved furin. The endo H-resistant furin did not run as a distinct band, but a smear of labeled proteins could be detected between 98 and 110 kD hinting at different amounts of sialic acid attached to its oligosaccharides. In addition to these two forms, a smaller band with an estimated molecular mass of 90 kD could be precipitated from the cell lysates, and a protein of similar size could also be detected in the supernatants of the cells (Fig. 2 B, top). During the following chase periods, the intracellular level of labeled furin decreased markedly and after 4 h it could no longer be detected. In contrast, the
amount of the soluble furin protein in the supernatant increased to the same extent which proved that furin was efficiently secreted from the cells.

The three forms of furin detected after propeptide removal could be precipitated from the cell lysates (Fig. 2 C) after 2 h labeling and 1-h chase period. The fluorogram showed two diffuse bands extending from 98 to 110 kD and from 80 to 90 kD and one sharp band at 98 kD. Endo H treatment revealed that the two heterogeneously migrating forms of furin had complex carbohydrate side chains, whereas the distinct band shifted from 98 kD in the untreated sample to ~92 kD. After deglycosylation, the larger band again shifted to the position of the 92-kD deglycosylated cleaved furin (see Fig. 2 A). The deglycosylated form of soluble furin, on the other hand, had an estimated molecular mass of 76 kD. From these results we conclude that the truncated, secreted furin was produced from the mature, membrane bound form by proteolytic cleavage and that this process might have taken place inside the cell in a late compartment of the exocytic pathway. These results indicate that the 85-kD furin isolated from MDBK cells represents the COOH-terminally truncated form of bovine furin.

In conclusion, pulse-chase labeling experiments revealed that propeptide removal from the 104-kD precursor having endo H–sensitive carbohydrate side chains starts soon after synthesis. After 40-min chase, pro-furin completely disappears. Cleaved furin bearing endo H–sensitive N-glycans with a molecular mass of 98 kD is then transported to the Golgi region, and about 50 min after pulse complex carbohydrate side chains can be found on the protein. The endo H–resistant, cleaved furin now migrating at 104 kD is then COOH-terminally truncated and, 4 h after the pulse, all of the labeled furin is found in the supernatant of cells as a 80–90-kD protein. This maturation pathway of furin is schematically shown in Fig. 8. It is now evident that maturation, transport, and secretion of bovine furin are performed in individual, consecutive steps which seem to be precisely scheduled and may be confined to distinct compartments in the exocytic pathway of cells.

**Mature Furin Is Sialylated**

To find out if the increase in the apparent molecular mass of furin from 98 kD to ~104 kD (Fig. 2 A and B) was due to sialylation, furin was coexpressed with the neuraminidase (NA) of FPV (Fig. 3). FPV NA hydrolyzes the a2-3 glycosidic bond between terminal sialic acid and galactose on glycoproteins and glycolipids. It is a membrane bound glycoprotein, matures in the exocytic pathway of cells and is transported to the plasma membrane. After coexpression with NA, furin had a molecular mass of 98 kD and was not sensitive to endo H treatment (Fig. 3, lanes 4–6). Thus, the increase in molecular mass observed after single expression results from the attachment of neuraminic acid to the carbohydrate side chains. After N-glycanase F treatment, furin obtained by single expression had the same electrophoretic mobility as furin obtained by coexpression with NA (Fig. 3, compare lane 3 with lane 6) indicating that sialic acid is attached to N-linked glycans but not to O-linked glycos. The observations that furin is sialylated indicates that furin enters the TGN where sialyltransferases are localized.

