A Second Mutant Allele of Furin in the Processing-incompetent Cell Line, LoVo

EVIDENCE FOR INVOLVEMENT OF THE HOMO B DOMAIN IN AUTOCATALYTIC ACTIVATION*

(Received for publication, July 6, 1995, and in revised form, August 25, 1995)

Senye Takahashi,a,b,c Tsutomu Nakagawa,a,c,d Kazuo Kasai,a Tomohiro Banno,a
Stephen J. Duguy,a Wim J. M. Van de Ven,a Kazuo Murakami,a,h
and Kazuhisa Nakayamaa)

From the a)Institute of Applied Biochemistry, b)Institute of Basic Medical Sciences, c)Tsukuba Advanced Research Alliance Center, and d)Institute of Biological Sciences and Gene Experiment Center, University of Tsukuba, Tsukuba, Ibaraki 305, Japan, the e)Howard Hughes Medical Institute and Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637, and the f)Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven, B-3000 Leuven, Belgium

Furin is a Golgi membrane-associated endoprotease that is involved in cleavage of various precursor proteins predominantly at Arg-X-Lys/Arg-Arg sites. Furin itself is synthesized as an inactive precursor, which is activated through intramolecular autocatalytic cleavage at an Arg-X-Lys-Arg site. We previously found that human colon carcinoma LoVo cells have a frameshift mutation within the homo B domain of furin and thereby lack processing activity toward Arg-X-Lys/Arg-Arg sites. In this study, however, we identified a second furin mutation in this cell line. The mutation, a replacement of a conserved Trp residue within the homo B domain with Arg, results in lack of processing activity of the mutant furin. The combination of both mutations can account for the recessive nature of the processing incompetence of LoVo cells. Immunofluorescence analysis with three distinct anti-furin monoclonal antibodies revealed that neither furin mutant underwent the autocatalytic activation or left the endoplasmic reticulum for the Golgi. These data indicate that the homo B domain as well as the catalytic domain is required for autocatalytic activation of furin.

A variety of biologically active peptides and proteins are synthesized as inactive precursors, which undergo endoproteolytic cleavage at sites marked by paired or multiple basic amino acids to yield mature products. A novel family of mammalian endoproteases homologous to the yeast Kex2 protease has been shown recently to play a key role in the proprotein processing. These include furin, PC1/3, PC2, PC4, PACE4, and PC5/6 (for review, see Seidah et al. (1991), Steiner et al. (1992), and Halban and Räminger (1994)). Furin is a membrane-associated protease that is predominantly localized to the trans-Golgi network (Misumi et al., 1991; Molloy et al., 1994). It is expressed ubiquitously and catalyzes cleavage of a wide variety of precursor proteins, such as those for growth factors, serum proteases, receptors, and viral envelope glycoproteins, at sites marked mainly by Arg-X-Lys/Arg-Arg (RXK/RR) consensus sequence (Brennahan et al., 1990; Hatzusawa et al., 1992; Hosaka et al., 1991; Misumi et al., 1991; Molloy et al., 1994). Furin itself is synthesized as an inactive zymogen and is activated by intramolecular autocatalytic removal of its propeptide through cleavage at the RXKR site (Leduc et al., 1992; Creemers et al., 1993). Most recent studies have revealed that the autocatalytic propeptide removal occurs in the endoplasmic reticulum (ER) and is a prerequisite for transport of furin out of this compartment to the trans-Golgi network (Molloy et al., 1994; Vey et al., 1994; Creemers et al., 1995). This is also the case with the yeast Kex2 protease (Gluschankof and Fuller, 1994).

Direct evidence for involvement of furin in precursor cleavage at RXK/RR sites has been provided by studies on processing-incompetent cell lines. A human colon carcinoma cell line, LoVo, has been shown to be incapable of cleaving endogenous precursor proteins for insulin and hepatocyte growth factor at the RXK/RR sites (Mandino et al., 1991; Komada et al., 1993). We (Takahashi et al., 1993) have recently shown that the processing incompetence is ascribed to a one-nucleotide deletion in the furin gene that shifts the open reading frame and causes an aberrant termination of the furin polypeptide in the homo B domain (also known as M or P domain). The homo B domain as well as the subtilisin-like catalytic domain has been shown to be essential for the endoproteolytic activity of furin and the yeast Kex2 protease (Hatzusawa et al., 1992; Creemers et al., 1993; Gluschankof and Fuller, 1994). The furin mutation in LoVo cells appears to be recessive, since transfection of furin compensated for the processing deficiency (Komada et al., 1993; Takahashi et al., 1993). On the other hand, Moehring and co-workers (Moehring and Moehring, 1983; Watson et al., 1991) established a mutant CHO-K1 cell line, named RPE-40, which is resistant to Pseudomonas exotoxin and various enveloped viruses. They have recently indicated that the resistance is ascribed to the lack of endoproteolytic cleavage of the toxin and viral envelope glycoproteins, which are normally cleaved by furin, although the molecular identity of the mutation in

