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Mammalian Subtilisin-Related Proteinases in Cleavage Activation of the Paramyxovirus Fusion Glycoprotein: Superiority of Furin/PACE to PC2 or PC1/PC3

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The fusion glycoprotein precursor of Newcastle disease virus is ubiquitously cleaved in the constitutive secretory pathway if it possesses an oligobasic cleavage motif (RRQR/KR), whereas the precursor is refractory to cleavage if the motif is monobasic (GR/KQGR). We examined the cleavage activity of the mammalian subtilisin-related proteinases furin/PACE, PC2, and PC1/PC3, which are thought to be responsible for proprotein processing in either the constitutive (furin/PACE) or the regulated (PC2 and PC1/PC3) secretory pathway, for the viral precursors with different cleavage motifs. Only furin/PACE was fully capable of cleaving the precursors with the oligobasic motif. PC2 and PC1/PC3 were incapable or only partially capable of cleaving at this motif. None of the proteinases cleaved the monobasic motif. These results suggest involvement of furin/PACE in viral protein processing in the constitutive secretory pathway.

Enveloped animal viruses usually possess a surface glycoprotein that mediates fusion between the viral envelope and host cell membrane and thereby enables initiation of infection. Biosynthesis of this glycoprotein often involves posttranslational endoproteolysis of the inactive precursor at the carboxyl side of a certain arginine by a host cell protease. This cleavage activation mechanism was first discovered for the fusion glycoprotein of Sendai virus, a paramyxovirus in mice (18, 43). Subsequent studies with a series of virulent and avirulent strains of Newcastle disease virus (NDV), an avian paramyxovirus, demonstrated that there are at least two distinct categories in their activation and that this variation determines the marked differences in viral spreading in the host and pathogenicity between strains (12, 31, 34, 36, 54, 55). In one group characteristic of avirulent strains, the fusion glycoprotein precursor (F_0) possesses a single arginine at the cleavage site and is cleaved only by certain cell types that express a special protease(s). Thus, infection by these strains is localized to those enzyme-expressing tissues without production of severe systemic symptoms. We recently isolated an endoprotease of this category from chicken embryos and demonstrated its identity with the activated form of blood clotting factor X (FXa) and strict correlation of its expression with viral tropism at the tissue level (13, 14, 39, 50). The same mechanism (FXa dependent) seems to be responsible for determining the tropism of Sendai virus and certain influenza A viruses in chicken embryos (13, 14, 39). The sequence motif Gln(Glu)-X-Arg (where X is Gly, Ser, or Thr), which resembles the cleavage site in prothrombin, the natural substrate of FXa, was commonly found at the cleavage site of these viruses. In another group characteristic of virulent NDV strains, the cleavage site consists of paired or multiple basic residues and cleavage occurs in a much greater number of tissues, such that the infection is pantropic (34, 54, 55). Such an oligobasic motif is almost universally present in pantropic paramyxoviruses, such as measles virus and mumps virus, and in other virus families, including avian influenza viruses (4, 5, 21, 59). However, none of the proteases involved in this category of activation have been identified or extensively characterized, except that they apparently function in the Golgi apparatus or the trans-Golgi network (30, 35, 41, 61). A number of cellular proproteins and prohormones are cleaved at an analogous di- or oligobasic site and at a similar intracellular location. Thus, this type of processing of either cellular or viral precursors has been of great interest in cell biology, and identification of the processing proteases has long been sought. A landmark in the field was discovery of the KEX2 protease, or kexin, which processes the α mating factor and killer toxin precursor protein in the yeast *Saccharomyces cerevisiae* and shares both sequence and organizational homologies with the prokaryotic subtilisins (6, 8, 10, 20, 26–28). Analysis of the KEX2 gene further led to the discovery of proteinases termed furin or paired basic amino acid-cleaving enzyme (furin/PACE) (9, 15, 16, 25, 40, 56), PC2 (15, 44, 48), and PC1/PC3 (15, 22, 37, 45, 47), which are responsible for processing in mammalian cells and constitute a unique family of mammalian subtilisins (for a review, see reference 2). Furin/PACE is thought to be a ubiquitous endoprotease which is present predominantly in the Golgi apparatus and catalyzes the processing of precursor proteins in the constitutive secretory pathway (CSP) (7, 16, 42). PC2 and PC1/PC3, on the other hand, are cloned from neuroendocrine cells and are thought to be responsible for processing in the regulated secretory pathway (RSP), which develops only in endocrine cells and secretes molecules in response to specific extracellular stimulation (15, 22, 37, 44–48, 52).

Cleavage of NDV F_0 yields two subunits, the C-terminal

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F_1 of 56 kDa and the N-terminal F_2 of 12 kDa (34, 54, 55). The N-terminal hydrophobic stretch of F_1 is thought to be the direct mediator of fusion activity (11). As described above, if the cleavage site is oligobasic, this processing occurs in the Golgi apparatus or the trans-Golgi network of vertebrate cells (30, 34, 35, 41, 61). In the present study, we examined whether these mammalian subtilisin-related proteinases, furin/PACE, PC2, and PC1/PC3, have the potential to cleave two viral precursors: the F_0 glycoprotein precursors of virulent NDV strains Sato and Beaudette C, which contain the F_0 cleavage motif RRQRR or RRQKR, respectively. The major experimental strategies employed include the use of lymphoblastoid cell line NALM-6, which is defective in full processing of the viral precursors (32, 41), and the use of recombinant vaccinia viruses that express those mammalian subtilisins (7, 51, 52).

