Crystallization of Biologically Active Hemagglutinin-Neuraminidase Glycoprotein Dimers Proteolytically Cleaved from Human Parainfluenza Virus Type 1

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We isolated, purified, and characterized the hemagglutinin-neuraminidase (HN) of human parainfluenza virus type 1, with the ultimate goal of producing crystals suitable for three-dimensional X-ray structure analysis. Pronase was used to cleave the globular head of the HN molecule directly from virus particles, forming HN monomers and dimers. The purified dimers retained neuraminidase and hemadsorption activity and were recognized by 14 anti-HN monoclonal antibodies, demonstrating intact HN antigenic structure and function. N-terminal sequence analysis of the dimers showed that cleavage had occurred at amino acid 136 or 137, freeing the C-terminal 438 or 439 amino acids. On electron micrography, the dimer appeared as two box-shaped structures, each approximately 5 by 5 nm. When the purified HN dimers were crystallized in hanging drops by vapor diffusion against 20% polyethylene glycol 3350, they formed both rectangular plates and needlelike crystals. The rectangular crystals diffracted X-rays, indicating an ordered atomic structure. However, the resolution was approximately 10 Å (1 nm), insufficient for three-dimensional structural analysis. Experiments to improve the resolution by increasing the size and quality of the crystals are in progress.

Human parainfluenza virus type 1 (hPIV-1), a major childhood respiratory pathogen (4), expresses hemagglutinin-neuraminidase (HN) and fusion (F) surface glycoproteins. The multifunctional HN protein attaches the virus to sialic acid-containing host cell receptors (10) and hydrolyzes sialic acid residues (18, 19, 21), agglutinates erythrocytes, and participates with the F protein in virus-host cell fusion (6, 11, 12, 14-16). Both HN and F proteins elicit neutralizing antibodies. From the nucleic acid sequence of the hPIV-1 HN gene (5), a three-domain HN structure is predicted: a large hydrophilic carbohydrate-containing domain outside the viral membrane, a small hydrophobic domain spanning the membrane, and a small hydrophilic domain inside the membrane (13). The large hydrophilic domain contains the functional sites for cell attachment and neuraminidase activity, as well as the antigenic sites recognized by monoclonal antibodies (MAbs). The hemagglutinin (cell-binding) and neuraminidase domains have not been precisely identified on the HN amino acid sequence.

Crystallography is the most powerful tool for delineating the detailed structure of proteins at atomic resolution, but to date, few viral membrane proteins have been crystallized. hPIV-1 and Sendai virus are reported to be closely related (5, 9). The HN protein of Sendai virus, when isolated by trypsin cleavage, yielded only amorphous material (20) until it was complexed with the antigen-binding fragments (Fab) of MAbs. The resulting needlelike crystals, however, were not suitable for X-ray diffraction analysis (8). A three-dimensional description of HN will increase our knowledge of the cell-binding and neuraminidase domains, antigenic structure, and structure-function relationships. This information may provide a basis for the design of new chemotherapeutic agents. To this end, we describe the first isolation and

purification of a soluble proteolytic digestion product of HN obtained directly from virus particles that retains its biological activity and antigenic activity and forms crystals that diffract X-rays.

Proteolytic cleavage and purification of the HN protein. To isolate a soluble form of HN, purified hPIV-1 (40 to 60 mg/ml in 0.01 M phosphate-buffered saline [PBS], pH 7.2) was treated with 20 µg of pronase (Calbiochem) per ml for 16 h at room temperature. Insoluble matter was removed by centrifugation at 120,000 × *g* (Beckman SW55 rotor) for 1 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions revealed that one dimeric and two monomeric forms of HN were released from virus particles (Fig. 1, lane 3). Figure 2A clearly shows the two monomeric forms. All three forms were recognized by anti-HN MAbs in Western immunoblot analyses (data not shown). SDS-PAGE under reducing conditions showed the molecular mass of the cleaved HN (c-HN) monomer to be about 10 kDa less than that of monomers released from dimeric c-HN by reduction (data not shown). To obtain homogeneous material for crystallization, we separated dimers from monomers by centrifugation through a 5 to 20% sucrose gradient in a VTi50 rotor at 170,000 × *g* for 8 h. Each fraction was analyzed for proteins by SDS-PAGE (7) and for neuraminidase (NA) activity (1) (Fig. 2). The fractions highest in dimeric c-HN (Fig. 2, lanes 15 to 22) were collected, pooled, and concentrated by dialysis against 70% saturated ammonium sulfate. The resulting product contained predominantly dimeric c-HN (Fig. 1, lane 4). The final concentration of dimeric c-HN, ranging from 7 to 19 mg/ml, was sufficient for crystallization trials. Further characterization and crystallization of the monomeric form were not attempted, since we were unable to separate the two monomeric forms of HN.

