Selective Dansylation of M Protein Within Intact Influenza Virions

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Exposure of purified influenza virions to [¹⁴C]dansyl chloride resulted in the covalent attachment of the dansyl chromophore to the virion. Gel electrophoresis revealed that the dansyl chromophore was specifically coupled to the internal membrane (M) protein. Purification of the M protein by gel filtration followed by cyanogen bromide cleavage and peptide fractionation revealed that four of six peptide peaks contained dansyl label. Acid hydrolysis of the separated peptide peaks followed by thin-layer chromatography revealed that dansyl label was coupled to lysine residues present in these peptides. The results of these investigations have demonstrated that the M protein molecule is the major viral polypeptide labeled when intact virions are exposed to dansyl chloride.

Influenza virus is a negative-stranded RNA virus composed of eight helical ribonucleoprotein species surrounded by a lipoprotein membrane. The polypeptides associated with the lipid bilayer are: (i) two glycoproteins, the hemagglutinin and neuraminidase, which form projections on the external surface; and (ii) a non-glycosylated polypeptide, termed the membrane or matrix (M) protein, associated with the internal surface of the viral membrane. The exact nature of the association of M protein with the lipid bilayer remains unclear, although suggestive evidence has been obtained that it may be interacting with lipids (23) and has lipid-like solubility characteristics (17). More recently (8, 18, 19), evidence was obtained that M protein is able to reassociate with lipid vesicles after detergent dialysis, indicating that it has some properties of an integral membrane protein.

In this paper, we report that exposure of intact influenza virions to dansyl chloride results in the covalent coupling of the dansyl chromophore to influenza virus M protein. Previous information on the location of the dansyl chromophore in lipid bilayers revealed that it is associated preferentially with the glycerol region of the bilayer membrane (31), thereby exposing its protein-reactive sulfonyl group to reactive amino and hydroxyl groups on membrane-associated proteins. The present results indicate that this chemical probe may be used to label proteins associated with lipid bilayers in biological membranes.

MATERIALS AND METHODS

Virus growth and purification. The WSN (HoN₁) strain of influenza A virus was grown in Madin-Darby bovine kidney (MDBK) cells (10), which were maintained in reinforced Eagle medium containing 10% newborn calf serum (3). Virus titers were measured by plaque assays on chicken embryo fibroblast monolayers (10). Hemagglutination titrations were performed by using 0.36% chicken erythrocytes in microtiter plates (13). Stock virus with a titer of at least 10⁸ PFU/ml was diluted 1:15 in Eagle medium containing 1% bovine serum albumin and applied to the confluent MDBK cells in 2-liter roller bottles. After a 2-h adsorption period, reinforced Eagle medium containing 2% calf serum and 3 μ Ci of [³H]leucine (60 Ci/mmol; Schwarz/Mann) per ml or ³H-amino acids (Schwarz/Mann) was added to the cultures, and the infection was allowed to proceed for 24 h at 37°C. Virus was then purified by polyethylene glycol precipitation and isolated from potassium tartrate gradients as previously described (15, 22).

Dansylation of intact virions. Purified virions were dialyzed against 0.01 M phosphate buffer (pH 7.2) and added to an Erlenmeyer flask on the bottom of which 25 μ Ci of [¹⁴C]dansyl chloride (104 mCi/nmol; Schwarz/Mann) had been dried. The sample was then stirred at room temperature for 2 h to allow for labeling.

Polyacrylamide gel electrophoresis. Ten percent polyacrylamide gels were electrophoresed in sodium dodecyl sulfate-phosphate buffer (pH 7.2) (9) and prepared for liquid scintillation counting by incubation in 10% water-protosol (12).

Purification and fractionation of M protein and CNBr peptides. Purification of the M protein, CNBr digestion, and peptide fractionation followed previously described procedures (26, 27) (see also legends to Fig. 2 and 3).