MATERIALS AND METHODS

Cells and viruses. NALM-6 and BHK-21 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and in Eagle's minimum essential medium supplemented with 10% bovine serum, respectively. NDV strains Sato, Beaudette C, La Sota, and Ulster were grown in 11-day-old chicken embryos. Recombinant vaccinia viruses that express human furin/PACE (VV:Fur), human PC2 (VV:PC2), or mouse PC1/PC3 (VV:PC3) were constructed by inserting each proteinase gene into the viral thymidine kinase gene as described elsewhere (7, 51, 52). These recombinant viruses and wild-type vaccinia virus strain WR (VV:Wt) were prepared by propagation in CV-1 cells.

In vitro assay of proteolytic cleavage of viral precursors. As enzyme sources, we used the media and extracts of BHK-21 cells infected with either VV:Wt, VV:Fur, VV:PC2, or VV:PC3 at an input multiplicity of 10 PFU per cell. The media were collected 24 h after infection. After a wash with phosphate-buffered saline, the extracts were prepared by lysing the cells with 1% Triton X-100 in PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (10 mM, pH 6.8) containing 100 mM KCl, 2.5 mM $MgCl_2$, and 0.3 M sucrose on ice. After centrifugation for 10 min at 10,000 × *g*, the clarified media and extracts were stored at −80°C until use. A soluble form of mouse furin was also used. This was produced by CHO cells transfected with a plasmid that expresses the proteinase without the transmembrane domain and was purified from the culture medium as previously described (17). The substrate NDV virions were grown in NALM-6 cells (32, 41). The infected cells were metabolically labeled with 500 kBq of EXPRE^{35}S^{35}S protein-labeling mix per ml (370 MBq/ml, >37 TBq/mmol; Du Pont-New England Nuclear, Wilmington, Del.), and the virions were isolated and purified from the culture medium (34). One hundred microliters of each of the extracts and media was mixed with an appropriate amount of NDV virions in 100 μl of 100 mM Tris-HCl (pH 6.8)–6 mM $CaCl_2$. The mixtures were incubated at 37°C for 2 h, subsequently immunoprecipitated with an anti-F monoclonal antibody (hybridoma clone 320 [1, 53]), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (23, 38). The protein bands on the gels were visualized by exposure to Kodak X-Omat X-ray films or by a Fujix BAS 2000 Image Analyzer (Fuji Photo Film, Tokyo, Japan).

In vivo assay of proteolytic cleavage of viral precursors. NALM-6 cells were coinfected with NDV (multiplicity of infection, 10) and either VV:Wt, VV:Fur, VV:PC2, or VV:PC3 (multiplicity of infection, 10). After 6 h, the cells were labeled for 15 h with 370 kBq of D-[1,6-^{3}H]glucosamine hydrochloride per ml (37.0 MBq/ml, 2,179.3 GBq/mmol; Du Pont-New England Nuclear). The cells were processed for immunoprecipitation with the anti-F monoclonal antibody, and the precipitates were analyzed by SDS-PAGE.

Infectivity titrations. Infectivity of NDV was determined by plaque formation on monolayers of BHK-21 cells grown in 35-mm-diameter plates as described elsewhere (34). The plaques were counted 2 days after infection. Under these conditions, no vaccinia virus plaques developed. However, polyclonal anti-NDV rabbit serum was added to certain plates to assess NDV titers in the specimens from coinfection studies with NDV and each vaccinia virus.

RESULTS

In vitro cleavage of NDV F_0 by mammalian subtilisin-related proteinases. We first examined the in vitro cleavage activity of the mammalian subtilisin-related proteinases as well as that of trypsin for NALM-6-grown virions, which contain a high level of F_0 (32, 41). As the source of these subtilisin-related proteinases, we used extracts and media of vaccinia virus-infected BHK-21 cells that expressed each proteinase gene and produced the respective enzymes in excess (7, 51, 52).

The extract from cells infected with VV:Fur efficiently cleaved the F_0 of both the Sato (Fig. 1A) and Beaudette C (data not shown) strains, whose cleavage site sequences are RRQRR and RRQKR, respectively. One of the cleavage products, F_2, is not shown here. None of the extracts from either uninfected cells or cells infected with VV:Wt, VV:PC2, or VV:PC3 manifested cleaving activity (Fig. 1A). The medium from VV:Fur-infected cells also was able to cleave F_0 (Fig. 1B). Immunoblotting analysis with rabbit anti-furin/PACE serum (60) revealed that the medium of VV:Fur-infected cells contains an immunoreactive protein whose molecular mass is a little lower than that of authentic furin/PACE (data not shown), in good agreement with the previous finding that suggested that the immunoreactive protein in the medium represents a truncated molecule that possibly results from furin/PACE autoproteolysis (60). Cleaving capability by a soluble form of furin/PACE was confirmed by using purified mutant mouse furin/PACE without the transmembrane domain, which is secreted from transfected CHO cells (17) (Fig. 1C). Although significant levels of vaccinia virus-derived PC2 and PC1/PC3 are secreted from infected cells (46), medium from PC2- and PC1/PC3-expressing cells exhibited no cleaving capacity (data not shown).