*This work was supported in part by grants from the Ministry of Education, Science and Culture of J. Japan, the Special Research Project on Circulation Biosystem in the University of Tsukuba, the Saneyoshi Scholarship Foundation, the Nissan Science Foundation, the Ciba-Geigy Foundation (J. Japan) for the Promotion of Science, and Sankyo Co., Ltd. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Present address: Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan.

† The first two authors contributed equally to this work.

‡ Recipient of a fellowship from the Japanes Society for the Promotion of Science for Japanese Junior Scientists.

§ To whom correspondence should be addressed: Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan. Tel.: 81-298-53-6356; Fax: 81-298-53-6006; E-mail: kazunaka@sakura.cc.tsukuba.ac.jp.

The abbreviations used are: ER, endoplasmic reticulum; RT-PCR, reverse transcriptase-polymerase chain reaction.
A Second Mutation of Furin in LoVo Cells

Fig. 1. Schematic representation of the structure of furin cDNA. The coding region is boxed. Relative positions of oligonucleotide primers used for RT-PCR are shown by arrows. Predicted epitope regions of antifurin monoclonal antibodies are shown by solid bars. SP, signal peptide; Cys-rich, Cys-rich domain; TMD, transmembrane domain.

RPE.40 cells is unknown (Moehring et al., 1993; Inocencio et al., 1994). Since our identification of the furin mutation in LoVo cells, many researchers have made use of this cell line for studies on precursor processing and demonstrated that furin is involved in processing of a wide variety of precursor proteins (for example, Tsuneoka et al. (1993), Ohnishi et al. (1994), Inocencio et al. (1994), Paquet et al. (1994), Hiromoto et al. (1994)). However, we have recently found by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis that LoVo cells possess not only one type of furin cDNA having the one-nucleotide deletion in the homo B domain but also another type with a sequence that appears to be normal around this mutation site. This is incompatible with the notion that the furin mutation in LoVo cells is recessive. We therefore started to address this problem.

EXPERIMENTAL PROCEDURES

RT-PCR—Total RNA of LoVo cells was isolated using an isogen RNA isolation reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Single-stranded cDNA was synthesized from the total RNA using a First-strand cDNA synthesis kit (Pharmacia LBK Biotechnology, Uppsala, Sweden). The cDNA was amplified using AmpliTaq DNA polymerase (Perkin Elmer) in a Perkin Elmer DNA Thermal Cycler for 40 cycles of denaturation (95°C, 1 min), annealing (60°C, 2 min), and extension (72°C, 3 min). Following sets of oligonucleotide primers were used for the PCR: primer 1 (5'-CAGCTTCTACCTCCTCCGAGC-3') and primer 3 (5'-GACCCGGGTCACGGTCTTCC-3') or primer 2 (5'-GGGCCGGAAATGGAGCC-3') and primer 4 (5'-GGTCGCCCTAAG-3'). Their relative positions are shown schematically in Fig. 1. The PCR product was blunt-ended with T4 DNA polymerase (Life Technologies, Inc.) and electrophoresed on a 5% polyacrylamide gel. A DNA fragment with an expected size was extracted from the gel, ligated into the EcoRV site of pBluescript-II (Stratagene), and subjected to sequence analysis using a BcaBEST Dideoxy Sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan).