**Autoprocessing of Pro-furin Is Ca2+ Dependent**

Previous studies had shown that substrate cleavage in the exocytic pathway of cells (Klenk et al., 1984; Nagahama et al., 1991; Oda, 1992) and the activity of furin in vitro are Ca2+ dependent (Bresnahan et al., 1990; Hatzuzawa et al., 1992; Molloy et al., 1992; Stieneke-Gröber et al., 1992). We now wanted to find out if autoprocessing also requires Ca2+ ions. To this end, furin was coexpressed with the HA of fowl plague virus and the autokatalytic and substrate processing activities were monitored in the absence or presence of different concentrations of Ca2+ ions after depletion of intracellular Ca2+ (Fig. 4 A). Furin labeled in the absence of Ca2+ for 1 h and chased for 1 h did not show detectable autocatalytic processing because only one sharp band with a molecular mass of 104 kD could be detected (Fig. 4 A, top, lane 0). Coexpressed HA predominantly appeared in its uncleaved form and only minor amounts of cleavage products, HA1 and HA2, were generated, proving that Ca2+ depletion interferes with substrate cleavage. In contrast, in the presence of Ca2+ ions (0.1 and 1.0 mM final concentration) both activities could be seen: furin was mainly processed into the 104-kD sialylated form (Fig. 4, A, top, lane 0.1 and 1.0), HA was almost completely converted into HA1 and HA2 (Fig. 4, bottom, lane 0.1 and 1.0) except for some remaining material which had previously been shown to be retained in the ER (Roberts et al., 1993). To further prove that the 104-kD furin band produced in the absence of Ca2+ really represented pro-furin, the protein was treated with endo H and N-glycanase F (Fig. 4 B). After complete deglycosylation (lane F), the protein shifted to the position of the deglycosylated profurin proving that no autoprocessing had occurred. The same shift was observed after endo H treatment (lane H) indicating that the uncleaved furin had not reached the medial Golgi region where complex carbohydrates are generated. Studies employing the ionophore A23187 have shown that vesicular transport between the ER and Golgi region is not blocked in the absence of Ca2+ and glycoproteins acquire normal carbohydrate modifications (Klenk et

Figure 3. Mature furin is sialylated. Fluorogram of bovine furin coexpressed with the fowl plague virus neuraminidase. 4.5 h after coinfection of CV-1 cells with vaccinia viruses encoding bovine furin (VV:bfur) and the neuraminidase of FPV (VVNA), cells were labeled with 35S-methionine for 1 h and chased for 1 h with cold methionine. Furin was immunoprecipitated from cell lysates and before SDS-PAGE on a 10% gel aliquots of proteins were digested with endoglycosidase H or N-glycanase F. Furin expressed in the absence of neuraminidase served as control. Arrow indicates position of the molecular weight marker; sialylated, autokatalytically cleaved furin; d, deglycosylated form of the marked protein.
with rabbit antiserum raised against FPV high marmose carbohydrate side chains; ~1 indicates autocatalytically cleaved furin with complex, sialylated sugar moieties; d symbolizes 0.1, or 1 mM CaCl₂. Cell lysates were divided and one half was incubated with antiserum specific for furin deglycosylated form of the marked protein.

rows on the left of fluorograms mark positions of molecular mass standards; arrows on the right mark positions of the precursor and cleavage products (HA/and ... 104-kD protein (Fig. 5 B, left). After extensive washing with DTT-free medium and an additional chase period in the absence of this chemical (Fig. 5 B, right) pro-furin was processed to the cleaved 98-kD furin. Compared to autoprocessing of nascent pro-furin kinetics of autocatalysis seemed to be slowed down after the release of the DTT shock (Fig. 1 A). This delay might reflect repair of incorrect disulfide bridges. The cysteine-rich region that precedes the transmembrane domain might play a critical role in these early folding processes. In conclusion, this experiment demonstrates that the oxidizing conditions in the ER are prerequisite for autoprocessing of pro-furin to occur.

**Inhibition of Pro-furin Processing Is Reversible**

Retention of furin in the ER or early Golgi under conditions of Ca²⁺ depletion may result from irreversible aggregation as described for other misfolded proteins (Gething et al., 1986; Roberts et al., 1993). To check if inhibition of pro-furin autoprocessing is reversible, bovine furin was labeled in the absence of Ca²⁺ for 1 h and then chased for 1 h in the presence of Ca²⁺ ions (Fig. 5 A). No processing and no transport of pro-furin could be detected after pulse labeling (Fig. 5 A, left). After the chase, however, autocatalytic cleavage (Fig. 5 A, right) and transport of furin occurred. Thus, the transport block of furin can be released by the addition of Ca²⁺ ions.

**Disulfide Bridge Formation Is Critical for Pro-furin Processing**

Conformation of glycoproteins that enter the exocytic pathway of cells is mainly stabilized by disulfide bridges formed during and shortly after protein synthesis. We wanted to find out if disulfide bridge formation contributes to the correct processing of pro-furin, too. Studies on the folding of influenza hemagglutinin had employed the reducing agent DTT for reversible inhibition of oxidation of sulfhydryl groups (Braakman et al., 1992). In analogy, we added the reducing agent to CV-1 cells expressing bovine furin (Fig. 5 B). In the presence of DTT, pro-furin showed no autoprocessing during a 1-h labeling period as could be demonstrated by the presence of the endo H-sensitive, 104-kD protein (Fig. 5 B, left). After extensive washing with DTT-free medium and an additional chase period in the absence of this chemical (Fig. 5 B, right) pro-furin was processed to the cleaved 98-kD furin.