To determine whether furin/PACE cleaved viral precursors at the correct site with the motif of RRQR/KR, we first attempted to determine the N-terminal amino acid sequence of F_1 generated by furin/PACE. However, NDV-infected NALM-6 cells are poor virus producers and consequently we were unable to obtain sufficient amounts of F_1 substrates for sequencing. Second, we used alternate strains of NDV as the substrate. These strains possess different cleavage motifs, i.e., GRQGR (strain La Sota) and GKQGR (strain Ulster). We found that neither furin/PACE, PC2, nor PC1/PC3, in either the extract, the medium, or a soluble purified form, was capable of cleaving the F_0 of strains La Sota (Fig. 1A, B, and C) and Ulster (data not shown), indicating requirement of the RRQR/KR motif for processing by furin/PACE. Trypsin correctly cleaves at this motif (Fig. 1A, B, and C), as reported previously (33, 34). Third, we examined whether the medium from VV:Fur-infected cells has the

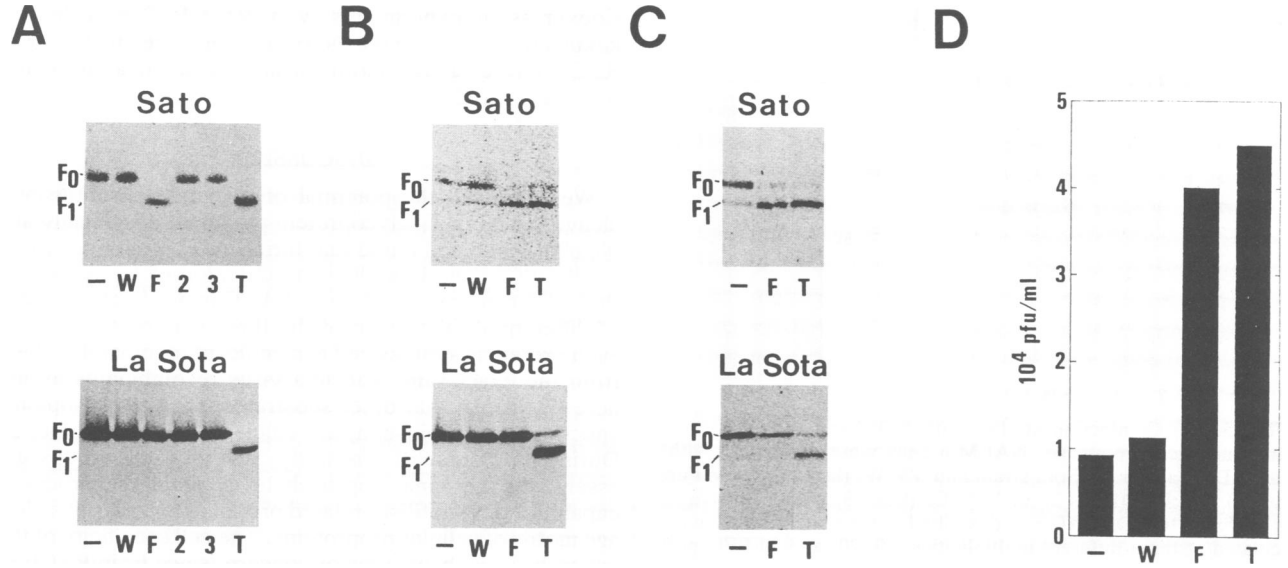

FIG. 1. Effect of mammalian subtilisin-related proteinases on the cleavage of F_0 glycoprotein of NDV strains Sato and La Sota (A to C) and on the infectivity of strain Sato (D). NALM-6-grown, EXPRE^{35}S^{35}S-labeled NDV virions were treated with the extract (A) or the medium (B) of BHK-21 cells infected with either VV:Wt (lanes W), VV:Fur (lanes F), VV:PC2 (lanes 2), or VV:PC3 (lanes 3) and analyzed by SDS-PAGE as described in the text. The virions were also treated with a soluble form of mutant furin/PACE (see text) (1 μg/ml) for 90 min and similarly analyzed (C). (D) The infectivity of strain Sato, treated as for panel B, was determined by plaque formation on BHK-21 cell monolayers. Lanes: T, treated with trypsin (4 μg/ml) for 10 min; –, treated with the extract or medium of mock-infected BHK-21 cells (A and B) or untreated (C and D).

