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Engineered Serine Protease Inhibitor Prevents Furin-Catalyzed Activation of the Fusion Glycoprotein and Production of Infectious Measles Virus

MICHIKO WATANABE,[1] AKIKO HIRANO,[1] STEPHEN STENGLEIN,[2] JAY NELSON,[2] GARY THOMAS,[3] AND TIMOTHY C. WONG[1]*

Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195,[1] and The Vollum Institute for Advanced Biomedical Research[3] and Department of Microbiology and Immunology,[2] Oregon Health Sciences University, Portland, Oregon 97201

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We have identified the major cellular endoprotease that activates the fusion (F) glycoprotein of measles virus (MV) and have engineered a serine protease inhibitor (serpin) to target the endoprotease and inhibit the production of infectious MV. The F-protein precursor of MV was not cleaved efficiently into the mature F protein in human colon carcinoma cells lacking functional furin, indicating that furin is the major enzyme responsible for activation of the MV F protein. A human serpin α_1-antitrypsin variant was engineered to specifically inhibit furin. When expressed from a recombinant vaccinia virus in primate cells infected by MV, the engineered serpin (α_1-PDX) specifically inhibited furin-catalyzed cleavage of the F-protein precursor without affecting synthesis of other MV proteins. We generated human glioma cells stably expressing α_1-PDX. MV infection in these cells did not result in syncytia. The infected cells produced all the MV proteins, but the F-protein precursor remained largely uncleaved. This did not prevent virus assembly. However, the released virions contained inactive F-protein precursor rather than mature F protein, and infectious-virus titers were reduced by 3 to 4 orders of magnitude. These results show that a mature F protein is not required for the assembly of MV but is crucial for virus infectivity. The engineered serpin may offer a novel molecular antiviral approach against MV.

Measles virus (MV) causes a highly contagious acute infection which affects tens of millions of the world population and causes over a million deaths each year (16, 25). A significant number of the recent measles cases have occurred in unvaccinated individuals or infants who are difficult to effectively vaccinate because of maternal antibodies (25, 28). Currently, there is no antiviral agent available that can specifically interrupt the replication of MV. There is a great need to develop potential antiviral agents for this virus.

MV, a negative-stranded RNA virus in the *Morbillivirus* genus of the *Paramyxoviridae* family (16), produces two essential glycoproteins called hemagglutinin (H) and fusion (F) proteins. The H protein serves for attachment to the host cells. The F protein allows virus entry and cell-to-cell transmission of the virus by inducing membrane fusion (14). The F protein of MV is synthesized in the endoplasmic reticulum as an inactive precursor, F_0. The nascent F_0 acquires three asparagine-linked oligosaccharide chains sequentially and undergoes a conformational change (1, 27). During transport to the plasma membrane, the fully glycosylated F_0 is cleaved with a half-life of about 1 h into the F_1 and F_2 subunits, which are joined by disulfide bonds to form the functional F protein (1, 22, 27). The cleavage of F_0 is critical for the infectivity and pathogenicity of paramyxoviruses (15). Thus, a possible approach to reduce the infectivity of MV is to develop molecular antagonists to inhibit the protease that cleaves the F_0 of MV.

Cleavage of the F_0 of MV occurs on the carboxyl side of a sequence Arg-His-Lys-Arg (21). This sequence conforms to the minimal consensus sequence Arg-X-X-Arg recognized by furin, a cellular calcium-dependent subtilisin-like endoprotease in the trans-Golgi network (8, 11, 12). Furin cleaves many cellular protein precursors, including the precursors of β nerve growth factor (3) and complement protein C3 (10). Furin also catalyzes the cleavage of many viral glycoproteins, including the gp160 of human immunodeficiency virus type 1 (4, 6, 13), the H protein of avian influenza virus (23, 26) and the F protein of Newcastle disease virus (5, 17).

