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Isolation of the Measles Virus Hemagglutinin Protein in a Soluble Form by Protease Digestion

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The hemagglutinin (H) glycoprotein was isolated in a soluble form by digesting measles virus particles with an endoproteinase, Asp-N (from a *Pseudomonas fragi* mutant). Digestion of H with Asp-N brought about glycopeptides in three different forms, depending on the cleaving site: AHD, which has an M_r of 66,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and which formed a disulfide-linked homodimer with an M_r of 132,000, and two monomeric digestion products, AHM-1 (with an M_r of 64,000) and AHM-2 (with an M_r of 58,000). The susceptibility of the H glycoprotein to the protease depended on the enzyme concentration. AHD was readily formed at a low concentration of Asp-N, while AHM-1 and AHM-2 required higher and even higher protease concentrations, respectively. All of the cleavage products reacted with monoclonal antibodies to various epitopes of the H protein; however, only AHD showed a significant hemagglutinin activity on African green monkey erythrocytes. The hemagglutinin activities of AHM-1 and AHM-2 were restored after a monoclonal antibody lacking the hemagglutination-inhibiting activity was added to the reaction mixture. AHDs purified by size-exclusion high-pressure liquid chromatography had two associating forms; one had an M_r higher than and the other an M_r as high as that of a tetramer. The former was associated noncovalently in addition to having two intermolecular disulfide bonds, and the latter was associated covalently with a single intermolecular disulfide bond and was also duplicated through a noncovalent association. In addition, both AHM-1 and AHM-2, having no intermolecular disulfide bond, were in a dimer form. These results suggest that AHM-1 and AHM-2 are monovalent in the hemagglutinin activity, while AHDs are divalent. Comparative analyses of the N termini of these soluble glycopeptides with the sequence of H suggested that the cysteine residue at position 139 was responsible for the intermolecular disulfide bonding between the monomeric H glycoproteins. The cysteine at position 154 was also suggested to participate in the forming of the intermolecular disulfide bond.

Measles virus has two surface glycoproteins, the hemagglutinin (H) and fusion (F) proteins, which are anchored in the viral envelope at the hydrophobic regions. The amino acid sequences of both the H (1) and F (12) glycoproteins of the Edmonston strain of measles virus were deduced from the nucleotide sequences of the cDNAs of cloned genes. The functions of the H protein include attachment of the virus to the host cells and agglutination of erythrocytes, while those of the F protein include cell fusion and hemolysis. The functions of both the H and F proteins are essential for virus infectivity.

Soluble forms of the envelope glycoproteins of measles virus which may provide tools for the understanding of the biological activities and steric structures of the glycoproteins have been isolated by detergent treatment of virus particles (2, 3, 5, 6, 9, 17, 22), and the purified molecules possessed the biological activities of the H and F proteins. Removal of the detergent, however, resulted in aggregation of the oligomers, involving the hydrophobic region that interacts with the reassorted viral envelope (22).

Partially degraded envelope proteins were successfully obtained from influenza virus by protease digestion, and the steric structures for hemagglutinin (23) and neuraminidase (21) proteins were determined. Recently, attempts similar to this have been made with respect to the hemagglutinin-neuraminidase (HN) protein of a paramyxovirus (8), but X-ray diffraction analysis has not been made. Several investigators have removed measles virus glycoproteins from the surfaces of infected cells or from virions by treatment with various proteolytic enzymes (6, 7, 11, 14, 16), but the structures or biological activities of these soluble glycoproteins have not been elucidated.

In the present study, we isolated the H glycoprotein in various forms from measles virus by releasing the hydrophobic membrane-spanning domain with a proteolytic enzyme. The purified H proteins were analyzed for biological and biochemical properties.