DNA Construction, Transfection, and Immunoprecipitation Analysis—A cDNA fragment covering the entire coding sequence of wild type human furin (a kind gift from Dr. Yukio Ikehara, Fukuoka University School of Medicine, Japan; Misumi et al. (1991)) was subcloned into the pCDNA3 mammalian expression vector (Invitrogen); the resulting vector was named pCDNA-hFur. To construct chimeras of wild type and LoVo furin cDNA, a cDNA fragment of LoVo furin cDNA either from its 5'-terminus to a unique Xhol site (see Fig. 1) or from the Xhol site to the 3'-terminus was substituted for the corresponding fragment of pCDNA-hFur. The wild type or chimeric furin vector along with an expression vector for mutant prorenin with an RXKR cleavage site sequence (Hosaka et al., 1991) was transfected into LoVo cells in a well of a six-well cluster dish using a CellPhect transfection kit (Pharmacia Biotech Inc.). After a 48-h incubation, the cells were labeled for 8 h with 0.1 mCi/ml EXPRE35S35S (DuPont NEN). The culture medium was collected and mixed with 4 mL of 10% trichloroacetic acid (TCA). The precipitates were dissolved in 500 μL of 1% sodium dodecyl sulfate (SDS) and 0.1% 3-(N-morpholino)propanesulfonic acid (MOPS) and electrophoresed on a 5% SDS-polyacrylamide gel. The gel was fixed in 50% methanol, 10% acetic acid, and 10% methanol, 10% acetic acid, and 10% SDS, and autoradiographed. For the immunoprecipitation analysis, the supernatants of the transfected LoVo cells were immunoprecipitated with anti-renin antiserum and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described previously (Hosaka et al., 1991; Takahashi et al., 1993).

Indirect Immunofluorescence Microscopy—COS-7 cells 24 h after transfection of the wild type or chimeric furin vector were trypsinized, plated onto wells of an 8-well Lab-Tek chamber slide (Nunc, Roskilde, Denmark), and cultured for 12 h. The cells were fixed, permeabilized, and blocked as described previously (Tori et al., 1995). The cells were double-labeled with mouse monoclonal anti-furin antibody (MON-148, 150, 152; Van Duijnhoven et al., 1992) and rabbit polyclonal anti-BIP antibody (kind gift from Dr. Yasunori Kozutsumi, Faculty of Pharmaceutical Sciences, Kyoto University, Japan) followed by Texas Red-labeled donkey anti-mouse Igs (Jackson Immuno Research Laboratories, Inc.) and FITC-labeled donkey anti-rabbit IgG (Chemicon, Temecula, CA).

RESULTS AND DISCUSSION

In the earlier study, we showed that the lack of precursor-processing activity toward RXKR/RK sites of human colon carcinoma LoVo cells is ascribed to a mutation in furin (Takahashi et al., 1993). The mutation is a one-nucleotide deletion; while four consecutive T residues are present from nucleotide residues 1,283 to 1,286 (the numbering is according to Van den Ouweland et al. (1991)) in wild type human furin cDNA, three consecutive ones are in the LoVo furin cDNA (see Fig. 1). This deletion shifts the open reading frame with an altered amino acid sequence beginning with the codon for amino acid 429 within the homo B domain, which, in addition to the substituting-like catalytic domain, is thought to be essential for furin activity (Hatsuzawa et al., 1992; Creemers et al., 1993). We hereafter refer to this mutant furin as 429FS (for frame shift from amino acid 429). However, by RT-PCR analysis of LoVo cell RNA, we recently noticed the presence of another type of cDNA, which had four consecutive T residues. If the furin molecule encoded by the second cDNA has endoproteolytic activity, the finding would be incompatible with the recessive nature of LoVo cells with respect to the processing deficiency. We therefore pursued analysis of additional furin cDNAs from LoVo cells.

We first amplified a cDNA region from LoVo cell RNA by RT-PCR using a set of primers; one corresponding to a part of 5'-untranslated region (primer 1) and the other complementary to a region downstream of the 4T/3T site (primer 2) (see Fig. 1). The amplified fragment was subcloned into a vector and subjected to partial sequence analysis. Of six independent clones analyzed, five clones had four T residues as in wild type cDNA, while one had three T residues as in 429FS cDNA. We therefore constructed a chimeric furin cDNA vector with a substitution of a fragment from the 5'-terminus to the unique Xhol site (see Fig. 1) of each clone having four T residues for the corresponding fragment of wild type cDNA. To determine if the chimeric furin has endoproteolytic activity, LoVo cells were co-transfected with the chimeric construct and a vector for prorenin with an RXKR cleavage site. Although the data are not shown, all the chimeras examined showed endoproteolytic activity toward the RXKR substrate. This data indicated that, at least within the region from 5'-terminus to the Xhol site, the LoVo cDNA of 4T type does not have any mutation that affects the furin activity.

