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Proprotein-Processing Endoproteases PC6 and Furin Both Activate Hemagglutinin of Virulent Avian Influenza Viruses

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Among the proprotein-processing subtilisin-related endoproteases, furin has been a leading candidate for the enzyme that activates the hemagglutinin (HA) of virulent avian influenza viruses. In the present study, we examined the cleavage activity of two other recently isolated ubiquitous subtilisin-related proteases, PACE4 and PC6, using wild-type HA of A/turkey/Ireland/1378/83 (H5N8) and a series of its mutant HAs. Vaccinia virus-expressed wild-type HA was not cleaved in human colon adenocarcinoma LoVo cells, which lack active furin. This processing defect was corrected by the expression of furin and PC6 but not of PACE4 and a control wild-type vaccinia virus. PC6 showed a sequence specificity similar to that with the endogenous proteases in cultured cells. When LoVo cells were infected with a virulent avian virus, A/turkey/Ontario/7732/66 (H5N9), only noninfectious virions were produced because of the lack of HA cleavage. However, when the cells were coinfected with vaccinia virus that expressed either furin or PC6, the avian virus underwent multiple cycles of replication, indicating that both furin and PC6 specifically cleave the virulent virus HA at the authentic site. These data suggest that PC6, as well as furin, can activate virulent avian influenza viruses in vivo, implying the presence of multiple HA cleavage enzymes in animals.

A link between hemagglutinin (HA) cleavability and the virulence of avian influenza viruses is well established (see references 19 and 51 for reviews). The HAs of virulent viruses, which contain multiple basic amino acids at the cleavage site, are cleaved intracellularly by endogenous proteases, whereas those of avirulent viruses, which contain only a single arginine, are not (3, 13). Thus, the tissue tropism of viruses may be determined by the availability of proteases responsible for the cleavage of different HAs, leading to a difference in virulence.

Two groups of proteases appear responsible for HA cleavage. One includes enzymes able to cleave avirulent-type HAs, such as plasmin (21), blood-clotting factor X-like protease (8), tryptase Clara (17), and bacterial proteases (43). The second group comprises proteases that cleave only virulent-type HAs with multiple basic residues at the cleavage site, although this type of enzyme's responsibility in vivo has not been determined yet.

Studies with HA cleavage mutants have demonstrated that the number of basic amino acids at the cleavage site and the presence or absence of a nearby carbohydrate affect HA cleavability in an interrelated manner (12, 14, 15, 30, 45, 46). The proposed sequence requirement for HA cleavage by endogenous proteases, when a carbohydrate is nearby, is X-X-R-X-R/K-R (X represents nonbasic residues); otherwise, it is R/K-X-R/K-R. The HA cleavage enzyme appears to be located in either the medial or *trans*-Golgi apparatus, to be calcium dependent, and to have an acidic pH optimum (48).

Many bioactive peptides and proteins are produced from large precursors through limiting endoproteolysis, which occurs usually at paired or multiple basic amino acids (see references 2, 37, and 40 for reviews). Six mammalian subtilisin-related endoproteases for prohormone and proprotein activation have been identified, including PC3 (also called PC1 [20, 28, 36, 38, 39]), PC2 (35, 39), furin (also called PACE [11, 44]), PC4 (29), PACE4 (18), and PC6 (also called PC5 [22, 26, 27]). Among them, furin is ubiquitously expressed in many organs and catalyzes the processing of precursor proteins through the constitutive pathway. The recently isolated PACE4 and PC6 are also expressed ubiquitously, but their levels of expression among tissues differ from that of furin, and their subtilisin-catalytic domains have only a 76% amino acid sequence identity with each other and only 69 and 57% identity, respectively, with furin (18, 26). Because of its tissue distribution and biological properties, furin has been thought to be the HA cleavage enzyme in cells infected with virulent avian influenza viruses (48), and purified furin showed a sequence requirement similar to that of endogenous proteases in a variety of cultured cells (47). Moreover, an HA cleavage enzyme partially purified from Madin-Darby bovine kidney (MDBK) cells antigenically cross-reacts with furin (41). These data suggest that furin is a prime candidate for the cleavage of virulent virus HAs in vivo, although direct evidence for the authenticity of HA cleavage by furin, such as direct amino acid sequencing at the cleaved site, has not been reported. Whether other ubiquitous endoproteases, such as PACE4 and PC6, have a role in the processing of virulent influenza virus HAs remains unknown.