To test whether furin is responsible for cleavage of the F protein of MV, we infected LoVo (human colon carcinoma) cells or HeLa (human cervical carcinoma) cells with the Nagahata strain MV (29) at a multiplicity of infection (MOI) of 5 and studied the synthesis and processing of viral proteins by pulse-chase analysis. LoVo cells lack functional furin because of a single-base deletion in the furin gene causing translational frameshift (24). HeLa cells, which produce furin, served as a control. In both cell types, MV produced similar H, phosphoprotein (P), nucleocapsid (N), and matrix (M) proteins immunoprecipitated by the green monkey (GM) antiserum (7) against total Nagahata strain MV proteins during 1 h of labeling (Fig. 1, lanes b and d). The turnover rates of these viral proteins were also similar in a 3-h chase (Fig. 1, lanes c and e). The GM antiserum did not react well with F protein. We therefore immunoprecipitated the same samples with F-specific polyclonal antiserum (7). The F-protein precursor was cleaved at markedly different rates in the two cell types. In HeLa cells, most of the glycosylated F_0 synthesized in 1 h of labeling was cleaved into the F_1 and F_2 subunits after a 3-h chase (Fig. 1, lanes f and g, respectively). The F_0 was also synthesized in MV-infected LoVo cells in 1 h of labeling (Fig. 1, lane h), although the level of F_0 appeared to be lower than in MV-infected HeLa cells (Fig. 1, lane f). More importantly, the F_0 in LoVo cells was not cleaved efficiently into F_1 and F_2. After a 3-h chase, much of the F_0 in MV-infected LoVo cells

* Corresponding author. Phone: (206) 685-2162. Fax: (206) 543-8297.

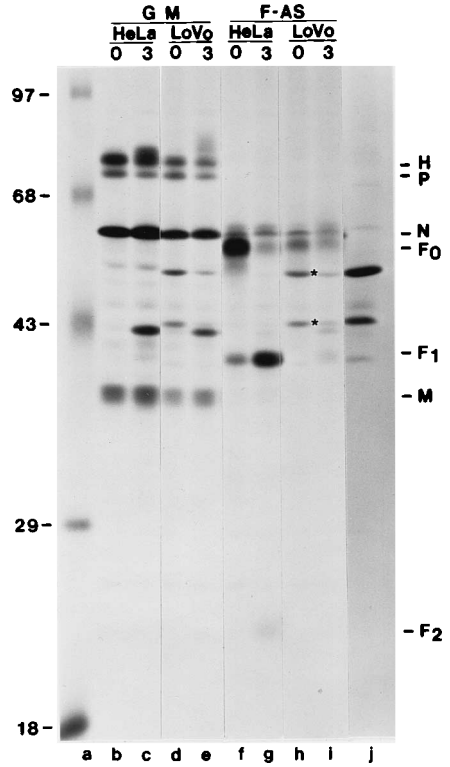

FIG. 1. Differential cleavage of the MV F_0 in LoVo and HeLa cells. Parallel cultures of LoVo or HeLa cells were infected with Nagahata strain MV (29) (MOI of 5). When the HeLa cells showed extensive cytopathic effects (18 h postinfection), the cultures were starved for 1 h in methionine-free medium and labeled with [^{35}S]methionine for 1 h. One culture from each set was lysed immediately (lanes b, d, f, and h), and the remaining cultures were washed with excess L-methionine and chased with unlabeled medium for 3 h and then lysed (lanes c, e, g, and i). The lysates were immunoprecipitated with the GM antiserum (7) against total Nagahata MV proteins (lanes b through e) or the F antiserum (F-AS) (7) (lanes f through i) and analyzed by electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gel. A lysate of ^{35}S-labeled uninfected LoVo cells was immunoprecipitated with the F antiserum and analyzed in parallel (lane j). Lane a shows molecular mass markers (in kilodaltons). Asterisks in lane i mark extraneous bands not related to F protein but probably of cellular origin.