ability to enhance viral infectivity. As shown in Fig. 1D, treatment of NDV strain Sato with medium from VV:Fur-infected cells resulted in an about fourfold increase of infectivity, while medium from VV:Wt-infected cells (Fig. 1D) failed to exhibit this enhancing activity. The precursor-cleaving activity and infectivity-enhancing capacity of furin/PACE were found to be comparable to those of trypsin (Fig. 1A and D), which has often been used for in vitro cleavage activation studies of viral precursor proteins. The degree of activation by furin/PACE or trypsin might be lower than that found for avirulent NDV strain La Sota by trypsin treatment, which resulted in an at least six- to sevenfold increase of the level of infectivity (34). However, activation by furin/PACE, as well as that by trypsin, was consistently found in more than 10 independent, similar experiments. Thus, the results were highly reproducible. Cleavage of only a fraction of the F_0 molecules on a virion surface is probably sufficient for the virus to become infectious, since a small but significant amount of cleaved F always coexisted with the F_0 in the virus samples prepared from infected NALM-6 cells (Fig. 1A, B, and C). In addition, the calcium ions and pH range (6.0 to 8.5) required for optimal action of furin/PACE in F_0 cleavage (Fig. 2) were similar to those found with the fluorogenic substrate Boc (*t*-butoxycarbonyl)-Gly-Lys-Arg-MCA (4-methylcoumarin-7-amide) (7). Thus, the available data suggested the specificity of furin/PACE in cleavage of the F_0 of virulent NDV strains.

In vivo cleavage activation of NDV F_0 by mammalian subtilisin-related proteinases. To examine whether furin/PACE can also catalyze the cleavage of F_0 in vivo, coinfection with NDV and VV:Fur was performed and the maturation of the F_0 precursor was analyzed by labeling with [^{3}H]glucosamine, followed by immunoprecipitation and SDS-PAGE. Furin/PACE was found to accelerate conversion of F_0 to F_1 and F_2 (the latter is not shown) for both strains Sato (Fig. 3A) and Beaudette C (data not shown). Recovery of F_1 from furin/PACE-expressing cells was low relative to that of the other cell extracts. One may interpret this result as loss of F_0 rather than conversion. However, this is quite unlikely because the F_0 of strain La Sota, which is highly homologous to strain Sato or Beaudette F_0, particularly to the latter, with a difference of only several amino acids, including the two residues at the cleavage site (51, 54), was recovered in a large amount (Fig. 3A). In contrast to furin/PACE, PC2 was found to be ineffective in vivo (Fig. 3A), as in the in vitro activation studies (Fig. 1A). Unlike the in vitro results, which showed almost absolute lack of cleavage, the PC1/PC3 expressed appeared to be effective to some extent (Fig. 3A). Presumably, the intracellular milieu might have contributed to this partial cleavage. However, the incompleteness of in vivo cleavage by PC1/PC3 was not overcome by increasing the input multiplicity of VV:PC3

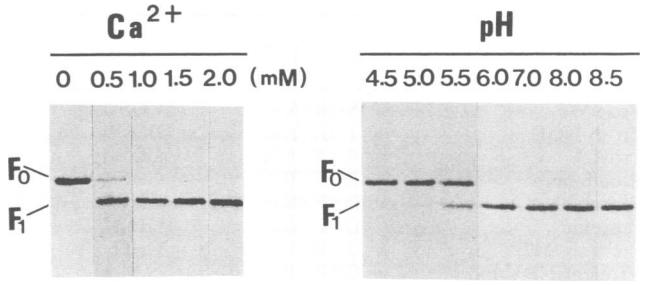

FIG. 2. Effects of Ca^{2+} and pH on cleavage of F_0 of NDV strain Sato by furin/PACE. Purified mutant mouse furin/PACE lacking the transmembrane domain was used as the enzyme source. Tris-HCl (pH 7.0 to 8.5) and Tris-acetate (pH 4.5 to 6.0) buffers were used to determine pH dependency.

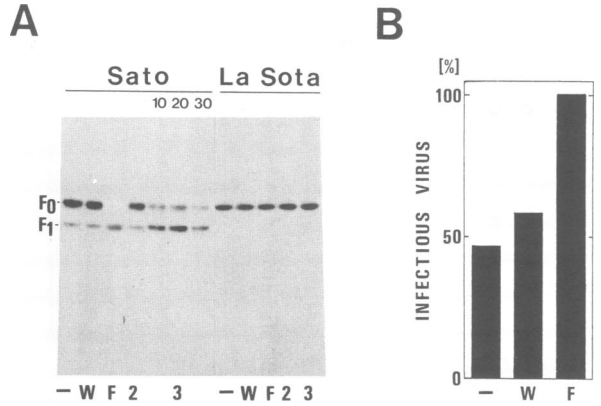

FIG. 3. (A) Coinfection studies with NDV and vaccinia virus expressing each proteinase. NALM-6 cells were coinfected with either NDV Sato or La Sota strain and VV:Wt (lanes W), VV:Fur (lanes F), VV:PC2 (lanes 2), or VV:PC3 (lanes 3), and F_0 cleavage was analyzed by SDS-PAGE as described in the text. VV:PC3 was inoculated at three different multiplicities (10, 20, and 30 PFU per cell), as indicated at the top of the gel, while the multiplicity was 10 PFU/cell for the other vaccinia viruses. −, infection with NDV only. (B) Relative NDV strain Sato infectivity before and after in vitro trypsin treatment of the virions obtained by coinfection with VV: Fur (lane F) or VV:Wt (lane W) as for panel A. The relative NDV infectivity was the average of four separate experiments.

