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SHORT COMMUNICATION

The Hemagglutinin Cleavability of a Virulent Avian Influenza Virus by Subtilisin-like Endoproteases Is Influenced by the Amino Acid Immediately Downstream of the Cleavage Site

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Many viral membrane glycoproteins are post-translationally processed by intracellular endoproteases such as subtilisin-like proteases. These proteases recognize a cleavage site sequence comprising basic amino acids positioned upstream of the cleavage site of the viral proteins. Here, we mutated the glycine residue immediately downstream of the cleavage site (P1) of hemagglutinin (HA) from a virulent avian influenza virus, A/turkey/Ontario/7732/66 (H5N9) (R–R–R–K–K–R/G), to examine the effect of this mutation on its clevability. Substitution of Gly with Ile, Leu, Val, or Pro, but not Ala, Asp, Phe, His, Ser, or Thr, resulted in substantial reduction of HA cleavage by endogenous endoproteases in CV-1 cells and by vaccinia-expressed PC6 and, albeit to a lesser extent, furin. We conclude that HA cleavage by subtilisin-like proteases is influenced by the downstream P1 amino acid in the absence of upstream cleavage site sequence alterations.

Many viral membrane proteins require post-translational modification to produce replication-competent viruses [reviewed in (*1*)]. In influenza viruses, proteolytic cleavage of the precursor hemagglutinin (HA) molecule (HA0) into HA1 and HA2 subunits, which generates a fusogenic domain at the amino terminal region of the HA2, is essential for entry of the virus into cells (*2, 3*). Similarly, cleavage of other viral glycoproteins, such as the fusion protein (F0) of paramyxoviruses and envelope protein (gp160) of human immunodeficiency virus type 1 (HIV-1), is essential to generate fusogenic domains for viral replication (*4, 5*). Virulent avian influenza viruses and Newcastle disease viruses (NDV) replicate systemically, because, unlike avirulent viruses, their HA0 and F0 are cleaved by ubiquitous intracellular proteases (*6–8*). Thus, glycoprotein cleavability determines both tissue tropism and viral virulence.

The host cell proteases that mediate viral glycoprotein processing have not been fully characterized. However, some subtilisin-like endoproteases, which process bioactive peptides such as neuropeptides, growth factors, and hormones, also cleave viral glycoproteins. A total of seven mammalian subtilisin-like endoproteases have been identified (*9–12*): PC3 (also called PC1), PC2, furin (also called PACE), PC4, PACE4, PC6 (also called PC5), and PC7. Furin is ubiquitously expressed and cleaves HA0 of virulent avian influenza viruses, as well as F0 of virulent NDV, Sindbis virus PE2, and HIV-1 gp160 (*13–18*). PC6 is also ubiquitously expressed and activates HA0 of virulent avian influenza viruses (*19*), whereas PC1, expressed in a lymphoblastoid cell line, cleaves HIV-1 gp160 (*13*).

These subtilisin-like endoproteases cleave substrates at dibasic or multiple basic residues (*9–11*). A minimal sequence requirement for cleavage by furin has been defined as R–X–X–R (X = nonbasic residues) (*20, 21, 22*). This motif is found at the cleavage site of many viral glycoproteins sensitive to intracellular proteases. Structural features other than this cleavage site sequence also affect viral glycoprotein cleavability by proteases. For example, a carbohydrate side chain in the vicinity of the HA cleavage site of avian influenza viruses inhibits protease accessibility (*23, 24*). Additionally, cleavage of R–R–Q–K–R/*F*-containing F0 from virulent NDV in COS cells is diminished if the Phe residue is replaced by Leu (*25*), demonstrating the importance of an amino acid immediately downstream of the cleavage site (designated the P1 position) for protease recognition. Similarly, altering the P1 amino acid of prorenin and proalbumin to hydrophobic aliphatic residues (Leu, Ile, and Val) abolishes their cleavability by intracellular proteases (*26, 27, 28*). These studies were done mostly by examin-

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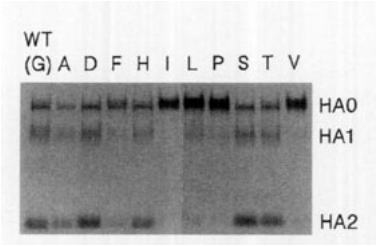