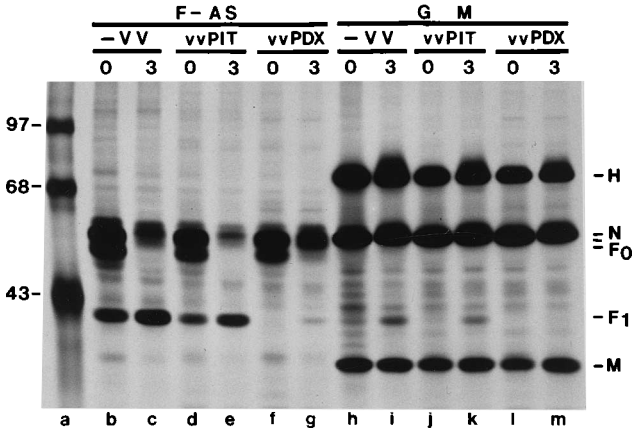

FIG. 2. Effects of α_1-PDX expressed from recombinant VV on cleavage of the MV F_0. CV-1 cells were infected with Nagahata strain MV (MOI of 5). At 14 h postinfection, the cells were superinfected with recombinant VV expressing α_1-PIT (vvPIT) or α_1-PDX (vvPDX). Parallel MV-infected CV-1 cells were mock superinfected (−VV). At 18 h post-MV infection, the cells were starved for 1 h in methionine-free medium and labeled with [^{35}S]methionine for 1 h. One culture from each pair was lysed immediately (lanes b, d, f, h, j, and l), and the remaining cultures were washed with excess L-methionine and chased with unlabeled medium for 3 h and then lysed (lanes c, e, g, i, k, and m). The cell lysates were immunoprecipitated with the F antiserum (F-AS) (lanes b through g) or GM antiserum (lanes h through m). Lane a shows molecular mass markers (in kilodaltons).

remained uncleaved, and only a very faint band was visible in the expected position of F_1 (Fig. 1, lane i). The extraneous bands were not related to F protein but were probably of cellular origin, since they were also precipitated nonspecifically from uninfected LoVo cells (Fig. 1, lane j). Thus, the F_0 of MV is not cleaved efficiently in cells lacking furin, indicating that furin is the major cellular enzyme responsible for proteolytic activation of the MV F protein. It is possible that the MV F_0 can be cleaved by other endoproteases besides furin. However, judging from the inefficient cleavage of F_0 in LoVo cells, those other endoproteases probably do not play a major role in normal processing of the MV F protein.

Because furin is the major cellular enzyme responsible for the cleavage of the MV F_0, it may be possible to inhibit the infectivity of MV by inhibiting furin activity. Two different approaches have been used to inhibit furin-catalyzed cleavage of viral glycoproteins. One approach made use of synthetic peptidyl-chloromethylketones, which are quite toxic to the cells, to inactivate furin (6). We chose a second approach, that of modifying the specificity of a natural serine protease inhibitor (serpin) to target furin. The human serpin α_1-antitrypsin is a serum glycoprotein which normally serves to prevent the detrimental effects of excess neutrophil elastase by forming a complex with elastase through interactions between Met-358 in the serpin and the hydroxyl group of Ser-195 in elastase (9, 19). A natural mutant of α_1-antitrypsin called α_1-antitrypsin-Pittsburgh (α_1-PIT) carries a point mutation from Met-358 to Arg-358 that changes the serpin from an elastase inhibitor to a potent inhibitor of thrombin, causing severe thrombin deficiency in the affected patient (18). This provided clues to the amino acid determinants for the specificity of the serpin. We further mutated Ala-355 in α_1-PIT to Arg-355, so that the active site sequence Arg-355–Ile–Pro–Arg-358 mimicked the consensus recognition sequence for furin. The engineered serpin, called α_1-PDX, is a potent inhibitor of furin. In an in vitro assay, α_1-PDX specifically prevented furin from cleaving a synthetic substrate (butoxycarbonyl-Arg-Val-Arg-Arg-4-methyl-coumaryl-7-amide) but did not prevent elastase and thrombin from cleaving their respective substrates (2). When α_1-PDX was expressed from a recombinant vaccinia virus (VV), it inhibited furin-catalyzed cleavage of β nerve growth factor and the human immunodeficiency virus type 1 gp160 expressed from separate recombinant VV vectors in cultured cells (2). How α_1-PDX may affect virus replication and assembly remains to be determined.