(Fig. 3A). Thus, it appears that PC1/PC3 is principally incapable of full processing or is much less active than furin/PACE. To resolve these problems, however, more detailed studies are necessary, including intracellular localization of the expressed PC1/PC3, as well as pH dependency, ionic requirements, and other factors in its in vitro functioning.

Enhancement of viral infectivity by furin/PACE was also observed in this in vivo experiment. As shown in Fig. 3B, NDV strain Sato produced by NALM-6 coinfected with VV:Fur was fully infectious before in vitro treatment with trypsin. In contrast, at least 50% of the progenies were noninfectious when coinfected with VV:Wt and became infectious after in vitro trypsin treatment. Coinfection with VV:Fur, however, neither cleaved the F_0 of strain La Sota (Fig. 3A) or Ulster (data not shown) nor activated the infectivity of the virus (data not shown). Fur/PACE, PC2, and PC1/PC3 were originally cloned as homologs of kexin. Coexpression experiments with VV:KEX2 revealed that kexin could also cleave NDV F_0 with specificity for the RRQR/KR cleavage motif, which is identical to that of furin/PACE (32).

DISCUSSION

We examined the potential of mammalian subtilisins to cleave the NDV F_0 glycoprotein precursor specifically at an oligobasic site and found that furin/PACE is superior to PC2 or PC1/PC3. The less efficient activation of F_0 by PC2 and PC1/PC3 compared with furin/PACE is not likely the result of differential expression of the three endoproteases, since each protein is synthesized at significant (comparable) levels from the exact same vaccinia virus recombinants as used here (7, 46), so that other substrates, such as pro-opiomelanocortin and proinsulin, are efficiently processed (46, 52). During the course of this study, several other groups used essentially the same approach to evaluate the processing capabilities of subtilisin-related proteinases at various cleavage motifs of cellular proproteins. The cell specificity of their action has also been a major concern, since furin/PACE is a ubiquitous protease in the CSP while PC2 and PC1/PC3 are specific for the RSP characteristic of endocrine cells. The available results, together with those presented in this report, are summarized in Table 1. When furin/PACE was expressed in cells that likely lack the RSP, namely, COS-1, BSC-40, CHO, and NALM-6 cells, most of the substrates were processed. These substrates, except for albumin, possess an arginine at the P4 position. In albumin, an arginine occupies P6. Prorenin, which has a dibasic motif but not the P4 arginine, was not processed by furin/PACE. However, furin/PACE did cleave a mutant prorenin in which the phenylalanine at P4 was replaced with arginine (19). Thus, a motif generalized as RXR/KR, with the P4 arginine, seems to be a prerequisite for processing in the CSP (19). For proalbumin processing, the P6 arginine seems to be important (58). The general importance of RXR/KR for processing in the CSP seems to be further supported by the fact that all but 1 of the 20 viral precursors readily cleavable there possess this cleavage motif (32). Numerous mutagenesis studies have also shown that for the substrate with a P4 basic residue, replacement of this residue with a nonbasic amino acid can fully inhibit processing in vivo (see, for example, reference 3). Analysis of the cleavage site specificity of purified furin/PACE in vitro similarly shows that P4 arginine is critical for efficient endoproteolysis of a candidate furin/

TABLE 1. Processing of cellular and viral proproteins by subtilisin-related proteinases

Proprotein	Processing motif (P6 to P1)	Cell line	Cleavage[a] by:				Reference(s)
			Kexin	Furin/PACE	PC2	PC1/PC3	
Pro-vWF	SHRSKR	COS-1	NT	+	NT	NT	57, 60
Pro-β-NGF	THRSKR	BSC-40	+	+	NT	NT	7
Pro-C3	AARRRR	COS-1	NT	+	NT	NT	24
Proalbumin	RGVFRR	COS-1	+[b]	+	NT	NT	24
Prorenin	DVFTKR	CHO	NT	−	NT	−	19
Prorenin	DVFTKR	GH_4C_1	NT	−	NT	+	19
Prorenin M2R^{-4}	DVRTKR	CHO	NT	+	NT	−	19
Virulent NDV F_0	GRRQRR	NALM-6	+	+	−	±	This work
Virulent NDV F_0	GRRQKR	NALM-6	+	+	−	±	This work
Avirulent NDV F_0	GGRQGR	NALM-6	−	−	−	−	This work
Avirulent NDV F_0	GGKQGR	NALM-6	−	−	−	−	This work

[a] Proteinases and proprotein substrates were coexpressed in the respective cells. +, fully cleavable; −, uncleavable; ±, partially cleavable; NT, not tested.
[b] In vitro activity.

PACE substrate, anthrax protective antigen (29), and several artificial substrates (17). Interestingly, however, the basic residue at position P2 sometimes seems to be less critical. For example, replacement of the P2 lysine in the insulin proreceptor by alanine did not block cleavage (62). Similarly, substitution of the P2 and P3 lysines with alanines (RAAR) in the anthrax protective antigen precursor only partially affected furin/PACE-catalyzed processing in vitro. However, our NDV strains (La Sota and Ulster) with an R/KXXR motif were found to be poor substrates for furin/PACE (Table 1). Thus, the P2 basic amino acid (arginine or lysine) was also critical in this viral substrate. In agreement with this view, although furin/PACE can also cleave an artificial substrate, pyro Glu-Arg-Thr-Gln-Arg-MCA, its efficiency for an RXXR motif is ~100 times lower than that for an RXR/KR motif (17).