FIG. 1. Cleavage of wild-type and mutant HAs in CV-1 cells. HA cleavage was examined by infecting cells with recombinant vaccinia virus expressing T7 polymerase (vTF7.3) at an m.o.i. of 5, followed by lipofectamine (GIBCO BRL)-mediated transfection of mutant HA genes 30 min later. Cells were exposed to a 15-min pulse of Tran^{35}S-label (ICN) at 5 hr post-transfection, followed by a 2-hr chase. Cells were then lysed, immunoprecipitated with anti-H5 monoclonal antibodies, and analyzed on a sodium dodecyl sulfate–polyacrylamide gel (10%) as described previously (*19*). WT, wild type; P1 residues of the mutant HAs are given at the top of each lane.

ing the sensitivity of expressed mutant proteins to endogenous endoproteases in cultured cells with the exception of a limited study using purified furin (*29*). Thus, the inhibitory effects of the P1 amino acid on proprotein cleavage by individual subtilisin-like proteases remain unknown. Here, we tested the sensitivity of a series of HA P1 mutants of a virulent avian influenza virus to cleavage by the endogenous intracellular proteases in CV-1 cells and by the transiently expressed subtilisin-like proteases furin, PACE4, and PC6.

Mutant HA genes were constructed by cassette mutagenesis of the HA cleavage site using the pT3(MO-C) plasmid, which contains the HA gene of the virulent virus A/turkey/Ontario/7732/66 (H5N9) [Ty/Ont] described earlier (*30*). Nucleotide codons used for mutagenesis at the P1 position were: *gcc* for Ala (A), *gat* for Asp (D), *ttc* for Phe (F), *cac* for His (H), *atc* for Ile (I), *ctc* for Leu (L), *ccc* for Pro (P), *agc* for Ser (S), *acc* for Thr (T), and *gtc* for Val (V) from *ggc* for Gly (G). Resulting plasmids were digested with *Bam*HI and the HA gene fragments ligated into the *Bam*HI site of pGEM-7Zf(−). HAs were expressed by the T7–vaccinia system (*31*) by transfecting the plasmids and infecting cells with a recombinant vaccinia virus (vTF7.3) expressing T7 polymerase.

To determine cleavability of mutant HAs, plasmids with mutant HA genes were transfected into CV-1 cells preinfected with vTF7.3. Cells expressing HAs were pulse-radiolabeled, chased, and lysed for radioimmunoprecipitation (RIP) analysis (*19*) (Fig. 1; Table 1). Substitution of the P1 Gly to Ala, Asp, His, Ser, or Thr did not affect HA cleavability in CV-1 cells, whereas substitution of Gly to Pro, Ile, Val, or Leu abolished nearly all cleavage, and substitution to Phe slightly reduced HA cleavage. Taken together, these results indicated that substitution of Gly with Pro, Ile, Val, or Leu structurally alters the HA cleavage site, affecting protease recognition or catalyzation. Notably, three of these amino acids are hydrophobic aliphatic residues.

To determine susceptibility of mutant cell surface HAs to tosylsulfonyl chloromethyl ketone (TPCK)-treated trypsin, cells were incubated with the protease (2.5 μg/ml) at 37° for 30 min after being pulse-radiolabeled and chased for 2 hr. Cells were then washed twice with phosphate-buffered saline that contained a trypsin inhibitor prior to being lysed for the RIP assay. Under these conditions, the level of cleavage differed slightly among the HAs (Table 1); the most cleavage (84%) occurred with wild-type HA, while the least cleavage (62%) was obtained with the mutant HA that contained Pro. These results show that at least 62% of the HAs were expressed on the cell surface and that they were susceptible to trypsin digestion.

To rule out the possibility that the decreased cleavage of the mutants was due to protein misfolding and a resulting inability to migrate to the cleavage site (the *trans* Golgi network), the level of HA cell surface expression was examined by immunoperoxidase detection (*19*) and hemadsorption with guinea pig erythrocytes, which are agglutinated by the influenza virus HA but not by vaccinia virus HA (*32*). Similar levels of HA were detected in both assays. Moreover, hemadsorption was specifically inhibited if the cells were pretreated with anti-H5 antibodies (data not shown). These results indicate that the mutation at P1 does not drastically affect the proper folding and transport of these molecules to the cell surface.