We then amplified the other cDNA region from LoVo cell RNA using primer 2 corresponding to a region upstream of the 4T/3T site and primer 4 complementary to a part of 3'-untranslated region (see Fig. 1). Of 10 independent clones subjected to partial sequence analysis, seven clones were 4T type, while three clones were 3T type. Since the partial sequence analysis revealed that the 4T clones did not have any mutation from the 5'-terminus of the cloned cDNA fragment to the unique Xhol site, we prepared chimeric constructs as shown schematically in Fig. 2A; a region of each LoVo furin cDNA from the Xhol site...
to the 3'-terminus was substituted for the corresponding one of wild type cDNA. The construct was then transfected into LoVo cells in combination with the RXKR prorenin vector to determine if the chimeras had endoproteolytic activity. As shown in Fig. 2, LoVo cells transfected with a chimeric construct of wild type furin cDNA and a LoVo cell cDNA clone 10 of 3'T type, cleaved the prorenin substrate like those transfected with the wild type furin vector. This indicates that, as shown in our previous study (Takahashi et al., 1993), the 429FS-type cDNA has no mutation that affects the furin activity within the substituted region; that is, from the Xho I site to the 3'-terminus. By contrast, transfection of either chimera of wild type furin and each 4T-type LoVo furin cDNA (clone 1, 4, or 7) did not give rise to cleavage of the substrate at the RXKR site. These observations indicated that the region from the Xho I site to the 3'-terminus derived from each LoVo cell cDNA clone, which appeared to be normal, at the 4T/3'T site, had some mutation that affects the furin activity.

Sequence analysis revealed that there was one nucleotide substitution within a region covering the homo B domain; the T residue at nucleotide 1,639 was replaced by C (Fig. 3A). This nucleotide substitution causes replacement of a codon for Trp (TGG) at amino acid 547 by that for Arg (CGG); therefore, we hereafter call this type of mutation as W547R. All the sequenced cDNAs, which appeared to be normal with respect to the 4T/3'T site, had the mutation of T to C, whereas all the 3'T type cDNAs did not have the W547R substitution. We confirmed that there is not any mutation in the cloned region other than the mutation of T to C by sequencing of three 4T cDNAs. Furthermore, the mutation was also found in genomic DNA of LoVo cells; 4T-type genomic DNA had the T to C mutation, while 3'T-type DNA did not have the mutation (data not shown). Taken together with our previous data for 429FS-type mutation (Takahashi et al., 1993), we conclude that the presence of two mutant alleles of furin, one for 429FS and the other for W547R, results in the processing incompetence of LoVo cells, being compatible with the recessive nature of the incompetence.

As shown schematically in Fig. 3C, the W547R mutation is positioned in the COOH-terminal part of the homo B domain. Comparison with other Kex2 family members revealed that the Trp residue within the homo B domain is completely conserved in this family (Fig. 3B). Around the region including the Trp residue, there is a consensus motif, WX\_WX\_X\_GXW (X for any amino acid); the last W is the residue mutated in furin of LoVo cells. It is noteworthy that, in this motif, three Trp residues are spaced at intervals of eight residues, although the significance is unknown since data base analysis failed to show the existence of other proteins having this motif outside the Kex2 family.