Vero cells were grown in roller bottles (each with an 850-cm^2 surface area) (Falcon No. 3027; Becton Dickinson, Lincoln Park, N.J.) in Eagle's minimum essential medium supplemented with 5% heat-inactivated calf serum and 10% tryptose phosphate broth. The subconfluent monolayer in each roller bottle received 0.5 g of Cytodex-1 microcarrier (Pharmacia-LKB Biotechnology, Tokyo, Japan), and then a fresh Vero cell suspension in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal calf serum was added. The monolayers were infected with the Toyoshima strain of measles virus (20). The virus-containing culture was harvested daily from day 2 to day 10. The virus was purified as described previously (7).

Endoproteinase Asp-N was dissolved in phosphate-buffered saline (PBS). Virus samples (6 mg/ml) in PBS were mixed with an equal volume of the protease at various concentrations, with enzyme-to-virus ratios ranging from 1:375 to 1:48,000, and the mixtures were incubated for 16 h at 37°C. The virus cores were

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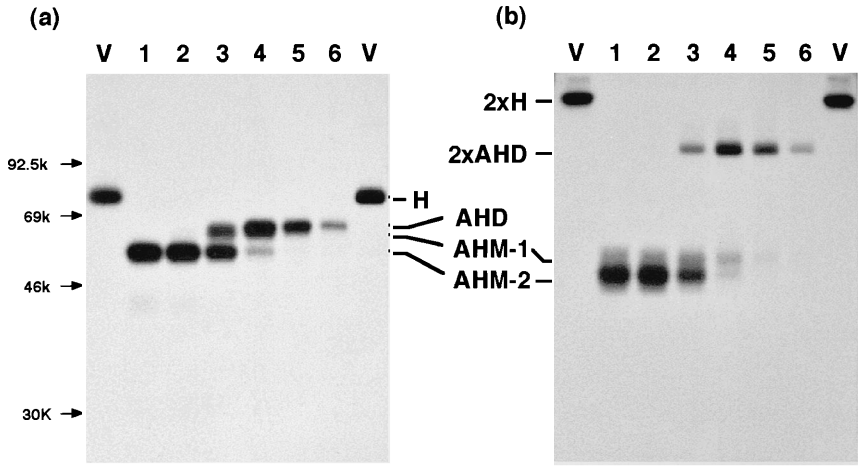

FIG. 1. Solubilization of H glycoprotein of measles virus by Asp-N digestion. Measles virus metabolically labeled with 0.074 MBq of [^{35}S]methionine (Du Pont NEN) per ml was harvested after 12 h of incubation and purified as described in the text. The purified virus was treated with Asp-N at various concentrations (lanes 1 to 6) for 16 h at 37°C, and undigested virus particles were removed from the incubation mixture by centrifugation. The soluble H protein in the supernatant was immunoprecipitated with anti-H MAb B5 (13) for analysis by SDS-PAGE on 10% acrylamide gels under reducing (a) or nonreducing (b) conditions. Lanes: 1, 2 μg/ml; 2, 1 μg/ml; 3, 0.5 μg/ml; 4, 0.25 μg/ml; 5, 0.125 μg/ml; 6, 0.0625 μg/ml. In the lanes labeled V, [^{35}S]methionine-labeled virus was solubilized with radioimmunoprecipitation assay buffer and immunoprecipitated with anti-H MAb B5. 2xH, dimeric form of the intact H; 2xAHD, dimeric form of Asp-N-released H. Molecular weight markers are shown in thousands along the left margin.

removed from the incubation mixture by centrifugation at 200,000 × *g* in a TL-100 centrifuge (Beckman, Palo Alto, Calif.) for 30 min. The supernatant was used as a soluble form of the H glycoprotein.

Monoclonal antibodies (MAbs) directed to measles virus H with immunoglobulin G contents of 3.5 mg/ml (B5), 600 μg/ml (B69) (13), and 2.3 mg/ml (E39) were obtained from mouse ascites. E39 was a new monoclonal antibody against the H protein of the Toyoshima strain. Its isotype was IgG2a, and it had neither hemagglutination inhibition nor virus-neutralizing activity. Competitive binding assays revealed that E39 belonged to an additional group recognizing an epitope different from those determined in the previous study (13).