**Autocatalysis, but Not Substrate Cleavage, Takes Place in the Endoplasmic Reticulum**

The results of pulse-chase labeling had shown that autoproteolytic processing probably occurred in the ER which is in contrast to previous studies postulating a post-ER compartment for cleavage as well as for autoprocessing (Rehemtulla et al., 1992). We therefore disintegrated the Golgi region by application of the fungal metabolite brefeldin A (BFA) (Lippincott-Schwartz et al., 1989), which, as a net result, blocked exit of the newly synthesized glycoproteins out of the ER (Klausner et al., 1992), and studied autoprocessing and substrate cleavage by coexpression with FPV hemagglutinin (Fig. 6 A). Pulse-chase labeling in the absence of BFA (Fig. 6 A, left) showed that during a 15-min pulse autocatalysis had already started but that hemagglutinin had not been cleaved by furin. There are at least three reasons why substrate cleavage had not occurred. Firstly, HA may not have acquired the correct conformation competent for cleavage by furin which may require trimerization or other...
A). Thirdly, some kind of chaperone may inhibit furin activity. Conditions of early vaccinia virus infection, not only the proteinase activity contributed to the HA cleavage. Apart from that, this control experiment confirmed that HA cleavage under normal conditions takes place when HA has passed the Golgi apparatus. When coexpressed in the presence of BFA, on the other hand, newly synthesized bovine furin could not be exported from the ER, and posttranslational modifications normally performed in this compartment should have also taken place (Russ et al., 1991). Pulse (15 min)-chase (60 min) labeling (Fig. 6 A, right) revealed that in the presence of BFA autoprocessing of pro-furin occurred with reduced kinetics, but most of the proprotein had been converted into the cleaved form (compared Fig. 6 A, top). Pulse labeled, coexpressed HA under these conditions is not cleaved during the 15-min pulse and even after a 60-min chase (Fig. 6 A, bottom right). Since autocatalytic cleavage of pro-furin is not inhibited in the presence of BFA, significant amounts of furin must have been produced during the preincubation period before the pulse and the chase but this cleaved furin was not active. The small shift of the HA precursor band which was visible after the chase had been caused by carbohydrate trimming. This experiment indicates that, although autoprocessing of pro-furin occurs in the endoplasmic reticulum, cleaved furin is not able to process substrates in trans.

**Transport to the Golgi Apparatus Is Necessary for Furin Activity**

Early studies on the localization of furin demonstrated a concentration in the Golgi region (Bresnahan et al., 1990; Misumi et al., 1991) and recent findings show that it colocalizes with the trans-Golgi network protein TGN 38 (Molloy et al., 1994). The presence of sialic acid residues on the N-linked glycans of mature furin (Fig. 3) further underlines that a late compartment might be the site of accumulation and action of furin. Coexpression studies in the presence of monensin, which inhibits transport of proteins from cis- and medial-Golgi cisternae to the trans region should give an answer to the question in which compartment of the Golgi region furin can activate substrates. Pulse-chase analysis (Fig. 6 B) in the absence of monensin showed that, regardless of coexpression or single expression, furin and HA matured normally (Fig. 6 B, right top and bottom): furin was cleaved and extensively sialylated which was also true for HA. In the presence of monensin (Fig. 6 B, top left), pro-furin was completely converted into the cleaved form, but no high molecular mass furin could be detected because it had not reached the compartment where sialic acid is attached to the N-linked glycans. Endo H treatment (data not shown) proved that this cleaved form has reached the medial Golgi cisternae because no shift was detected. Although under these conditions the endogenous furin of CV-1 cells is present in the TGN, HA could not be activated during single expression because it had not reached this compartment and no or inadequate amounts of endogenous furin had been synthesized during the monensin incubation period. On the contrary, newly synthesized bovine furin had accumulated in the early and medial-Golgi region and cleaved the coexpressed HA into HA1 and HA2. Whereas HA1 produced in the absence of monensin migrated as a diffuse band (Fig. 6 B, bottom, third and forth lane), HA1 produced in the presence of the ionophore ran as a sharp band, proving that cleavage occurred before the compartment in which sialic acid is attached was reached. These results show that furin can process substrates in the early Golgi region even before it arrives in the TGN where it is temporarily concentrated.

