A Polar Octapeptide Fused to the N-Terminal Fusion Peptide Solubilizes the Influenza Virus HA_2 Subunit Ectodomain†

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ABSTRACT: As a step toward studying membrane fusion with a simplified molecule, the ectodomain, residues 1−185, of the membrane-anchored subunit HA_2 of the influenza virus haemagglutinin (HA) was solubilized by adding the very polar FLAG octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) to the N-terminal HA_2 fusion peptide. The resulting chimeric protein, F185, when expressed in bacteria, folded spontaneously into a soluble trimer, with a high α-helical content and a high melting temperature, structural characteristics of the low-pH-induced conformation of HA_2. Removal of the FLAG octapeptide by proteolysis with enterokinase converted the soluble molecule to one that aggregated, bound nonionic detergent, and bound to lipid vesicles, properties of the low-pH-induced conformation of HA. Thermolysin treatment of the aggregated protein removed the nonpolar fusion peptide, regenerating soluble trimers of HA_2 (residues 24−185), which is analogous to thermolysin treatment of HA in the low-pH-induced conformation. Thermolysin treatment also dissociates F185 from the detergent−protein complex by removing the fusion peptide. These results suggest that highly polar peptides can be fused to the membrane-binding regions of membrane proteins to increase their solubility. They also indicate that ectodomains of HA_2 made in bacteria have membrane-binding properties similar to those of the same ectodomain generated by low-pH treatment of HA isolated from virus.

The membrane fusion glycoprotein of the influenza virus, the hemagglutinin (HA[1]), is a trimer of identical subunits each composed of two disulfide-linked polypeptide chains, HA_1 and HA_2 (*1*) (Figure 1). The HA_2 polypeptide contains a hydrophobic membrane anchor sequence at its C terminus, which can be removed with bromelain to produce soluble BHA (*2*) (Figure 1), and an uncharged sequence at its N terminus known as the "fusion peptide". The membrane fusion potential of HA, which is essential for infectivity, is activated at the low pH of endosomes in a process that involves extensive refolding and conformational rearrangements of the HA structure (*3*). As a consequence, the hydrophobic fusion peptide is displaced from a buried location in the trimer interface to the top of a 100 Å long α-helical coiled coil, a position in which it could interact directly with a target membrane (*3*−*5*). Photolabeling (*6*, *7*) and proteolysis experiments (*8*) indicate that BHA interacts with liposomes or detergent micelles at low pH through the fusion peptide. The details of the low-pH structural transition were determined by comparing the X-ray structure of a proteolytic fragment of HA, $TBHA_2$ (*5*), with that of BHA (*9*, *10*) (Figure 1). BHA aggregates at the fusion pH, and the soluble fragment $TBHA_2$ is derived by digesting the aggregates first with trypsin, which removes residues 28−328 of HA_1 (*3*), and then with thermolysin, which solubilizes the aggregates by removing 37 N-terminal residues of HA_2, including the fusion peptide (*8*, *11*).

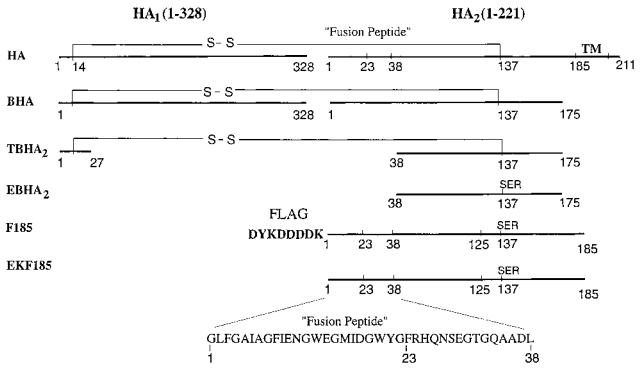

FIGURE 1: Schematic structure of influenza HA and HA_2 ectodomains, including the sequence of the fusion peptide and the FLAG tag. HA_1 and HA_2 chains in viral HA, BHA, and $TBHA_2$ are linked by a disulfide bond. $EBHA_2$ and F185 are recombinant proteins expressed in *Escherichia coli*. EKF185 is the F185 construct after removal of the FLAG tag with enterokinase.