In the present study, we examined the HA cleavage activity of PACE4 and PC6 using a virulent avian influenza virus HA and a series of its mutant HAs in human colon adenocarcinoma LoVo cells, which lack active furin (42), in a vaccinia virus expression system. We also determined whether these proteases, as well as furin, can proteolytically activate virulent avian influenza viruses.

Wild-type and mutant HAs of a virulent avian influenza

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virus are not cleaved in LoVo cells. When we previously examined the cleavability of the A/turkey/Ireland/1378/83 (H5N8) (Ty/Ire) HA, which has R-K-R-K-K-R at the cleavage site, using the simian virus 40 or vaccinia virus system, we found that the wild-type HA was processed by endogenous proteases in all cultured cells examined, suggesting the ubiquitous distribution of cellular proteases responsible for HA cleavage in diverse cell types (47). LoVo cells (human colon adenocarcinoma, ATCC CCL 229) (7) are reported to lack the active form of furin as a result of a nucleotide deletion in the gene region of the Homo B domain flanking the catalytic domain (42). Hence, LoVo cells were infected with recombinant vaccinia virus expressing the wild-type HA and HA processing was assessed with a radioimmunoprecipitation assay (Fig. 1A). Only the uncleaved form (HA0) of the wild-type protein was observed, indicating the lack of endogenous proteases responsible for HA cleavage. Similarly, none of the mutant HAs listed in Table 1 were processed in LoVo cells (Fig. 1A). These data suggest that the absence of active furin in this cell line results in the failure of HA cleavage.

Exogenously expressed furin and PC6, but not PACE4, correct the HA cleavage in LoVo cells. To validate that the uncleaved HA made in LoVo cells is susceptible to HA cleavage enzymes and thus can be used as a substrate for assessing the cleavage activity of PC6 and PACE4, we first examined the cleavability of the wild-type HA in LoVo cells with vaccinia virus-expressed furin. LoVo cells were coinfected with recombinant vaccinia viruses expressing both the HA and furin. The wild-type HA was readily cleaved into HA1 and HA2 subunits (Fig. 1B) but was not cleaved in cells coinfected with a control wild-type vaccinia virus (Fig. 1E), indicating that furin cleaved the HA. The cleavage profile of mutant HAs with overexpressed furin in LoVo cells (Fig. 1B; Table 1) was broader than those with purified furin or the endogenous proteases (47).

We next exploited the lack of endogenous HA cleavage enzymes in LoVo cells to test the processing activity of PACE4 and PC6. Vaccinia virus-expressed PACE4 did not cleave the wild-type or any mutant HA under conditions similar to those used in tests of furin (Fig. 1C; Table 1). However, very faint cleavage products of the wild-type HA in the PACE4 but not wild-type vaccinia virus samples did appear upon longer exposure of the radioimmunoprecipitation gel (data not shown). By contrast, vaccinia virus-expressed PC6 cleaved the wild-type HA nearly completely (83%), just as furin had done (89%) (Fig. 1D; Table 1). In studies with a series of mutant HAs (Fig. 1D), the PC6 protease showed a sequence requirement similar to those of endogenous proteases in a variety of cultured cells or of purified furin (47) (Table 1). It was not, however, as broad as that of overexpressed furin.

Furin and PC6 proteolytically activate the wild-type HA. Although furin is a leading candidate for the endogenous protease responsible for HA cleavage, the authenticity of furin-induced cleavage remains in question. Moreover, even though we showed that PC6 can cleave the wild-type Ty/Ire HA and that the electrophoretic mobilities of the cleaved forms appear similar to those cleaved by furin (Fig. 1), the authenticity of the cleavage sites was uncertain. We therefore investigated whether furin and PC6 could activate virulent avian influenza viruses by processing their HAs in LoVo cells.