**COOH-terminal Truncation of Furin Depends on Low pH and Ca2+ Ions**

Pulse-chase labeling (Fig. 2 C) strongly hinted at an intra-
with a furin-specific antiserum and the other one with an antiserum raised against FPV. After immunoprecipitation proteins were analysed by SDS-PAGE on 10% gels and fluorography. (B) 4.5 h after infection with recombinant vaccinia viruses encoding bovine furin (VV:bfur) or FPV hemagglutinin (VVHAWt) or coinfection with both of these viruses, CV-1 cells were labeled with 35S-methionine for 15 min and chased for 60 min with cold methionine. During starvation, labeling and chase period cells were either incubated in the presence or absence of monensin (2 μg/ml). Cell lysates of the single infections were incubated with the respective antisera whereas lysates of coinfected cells were divided up into two halves one of which was incubated with the furin-specific and the other one with an antiserum raised against FPV. For further treatment of samples and for legends see A. Arrows on the left indicate positions of molecular mass standards. Arrows on the right mark the positions of precursor (HA) and cleavage products (HA1 and HA2) of the FPV hemagglutinin; c, pro-furin; /, autcatalytically cleaved furin; \/, autcatalytically cleaved furin with siaylated carbohydrate side chains.

Discussion

A general feature of subtilisin and subtilisin-like proteases of eukaryotes is their synthesis as prepro-enzymes which are proteolytically processed by an intramolecular cleavage event removing a propeptide from the catalytic domain (Power et al., 1986; Brenner and Fuller, 1991; Leduc et al., 1992; Creemers et al., 1993). Whereas autoprocessing of subtilisin leads to secretion of the active protease, the eukaryotic enzymes kexin and furin remain temporarily mem-
brane bound because of a COOH-terminal hydrophobic anchor domain. It was of interest to see where pro-kexin and pro-furin enzyme activation occurs and where in the exocytic pathway of cells the mature enzymes finally end up. For kexin, the yeast homologue of furin, maturation, transport, and intracellular concentration are well studied, and different compartments in the exocytic pathway of cells could be defined in which individual steps of these processes take place. Thus, autocatalytic removal of the propeptide of kexin occurs at the endoplasmic reticulum (Wilcox and Fuller, 1991). The cleaved enzyme then reaches the yeast equivalent of the Golgi compartment, whereas uncleaved precursor is retained in the ER. The intracellular site of kexin concentration and substrate cleavage on the other hand is a late Golgi compartment (Redding et al., 1991). The data presented in this paper provide information on intracellular maturation and transport of furin.

Soon after translation of pro-furin, autoprocessing occurs with a half time of less than 15 min (Fig. 2 A). During the whole process, no conversion of the high mannose type N-glycans of pro-furin and cleaved furin occurs, indicating that the ER is the primary site of autoprocessing. Even after this process, the N-glycans of cleaved furin remain endo H sensitive for more than 30 min. The experiments employing brefeldin A underlined the important role the ER plays during these early events. The observation that in the presence of BFA, which prevents exit of proteins from the ER (for review see Klausner et al., 1992), profurin cleavage occurs (Fig. 6 A) indicates that no post ER compartment is involved in autocatalysis. These results are in contrast to a previous report postulating that autoprocessing might be a late event. This was inferred from studies on a soluble furin mutant with an attached KDEL sequence that mediated ER retention (Rhemtulla et al., 1992). However, studies of Molloy et al. (1994) who used soluble, epitope tagged KDEL- and membrane-bound, epitope-tagged furin mutants and our data on unmutated bovine furin independently show that furin does not have to enter a post ER compartment for pro-furin propeptide removal. In this respect compartmentalization of pro-furin conversion resembles maturation of the yeast protease kexin (Wilcox and Fuller, 1991) very closely and therefore this conservation of the intracellular location of propeptide removal seems to be typical for maturation of these subtilisin-like proteases in the secretory pathway of eukaryotic cells.

Ca2+ ions have been shown to be indispensable for substrate cleaving activity (Klenk et al., 1984; Bresnahan et al., 1990; Hatsuzawa et al., 1992; Molloy et al., 1992; Stieneke-Grober et al., 1992). Our experiments employing the ionophore A23187 reveal that autocatalytic cleavage is strictly dependent on Ca2+ ions, too.