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[1] Abbreviations: HA, hemagglutinin; HA_2, membrane-anchored subunit of HA; $TBHA_2$, proteolytic fragment of the low-pH-induced conformation of HA; $EBHA_2$, *Escherichia coli*-expressed HA_2 residues 38−175; F185, *E. coli*-expressed HA_2 residues 1−185 with the FLAG tag at the N terminus; mAb, monoclonal antibody; IPTG, isopropyl β-D-thiogalactopyranoside.

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Previously, we have described the expression in bacteria, purification, and characterization of the segment of HA_2, residues 38−175, found in $TBHA_2$ (termed $EBHA_2$, Figure 1) (*12*). $EBHA_2$ was found to fold spontaneously in bacteria into a soluble trimer, with the same structural features as the low-pH-induced $TBHA_2$. Both $EBHA_2$ and $TBHA_2$ have similar α-helical content and high thermal stability and form the same products under mild proteolytic treatment, characteristics consistent with both the extensive coiled-coil structure and the location of flexible loops (*12*). Monoclonal antibodies, which specifically recognize HA in the fusogenic state, bind equally well to $EBHA_2$ and $TBHA_2$, and electron micrographs of $EBHA_2$ and $TBHA_2$ show indistinguishable long rods with a knob visible at one end (*12*). These results provided further evidence that the low-pH-induced conformation of HA_2 is its lowest free energy state and that this state can be achieved at neutral pH when expressed without the constraints imposed by HA_1 (*12*). Other evidence suggesting that native HA was in a metastable state, and the low-pH-induced conformation was more stable, includes the following: (1) the original observation that the conformational transition was not reversible (*3*), either for viral HA or for proteolytic products BHA and $TBHA_2$ (Figure 1); (2) the fact that $TBHA_2$ denatured at a higher temperature than native HA (*8*); and (3) the fact that temperature (*8*, *13*, *14*) or the chemical denaturant urea (*15*) could replace low pH in inducing the conformational change.

Here we report the expression and purification of a soluble, complete HA_2 ectodomain in the fusion pH conformation by N-terminal addition of a very polar peptide to solubilize the membrane-binding fusion peptide, and we show that proteolytic removal of the capping peptide results in membrane-binding activity similar to that of the low-pH-induced conformation of viral HA. The molecule F185 (Figure 1), like $EBHA_2$, lacks the C-terminal membrane anchor sequence but is 10 residues longer, extending to the beginning of the transmembrane sequence. The results of expression, purification, and initial structural analyses of this molecule are presented here. Our results are an addition to the cases (*16*−*18*) where sequence engineering has been used to solubilize a membrane-binding protein.

MATERIALS AND METHODS

Bacterial Expression and Protein Purification. cDNA encoding HA_2 residues 1−185 (*19*) of influenza A virus strain X31 was amplified by PCR. The initiating methionine codon and the FLAG peptide codon were inserted using the 5′-oligonucleotide primer CTAGAGGATCCATAAGGAGGATATTAAAATGGACTACAAGCACGATGACAAAGGCCTATTCGGCGCAATAG. This primer also contains a ribosomal binding site and a translational spacer element (*20*). Three rare arginine codons (Arg123, -124, and -127) were changed from AGG to CGT to improve the expression level, and the unpaired cysteine 137 was changed to serine by overlap PCR and restriction fragment replacement (*21*). Plasmid pLM1 with the T7 promoter and bacterial strain BL21(DE3) were used for expression. The correct DNA sequence was confirmed on both strands of the plasmid. Cells transformed with the expression plasmid were grown to the log phase, induced with 1 mM IPTG, and harvested 3 h after induction by centrifugation. The cell pellets were suspended in 10 mM phosphate buffer (pH 7.5) with 150 mM NaCl, 0.5 μg/mL pepstatin, 7 μg/mL PMSF, 1 μg/mL leupeptin, and 1 mM EDTA and lysed by sonication. The cell lysate was centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was loaded on an M2 anti-FLAG affinity column (Biozyme, South Wales, U.K.) under gravity flow. After being washed with 5 column volumes of PBS and being pre-eluted with 50 mM glycine buffer (pH 4), the bound F185 was eluted with 50 mM CAPS (pH 11.5), and 10 mL fractions were collected into vials containing 0.5 mL of 0.5 M Tris-HCl buffer (pH 6.8). Further purification was achieved by size exclusion chromatography (Superdex 200).