Amino-terminal sequence analysis of c-HN. SDS-PAGE indicated that pronase had cleaved an approximately 19-kDa

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FIG. 1. Nonreducing SDS-PAGE analysis of cleaved HN protein. Lanes: 1, 15 μ g of hPIV-1; 2, 15 μ g of pronase-treated hPIV-1; 3, c-HN before purification by sucrose gradient; 4, purified c-HN dimer. The loading buffer did not contain β -mercaptoethanol. The gel was stained with Coomassie brilliant blue.

peptide from the intact HN molecule (data not shown). To identify the precise cleavage site, we sequenced the N-terminal amino acids of c-HN by automated Edman degradation on a gas-phase sequencer (Applied Biosystems). Two sequences were obtained: ICENTIAHHADGIS and CEN TIAHHADGISP. By comparing these two sequences with the complete protein sequence predicted from hPIV-1 HN cDNA (5), we found that pronase had cleaved the HN molecule at Gln-136 or Ile-137 (Fig. 3). This finding confirmed that c-HN no longer contained the hydrophobic anchor region (residues 35 to 60) and was consistent with the migration of c-HN on SDS-PAGE.

Biological and antigenic characterization of c-HN. We characterized the cleaved HN to ensure that it retained the structure and functions of intact HN protein. SDS-PAGE and NA activity assays of the sucrose gradient fractions (Fig. 2) indicated that both monomeric and dimeric c-HN retained NA activity. The A_{549} of purified c-HN dimers

FIG. 3. Schematic diagram of the N-terminal sequence of c-HN. The amino acid residues sequenced are shown.

(0.641) was almost identical to that of the same amount of intact HN protein (0.613), establishing that the level of NA function had not been altered by cleavage and purification.

Purified c-HN dimers should possess two erythrocyte-binding sites, but it has been suggested that their proximity sterically interferes with agglutination (20). As expected, purified c-HN did not agglutinate chicken erythrocytes

FIG. 2. Sucrose gradient purification of c-HN dimers. (A) Nonreducing SDS-PAGE analysis of gradient fractions 1 to 39, visualized by Coomassie brilliant blue staining; lane M (marker) contains centrifuge supernatant of pronase-treated hPIV-1. (B) NA activity of each fraction.

FIG. 4. Rectangular and needlelike crystals of hPIV-1 c-HN dimers were observed in the same hanging drop. Bar, 0.5 mm.

(cRBC) at 4°C. As an alternative means of examining cell-binding activity, we tested the ability of c-HN to compete with intact virus for cRBC-binding sites. Purified c-HN was incubated with cRBC at 4°C for 1 h, and then 4 hemagglutination units of intact virus was added. The agglutination of cRBC by intact virus was completely inhibited in the presence of 0.5 mg of c-HN per ml, indicating that c-HN retained its host-cell-binding activity.

To determine whether the antigenic structure of HN had been altered by cleavage and purification, we tested its reactivity with 14 anti-HN MAbs spotted onto dry cellulose acetate strips. All 14 MAbs recognized the purified c-HN dimers (data not shown), demonstrating that the antigenic structure was intact and that all the epitopes recognized by these MAbs are located downstream from residue 136. The purified c-HN dimers visualized by electron microscopy consisted of many small unaggregated molecules containing two subunits, each (presumably the c-HN monomer) measuring approximately 5 by 5 nm (data not shown).

Crystallization of c-HN dimers. Vapor-diffusion crystallization was performed in hanging drops. A mixture with equal amounts of c-HN and precipitant (8% polyethylene glycol 3350 in 0.1 M NaH_2PO_4) was suspended in a drop onto a glass plate placed over 20% polyethylene glycol 3350 in 0.15 M NaCl. Two forms of crystals were obtained: rectangular crystals, either single crystals or in clusters (Fig. 4, top), and long needlelike crystals (bottom). After being washed and dissolved in PBS, the material from the rectangular crystals migrated to the position of purified c-HN dimers on SDS-PAGE and demonstrated neuraminidase activity (data not shown), indicating that the rectangular crystals were composed of pure, enzymatically active HN protein. The needlelike crystals were not suitable for X-ray analysis. The rectangular crystals diffracted X-rays to about 10 Å (1 nm) (19a), indicating an ordered atomic structure, but

the resolution was not sufficient to determine three-dimensional structure.

We report the successful isolation, purification, and characterization of a soluble form of hPIV-1 HN and the first crystallization of a paramyxovirus HN molecule without complexing with MAbs. These steps are a prerequisite to the X-ray structure analysis of the protein, an approach to structure-function studies that has proved successful with influenza virus HA and NA (2, 17, 22–26). Multiple forms of crystals were obtained when influenza B virus NA was crystallized by using a variety of concentrations of precipitant or salt (3). We are optimistic that we can improve the resolution of hPIV-1 HN crystals for structural determination by exploring various crystallization conditions.