Identification of dansylated residues. Peptides containing the [¹⁴C]dansyl chromophore were hydrolyzed in 6 N HCl for 18 h. After drying to remove the HCl,

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FIG. 1. Polyacrylamide gel electrophoresis of virion polypeptides after exposure of [^3H]leucine-labeled influenza virions to [^{14}C]dansyl chloride for 2 h at room temperature.

the dansyl-coupled residues were extracted with 50% aqueous pyridine and, after adding a standard mixture of unlabeled dansyl amino acids, were spotted on 7.5-by 7.5-cm polyamide plates. The derivatives were then separated by chromatography in 1.5% formic acid in one direction, followed by perpendicular chromatography in benzene-acetic acid (9:1) (33). The separated derivatives were subjected to autoradiography for 2 weeks at -20°C with Royal X-Omat film. The labeled residues were identified by overlaying the developed film on the original plates with the marked derivatives.

RESULTS

Labeling of influenza virion proteins by dansyl chloride. The exposure of intact virions in phosphate buffer (pH 7.2) to [^{14}C]dansyl chloride resulted in the linear uptake of the dansyl label into trichloroacetic acid-insoluble material for 1.5 to 2 h, at which time the incorporation reached a plateau (data not shown). Analysis of the resulting dansyl-labeled virus by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed coupling of the dansyl chromophore to the M protein and perhaps the HA2 moiety but no significant labeling of the external HA1 and NA glycoproteins (Fig. 1). This observation suggested that the dansyl label diffused into the lipid bilayer by virtue of its hydrophobic ring structure and labeled a portion of the M or HA2 protein which is in proximity to the lipid bilayer. This conclusion was reinforced by the observation that the viral nucleocapsid protein, which is known not to interact with the lipid bilayer, remained unlabeled. To investigate the sites of labeling, dansyl-labeled membrane-protected polypeptides were isolated and the labeled CNBr peptides were characterized.

Purification of dansyl-labeled polypeptides. Virions which had been exposed to [^{14}C]dansyl chloride for 2 h were treated with chymotrypsin to digest the external glycoproteins and any disrupted particles and repurified on a potassium tartrate gradient. The spikeless particles were then disrupted with guanidine-hydrochloride, and the remaining polypeptides were fractionated on a BioRad A5M column in 5 M guanidine (Fig. 2). Two peaks of ^3H -amino acid label were resolved, and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown) demonstrated that they corresponded to the NP and M protein, respectively. An initial peak containing [^{14}C]dansyl label and no ^3H label probably consists of free dansyl label associated with lipids from the viral membrane, since no high-molecular-weight ^{14}C label was found in samples which were treated with alcohol before gel electrophoretic separation (Fig. 1). The only other component containing [^{14}C]dansyl label is the M protein. Since no peak of [^{14}C]dansyl label was found in the included volume of the column, it appeared that no label was present in the membrane-protected portion of the HA2 polypeptide. We calculated that approximately 0.04 to 0.06 mol of dansyl chloride was coupled per mol of M protein.

Fractionation of dansyl-labeled peptides. The labeled M protein was concentrated, treated with CNBr, and analyzed by gel filtration on a Bio-Gel P-6 column to determine which peptide(s) contained [^{14}C]dansyl label. In previous work, we had demonstrated that Bio-Gel P-6 fractionation of [^3H]leucine-labeled CNBr peptides from the M protein yielded six distinct peaks, the third of which contained multiple

FIG. 2. BioRad A5M gel filtration in 5 M guanidine-hydrochloride–0.01 M Tris-hydrochloride (pH 7.2) of polypeptides of ^3H -amino acid-labeled influenza virus particles following [^{14}C]dansyl chloride labeling and chymotrypsin digestion. Purified virus in 0.01 M phosphate buffer (pH 7.2) was digested at 37°C overnight with chymotrypsin at an enzyme-to-viral protein ratio of 5:2 to remove the outer glycoproteins (23). The resulting spikeless particles were rebanded on a 5 to 40% continuous potassium tartrate gradient, dialyzed against 0.01 M sodium phosphate buffer (pH 7.2), and pelleted. The pellet was then disrupted with 8 M guanidine in 0.01 M Tris (pH 7.2). After incubating the guanidine-solubilized particles at 60°C for 3 h, followed by boiling for 1 min, the sample was centrifuged at 2,000 rpm to remove aggregated material and applied to a BioRad A5M column (1.5 by 80 cm) in 5 M guanidine–0.01 M Tris (pH 7.2). Column dimensions, 1.5 by 90 cm; fractions, 1 ml; flow rate, 3 ml/h.