Cross-Linking Experiments. F185 (0.30 mg/mL in PBS) was chemically cross-linked with bis(sulfosuccinimidyl) suberate (BS^3) (Pierce, Rockford, IL) (0−10 mM) at room temperature for 1 h followed by incubation for 0.5 h at 4 °C.

CD Spectra. CD spectra of F185 [100 μg/mL in 150 mM KF (pH 7.0)] were obtained with an Aviv 62 DS spectropolarimeter equipped with a thermoelectric temperature controller. The thermal denaturation curve was obtained by monitoring the change in the CD signal at 222 nm using a scan rate of 1 °C/min in the range of 20−95 °C.

Western Blot Analysis. Proteins separated by SDS−PAGE were eletrophoretically transferred onto an Immobilon-P membrane (Millipore, Bedford, MA) using a semi-dry blot apparatus (Owl Scientific, Mansfield, OH) for 30 min at 400 mA in transfer buffer [48 mM Tris-HCl (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol]. The membrane was blocked with 2% nonfat milk in PBS for 1 h followed by incubation with antibody. The blotted membrane was developed using the Protoblot western blot alkaline phosphatese system (Promega, Madison, WI).

Protease Digestion. Incubation of F185 (2 μg/mL in TBS, with 1 mM $CaCl_2$) with enterokinase (1 unit/μL) at 37 °C for 20 h cleaves the FLAG tag, producing an HA_2 polypeptide of residues 1−185. The digestion was terminated by the addition of EDTA (20 mM).

Thermolysin was used to cleave off the fusion peptide of HA_2, as previously described (*8*). The reaction mixture containing EKF185 (1.0 mg/mL in TBS, with 1 mM $CaCl_2$) and thermolysin (2%, w/w) was incubated at 37 °C for 2 h. The reaction was terminated with EDTA (20 mM).

Detergent Binding. F185 (1 mg/mL in TBS) was mixed with 0.5% polyoxyethylene 10 lauryl ether (Brij36T, Sigma, St. Louis, MO) and the mixture incubated at room temperature for 1 h. The mixture was analyzed by size exclusion chromatography (Biosep Sec3000, Phenomenex, Torrance, CA) in TBS containing 0.5% Brij36T. Fractions corresponding to each peak were collected and analyzed by SDS−PAGE.

Lipid Binding. Lipid vesicles (*22*) containing 80 mol % 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 20 mol % 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) (Avanti Polar Lipids, Alabaster, AL) were prepared by extruding the mixture [50 mM phosphate buffer and 1 M NaCl (pH 7.5)] through polycarbonate filters with a pore size of 100 nm (Avestin, Ottawa, Canada) five times. Lipid and protein were mixed at a 4×10^4 molar ratio and incubated in the presence or absence of enterokinase (5 units/μg of F185) at 37 °C for 30 h. At the end of the incubation, the mixtures were made 30% in sucrose (in TBS), overlaid with 5 mL sucrose gradients from 0 to 7.5%, and centrifuged at 100000*g* at 4