The hemagglutination test (HA) was performed on a 96-well microplate with 0.5% African green monkey erythrocytes. An enhanced hemagglutination test was carried out in the presence of anti-H MAb E39. Twenty-five microliters of each of serial twofold dilutions of a sample was incubated for 16 h at 4°C with an equal volume of E39 diluted to 1:5,000. Erythrocytes (50 μl) were then added, and the mixtures were incubated for 2 h at 37°C. The highest dilution of the test sample showing hemagglutination was expressed as HA and enhanced hemagglutination titers.

The soluble Hs were isolated by size-exclusion high-pressure liquid chromatography (HPLC) with a Waters 600 system (Waters, Milford, Mass.) equipped with a TSK G3000SW$_{XL}$ (Tosoh, Tokyo, Japan) and concentrated with a Centricon microconcentrator (Amicon, Danvers, Mass.). The concentrates were treated with a sodium dodecyl sulfate sample buffer containing 5% 2-mercaptoethanol and electrophoresed in a 10% (wt/vol) polyacrylamide gel (14). The separated polypeptides were transferred to a ProBlott membrane (Applied Biosystems, Foster City, Calif.) by the manufacturer's procedure. The peptide bands of the blots were subjected to N-terminal amino acid sequencing by automated Edman degradation in a gas phase sequencer (Applied Biosystems).

Asp-N was found to be suitable for solubilization, selectively releasing H proteins without affecting the other viral structural proteins. Treatment of [^{35}S]methionine-labeled measles virus with Asp-N at various concentrations yielded soluble H proteins in the centrifuge supernatants, and they were immunoreactive with an MAb to H (Fig. 1). At a low enzyme concentration (0.0625 μg/ml), a single band of the glycopeptide with an M_r of 66,000 was evident in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses under reducing conditions (Fig. 1a). As the Asp-N concentrations increased (from 0.125 to 0.5 μg/ml), the bands were broadened and an additional band with an M_r of 64,000 became detectable. At an enzyme concentration higher than 0.25 μg/ml, a third band with an M_r of 58,000 appeared while the 66,000 band disappeared. SDS-PAGE under nonreducing conditions showed that the bands of 64,000 and 58,000 obtained under reducing conditions remained at the original positions whereas the 66,000-molecular-weight protein migrated to the 132,000 position (Fig. 1b). This suggests that the Asp-N cleavage product with an M_r of 66,000 forms a homodimer by intermolecular disulfide bonding. This homodimer will hereafter be designated AHD. Those with M_rs of 64,000 and 58,000 are monomers and are designated AHM-1 and AHM-2, respectively.

To isolate and characterize H proteins in soluble forms, substantial amounts of the virions were treated with Asp-N at concentrations deemed to be suitable to obtain the desired peptides as major digestion products. This procedure, however, did not isolate any soluble H protein, because considerable amounts of by-products were formed at each protease concentration. The mixture of solubilized H proteins, therefore, was subjected to size-exclusion HPLC to purify the peptides. Four fractions containing soluble H proteins with distinct molecular sizes were obtained. Each fraction was analyzed for peptides by SDS-PAGE under nonreducing conditions (data not shown). A small amount of soluble H with an M_r larger than that of tetrameric AHD was obtained from the first fraction. This oligomer, however, was found to be tetrameric AHD by SDS-PAGE. The second fraction, corresponding to an M_r of 260,000, contained dimeric AHD, and the last fraction, corresponding to an M_r of 120,000, contained monomeric AHM-2. The third fraction contained a mixture of AHD dimer and AHM-1; the AHM-1 was not purified by HPLC. The overall results are summarized in Table 1.