Although available data are limited, PC2 and PC1/PC3 appear to display different specificities. When expressed in CHO or NALM-6 cells, they were incapable or only partially capable of processing precursors with an RXR/KR motif (Table 1). However, PC1/PC3 was able to process at the dibasic site of prorenin in GH_4C_1 cells, most likely in the RSP, but not in the CSP of CHO cells (Table 1). These results suggest that both cleavage motifs and cell types are critical for the action of PC2 and PC1/PC3. However, these experiments do not rule out the possibility that the expressed PC2 or PC1/PC3 was not properly incorporated into the CSP in CHO or NALM-6 cells or was inoperative, even if incorporated properly, in the CSP for some unknown reasons. This is unlikely, however, since the PC2 and PC1/PC3 expressed similarly in BSC-40 cells, which also lack the RSP, were able to process pro-opiomelanocortin at the KR or RR sites (52).

Unequivocal identification of the cellular processing protease is of great importance to our understanding of viral replication and pathogenesis, as well as of normal cell functions. Although we have been able to demonstrate the possible involvement of furin/PACE in viral glycoprotein processing, the data obtained are indirect, since the applicable methods are limited. The same problem has been encountered in other studies (Table 1). This is due mainly to the difficulty in using a truly genetic approach with vertebrate cells. This molecular genetic approach enabled unequivocal identification of kexin as the prototypic processing endoprotease of eukaryotes. Alternative strategies are required to ascertain the exact substrate specificities of the mammalian homologs in vivo. As this report was being completed, Stieneke-Gröber et al. reported the isolation from Madin-Darby bovine kidney cells of an endoprotease that cleaves the influenza fowl plague virus hemagglutinin at the oligobasic site and demonstrated its identity with furin/PACE (49). The results obtained by the two laboratories thus are in good agreement and strongly suggest the role of furin/PACE as a ubiquitous virus-activating protease.

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REFERENCES

1. **Abenes, G., H. Kida, and R. Yanagawa.** 1986. Antigenic mapping and functional analysis of the F protein of Newcastle disease virus using monoclonal antibodies. Arch. Virol. **90**:97–110.
2. **Barr, P. J.** 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell **66**:1–3.
3. **Bentley, A. K., D. J. Rees, C. Rizza, and G. G. Brownlee.** 1986. Defective propeptide processing of blood clotting factor IX caused by mutation of arginine to glutamine at position −4. Cell **45**:343–348.
4. **Bosch, F. X., W. Garten, H.-D. Klenk, and R. Rott.** 1981. Proteolytic cleavage of influenza virus hemagglutinin. Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza virus. Virology **113**:725–735.
5. **Bosch, F. X., M. Orlich, H.-D. Klenk, and R. Rott.** 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology **95**:197–207.
6. **Brenner, C., and R. S. Fuller.** 1992. Structural and enzymatic characterization of a purified prohormone-processing enzyme: secreted, soluble Kex2 protease. Proc. Natl. Acad. Sci. USA **89**:922–926.
7. **Bresnahan, P. A., R. Leduc, L. Thomas, J. Thorner, H. L. Gibson, A. J. Brake, P. J. Barr, and G. Thomas.** 1990. Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-β-NGF in vivo. J. Cell Biol. **111**:2851–2859.
8. **Fuller, R. S., A. Brake, and J. Thorner.** 1989. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca^{2+}-dependent serine protease. Proc. Natl. Acad. Sci. USA **86**:1434–1438.
9. **Fuller, R. S., A. J. Brake, and J. Thorner.** 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science **246**:482–486.
10. **Fuller, R. S., R. E. Sterne, and J. Thorner.** 1988. Enzymes required for yeast prohormone processing. Annu. Rev. Physiol. **50**:345–362.
11. **Gething, M. J., J. M. White, and M. D. Waterfield.** 1978. Purification of the fusion protein of Sendai virus: analysis of the NH_2-terminal sequence generated during precursor activation. Proc. Natl. Acad. Sci. USA **75**:2737–2740.
12. **Glickman, R. L., R. J. Syddall, R. M. Iorio, J. P. Sheehan, and M. A. Bratt.** 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J. Virol. **62**:354–356.
13. **Gotoh, B., T. Ogasawara, T. Toyoda, N. M. Inocencio, M. Hamaguchi, and Y. Nagai.** 1990. An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. EMBO J. **9**:4189–4195.
14. **Gotoh, B., F. Yamauchi, T. Ogasawara, and Y. Nagai.** 1992. Isolation of factor Xa from chick embryo as the amniotic endoprotease responsible for paramyxovirus activation. FEBS Lett. **296**:274–278.
15. **Hakes, D. J., N. P. Birch, A. Mezey, and J. E. Dixon.** 1991. Isolation of two complementary deoxyribonucleic acid clones from a rat insulinoma cell line based on similarities to kex2 and furin sequences and the specific localization of each transcript to endocrine and nonendocrine tissues in rats. Endocrinology **129**:3053–3063.
16. **Hatsuzawa, K., M. Hosaka, T. Nakagawa, M. Nagase, A. Shoda, K. Murakami, and K. Nakayama.** 1990. Structure and expression of mouse furin, a yeast Kex2-related protease; lack of processing of coexpressed prorenin in GH_4C_1 cells. J. Biol. Chem. **265**:22075–22078.
17. **Hatsuzawa, K., M. Nagahama, S. Takahashi, K. Takada, K. Murakami, and K. Nakayama.** Purification and characterization of furin, a Kex2-like processing endoprotease, produced in Chinese hamster ovary cells. J. Biol. Chem., in press.
18. **Homma, M., and M. Ohuchi.** 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural difference of Sendai viruses grown in eggs and tissue culture cells. J. Virol. **12**:1457–1465.
19. **Hosaka, M., M. Nagahama, W.-S. Kim, T. Watanabe, K.**

Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama. 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J. Biol. Chem. **266**:12127–12130.

20. **Julius, D. A., A. Brake, L. Blair, R. Kunisawa, and J. Thorner.** 1984. Isolation of the putative structural gene for the lysine-arginine cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell **37**:1075–1089.
21. **Klenk, H.-D., and R. Rott.** 1988. The molecular biology of influenza virus pathogenicity. Adv. Virus Res. **34**:247–281.
22. **Korner, J., J. Chun, D. Harter, and R. Axel.** 1991. Isolation and functional expression of a mammalian prohormone processing enzyme, murine prohormone convertase 1. Proc. Natl. Acad. Sci. USA **88**:6834–6838.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Misumi, Y., K. Oda, K. T. Fujiwara, N. Takami, K. Tashiro, and Y. Ikehara.** 1991. Functional expression of furin demonstrating its intracellular localization and endoprotease activity for processing of proalbumin and complement pro-C3. J. Biol. Chem. **266**:16954–16959.
25. **Misumi, Y., M. Sohda, and Y. Ikehara.** 1990. Sequence of the cDNA encoding rat furin, a possible propeptide-processing endoprotease. Nucleic Acids Res. **18**:6719.
26. **Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka, and H. Matsuo.** 1988. Yeast Kex2 gene encodes an endopeptidase homologous to subtilisin-like serine proteases. Biochem. Biophys. Res. Commun. **156**:246–254.
27. **Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka, and H. Matsuo.** 1989. Characterization of Kex2-encoded endoprotease from yeast *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **159**:305–311.
28. **Mizuno, K., T. Nakamura, K. Takada, S. Sakakibara, and H. Matsuo.** 1987. A membrane-bound, calcium-dependent protease in cell cleaving on the carboxyl side of paired basic residues. Biochem. Biophys. Res. Commun. **144**:807–814.
29. **Molloy, S. S., P. A. Bresnahan, K. Klimpel, L. Leppla, and G. Thomas.** Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen J. Biol. Chem., in press.
30. **Morrison, T. G., L. J. Ward, and A. Semerjian.** 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. J. Virol. **53**:851–857.
31. **Nagai, Y., M. Hamaguchi, and T. Toyoda.** 1989. Molecular biology of Newcastle disease virus. Prog. Vet. Microbiol. Immunol. **5**:16–64.
32. **Nagai, Y., N. M. Inocencio, and B. Gotoh.** 1991. Paramyxovirus tropism dependent on host proteases activating the viral fusion glycoprotein. Behring Inst. Mitt. **89**:35–45.
33. **Nagai, Y., and H.-D. Klenk.** 1977. Activation of precursor to both glycoproteins of Newcastle disease virus by proteolytic cleavage. Virology **77**:125–134.
34. **Nagai, Y., H.-D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology **72**:497–508.
35. **Nagai, Y., H. Ogura, and H.-D. Klenk.** 1976. Studies on the assembly of the envelope of Newcastle disease virus. Virology **69**:523–538.
36. **Nagai, Y., K. Simokata, T. Yoshida, M. Hamaguchi, M. Iinuma, K. Maeno, T. Matsumoto, H.-D. Klenk, and R. Rott.** 1979. The spread of a pathogenic and an apathogenic strain of Newcastle disease virus in the chick embryo as depending on the protease sensitivity of the virus glycoprotein. J. Gen. Virol. **45**:263–272.
37. **Nakayama, K., M. Hosaka, K. Hatsuzawa, and K. Murakami.** 1991. Cloning and functional expression of a novel endoprotease involved in prohormone processing at dibasic sites. J. Biochem. **109**:803–806.
38. **Naruse, H., Y. Nagai, T. Yoshida, M. Hamaguchi, T. Matsumoto, S. Isomura, and S. Suzuki.** 1981. The polypeptides of mumps virus and their synthesis in infected chick embryo cells. Virology **112**:119–130.
39. **Ogasawara, T., B. Gotoh, H. Suzuki, J. Asaka, K. Shimokata, R. Rott, and Y. Nagai.** 1992. Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. EMBO J. **11**:467–472.
40. **Roebroek, A. J. M., J. A. Schalken, J. A. M. Leunissen, C. Onnekink, H. P. J. Bloemers, and W. J. M. van de Ven.** 1986. Evolutionary conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. EMBO J. **5**:2197–2202.
41. **Sakaguchi, T., Y. Matsuda, R. Kiyokage, N. Kawahara, K. Kiyotani, N. Katunuma, Y. Nagai, and T. Yoshida.** 1991. Identification of endoprotease activity in the trans Golgi membranes of rat liver cells that specifically processes in vitro the fusion glycoprotein precursor of virulent Newcastle disease virus. Virology **184**:504–512.
42. **Schalken, J. A., A. J. M. Roebroek, P. P. C. A. Oomen, S. S. Wagenaar, F. M. J. Debruyne, H. P. J. Bloemers, and W. J. M. van de Ven.** 1987. fur gene expression as a discriminating marker for small cell and nonsmall cell lung carcinomas. J. Clin. Invest. **80**:1545–1549.
43. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:470–490.
44. **Seidah, N. G., L. Gaspar, P. Mion, M. Marcinkiewicz, M. Mbikay, and M. Chretien.** 1990. cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for prohormone processing proteinases. DNA Cell Biol. **9**:415–424.
45. **Seidah, N. G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, G. Beaubien, M. G. Mattei, C. Lazure, M. Mbikay, and M. Chretien.** 1991. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. Mol. Endocrinol. **5**:111–122.
46. **Smeekens, S. P., C. Albiges-Rizo, R. Carrol, S. Martin, S. Ohagi, L. A. Phillips, M. Benig, P. Gardner, A. G. Montag, H. H. Swift, G. Thomas, and D. F. Steiner.** Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2 and PC3. Proc. Natl. Acad. Sci. USA, in press.
47. **Smeekens, S. P., A. S. Avrruch, J. LaMendola, S. J. Chan, and D. F. Steiner.** 1991. Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc. Natl. Acad. Sci. USA **88**:340–344.
48. **Smeekens, S. P., and D. F. Steiner.** 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J. Biol. Chem. **265**:2997–3000.
49. **Stieneke-Gröber, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.-D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. **11**:2407–2414.
50. **Suzuki, H., A. Harada, Y. Hayashi, K. Wada, J. Asaka, B. Gotoh, T. Ogasawara, and Y. Nagai.** 1991. Primary structure of the virus activating protease from chick embryo; its identity with the blood clotting factor Xa. FEBS Lett. **283**:281–285.
51. **Thomas, G., B. A. Thorne, L. Thomas, R. G. Allen, D. E. Hruby, R. Fuller, and J. Thorner.** 1988. Yeast kex2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells. Science **241**:226–230.
52. **Thomas, L., R. Leduc, B. A. Thorne, S. P. Smeekens, D. F. Steiner, and G. Thomas.** 1991. Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: evidence for a common core of neuroendocrine processing enzymes. Proc. Natl. Acad. Sci. USA **88**:5297–5301.
53. **Toyoda, T., B. Gotoh, T. Sakaguchi, H. Kida, and Y. Nagai.** 1988. Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. J. Virol. **62**:4427–4430.
54. **Toyoda, T., T. Sakaguchi, H. Hirota, B. Gotoh, K. Kuma, T.**