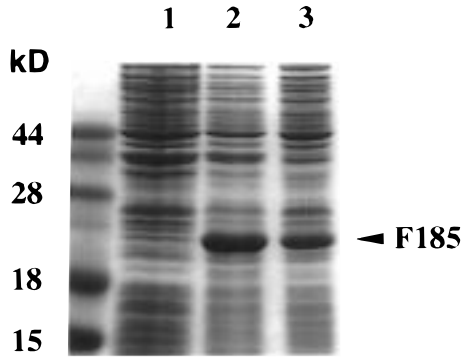

FIGURE 2: SDS−PAGE of expression of F185 in *E. coli*: lane 1, bacterial lysate from uninduced cells; lane 2, lysate from cells induced by 1 mM IPTG for 3 h; and lane 3, supernatant of the lysate centrifuged at 100000*g* at 4 °C for 1 h. The induced band at 21 kDa stays in the supernatant.

°C for 12 h. A high salt level was used to block electrostatic interaction between the highly charged FLAG peptide and the liposomes. Five fractions were collected from each sucrose gradient and analyzed by SDS−PAGE followed by Western blotting using anti-HA polyclonal antibody from rabbit.

RESULTS

HA_2 Residues 1−185 Can Be Solubilized by Fusion to a Very Polar Peptide. A chimera of HA_2 residues 1−185 with the octapeptide (FLAG), Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, on its N terminus was expressed in *Escherichia coli*. The FLAG peptide has been used as an epitope tag for purification and immobilization of recombinant proteins (*23*, *24*). It was chosen here on the basis of its highly charged, polar sequence and for the specific enterokinase cleavage site at its C terminus. SDS−PAGE of uninduced (lane 1 of Figure 2) and IPTG-induced cell lysates (lane 2 of Figure 2) from bacteria expressing the chimera demonstrates the overproduction of the protein, F185, after induction. After centrifugation, the protein was found in the soluble fraction (lane 3 of Figure 2). The gel filtration chromatogram of F185 (Figure 3A) shows two major peaks corresponding to soluble aggregates (peak 1 of Figure 3A) and trimeric F185 (67 kDa) (peak 2 of Figure 3A). Fractions corresponding to the trimeric F185 peak were collected, concentrated, and analyzed on a second size exclusion chromatography column (Figure 3B), where F185 eluted predominantly as a single peak at the expected elution volume of the F185 trimer. The F185 trimer was soluble to at least 20 mg/mL, suggesting that addition of the hydrophilic FLAG peptide suppresses aggregation of most of the protein.

The oligomeric state of F185 was confirmed by chemical cross-linking. Fractions corresponding to both peaks of Figure 3A were collected and cross-linked with BS^3. The reaction mixtures were analyzed by SDS−PAGE (Figure 3C). After chemical cross-linking, F185 collected from peak 1 of Figure 3A shifted to the top of the gel (lanes 1−3 of Figure 3C), indicating aggregation of these molecules. Unaggregated F185 collected from peak 2 of the gel filtration column cross-linked into two new species (lanes 4−6 of Figure 3C), migrating at 44 and 66 kDa, corresponding to a dimeric and trimeric form of the F185, respectively.

The FLAG Peptide−HA_2 Residues 1−185 Chimera Folds into the Low-pH-Induced Conformation. The CD spectrum