Human colon adenocarcinoma LoVo cells (*33*) contain endogenous protease(s) that cleaves HIV-1 gp160, but not virulent NDV F0 (*34*) or virulent avian influenza HA (*19*). All three glycoproteins have similar cleavage site sequences but different residues at the P1 position: R–K–K–R/*G* for influenza virus HA0, R–Q–K–R/*F* for NDV F0, and R–E–K–R/*A* for HIV-1 gp160. To investigate whether the P1 residues determine substrate sensitivity to endogenous protease(s) in LoVo cells, we examined mutant HAs for clevability in this cell line by RIP assay. Neither wild-type HA nor any of the mutant HAs was cleaved (Fig. 2A), suggesting that the P1 residue does not confer substrate sensitivity to LoVo cell protease(s) and that an unknown structural feature of HIV-1 gp160 must specify its sensitivity to the protease(s) in LoVo cells.

We also examined the cleavage profile of P1 mutant HAs coexpressed in LoVo cells with furin, PC6, or PACE4. Vaccinia-expressed furin cleavage of HA was abolished by substituting Pro for Gly and slightly reduced by substitution to hydrophobic aliphatic residues (Fig. 2B; Table 1). By contrast, vaccinia-expressed PC6 showed a similar cleavage profile to that of the endogenous proteases in CV-1 cells, although the percentage of HA mutant molecules containing hydrophobic aliphatic residues cleaved by PC6 was greater than that cleaved by the endogenous proteases in CV-1 cells (Fig. 2C; Table 1). PACE4 did not cleave any P1 mutants (Fig. 2D; Table 1).

These results indicate that aliphatic amino acids (Leu, Ile, and Val) at the P1 position greatly inhibit the cleavage

TABLE 1

The Effect on Cleavability of Mutating the P1 Residue at the Cleavage Site of Ty/Ont HA

HA	Sequence at cleavage site[a] HA1	HA2	% HA cleavage[b] CV-1 endogenous proteases	Vaccinia expressed Furin	PC6	PACE4	Trypsin[c]
Wild type	R R R K K R\	G L	74	91	68	—	84
P1 mutant	R R R K K R\	A L	72	92	71	—	79
		E	74	91	67	—	73
		F	52	85	67	—	76
		H	72	89	65	—	65
		I	10	70	33	—	68
		L	16	76	45	—	77
		P	15	18	15	—	62
		S	75	91	67	—	77
		T	68	80	58	—	72
		V	18	76	27	—	78

[a] Only sequence changes at P1 are shown for mutant HAs. The solidus indicates the cleavage site.

[b] Percentage of total HA, calculated from radioimmunoprecipitation gels scanned with a Phosphorimager (Molecular Dynamics); — denotes <10%.

[c] Intact cells expressing HA were treated with TPCK–trypsin (2.5 μg/ml, 37°, 30 min) prior to cell lysis for radioimmunoprecipitation assays.

of the virulent avian influenza virus Ty/Ont HA in CV-1 cells even if the cleavage site sequence per se is unaltered. These substitutions also inhibit HA cleavage by transiently expressed furin or PC6, although the effect was less remarkable with furin possibly due to its overexpression in the vaccinia system. Clearly, the introduction of a hydrophobic aliphatic amino acid at the P1 position causes structural alterations of the cleavage site, resulting in significant reduction of viral glycoprotein cleavage by subtilisin-like proteases. Indeed, none of the native subtilisin-like protease substrates has a hydrophobic aliphatic residue at the P1 position (*1, 20*).

The HA mutant with Pro at P1 was not cleaved in CV-1 cells or by subtilisin-like proteases, even though it was transported to the cell surface efficiently. This result does not support that of a recent study using a substrate phage display assay, wherein Pro at P1 did not affect furin cleavage (*22*). It may be that other structural features determine the effect of Pro at P1 on furin susceptibility or, as suggested by the authors of this study, that cleavage may have occurred immediately upstream (K/R–R ↓ R–P) rather than downstream of Arg (K/R–R ↓ P) (*22*).

The HA mutants with Pro or hydrophobic aliphatic residues at P1 were cleaved efficiently by trypsin at the cell surface, suggesting that substrate recognition by trypsin-like proteases is not affected by the P1 amino acids. The fact that the avirulent NDV F and the influenza C virus hemagglutinin–esterase proteins are both processed by trypsin-like proteases and have aliphatic residues at P1 (Leu and Ile, respectively) (*35–38*) supports this notion.