To investigate the effects of the engineered serpin on protein synthesis by MV, we infected African green monkey kidney (CV-1) cells with Nagahata strain MV at an MOI of 5. At 14 h after infection, the cells were superinfected with recombinant VV expressing either the furin inhibitor α_1-PDX or the thrombin inhibitor α_1-PIT. Five hours later, synthesis and processing of the MV proteins were examined by pulse-chase analysis and immunoprecipitation with the F and GM antisera. During 1 h of labeling, the cells infected with MV alone synthesized multiple forms of F_0 which migrated as distinct bands just below the N protein (Fig. 2, lane b). These multiple bands are the fully glycosylated F_0 with three oligosaccharide chains and the partially glycosylated F_0 with one or two chains, which we have characterized recently (27). Some F_1 cleavage product was also

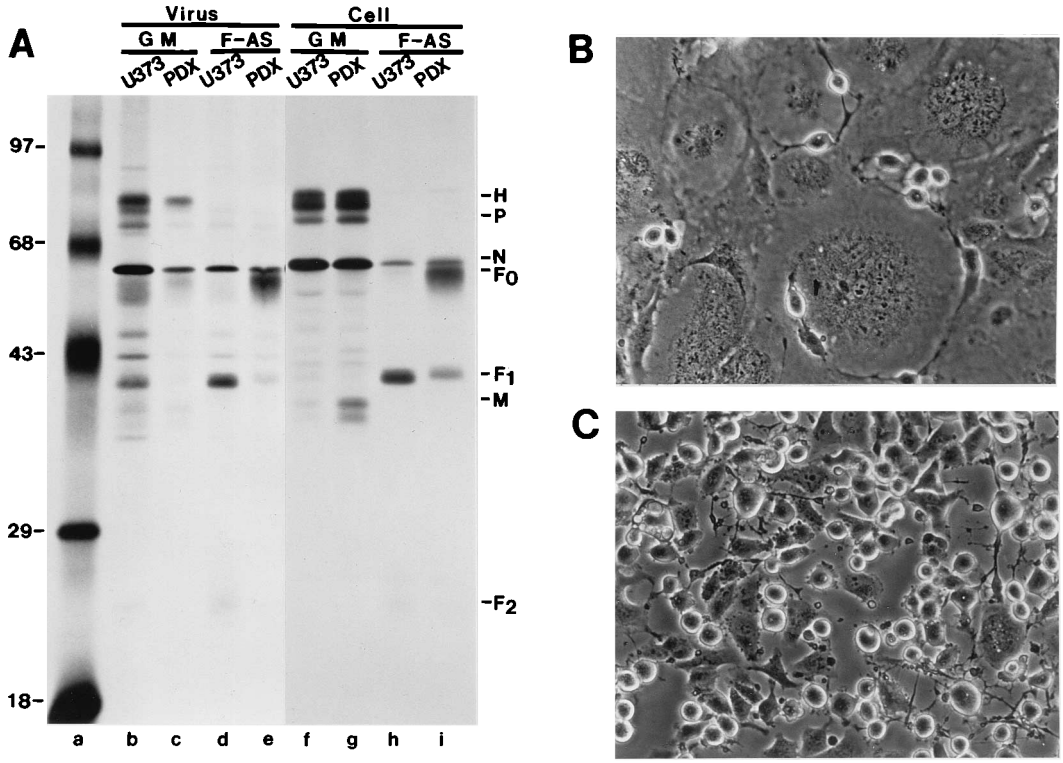

FIG. 3. Effects of stable expression of α_1-PDX on protein synthesis and production of MV. (A) Cultures of U373 or U373-PDX cells were infected with Nagahata strain MV (MOI of 5). After 1 h, the unadsorbed virus was inactivated by incubation with neutralizing antibodies for 45 min and removed by repeated washing. From 17 h postinfection, the cells were labeled with [^{35}S]methionine for 12 h. The culture fluid was cleared of cell debris by centrifugation at 2,000 $\times$ *g* for 10 min, and the extracellular virions were purified by pelleting through 25% glycerol at 100,000 $\times$ *g* for 180 min. The virion proteins and the intracellular viral proteins were immunoprecipitated with the GM antiserum or F antiserum (F-AS) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (B) Syncytium formation in MV-infected U373 cells 27 h after infection. (C) Lack of syncytia in MV-infected U373-PDX cells 27 h after infection.