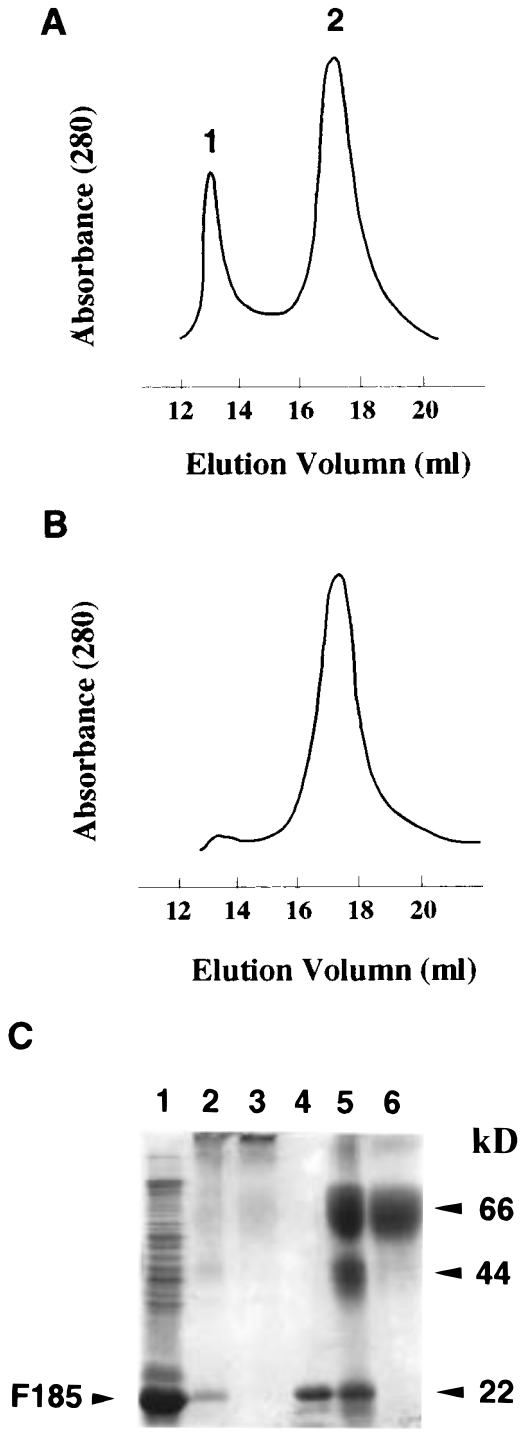

FIGURE 3: Oligometric states of F185. (A) FPLC gel filtration chromatograms of purified F185: peak 1, aggregates at void volume of the column; and peak 2, trimeric F185. (B) Peak 2 from the top chromatogram was collected and reloaded to the column. It elutes as a single trimeric peak. (C) SDS−PAGE of chemically cross-linked F185. Fractions collected from peak 1 (lanes 1−3) and peak 2 (lanes 4−6) were cross-linked with 0, 5, and 10 mM BS^3, respectively. Bands at 22, 44, and 66 kDa correspond to the monomer, dimer, and trimer, respectively.

of F185 (Figure 4A) shows the characteristic minima at 208 and 222 nm of an α-helix. Similar results were reported for $EBHA_2$ (*12*) and $TBHA_2$ (*8*). The thermal denaturation curve obtained by monitoring the change in the CD signal at 222 nm (Figure 4B) shows a single, cooperative transition centered at 80.5 °C. This is comparable to the thermal unfolding transition of $EBHA_2$ (T_m = 70 °C) (*12*), $TBHA_2$