PACE4 processes pro-von Willebrand factor (R–S–K–R/*S*), but does not process profactor IX (R–P–K–R/*Y*). Both factors are, however, cleaved by furin (*39–41*). Moreover, a serine protease inhibitor, α_1-antitrypsin Pittsburgh mutant, inhibits pro-von Willebrand factor cleavage by furin, but not by PACE4 (*40*). These findings indicate that PACE4 and furin have different yet overlapping substrate specificity and that PACE4 may recognize structural features other than the cleavage site sequence. When we substituted Ser for Gly at P1 of the influenza virus HA0, mimicking the pro-von Willebrand factor cleavage site, the mutant was not cleaved by PACE4, suggesting that structural features other than basic residues and Ser at P1 specify cleavage by PACE4.

All naturally occurring influenza A viruses have Gly at P1 of their HA cleavage sites. None of the mutant HAs described here could be rescued as infectious virus by reverse genetics (Horimoto and Kawaoka, unpublished data), suggesting that the mutations are lethal or affect virus replication to a level that inhibits the generation of such mutants. Our results show that the Gly at P1 was not essential for cleavage by subtilisin-like proteases and suggest that it may be necessary at other virus replication steps such as fusion. The Gly does not seem to be essential for correct HA folding or transport because all of our HA mutants appeared to be transported to the cell surface efficiently in CV-1 cells. Although thermolysin activation mutants of H7 seal virus were shown to contain Leu at the amino terminus of HA2, they all had an insertion of an amino acid in the fusion peptide (*42*). Thus, the Gly could be essential for efficient fusion when the fusion peptide is not altered. This concept is supported by the finding that site-specific substitution of Gly with Ala or Glu resulted in reduction of fusion activity in an SV40 recombinant virus system (*43, 44*). We were not able to determine the fusion activity of our P1 mutant

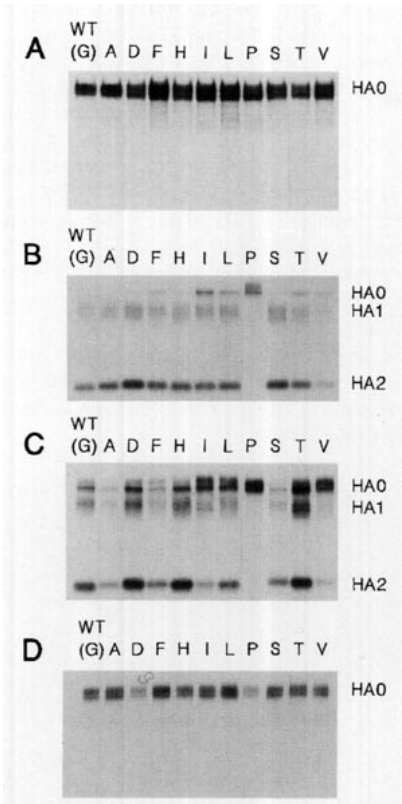

FIG. 2. Cleavage of wild-type and mutant HAs in LoVo cells by subtilisin-like proteases expressed in a vaccinia–T7 system. Recombinant vaccinia viruses expressing proteases were described earlier (*19*). Expression of functional PACE4 was confirmed using pro-von Willebrand factor as a substrate (kindly provided by Dr. J. Van Mourik, Red Blood Transfusion Service, Amsterdam, The Netherlands) (data not shown) (*39, 40*). HA cleavage by endogenous LoVo cell proteases (A) or by furin (B), PC6 (C), or PACE4 (D) was examined by infecting cells with vTF7.3 (m.o.i. = 5), or by coinfecting cells with vTF7.3 (m.o.i. = 5) and recombinant vaccinia virus expressing each of the proteases (m.o.i. = 5). Cells were then transfected with HA genes and analyzed using a radioimmunoprecipitation assay. The two species of HAs observed on the SDS–PAGE gels are presumably due to differences in glycosylation as have been observed by others (*45, 46*).

HAs because expression levels of the mutants in the T7–vaccinia system with our constructs were too low to perform fusion assays, even though the system is generally known for high expression levels of foreign proteins (*31*).

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