Mutants whose autocatalytic cleavage is severely impaired show that inhibition of autoprocessing leads to accumulation of pro-furin in the ER (Molloy et al., 1994; Creemers, J. W. M., M. Vey, W. Schäfer, T. A. Y. Ayoubi, A. J. M. Roebroek, H.-D. Klenk, W. Garten, and W. J. M. Van de Ven, manuscript submitted for publication). This observation suggests that propeptide removal might be a prerequisite for exit out of the ER. Our studies on wild-type furin reveal that carbohydrates of wild-type pro-furin remain endo H sensitive when autocatalysis is impaired, for example in the absence of Ca2+ ions or in the presence of DTT (Fig. 5), hinting at an arrest of pro-furin in an early compartment. As a consequence, only correctly cleaved furin can leave the ER and enter the Golgi region. This means that furin has to pass a quality control. A similar regulatory function of the ER has been described for the influenza virus hemagglutinin. During folding, hemagglutinin adopts intermediate conformations which interact with the chaperone BiP (Gething et al., 1986). BiP binding is Ca2+ dependent and, in the absence of Ca2+ ions, formerly retained molecules enter the Golgi apparatus (Suzuki et al., 1991). Pro-furin does not seem to interact with BiP, because in the absence of Ca2+ ions it does not bear complex N-glycans, indicating that it is still in the ER (Fig. 4 B). The recently discovered chaperone calnexin has been shown to bind to glycoproteins like the heavy chain of MHC class I molecules (Galvin et al., 1992) and the complement factor C3 (Ou et al., 1993). Thus, calnexin, which is ubiquitously and abundantly expressed in the ER, might be the candidate chaperone mediating pro-furin retention.
We further investigated the compartment of furin substrate cleavage. Previous studies showed that furin is concentrated in the Golgi compartment (Bresnahan et al., 1990; Misumi et al., 1991). Furthermore, it has been known for a long time that substrates are cleaved late after their synthesis (Klenk et al., 1974), and in recent subcellular fractionation experiments cleavage products of substrates produced by coexpressed furin could only be precipitated from Golgi fractions (Wasley et al., 1993), hinting at a late compartment of furin action. Furin mutants captured in the ER via a KDEL sequence were not able to process coexpressed substrates in this early compartment (Rehemtulla et al., 1992; Molloy et al., 1994). We coexpressed FPV hemagglutinin with wild-type furin in the presence of BFA and found that substrate cleavage did not occur (Fig. 6 A), proving that the ER and the intermediate salvage compartment do not allow substrate cleavage, whereas autoprocessing is not inhibited (Fig. 6 A). Thus, we conclude that furin is either blocked, maybe by binding to some kind of chaperone, or has to be further modified in order to gain substrate cleaving activity. In this respect furin resembles the kexin protease of *S. cerevisiae* because the yeast protease is not able to cleave substrates if it is captured in the ER (Julius et al., 1984b). Thus, furin and kexin seem to be not only related by a conserved structure of their catalytic domains but also their maturation in the secretory pathway seems to underly similarly conserved control mechanisms.

The next compartments furin enters are the cis- and medial-Golgi cisternae, and we asked whether furin can activate substrates there. Coexpression in the presence of monensin clearly showed that furin can cleave HA in the early Golgi region where no endogenous furin activity could be detected (Fig. 6 B). This finding demonstrates that furin gains substrate cleaving activity when it enters the Golgi region, even before it reaches the trans-Golgi cisternae and TGN where it is concentrated (Molloy et al., 1994; Schäfer et al., submitted). Therefore, the specific conditions in the TGN, including low pH and sugar modifications like sialylation, are not crucial for proteolytic activity. They may be of importance for another step in the intracellular lifecycle of furin (see Fig. 7 and Discussion below).

During transport to and concentration of furin in the TGN, the N-linked carbohydrate side chains of furin are processed and we have monitored these modifications by endoglycosidase digestions (Fig. 2). An earlier report on the sugar modifications of rat furin suggested that pro-furin and autocatalytically cleaved furin gain complex N-glycans (Misumi et al., 1991) very late after synthesis, which is not consistent with our results. In our studies, carbohydrates of pro-furin never showed endo H resistance. Even cleaved furin could hardly be detected in a 98-kD endo H-resistant form under normal conditions. Since rat furin was labeled early after onset of furin expression, whereas in our system furin was labeled early after onset of furin expression, differences in the assay systems may account for the different results. The endo H-resistant, cleaved furin that can be detected in our studies is sialylated and this modification leads to a significant shift in electrophoretic mobility (Figs. 2 and 3). Sialylation of furin proves that the protease has reached the trans-Golgi cisternae and the TGN, where sialyltransferases are located. Recently, evidence was provided that furin is not strictly retained in the TGN but it cycles between the plasma membrane and the TGN which implicates that furin appears in the endosomal compartment (Molloy et al., 1994) where it might activate exogenous substrates like the protective antigen of *B. anthracis* or exotoxin A of *Pseudomonas*.