TABLE 1. Hemagglutinating activity of soluble forms of H protein

Glycopeptide or glycoprotein	Associating form by:		HAU/25 μl[a]		HAU/μg of protein[b]		+/− ratio
	SDS-PAGE[c]	Gel filtration[d]	−	+	−	+	
AHD	Tetrameric	>Tetrameric	800	800	83.6	83.6	1.0
AHD	Dimeric	Tetrameric	1,280	2,560	113.8	227.6	2.0
AHD + AHM-1	Dimeric + monomeric		4,000	16,000	39.7	158.7	4.0
AHM-2	Monomeric	Dimeric	80	2,560	3.0	96.6	32.2
H[e]	Dimeric		3,200	3,200	24.9	24.9	1.0

[a] H was titrated in the absence (−) and presence (+) of anti-H MAb E39. HAU, hemagglutinating units.
[b] Protein concentration was determined by Protein Assay (Bio-Rad) according to the manufacturer's procedure, with bovine serum albumin as a standard.
[c] SDS-PAGE was carried out in 10 to 20% gradient gels under nonreducing conditions.
[d] Relative molecular weights were estimated by size-exclusion HPLC with β-galactosidase (465,000), immunoglobulin G (150,000), the Fab fragment from immunoglobulin G (50,000), and myoglobin from skeletal muscles (17,000) as standards.
[e] Isolated by treating virions with a detergent as described previously (7).

Table 1 also shows two biological activities associated with the isolated soluble glycoproteins. The proteins were tested by conventional HA assay and enhanced hemagglutination assay with anti-H MAb E39, which has no hemagglutination inhibition activity. Purified AHM-2 showed a very low hemagglutinating titer. To test whether AHM-2 is adsorbed on to erythrocytes, the HA assay was carried out with a trace amount of MAb E39 to bridge the monomeric forms. If AHM-2 adheres to erythrocytes, the divalent antibody should form lattices among the erythrocyte-glycopeptide complexes, resulting in hemagglutination. As shown in Table 1, a dramatically increased HA titer of 32-fold was demonstrated with AHM-2. The result clearly showed that AHM-2 adhered to the erythrocytes. In contrast, two types of AHD, which by itself had a considerable HA activity, were not or, if at all, only slightly enhanced with E39. The above results suggest that these soluble proteins are associated with an interaction other than disulfide bonding.

The N-terminal amino acid sequences of the respective peptide fragments were analyzed. The results are shown schematically in Fig. 2. Asp-N, which is known to cleave specifically the peptide bonds on the N-terminal side of aspartic or glutamic acid, was found to cleave the H protein at three sites, i.e., the aspartic acid residues at positions 135 (D-135) and 151 (D-151) and the glutamic acid residue at position 173 (E-173). Each cleavage site corresponded to AHD, AHM-1, and AHM-2, respectively. There are two cysteine residues close to the cleavage sites at positions 139 and 154. Either of the two cysteine residues may have participated in the forming of the intermolecular disulfide bond. The fact that the N terminus of AHD, which formed a disulfide-linked tetramer and dimer in SDS-PAGE, was D-135 and that for AHM-1, a monomeric form, was D-151 suggests that, at least, the cysteine residue at position 139 (C-139) is oxidized, forming an intermolecular disulfide bond. The presence of the tetrameric form of AHD suggests that C-154 also takes part in the disulfide bond formation. That no tetrameric or dimeric form of AHM-1 or AHM-2 was detected, even when the mixture of the two monomers was analyzed by SDS-PAGE under nonreducing conditions (Fig. 1), suggests that C-139 participates in the principal intermolecular disulfide bond formation and is followed by C-154. The M_r of AHM-2 was lower than that of AHM-1 by approximately 6,000 by SDS-PAGE (Fig. 1). However, the difference in size between the two peptides deduced from the cleaved sites was expected to be as low as 2,000. This gap in the magnitudes of the two M_rs might be interpreted by the estimation that one of the five potential glycosylation sites (N-168) which might be linked with an N-linked carbohydrate side chain with an approximate M_r of 3,500 to 4,000 was lost by the cleavage.