peptides (27). Dansyl label was found to coelute with peptides contained within peaks 1, 2, 3, and 5 (Fig. 3). The amino acid sequence of M deduced from nucleic acid sequencing (2, 6, 32) indicates that 14 peptides should result from CNBr cleavage, and our data indicate that the dansyl label was incorporated into at least 4 of these peptides. We attempted further to define the tryptic peptide(s) labeled within the P-6/3 peak by thin-layer chromatographic separation, as previously described by Schmer and Kreil (28), but due to the large amount of material needed to detect the [^{14}C]dansyl label, migration of all the peptides was drastically altered and adequate separation was not obtained.

Determination of dansyl-labeled residues within Bio-Gel-fractionated components. The coupling of dansyl chloride to a protein results in labeling of α -amino groups, the ϵ -amino group of lysine residues, and the hydroxyl group of tyrosine residues. Since our previous results (27) had indicated that the M protein had no free alpha amino group, the dansyl chloride was likely to be linked to the reactive groups of lysine or tyrosine residues. To determine the labeled residues, the [^{14}C]dansyl-labeled peptides obtained by Bio-Gel P-6 fractionation were subjected to acid hydrolysis, and the labeled residues were

FIG. 3. Bio-Gel P-6 gel filtration in 45% formic acid of ^3H -amino acid-labeled CNBr peptides containing coupled [^{14}C]dansyl label. The purified M protein was suspended in 70% formic acid, and a 30-fold excess by weight of CNBr was added. This solution was held at room temperature overnight to cleave the carboxy terminal side of methionine residues (20). The sample was then desalted on a Bio-Gel P-2 column (0.7 by 35 cm) in 50% formic acid to remove unreacted CNBr. The peptides which eluted were identified by following the tritiated amino acid label and, after pooling, were applied to a 0.7 by 75-cm Bio-Gel P-6 (200/400 mesh) column in 45% formic acid. Fractions (0.35 ml) were collected at a flow rate of 5 fractions per h.

FIG. 4. Autoradiograph of polyamide thin-layer separation of [^{14}C]dansyl amino acids from dansylated P6 peptides after acid hydrolysis (first dimension, 1.5% formic; second dimension, benzene-acetic acid 9:1). A, alanine; G, glycine; E, glutamic acid; D, aspartic acid; S, serine; T, threonine; K, lysine; H, histidine; R, arginine; Y, tyrosine; F, phenylalanine; L, leucine; I, isoleucine; P, proline; V, valine; W, tryptophan. Panel 1, peak 1; panel 2, peak 2; panel 3, peak 3; panel 5, peak 5. Arrow indicates the position of the hydroxyl-labeled tyrosine derivative.

chromatographed as described in Materials and Methods. The autoradiogram revealed that all four peptides contained dansyl label coupled to the ϵ -amino group of lysine residues (Fig. 4), with no label being found in the position of the tyrosine residues (Fig. 4, arrow). Assuming equivalent reactivity of all lysine residues, and based on the fact that the P-6/5 peptide contains only one lysine residue (27), we calculated that the potential number of lysine residues labeled in peaks 1, 2, and 3 were 2, 2, and 5, respectively.

DISCUSSION

The present studies were undertaken to investigate the use of dansyl chloride as a probe for labeling the protein molecules that interact with the virion lipid bilayer. Earlier, Bolognesi et al. (5) added acetone-solubilized dansyl chloride to a suspension of avian myeloblastosis virus and

found the dansyl group covalently coupled to the outer surface glycoproteins. Surprisingly, exposure of purified influenza virions to dansyl chloride under the conditions described resulted in heavy dansylation of the internal membrane protein relative to other components.