On the other hand, acidic compartments seem to be necessary for the final step of the intracellular lifecycle of furin which is discussed below. A schematic drawing (Fig. 8) illustrates the complex maturation pathway of furin.
Intracellular furin levels seem to be strictly controlled, because endogenous furin cannot be detected by immunocytochemical methods, and only catalytic amounts are present in the TGN, although furin mRNA seems to be continuously present in all cells tested so far. Other transcriptional or post-transcriptional regulatory mechanisms might be involved, but our experiments strongly hint at an important posttranslational control by a second proteolytic cleavage step that leads to secretion of an anchorless but active form. Truncation of furin, although less efficient than observed here, has been reported before (Wise et al., 1990; Rehemtulla et al., 1992; Vidricaire et al., 1993). Molloy et al. (1994) have recently reported efficient shedding of truncated furin from cells expressing wild type or a mutant that lacked the cytoplasmic tail. Our results with endogenous furin of MDBK cells now strongly suggest that truncation is a process by which endogenous furin is modified also under physiological conditions, because an 85-kD furin is the only furin-immunoreactive protein that can be detected in the enriched Golgi fractions. Earlier studies on this endogenous furin have shown that the electrophoretic mobilities of furin after elution from a molecular sieving column increased in subsequent fractions (Stieneke-Gröber et al., 1992) hinting at a similar modification of terminal sugars by sialylation which can be seen for mature, membrane bound and secreted bovine furin causing a heterogeneously migrating band in the SDS-PAGE (Figs. 2 C and 3). The studies on the overexpressed wild-type bovine furin further show that secretion of truncated furin is indeed a very efficient process by which cells lose this membrane-bound enzyme. After 4 h of chase (Fig. 2 B), the labeled protein completely disappeared from cells, and most of it was found in the media in a truncated and heavily sialylated form. In addition, the studies strongly hint at an intracellular site of cleavage because the COOH-terminally truncated furin could also be precipitated from cell lysates (Fig. 2). Furthermore, when we raised the pH of acidic compartments by addition of weak bases like chloroquine or ammonium chloride, truncation was inhibited. This observation suggests that concentration of furin in an acidic compartment like the TGN, on the one hand, might provide permanently low levels of active, intracellular furin by truncation of the membrane bound form. On the other hand, accumulation in the TGN does not interfere with cleavage activity because it occurs between pH 6 and 8 (Molloy et al., 1992; Stieneke-Gröber et al., 1992). With regard to substrate specificity low intracellular furin levels also make sense, because high levels of furin cause unspecific cleavage (Walker et al., 1993). So far no direct information on the enzyme(s) catalyzing this truncation reaction exist, but an earlier report (Rehemtulla et al., 1992) proposed an autocatalytic cleavage similar to propeptide processing. The restricted substrate specificities of propeptide processing (Leduc et al., 1992; Creemers et al., submitted) and substrate cleavage (Molloy et al., 1992; Nakayama et al., 1993; Watanabe et al., 1993) argue against an autocatalytic process, because no typical motif like R-X-K/R-R, R-X-X-R or R-X-X-X-K/R-R is present in the region where furin can be clipped off the membrane. Ca2+ dependence and inhibition by chloroquine, which both have been observed with substrate cleavage as well as truncation, do not provide direct evidence for an autocatalytic reaction, either. Identification of the exact peptide bond that is hydrolyzed and studies employing protease inhibitors might give some further information on the nature of the enzyme(s) involved.

In conclusion, our results and the recent studies on furin demonstrate that maturation of this TGN protease is a precisely tuned process, consisting of individual, successive steps which are performed in different compartments and depend on Ca2+ ions, oxidizing conditions and pH among other so far unidentified mechanisms.

The authors like to thank Dr. G. Thomas, Vollum Institute, Portland, Oregon, for providing the human furin recombinant vaccinia virus; Dr. P. C. Roberts for providing the FPV HA recombinant vaccinia virus; Dr. J. Hausmann for the FPV NA recombinant vaccinia virus; and Dr. M. Krause, Institut f. Molekularbiologie, Philipps-Universität Marburg, for synthesizing the oligonucleotides.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 286) and by the Fonds der Chemischen Industrie.

Received for publication 9 May 1994 and in revised form 30 August 1994.

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