To validate the assay system, we studied the infectivity of a virulent virus, A/turkey/Ontario/7732/66 (H5N9) (Ty/Ont), with R-R-R-K-K-R at the cleavage site in LoVo cells. Virus was inoculated into the cells at a multiplicity of infection (MOI) of 1 and adsorbed for 1 h. After washing out and inactivating unadsorbed viruses with anti-Ty/Ont serum, we

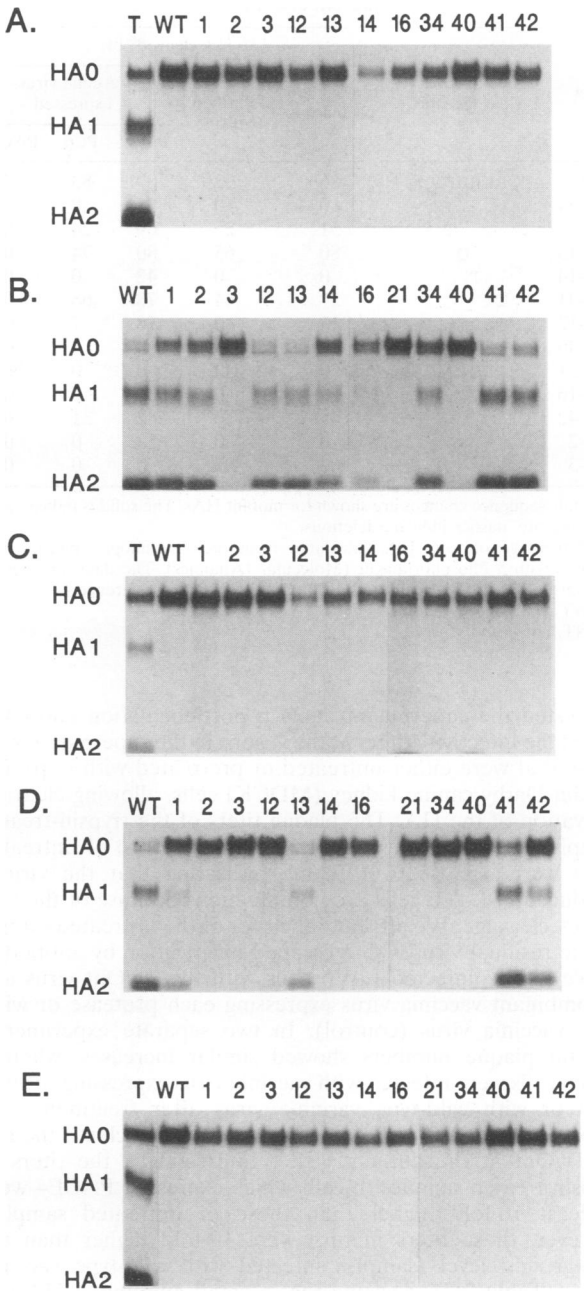

FIG. 1. Cleavage of wild-type and mutant HAs of Ty/Ire in LoVo cells by subtilisin-related proteases expressed in the vaccinia virus system. Recombinant vaccinia viruses expressing the HAs or proteases were generated as described in earlier reports (4, 5, 47). HA cleavage in LoVo cells (A) or by furin (B), PACE4 (C), PC6 (D), or a control wild-type vaccinia virus (WR strain) (E) was examined by infecting cells with recombinant vaccinia viruses expressing the HAs (MOI = 2) or by coinfecting cells with recombinant vaccinia viruses that express the HAs (MOI = 2) and each of the proteases (MOI = 5). Cells were exposed to a 15-min pulse of Tran^{35}S-label (ICN) at 5.5 h postinfection, followed by a 2-h chase. Cell lysates were immunoprecipitated with anti-H5 antiserum (50) and analyzed on a sodium dodecyl sulfate-polyacrylamide gel (10%). WT, wild type; numbers correspond to the mutant HAs in Table 1; T, trypsin treatment of wild-type HA (2.5 μg/ml for 10 min).

TABLE 1. Cleavage of wild-type and mutant HAs of Ty/Ire by different endoproteases

HA	Sequence at cleavage site[a]	% HA cleaved[b] by:				
		Endogenous protease in CV-1	Purified furin	Vaccinia virus-expressed		
				Furin	PC6	PACE4
WT[c]	PQRKRKKR/G	86	92	89	83	0
MT-34	K	0	0	51	0	0
MT-1	T	51	25	69	24	0
MT-13	Q	80	65	80	74	0
MT-14	T	0	0	42	0	0
MT-41	T	57	44	78	68	0
MT-12	TE	15	0	78	7	0
MT-40	TET	0	NT[d]	0	0	0
MT-21	TESP	0	NT	0	0	NT
MT-16	TQ	0	0	32	0	0
MT-42	-	45	6	82	31	0
MT-2	--	0	0	58	0	0
MT-3	-- T	0	NT	0	0	0

[a] Only sequence changes are shown for mutant HAs. The solidus indicates the cleavage site; dashes indicate deletions.