Conflicting reports have appeared as to whether M protein is exposed on the exterior of virions and infected cells. There have been reports of recognition by M-specific antibodies on the surface of virions and infected cells (1, 4, 7, 25), although recent evidence indicates that the exposed glycoproteins are responsible for both humoral and cellular immunological response by the infected host (21). Although the molecular interaction of the influenza M protein with the lipid bilayer is not well defined, previous morphological observations (14) and the resistance of M protein to digestion with proteases (11, 15, 29) suggested that it was localized as

FIG. 5. Amino acid sequence of PR8 influenza M protein derived from a nucleotide sequence (6, 32) and CNBr peptides derived from it. Placement of peptides sequenced (27) from WSN strain (HoN₁) are indicated by dashes (or the amino acid obtained) underneath the corresponding CNBr peptide. Peptides identified by sequencing: P6/2, residues 94 to 128; P6/4, residues 166 to 179; P6/5, residues 180 to 189. Theoretical placement of the remaining P6 peptides: P6/1, residues 44 to 93; P6/3, residues 1 to 43, 136 to 165, and 217 to 244; P6/6, a mixture of leucine- and non-leucine-containing peptides, including residues 129 to 135, 193 to 203, and 204 to 211. Areas containing 10 or more uncharged residues are designated by hatching; lysine residues are highlighted by arrows. Q, Glutamic acid; N, asparagine; C, cysteine; M, methionine. For other abbreviations, see the legend to Fig. 4.

a layer on the interior of the lipid bilayer. Since the [¹⁴C]dansyl-labeled fraction of M was also found to be resistant to protease, the selective labeling of the M protein by dansyl chloride supports these previous conclusions and indicates that it is not present in a minor fraction of disrupted virions or in molecules which have a unique surface location. As previous data have localized the dansyl chromophore to the glycerol region of the bilayer membrane (31), the label apparently has partitioned on both sides of the bilayer and is capable of labeling proteins in contact with either surface. Since it would be unlikely that many different segments of M protein are outside the bilayer but resistant to protease, we conclude that the dansyl label has coupled to regions of M protein in proximity to the interior of the lipid bilayer.

Figure 5 depicts schematically a linear map of the influenza M protein derived from its nucleotide sequence (2, 6, 32), showing the peptides resulting from CNBr cleavage and the location of lysine residues. An inspection of the M protein sequence reveals seven areas containing 10 or more uncharged residues (hatched areas), the minimum number of residues needed to traverse a lipid bilayer. Calculations based on the predictive method of Segrest and Feldman (30) indicate that none of these regions contain sequences thought to be capable of transmembrane association, which supports evidence that M protein does not span the lipid bilayer. Nevertheless, M protein has been shown to have an intrinsic ability to associate with lipid vesicles (8, 18) although the exact mechanism of this association is not obvious. With the exception of

the peak 5 peptide (residues 180 to 189), which may react by virtue of the tertiary conformation of the M protein within the virion, each potential dansyl-labeled CNBr peptide contains a lysine residue near the periphery of these uncharged stretches. Three of these uncharged areas, which contain potential dansylated residues (CNBr peptides 1, 2, and 3 from the amino terminus), were also implicated by Gregoriades and Frangione (19) as being areas of membrane association. As nothing is known about the tertiary structure of the M protein within the virion, it is difficult to conclude that preferential labeling of lysines associated with hydrophobic regions has occurred, since such a large number of lysines may be labeled.

The present results indicate that influenza M protein is intimately associated with the lipid bilayer and is capable of being covalently labeled by probes intercalated within the viral membrane. These results are consistent with recent observations by Gregoriades and Frangione with pyrenesulfonyl azide (19). In contrast to the results obtained with pyrenesulfonyl azide, which also labels the membranous segment of glycoproteins (19, 34), dansyl chloride was observed to selectively label the M protein of influenza virus, despite the fact that potential reactive lysine groups are located on either side of the hydrophobic segment of the hemagglutinin (16, 24). Although we cannot rule out the possibility that the external portion of HA2 was labeled, we found no detectable labeling of the external HA1 or NA molecule or the membrane-protected region of HA1. Our results indicate that the labeled M protein is present in at least a

10-fold molar excess over any other component, since a ^{14}C peak of more than 10% of the level of M would have been detected. Therefore, labeling with dansyl chloride may serve as a useful approach to further define areas of the molecules involved in membrane association.

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