Characterization of the proteins retaining the biological activities has provided information on the structure-function relationship. Even though the H glycoprotein can be isolated in its biologically active form by detergent solubilization, aggregated forms appear after the removal of detergents, disturbing crystallization. To avoid such aggregation, the hydrophobic membrane-anchoring region should be cleaved off. This attempt in the present study resulted in the release of H in a soluble form. The protease-released Hs retain the whole ectodomain of the glycoprotein. Our result proved that the measles viral H is a member of the membrane proteins that can

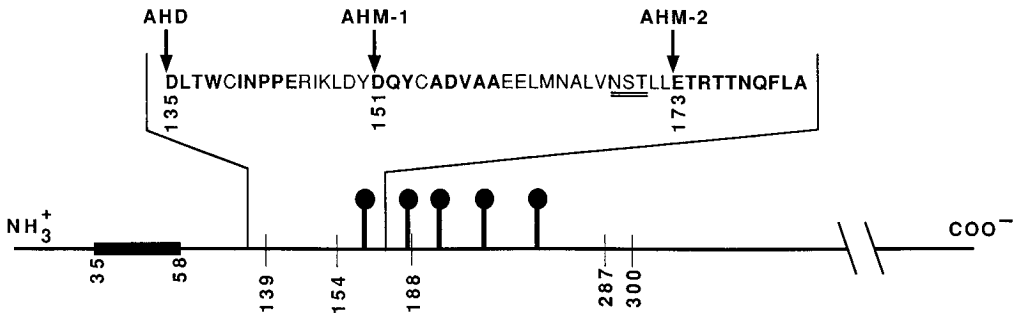

FIG. 2. Location of N-terminal amino acid sequence of H protein. The cleavage products of Asp-N are indicated (arrows and residue numbers). The locations of the potential sites for N-linked glycosylation are indicated by the double underline and tadpoles. Cysteine residues (vertical lines) and the transmembrane domain (heavy bar) are shown. The amino acid sequence of the H protein has been predicted by the complete cDNA sequencing of the H gene of the Toyoshima strain (unpublished data), which shows that the sequence from 135 to 252 is identical to that of the Edmonston strain (1). The amino acids not directly determined by sequencing analysis are designated in lightface.

be released from the envelope by releasing a small peptide tail near the amino terminus (1).

Amino acid residues 1 to 34 of the H polypeptide are proposed to form a cytoplasmic domain, while residues 35 to 58 are buried in the lipid bilayer, forming a transmembrane domain (1). The residues from 59 to 181 are estimated to compose a slender stalk. Kinetic analysis of the enzymatic digestion demonstrated that the small region 135 to 181 within this stalk is extremely sensitive to various proteases, including Asp-N (this paper), chymotrypsin, and trypsin (unpublished observations). This suggests that the region is exposed to the outside and forms a hinge of the molecule. The disulfide bond at the C-139 residue should act as an axis of the hinge.

The cleaved monomers and dimers retained the activity to bind to erythrocytes as well as their antigenicity, suggesting that the globular head of the H molecule is protease resistant, although this could not be confirmed because the C-terminal peptide has not been sequenced.

It is very interesting that all soluble H fragments interact with erythrocytes. Several attempts to obtain the H proteins of paramyxoviruses in soluble form have been made by proteolytic cleavage (15, 19). The HN protein of SV5 was the first paramyxovirus glycoprotein that was isolated by treating the Triton X-100-solubilized peplomer with chymotrypsin (15). Scheid et al. (15) reported that the dimeric form of SV5 HN did not agglutinate erythrocytes, but it retained the neuraminidase activity. This was attributed to the univalency of the cleaved HN dimer. Similarly, the HN of Sendai virus was isolated in dimeric and tetrameric forms by detergent solubilization followed by trypsin treatment (18, 19). These cleavage products were shown to be capable of hemagglutination, in contrast to the findings with SV5. Quantitative analysis of the tetrameric measles viral H fragment in the present study agrees with the presence of hemagglutinating activity, suggesting that AHDs are divalent (4, 10). This finding suggests that AHM-1 and AHM-2 that adhere to erythrocytes are monovalent in hemagglutination. That the hemagglutinating titers of AHM-1 and AHM-2 were increased to a great extent with divalent MAbs provides evidence showing the capability of the monomers to adhere to the receptors on erythrocytes.

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