[b] Percentage of total HA, calculated from radioimmunoprecipitation gels scanned with a Phosphorimager (Molecular Dynamics). The data for endogenous proteases in CV-1 cells and purified furin are from reference 47.

[c] WT, wild type.

[d] NT, not tested.

TABLE 2. Activation of a virulent avian influenza virus by vaccinia virus-expressed endoproteases in LoVo cells

Expt. and vaccinia virus-expressed endoprotease[a]	Infectivity (PFU/ml)[b]	
	Try+	Try−
Expt. 1		
None (WR)	5.0×10^3	1.0×10^1
Furin	7.2×10^3	6.4×10^3
PACE4	3.9×10^3	3.3×10^2
PC6	6.0×10^3	5.6×10^3
Expt. 2		
None (WR)	7.0×10^3	2.0×10^1
Furin	2.8×10^4	2.1×10^4
PACE4	1.6×10^4	2.0×10^2
PC6	1.5×10^4	1.1×10^4

[a] LoVo cells were coinfected with a recombinant vaccinia virus expressing one of the indicated endoproteases or wild-type vaccinia virus (WR strain) (MOI = 5) and a virulent virus [Ty/Ont, R(36-2)] (12) (MOI = 1). After 1 h of adsorption, the inocula were removed and cells were washed five times. To inactivate the remaining unadsorbed viruses, anti-Ty/Ont serum was added to medium 1 h later, incubated for 30 min, and washed out. Supernatants were harvested at 24 h postinoculation and titrated. Results from two separate experiments are shown.

[b] The infectivities of the supernatants were determined by plaque titration in MDCK cells, after treatment with (Try+) (2.5 μg/ml, 10 min at 37°C) or without (Try−) trypsin. The Ty/Ont plaques were detected specifically by immunostaining with anti-H5 monoclonal antibodies, biotinylated anti-mouse immunoglobulin G, a preformed avidin-biotinylated horseradish peroxidase complex (ABC kit; Vector Laboratories), and 3,3′-diaminobenzidine–H_2O_2 as a substrate solution.

harvested the supernatant at 24 h postinoculation and compared the infectivity titers of the virions in the supernatant with those that were either untreated or pretreated with trypsin in Madin-Darby canine kidney (MDCK) cells, allowing cleavage activation of the HA. The plaque titers of the trypsin-treated samples were 1.5×10^4 PFU/ml, whereas those of untreated ones were 6.5×10^1 PFU/ml, indicating that the viruses produced in LoVo cells are noninfectious because of the lack of HA cleavage. We attribute the titer of the untreated sample to the residual viruses that escaped inactivation by antibody.

We next coinfected LoVo cells with the Ty/Ont virus and recombinant vaccinia virus expressing each protease or wild-type vaccinia virus (control). In two separate experiments, Ty/Ont plaque numbers showed similar increases, whether LoVo cells were infected with vaccinia virus expressing furin or PC6 or with wild-type vaccinia virus after treatment with trypsin (Table 2). Thus, either furin or PC6 can cleave the HA of Ty/Ont at the authentic site. Interestingly, the titers of trypsin-treated samples in cells which expressed PACE4 were at least 10-fold higher than those of untreated samples; however, those titers in turn were 10-fold higher than the background level (samples infected with wild-type vaccinia virus) (Table 2), suggesting that a small amount of HA was activated by PACE4. We were unable to determine the percentage of cleaved HA on the Ty/Ont virions because of the limited number of virions produced in LoVo cells. Conceivably, the virions produced in LoVo cells expressing PACE4 contain a small but sufficient number of cleaved HAs on the envelope to render some virions infectious.

We have shown that furin and PC6 can both proteolytically activate a virulent avian influenza virus by cleaving its HA, providing evidence for the presence of multiple HA cleavage enzymes in vivo. Furin has been a prime candidate for the enzyme that processes the precursors of many viral glycoproteins, including the HA of virulent influenza viruses, all of which possess similar sequence motifs consisting of multiple basic residues at the cleavage site (9, 10, 31, 41, 47). Recently, it was demonstrated that furin proteolytically activates the Sindbis virus PE2 and virulent Newcastle disease virus F (23). These observations combined with results of the present study suggest that furin can activate numerous viral glycoproteins as well as many other bioactive proteins in vivo.

