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A Mutant CHO-K1 Strain with Resistance to *Pseudomonas* Exotoxin A Is Unable To Process the Precursor Fusion Glycoprotein of Newcastle Disease Virus

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RPE.40, a mutant strain of CHO-K1 cells isolated for resistance to *Pseudomonas* exotoxin A and cross-resistant to alphaviruses, is also highly resistant to virulent strains of Newcastle disease virus. The resistance of RPE.40 cells to Newcastle disease virus results from the failure to cleave the viral envelope precursor glycoprotein Fo to fusion glycoprotein F1 at the consensus sequence (Lys/Arg)-Arg-Gln-(Lys/Arg)-Arg.

RPE.40 is a mutant strain isolated from CHO-K1 Chinese hamster ovary cells. We previously reported that these cells are resistant to *Pseudomonas* exotoxin A and to certain alphaviruses (6). We have shown that the resistance of RPE.40 to Sindbis virus is related to its inability to properly cleave Sindbis virus proprotein PE2 to produce viral membrane glycoprotein E2 (20). Like Sindbis virus and other alphaviruses, the avian paramyxovirus Newcastle disease virus (NDV) possesses a surface glycoprotein which mediates fusion between the viral envelope and the host cell membrane. This F glycoprotein is formed by posttranslational cleavage of the biologically inactive 68-kDa precursor protein Fo. Proteolytic cleavage occurs at the carboxyl side of the amino acid sequence Arg-Gln-(Lys/Arg)-Arg to yield two subunits: the 12-kDa N-terminal F2 and the 56-kDa C-terminal F1 (11, 17, 18). Many viral envelope proteins which are cleaved in the constitutive secretory pathway, including those of the alphaviruses and paramyxoviruses, share the highly conserved Arg-X-(Lys/Arg)-Arg sequence, in which cleavage occurs after the final Arg residue (3, 7, 9, 14, 16). The presence of paired or multiple basic amino acid residues in the Fo precursor glycoprotein is an important determinant of virulence in NDV strains (4). Found in highly virulent strains, such as NDV Texas GB and NDV California RO, this particular processing site sequence is cleaved by one or more proteases present in virtually all types of cells tested thus far (10, 11). The Fo of avirulent NDV strains, which contains only a single Arg at the cleavage site, is cleaved by only a few limited tissue types that express a specific protease(s) (8, 9, 11, 12) (Table 1). Chicken embryo tissues possess this activity.

We examined the response of wild-type CHO-K1 (American Type Culture Collection, Rockville, Md.) and mutant RPE.40 cells to infection with virulent and avirulent NDV strains (obtained from the U.S. Department of Agriculture National Veterinary Services Laboratory, Ames, Iowa). All NDV stocks were grown in chicken eggs, and all experiments were done in U.S. Department of Agriculture-inspected facilities at the University of Vermont. Cells were grown to confluent monolayers in a DME/F12 mixture (Sigma, St. Louis, Mo.) containing 5% fetal bovine serum, at 37°C, in an atmosphere of 5% CO_2 in air. Monolayers were infected with virus at a multiplicity of infection of 10 PFU per cell. After a 1-h incubation at 37°C, the cells were washed with Dulbecco's phosphate-buffered saline (PBS) (GIBCO, Grand Island, N.Y.) and fresh growth medium was added. After 12 to 24 h of incubation, the cells were examined by light microscopy and the cytopathic effect (CPE) was determined. As expected, CHO-K1 cells infected with the avirulent NDV strains La Sota and Ulster showed no typical viral CPE even after several days. In CHO-K1 cells infected with the virulent NDV strains Texas GB and California RO, extensive fusion (90 to 100%) causing subsequent death of the cells was observed within 12 h. In contrast, RPE.40 mutant cells infected with the same virulent NDV strains showed no typical viral CPE after 12 h or more. The viability of the cells was unimpaired, as the addition of fresh medium caused further proliferation of the mutant cells during the period of time when death of all wild-type cells was observed.