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REFERENCES

1. Aymard-Henry, M., M. T. Colman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenza virus neuraminidase and neuraminidase-inhibition test procedures. *Bull. W.H.O.* 48:199–202.
2. Baker, A. T., J. N. Varghese, W. G. Laver, G. M. Air, and P. M. Colman. 1987. Three-dimensional structure of neuraminidase of subtype N9 from an avian influenza virus. *Proteins Struct. Funct. Genet.* 2:111–117.
3. Burmeister, W.-P., R. S. Daniels, S. Dayan, J. Gagnon, S. Cusack, and R. W. H. Ruigrok. 1991. Sequence and crystallization of influenza virus B/Beijing/1/87 neuraminidase. *Virology* 180:266–272.
4. Glezen, W. P., and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. *N. Engl. J. Med.* 288:498–505.
5. Gorman, W. L., D. S. Gill, R. A. Scroggs, and A. Portner. 1990. The hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus type 1 and Sendai virus have high structure-function similarity with limited antigenic cross-reactivity. *Virology* 175:211–221.
6. Heath, T. D., F. J. Martin, and B. A. Macher. 1983. Association of ganglioside-protein conjugates into cell and Sendai virus. Requirement for the HN subunit in viral fusion. *Exp. Cell Res.* 149:163–175.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London)* 227:680–685.
8. Laver, W. G., S. D. Thompson, K. G. Murli, and A. Portner. 1989. Crystallization of Sendai virus HN protein complexed with monoclonal antibody Fab fragments. *Virology* 171:291–293.
9. Lyn, D., D. G. Gill, R. A. Scroggs, and A. Portner. 1991. The nucleoproteins of human parainfluenza virus type 1 and Sendai virus share amino acid sequences and antigenic and structural determinants. *J. Gen. Virol.* 72:983–987.
10. Markwell, M. A. K. 1991. New frontiers opened by the exploration of host cell receptors, p. 407–425. In D. W. Kingsbury (ed.), *The paramyxoviruses*. Plenum Press, New York.
11. Merz, D. C., and J. S. Wolinsky. 1983. Conversion of non-fusing mumps virus infections to fusing infections by selective proteolysis of the HN glycoprotein. *Virology* 131:328–340.
12. Miura, N., T. Uchida, and Y. Okada. 1982. HVJ (Sendai virus)-induced envelope fusion and cell fusion are blocked by monoclonal anti-HN protein antibody that does not inhibit hemagglutination activity of HVJ. *Exp. Cell Res.* 141:409–420.
13. Morrison, T., and A. Portner. 1991. Structure, function, and intracellular processing of the glycoproteins of *Paramyxoviridae*, p. 347–382. In D. W. Kingsbury (ed.), *The paramyxoviridae*.

- ruses. Plenum Press, New York.
14. Örvell, C., and M. Grandien. 1982. The effects of monoclonal antibodies on biologic activities of structural proteins of Sendai virus. *J. Immunol.* **129**:2779–2787.
 15. Portner, A. 1984. Monoclonal antibodies as probes of the antigenic structure and functions of Sendai virus glycoproteins, p. 345–350. *In* D. H. L. Bishop and R. W. Compans (ed.), *Nonsegmented negative strand viruses*. Academic Press, Orlando, Fla.
 16. Portner, A., R. A. Scroggs, and D. W. Metzger. 1987. Distinct functions of antigenic sites of the HN glycoprotein of Sendai virus. *Virology* **158**:61–68.
 17. Sauter, N. K., G. D. Glick, R. L. Crowther, S.-J. Park, M. B. Eisen, J. J. Skehel, J. R. Knowles, and D. C. Wiley. 1992. Crystallographic detection of a second ligand binding site in influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA* **89**:324–328.
 18. Scheid, A., L. Caliguri, R. W. Compans, and P. W. Choppin. 1972. Isolation of paramyxovirus glycoproteins. Association of both hemagglutinin and neuraminidase activities with the larger SV5 glycoprotein. *Virology* **50**:640–652.
 19. Scheid, A., and P. W. Choppin. 1973. Isolation and purification of the envelope proteins of Newcastle disease virus. *J. Virol.* **11**:263–271.
 - 19a. Taylor, G. Personal communication.
 20. Thompson, S. D., W. G. Laver, K. G. Murti, and A. Portner. 1988. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *J. Virol.* **62**:4653–4660.
 21. Tozawa, H., M. Watanabe, and N. Ishida. 1973. Structural components of Sendai virus: serological and physicochemical characterization of hemagglutinin subunit associated with neuraminidase activity. *Virology* **55**:242–253.
 22. Tulip, W. R., J. N. Varghese, A. T. Baker, A. van Donkelaar, W. G. Laver, R. G. Webster, and P. M. Colman. 1991. Refined atomic structures of N9 subtype influenza virus neuraminidase and escape mutants. *J. Mol. Biol.* **221**:487–497.
 23. Varghese, J. N., and P. M. Colman. 1991. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2 Å resolution. *J. Mol. Biol.* **221**:473–486.
 24. Varghese, J. N., W. G. Laver, and P. M. Colman. 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature (London)* **303**:35–40.
 25. Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley. 1988. Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. *Nature (London)* **333**:426–431.
 26. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (London)* **289**:366–373.