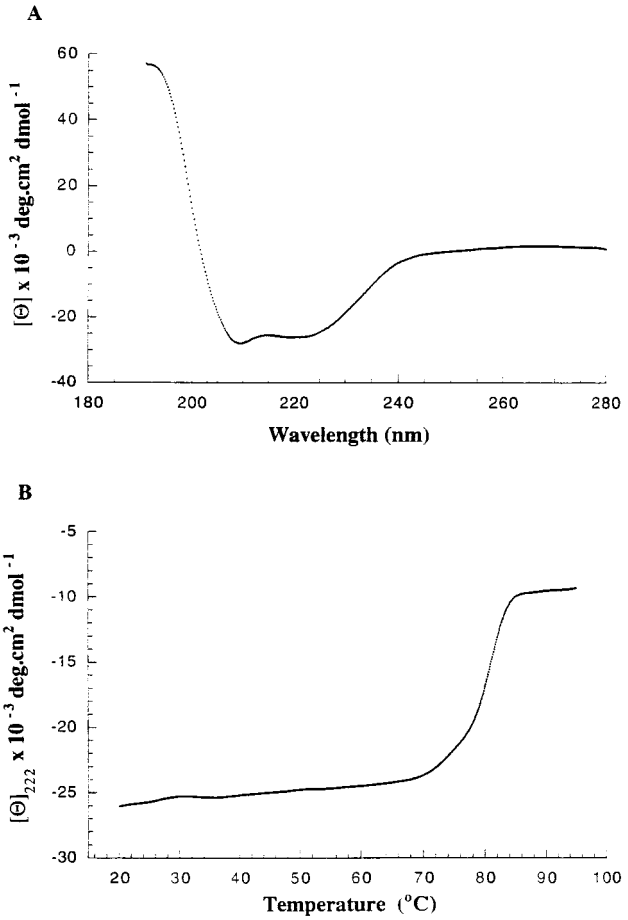

FIGURE 4: (A) Circular dichroism spectrum of F185. Minima at 208 and 222 nm are characteristic of α-helices. (B) Thermal denaturation curve of F185 at pH 7.0.

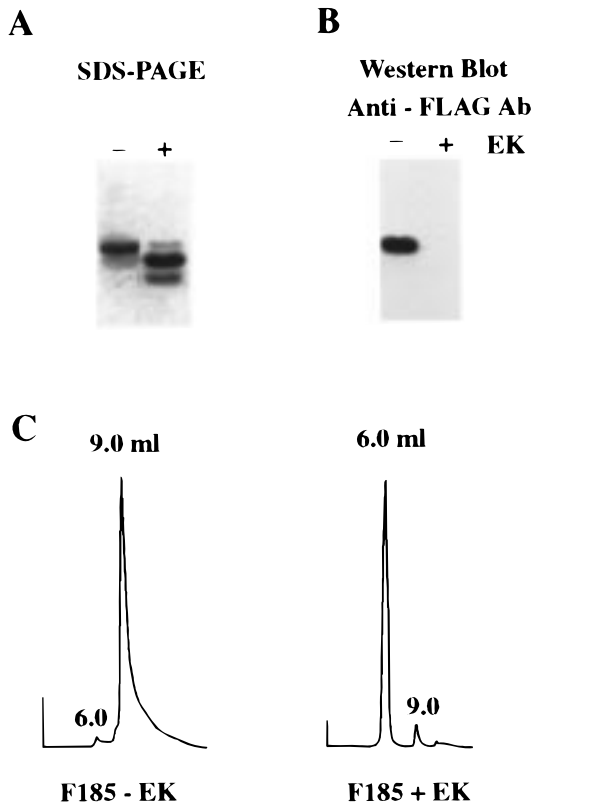

FIGURE 5: Removal of the FLAG octapeptide of F185 by enterokinase digestion. (A) SDS−PAGE of F185 with or without enterokinase (EK) treatment. (B) Immunoblotting analysis of F185 before and after enterokinase treatment. Proteins from SDS−PAGE were blotted electrophoretically onto nitrocellulose paper. After separate incubations with monoclonal antibodies, immunocomplexes were detected using anti-mouse Ig conjugated to horseradish peroxidase. (C) HPLC gel filtration chromatography elution patterns of F185 and F185 cleaved with enterokinase. About 30 μg of the protein was loaded onto the analytical size exclusion column (Biosep Sep3000) and eluted in TBS [10 mM Tris buffer with 150 mM NaCl (pH 7.5)]. Aggregated molecules after enterokinase treatment (EKF185) elute in the void volume at 6.0 mL.

(T_m = 76 °C) (*8*), and the low-pH-treated BHA_2 (T_m = 78 °C) (*8*).