By comparison with furin (see reference 40 for a review; 24), the biological properties (e.g., substrate specificity or intracellular localization) of other ubiquitous proteases, such as PACE4 and PC6, are poorly characterized. Here, we show that the substrate specificity of overexpressed PC6 in HA cleavage, although similar to that of endogenous proteases in a variety of cell cultures or purified furin (47), is not as broad as that of overexpressed furin, raising the possibility of differences in sequence requirements between the two enzymes, in agreement with a previous report (6). It will be important to determine the specificity of PC6 at its physiological concentrations to clarify its role in the activation of virulent influenza viruses in vivo.

By contrast to findings with PC6, the PACE4 protease did not correct efficiently the HA processing defect in LoVo cells, even though its catalytic domain is more closely related to furin (69%) than is PC6 (57%) (18, 26). Yet, PACE4 did appear to cleave slightly the wild-type HAs of virulent influenza viruses, as suggested by long-term radioimmunoprecipitation gels and infectivity-complementation assay. Such limited activation of the HA may have been caused by overexpression of the proteases, so that PACE4 does not, in fact, participate in the cleavage activation of Ty/Ire or Ty/Ont in vivo. In previous studies, PACE4 was shown to process pro-von Willebrand factor, but not pro-factor IX, both of which contain R-X-K-R at the cleavage site recognized by furin (6, 32, 49). Moreover, a serine protease inhibitor, α_1-antitrypsin Pittsburgh mutant (α_1-PIT), inhibited the cleavage of pro-von Willebrand factor by furin but not by PACE4 (32). These findings indicate that PACE4 and furin have different yet overlapping substrate specificities. Hence, PACE4 may recognize structural features other than the cleavage site sequence, so that it could be involved in HA cleavage activation of other virulent influenza

virus strains or other viral glycoproteins in viruses possessing the requisite ancillary recognition sites.

Virulent avian influenza viruses replicate in a variety of organs in birds, implying the presence of HA cleavage enzymes in these organs, yet very little is known about the intracellular proteases of avian species. Indeed, neither furin, PACE4, nor PC6 has been isolated from chickens. The existence of multiple enzymes responsible for HA cleavage could provide an advantage for the replication of virulent viruses in vivo. Previous studies have shown that, in rat brain, PC6 is primarily expressed in the cortex, hippocampus, hypothalamus, and thalamus, with much lower levels found in the cerebellum and the atriatum (22), in marked contrast to the distribution of furin in the same organ (34). We propose that the distribution of HA cleavage enzymes within an organ could affect the virulence of a virus. Determining the tissue distribution of furin and PC6 in each organ of birds may well lend insight into the pathogenesis of avian influenza.

Could additional enzymes participate in HA cleavage in vivo? An endoprotease unrelated to subtilisin-like enzymes was recently purified from human $T4^+$ lymphocytes and shown to cleave human immunodeficiency virus type 1 (HIV-1) gp160 at the authentic site (16). Furin appears to cleave the HIV-1 gp160 at the authentic site (25), but its level of expression in $T4^+$ lymphocytes was relatively low (52), suggesting the importance of the former protease for HIV-induced pathogenesis. Such information also suggests the presence of uncharacterized proteases that are different from furin or PC6 and may play an important role in the regulation of influenza virus virulence.

There have been reports of peptide-based inhibitors of processing enzymes that blocked replication of an H7 avian virulent virus and HIV-1 in cell culture (10, 41). Health applications of this finding are unlikely because of potentially adverse side effects that could be produced by inhibition of normal protein processing (e.g., prohormone processing). More recently, protein-based inhibitors (α_1-PIT variant α_1-PDX) that block the processing of HIV-1 gp160, whose tissue-specific expression in vivo is controlled by a promoter, were described (1). These agents could provide a novel strategy for the development of virus-specific inhibitors for in vivo use, leading perhaps to the production of virus-resistant animals by gene transfer. Continued efforts to identify enzymes involved in HA cleavage are warranted, not only to promote understanding of viral diseases that may share sequence motifs at the cleavage sites of viral glycoproteins (see reference 33 for a review) but also to stimulate the development of antiviral strategies.

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