Our previous studies have shown that the homo B domain as well as the subtilisin-like catalytic domain is essential for furin activity (Hatsuzawa et al., 1992; Creemers et al., 1993). With respect to the catalytic domain, it is known that a mutation at either the active site Asp, His, or Ser residue blocks intramolecular autocatalytic cleavage of the furin propeptide, whereas an oxyanion hole mutant and a negative side chain mutant do undergo the autocatalytic maturation but lack substrate processing activity (Creemers et al., 1993, 1995). Furthermore, the autoproteolytic removal of the propeptide has been shown to be prerequisite for transport of furin out of the ER to the Golgi (Molloy et al., 1994; Vey et al., 1994; Creemers et al., 1995). However, it remained to be determined whether the homo B domain is required for substrate processing activity or autocatalytic activation. To address this issue, we expressed wild type furin and 429FS and W547R mutants in COS-7 cells and stained these cells with three distinct monoclonal antibodies to furin, which recognize distinct epitopes of furin polypeptide (Van Duijnhoven et al., 1992). As shown schematically in Fig. 1A, epitopes of these antibodies are within the following regions: MON-148, the catalytic domain; MON-150, the propeptide region; MON-152, a region COOH-terminal to the homo B domain. When stained with the antibody to the catalytic domain, MON-148 (Fig. 4), COS-7 cells expressing wild type furin gave a perinuclear staining characteristic of the Golgi. By contrast, the cells expressing 429FS or W547R gave a reticular staining throughout the cytoplasm but not a Golgi-like one. The reticular staining was well correlated with that produced by an antibody to BiP, a well characterized marker protein for the ER, indicating that neither mutant exited the ER. The data also indicate that the region downstream of amino acid 429 is not required for retention of the furin precursor in the ER. MON-152 produced a Golgi-like and an ER-like staining pattern for wild type and W547R furin, respectively, as those observed with MON-148, but did not produce any significant staining for 429FS (Fig. 5). This is in agreement with the fact that 429FS lacks the region containing the epitope of MON-152 due to the frameshift mutation. When stained with the propeptide antibody, MON-150 (Fig. 6), cells expressing wild type furin did not produce any significant staining. This observation together with those shown in Figs. 4 and 5 indicate that all the wild type furin molecules have left the ER for the Golgi after autocatalytic removal of the propeptide. By contrast, an ER-like staining but not a Golgi-like one was observed with MON-150 in cells expressing 429FS or W547R that had MON-148. These observations together with those in Figs. 4 and 5 indicate that neither mutant is able to go out of the ER due to the lack of autoproteolytic removal of the propeptide.

As described so far, the present study establishes the following. First, LoVo cells lack processing activity toward RXK/RK sites due to the presence of two distinct mutant alleles of furin, 429FS and W547R. The combination of the two alleles can account for the recessive nature of the processing incompetence of LoVo cells. The detailed characterization will make this processing-deficient cell line a more useful tool for processing studies.
Second, the homo B domain is essential for autoproteolytic activation of furin, so that neither mutant can leave the ER. Surprisingly, the mutation of only one amino acid (Trp at amino acid 547) within this domain completely blocks the autocatalytic activation. To date, researchers have distinguished, for convenience sake, between the subtilisin-like catalytic and homo B domains, since the former but not the latter one is present in the prototypical proteases, bacterial subtilisins. However, our previous (Hatsuzawa et al., 1992; Creemers et al., 1993) and present studies using furin and the study of Gluschankof and Fuller (1994) using the yeast Kex2 protease have shown that the homo B domain is also essential for the auto-
catalytic activation process. Kex2-like proteases have more strict substrate specificity than subtilisins, making it tempting to speculate that an additional region is required for keeping the strict specificity. Although the three-dimensional structure of furin has been modeled on the basis of x-ray crystallographic data of subtilisin BPN’ and thermolysin, this model is restricted to the subtilisin-like domain (Van de Ven et al., 1990; Siezen et al., 1994). To understand the overall structure and thereby the molecular mechanisms that underlie the autocatalytic activation and substrate cleaving processes of Kex2 family proteases, it must therefore await x-ray crystallographic analysis of the proteases themselves.

Finally, the region downstream of amino acid 429, including a most portion of the homo B domain, and the Cys-rich, transmembrane, and cytoplasmic domains, is not implicated in the ER retention of furin. Especially, this indicates that association of the furin molecule with membranes is not implicated in the precursor retention. Although the molecular mechanism that underlies the ER retention of furin is currently unknown, the presence of an ER-resident protein that interacts with the pro- but not mature form of furin could account for the ER retention (Creemers et al., 1995). It is tempting to speculate that such a retention protein might be a molecular chaperone essential for correct folding of furin. Identification of an ER protein(s) that interacts with the furin precursor will be required to understand the mechanism of the ER retention.

Acknowledgments—We would like to thank Dr. Yukio Ikehara for providing the human furin cDNA, Dr. Yasunori Kozutsumi for providing anti-BiP antibody, and Dr. Kaichiro Yanagisawa for encouragement.

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Fig. 6. Intracellular localization of wild type and mutant furin detected with MON-150. COS-7 cells transiently transfected with a construct for wild type (WT), 429FS, or W547R furin were fixed and double-stained with a monoclonal antibody to furin MON-150, which recognizes an epitope within the propeptide region (see Fig. 1), and anti-BiP antibody as described under “Experimental Procedures.”