The absence of CPE in NDV-infected RPE.40 cells could be caused by the failure of these cells to properly process the viral fusion glycoprotein. If viruses with uncleaved glycoproteins were released, the normally observed fusion of infected cells would not occur. This is the case when most cells are infected with nonvirulent NDV strains, such as La Sota and Ulster, which have a monobasic cleavage motif at their processing site. It has been shown that the isolated noninfectious virions of avirulent NDV strains can be activated and made infectious in vitro by exposing them briefly to trypsin (8, 12, 19). In order to determine, first, whether noninfectious virions were released from infected RPE.40 cells and, second, whether these virions could be activated, we conducted trypsin experiments.

Confluent monolayers of wild-type and mutant cells were grown in 100-mm tissue culture plates. They were then infected with NDV strains, as described above. After 30 h of incubation, trypsin was added to a final concentration of 10 μg/ml [trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; Worthington, Freehold, N.J.) and incubation was continued for 10 min before the supernatant was harvested. Soybean trypsin inhibitor (Sigma) to a concentration of 12 μg/ml and fetal bovine serum to 1% were added to inactivate the trypsin. The titer and infectivity of

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TABLE 1. Sequences and cleavage sites of the fusion glycoproteins of virulent and avirulent strains of NDV and Sindbis virus

Strain	Sequence[a] and cleavage site[b]
NDV	↓
Texas GB	SGGRRQKR FIGAIIGG
California RO	SGGRRQRR FIGAIIGS
La Sota	SGGGRQGR LIGAIIGG
Ulster	SGGGKQGR LIGAIIGG
Sindbis virus	GSSGRSKR SVIDGFTL

[a] References for the sequence data (presented in single-letter notation): Texas GB (8), California RO, La Sota, Ulster (4), Sindbis (13).
[b] Arrow points to cleavage sites.

virions were evaluated by plaque assay on Vero African green monkey kidney cells (ATCC CCL 81) grown to confluency in 60-mm plates. An overlay medium of 0.9% agarose (type V) (Sigma) in minimum essential medium (MEM) (Sigma) containing 5% fetal bovine serum was used, and plaques were detected by neutral red staining after 2 to 3 days (19). In order to enumerate virus particles from avirulent strains, it was necessary to include 10 μg of trypsin per ml in the overlay medium.

As shown in Table 2, the addition of trypsin had little or no effect on virions produced by CHO-K1 cells. In contrast, trypsin treatment caused increases of more than 10- and 16-fold, respectively, in viral titers of the virulent NDV strains Texas GB and California RO produced by RPE.40 cells. These data were compatible with the hypothesis that RPE.40 cells produce and release noninfectious virus particles containing uncleaved glycoprotein Fo.

To further assess the processing of the fusion glycoprotein in these cells, NDV produced by CHO-K1 and RPE.40 cells was labeled metabolically. Cells grown to confluency in 60-mm tissue culture plates were infected with virulent strains of NDV at a multiplicity of infection of 10 PFU per cell. After 5 to 6 h of incubation at 37°C, cells were pulse-labeled. For labeling of all viral proteins, cultures were exposed for 10 min to methionine-free Eagle MEM containing 20 μCi of Tran^{35}S-label (ICN, Irvine, Calif.) per ml and then washed three times with complete MEM. For labeling of viral glycoproteins, cultures were incubated with MEM containing 40 μCi of D-[^{3}H]glucosamine-HCl (ICN) for 2 h. Radioactivity was then chased for 3 h by incubating cells in glucose-depleted MEM with 5% fetal bovine serum. Cells were then washed once with PBS and twice with growth

TABLE 2. Effect of trypsin on infectivity of virulent and avirulent strains of NDV produced by CHO-K1 and RPE.40 cells

NDV strain	Virus production (10^5 PFU/ml) in:			
	CHO-K1 cells		RPE.40 cells	
	Without trypsin	With trypsin	Without trypsin	With trypsin
Virulent				
Texas GB	6.1	8.6	1.3	13.0
California RO	3.0	4.0	0.5	9.0
Avirulent				
La Sota	0	7.0[a]	0	6.8
Ulster	0	5.3	0	5.4

[a] Trypsin incorporated in overlay medium for plaque assay of avirulent strains; values for controls without this = 0.