Monoclonal antibody IIF4 was reported to recognize the low-pH-treated viral HA but not the neutral-pH form (*25*, *26*). Immunoprecipitation of F185 from solution by Protein-A beads coupled with mAb IIF4 indicates that F185 has a high affinity for IIF4 (data not shown).

Removal of the FLAG Peptide Results in EKF185. Enterokinase cleaves after the five-amino acid recognition sequence at the C terminus of the FLAG octapeptide and was used to remove the FLAG peptide from F185. Enterokinase-treated F185 migrates as a slightly lower-molecular mass band (+ lane of Figure 5A) than F185 (− lane of Figure 5A) on SDS−PAGE, suggesting removal of the FLAG peptide. Removal was further demonstrated by immunoblotting with a monoclonal antibody specific for the FLAG peptide, which only recognized F185 (− lane of Figure 5B), not the enterokinase-treated F185 (+ lane of Figure 5B), indicating quantitative removal of the FLAG by enterokinase treatment. HPLC size exclusion chromatography was used to analyze the oligomeric state of F185 before and after the treatment with enterokinase (Figure 5C). Results show that before the enterokinase treatment, trimeric F185 eluted at the expected position of the trimer (9.0 mL) (F185 − EK in Figure 5C). The enterokinase-digested product, which has a lower molecular mass as shown by the mobility on SDS−PAGE (+ lane of Figure 5A), eluted at the void volume of the column (6.0 mL) (F185 + EK in Figure 5C), indicating that in the absence of the FLAG peptide EKF185 aggregates. Thus, enterokinase treatment shifted the elution volume of trimeric F185 from 9.0 to 6.0 mL, which is consistent with the conclusion that the loss of the FLAG peptide causes the HA_2 (residues 1−185) molecule, termed EKF185, to aggregate.

Thermolysin Removes the Fusion Peptide and Solubilizes the Aggregated EKF185. Digestion of low-pH BHA_2 protein micelles with thermolysin results in removal of the N-terminal residues of HA_2 and solubilization of the aggregates (*8*). Thermolysin first cleaves low-pH-treated BHA_2 at residue 24 (*8*). Increasing the time of digestion yields fragments identified as HA_2 residues 38−175 (*8*) and with further digestion a 10 kDa fragment, residues 38−125 (*26*). Thermolytic digestion of aggregated trimers of EKF185, generated by enterokinase treatment of F185, also produced soluble fragments (Figure 6). These fragments were separated by gel filtration chromatography and analyzed by SDS−PAGE. The first peak of the gel filtration chromatogram contained undigested EKF185 as well as some partially digested trimers where one or two monomers have been cleaved (lane 1 of Figure 6B). The second peak fraction eluted at the position expected for a trimer and contained a 19 kDa fragment and a 10 kDa fragment (lane 2 of Figure