Miyata, and Y. Nagai. 1989. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology **169:**273–282.

55. **Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virology **158:**242–247.
56. **van den Ouweland, A. M. W., J. L. P. van Duijnhoven, G. D. Keizer, L. C. J. Dorssers, and W. J. M. Van de Ven.** 1990. Structural homology between the human fur gene product and the subtilisin-like protease encoded by yeast KEX2. Nucleic Acids Res. **18:**664.
57. **van de Ven, W. J. M., J. Voorberg, R. Fontijn, H. Pannekoek, M. W. van den Ouweland, H. L. P. van Duijnhoven, A. J. M. Roebroek, and R. J. Siezen.** 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. Mol. Biol. Rep. **14:**265–275.
58. **Watanabe, T., T. Nakagawa, J. Ikemizu, M. Nagahama, K. Murakami, and K. Nakayama.** 1992. Sequence requirement for precursor cleavage within the constitutive secretory pathway. J. Biol. Chem. **267:**8270–8274.
59. **Webster, R. G., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50:**665–666.
60. **Wise, R. J., P. J. Barr, P. A. Wong, M. C. Kiefer, A. J. Brake, and R. J. Kaufman.** 1990. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. Proc. Natl. Acad. Sci. USA **87:**9378–9382.
61. **Yoshida, T., Y. Nakayama, H. Nagura, T. Toyoda, K. Nishikawa, M. Hamaguchi, and Y. Nagai.** 1986. Inhibition of the assembly of Newcastle disease virus by monensin. Virus Res. **4:**179–195.
62. **Yoshimasa, Y., J. I. Paul, J. Whittaker, and D. F. Steiner.** 1990. Effects of amino acid replacements within the tetrabasic cleavage site on the processing of the human insulin receptor precursor expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**17230–17237.