detected (Fig. 2, lane b). Most of the F_0 was cleaved into F_1 after a 3-h chase (Fig. 2, lane c; F_2 not shown). In CV-1 cells infected with MV and superinfected with the recombinant VV expressing α_1-PIT, F_0 was also cleaved into F_1 (Fig. 2, lanes d and e). By contrast, the cells infected with MV and superinfected with the recombinant VV expressing α_1-PDX produced normal amounts of F_0 but no F_1 during 1 h of labeling (Fig. 2, lane f), and very little F_1 appeared after a 3-h chase (Fig. 2, lane g). Immunoprecipitation with the GM antiserum showed that the recombinant VV expressing α_1-PDX or α_1-PIT did not inhibit synthesis of the H glycoprotein, which does not require furin for processing, or of the nonglycosylated proteins of MV, such as the N and M proteins (Fig. 2, lanes h through m). This indicates that the inhibition of F-protein cleavage by α_1-PDX is not due to the suppression of translation or the blockade of glycoprotein synthesis through the cellular exocytic pathway. Therefore, α_1-PDX is a potent and specific inhibitor of furin-catalyzed cleavage of F_0. The specificity of this inhibition is remarkable, since α_1-PIT, which differs from α_1-PDX by only a single amino acid (Ala-355 instead of Arg-355) at the reactive site (2), does not inhibit F_0 cleavage (Fig. 2, lane e).

In order to study the effects of the engineered serpin on MV replication, we generated cells stably expressing α_1-PDX. The α_1-PDX gene was placed under control of a cytomegalovirus promoter in a modified pCEP4 vector (Invitrogen) in which the EBNA-1 gene was deleted. This plasmid was introduced into human glioma (U373) cells, and cells stably expressing α_1-PDX (U373-PDX cells) were selected with hygromycin. The U373-PDX cells and the parental U373 cells were infected with Nagahata strain MV at an MOI of 5. The MV-infected U373 culture developed typical cytopathic effects characterized by multinucleated giant cells (syncytia) (Fig. 3B). By 27 h postinfection, nearly all the MV-infected U373 cells were fused. By contrast, the MV-infected U373-PDX cells appeared normal until late infection, when some of the infected U373-PDX cells showed rounding but not syncytia (Fig. 3C).

To understand the molecular basis of the lack of typical cytopathic effects in the MV-infected U373-PDX cells, we labeled the MV-infected cells for 12 h and studied the steady-state viral proteins in the cells by immunoprecipitation with the GM and F antisera. Both the MV-infected U373 and U373-PDX cells synthesized equivalent levels of intracellular viral proteins detectable by the GM antiserum (Fig. 3A, lanes f and g). However, most of the steady-state F protein in the U373 cells was cleaved into F_1 and F_2 (Fig. 3A, lane h), whereas the uncleaved F_0 was the predominant F-protein species in the U373-PDX cells (Fig. 3A, lane i). The GM antiserum detected different levels of M protein in the two cell types (Fig. 3A, lanes f and g). This was not consistently seen in repeated experiments and should be considered fortuitous until confirmed by M-specific antisera. Therefore, the lack of typical cytopathic effects in the MV-infected U373-PDX cells is most likely due to a failure to cleave F_0 into F_1 and F_2 to form a functional F protein.