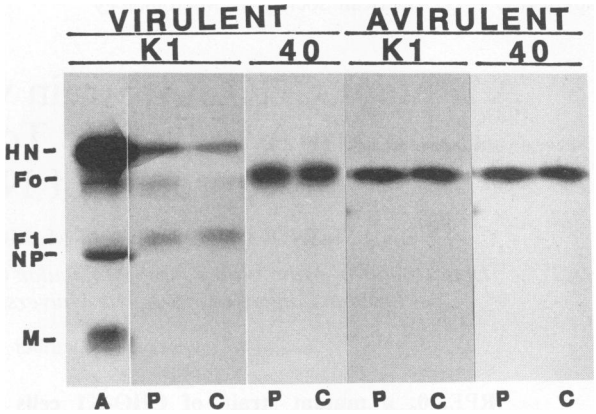

FIG. 1. Polypeptides of NDV and cleavage of fusion glycoprotein precursor (Fo) in CHO-K1 and RPE.40 cells. Viral proteins were pulse-labeled with [^{35}S]methionine and immunoprecipitated with polyclonal anti-NDV serum (lane A). Viral glycoproteins were pulse-labeled (P) with [^{3}H]glucosamine-HCl or pulse-labeled and then chased (C) in glucose-depleted medium. F-related glycoproteins were immunoprecipitated with anti-F monoclonal antibody and analyzed by SDS-polyacrylamide gel electrophoresis as described in the text. F1, fusion glycoprotein; HN, hemagglutinin-neuraminidase protein; NP, nucleocapsid; M, matrix protein.

medium. Cell extracts were prepared by adding 0.5 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride) and centrifuging at 8,000 × *g* for 5 min in an Eppendorf microcentrifuge. Viral glycoproteins were then immunoprecipitated from the supernatants by using NDV polyclonal antibodies (received from Y. Nagai, Nagoya University, Nagoya, Japan) or anti-F monoclonal antibodies (1) (received from H. Kida, Hokkaido University, Sapporo, Japan), and proteins were analyzed by SDS-polyacrylamide gel electrophoresis, with 10% resolving gels, as previously described (5, 11, 14).

Figure 1 shows the viral proteins and glycoproteins produced by CHO-K1 and RPE.40 cells. The pulse-chase data demonstrated the conversion of the glycoprotein precursor Fo to fusion glycoprotein F1 in NDV-infected CHO-K1 cells. The efficiency of conversion of Fo to F1 in CHO-K1 cells was similar to that in other cells known to be highly susceptible to NDV virulent strains, such as Vero and BHK-21 cells (8, 11). In contrast, RPE.40 cells produced and accumulated the fusion glycoprotein precursor Fo with no conversion to F1, even after more than 3 h of chase.

We report here that RPE.40, a mutant strain of CHO-K1 cells selected for resistance to *Pseudomonas* exotoxin A and cross-resistant to alphaviruses, is also highly resistant to the paramyxovirus NDV. Metabolic labeling and trypsin activation experiments have shown that this resistance is the direct result of a failure to cleave the NDV virulent strain precursor glycoprotein Fo to fusion glycoprotein F1. It has been well established that the Fo glycoprotein of virulent strains of NDV is cleaved by host cell proteases at a consensus sequence containing pairs of basic amino acids [Arg-X-(Lys/Arg)-Arg] in all cell types studied thus far (11). Proteolytic cleavage at this consensus sequence has been recognized as the determinant of its virulence and tissue specificity. We previously demonstrated that the resistance of RPE.40 cells to alphaviruses was caused by their inability to cleave

membrane precursor glycoproteins containing similar paired basic amino acid residues. Therefore, we now have identified an endoprotease activity, mutated in RPE.40 cells, that is required for the processing of the E2 precursor glycoprotein of alphaviruses and Fo of virulent NDV, as well as for the toxicity of *Pseudomonas* exotoxin A.

It has recently been shown that furin, the subtilisin-like endoprotease recently discovered in human and mouse tissues and implicated in the cleavage of proproteins secreted via the constitutive pathway (2), is responsible for the activation of the hemagglutinin of fowl plague virus (15). It does this by cleavage of the precursor hemagglutinin at an amino acid consensus sequence similar to those found in PE2 and Fo. We believe that the mutation in RPE.40 cells disrupts the function of furin or a furin-like endoprotease. Experiments are currently under way in our laboratory to identify additional substrates whose processing is altered by the mutation and to identify and characterize the proteolytic activity that is impaired in RPE.40 cells.

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