To see how α_1-PDX may affect virus assembly, we purified the virions produced during the labeling period by pelleting through 25% glycerol and analyzed the labeled virion proteins by immunoprecipitation. Both the MV-infected U373 and

TABLE 1. Effects of α_1-PDX on MV production

Expt	Cell type[a]	F_1/F_0 ratio (%)[b]		Titer (PFU/ml)[c]	Relative titer (%)
		Intracellular	Virion		
1	U373	ND	ND	1.3×10^4	100
	U373-PDX	ND	ND	5×10^0	0.04
2	U373	100	100	1.1×10^4	100
	U373-PDX	0.7	0.2	25×10^0	0.2

[a] U373 or U373-PDX cells were infected with Nagahata strain MV (MOI of 5).
[b] F_1/F_0 ratios were determined by laser densitometry and PhosphorImager analysis. ND, not determined.
[c] Average plaque-forming titers from triplicate assays on CV-1 cell cultures.

U373-PDX cells were able to produce virions, as shown by the presence of H, P, N, and M proteins in the virus pellets (Fig. 3A, lanes b and c, respectively). The U373-PDX cells appeared to produce less virion-associated protein than the U373 cells (Fig. 3A, compare lanes b and c). However, this difference could be simply due to lysis of the MV-infected U373 cells, which might allow more efficient release of virions than the intact U373-PDX cells (Fig. 3B and C). More importantly, the virions released from the U373 cells invariably contained the mature F_1 and F_2 peptides, whereas the virions from the U373-PDX cells contained predominantly the uncleaved F_0 (Fig. 3A, lanes d and e, respectively).

To test the infectivity of these virions, the virus titers in culture fluid were determined by plaque-forming assay. In two separate experiments, the MV-infected U373 cells produced 1.1×10^4 to 1.3×10^4 PFU of infectious MV per ml (Table 1). By contrast, infectious-virus titers from the MV-infected U373-PDX cells were drastically reduced by 3 to 4 orders of magnitude to 5 to 25 PFU/ml (Table 1). We also determined virus titers in the culture fluid from cells coinfected with MV and recombinant VV expressing α_1-PDX or α_1-PIT (Fig. 2) in the presence of rifampin (100 μg/ml), which preferentially inhibited replication of VV over MV. MV titers were reduced by VV expressing α_1-PDX more than by VV expressing α_1-PIT or VV alone (data not shown). Therefore, the reduction of infectious MV titers from the U373-PDX cells can be attributed to α_1-PDX and is not due to other factors that might have been selected in the U373-PDX cells.

Furin may not be the only cellular enzyme capable of cleaving the MV F_0. For example, PACE4 is another ubiquitous subtilisin-like protease with a substrate specificity overlapping but not identical to that of furin (20). The presence of alternative cellular enzymes like PACE4 may explain the background levels of F_0 cleavage in MV-infected LoVo cells, which lack furin (Fig. 1, lane i). However, the inefficient cleavage of F_0 in LoVo cells indicates that furin is the major cellular enzyme responsible for the activation of the MV F protein. Notably, α_1-PDX inhibits both furin and PACE4 (24a), which may account for the potent inhibitory effects of α_1-PDX on F_0 cleavage.

Since there are few natural mutants of MV available for specifically studying the role of a functional F protein in the assembly and pathogenesis of MV, α_1-PDX provides a useful tool for understanding these aspects of measles virology. Inhibiting the cleavage of the F_0 of MV does not prevent transport of the uncleaved F_0 to the cell surface and assembly of virus particles. The uncleaved F_0 is incorporated into noninfectious virions, and the ratios of F_1 to F_0 in the virions and in the cells are comparable (Table 1). Therefore, as in other paramyxoviruses previously studied (15, 17), there seems to be no mechanism to preferentially utilize the mature form of F protein for MV assembly, and a mature F protein is not required for the virus assembly process but is critical for the infectivity and syncytium-forming function of MV. Finally, α_1-PDX appears to be strongly inhibitory to MV but is completely nontoxic to the host cells, since cells stably expressing α_1-PDX appear normal and can be propagated indefinitely. The nontoxic nature and the potent antiviral effects of α_1-PDX make it an attractive candidate for development into a possible agent against not only MV but also a wide range of other viruses that rely on cellular furin for the production of a functional viral protein. In this context, an important question is whether inhibiting F-protein cleavage alone can lead to a persistent MV infection. Long-term studies of the virus-cell interaction in the presence of α_1-PDX may provide new insights into this question.

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