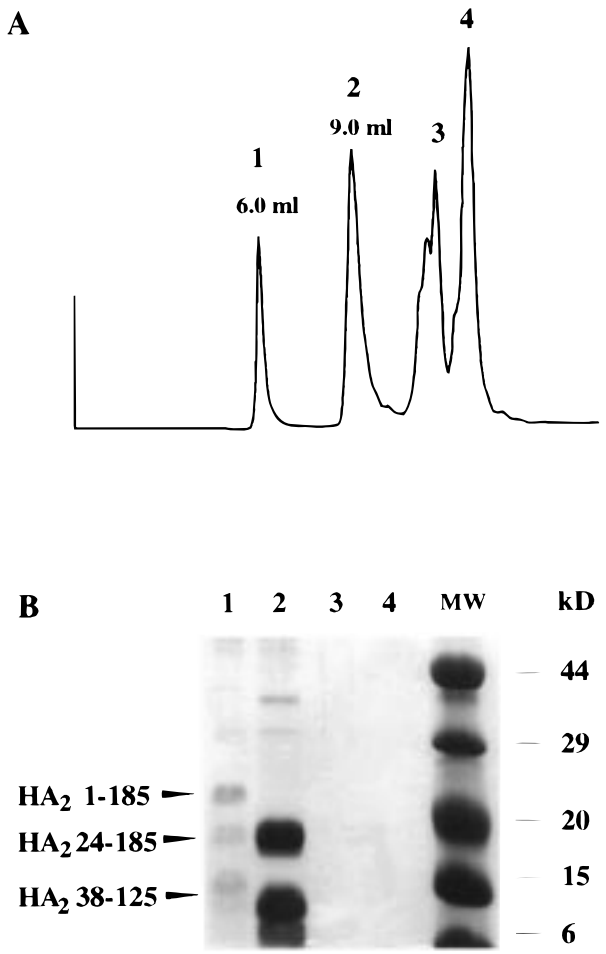

FIGURE 6: Thermolysin digestion of EKF185. (A) HPLC size exclusion chromatography of a thermolysin-cleaved HA_2 mixture: peak 1, uncleaved HA_2 aggregates; peak 2, soluble fragments of HA_2 residues 24−185 and HA_2 residues 38−125; and peaks 3 and 4, unidentified peptides. (B) SDS−PAGE of peaks 1−4.

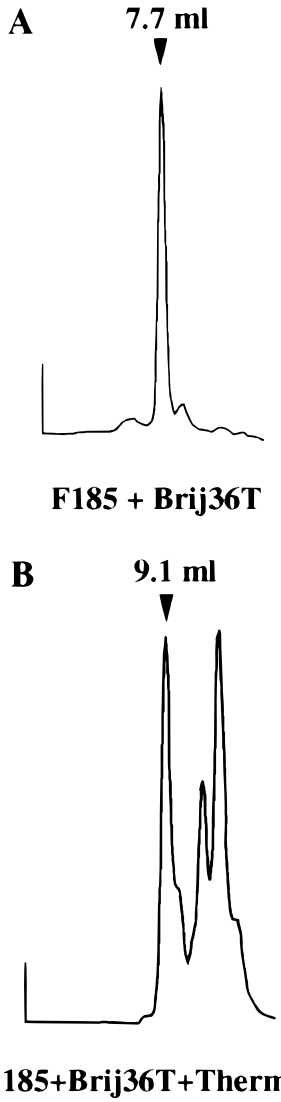

FIGURE 7: HPLC gel filtration elution patterns of F185 in detergent. (A) F185 incubated with Brij36T elutes with the detergent micelles at a position corresponding to 250 kDa (7.7 mL). (B) Thermolysin-cleaved HA_2. The peak from panel A was collected and treated with thermolysin. The peak at 9.1 mL is the size of trimeric HA_2 with the fusion peptide removed. Thermolysin treatment dissociates F185 from the detergent−protein complex by removing the fusion peptide.

6B). N-Terminal amino acid sequencing and mass spectrometry identified the 19 kDa fragment in the trimer peak as HA_2 residues 24−185. Judged by the mobility on SDS−PAGE, the second band was the same as the 10 kDa fragment of the thermolysin digestion product observed in the BHA_2 digestion, HA_2 (residues 38−125) (*26*). No protein was visible for fractions corresponding to peaks 3 and 4 on SDS−PAGE (lanes 3 and 4 of Figure 6B), which probably contain short peptides from the thermolysin cleavage.

F185 Binds to Nonionic Detergent. Low-pH treatment of BHA in the presence of nonionic detergent results in binding of BHA to detergent micelles by the exposed fusion peptide (*3*). Incubation of F185 with Brij36T and subsequent analysis of the mixture by gel filtration chromatography (Figure 7A) indicated that F185 bound detergent at neutral pH. Detergent−F185 complexes eluted from a gel filtration column as a single peak at a 7.7 mL elution volume, which corresponds to a molecular mass that is larger than that of the trimeric F185 (9.0 mL, Figure 5C) and smaller than that of aggregated trimers (6.0 mL, Figure 5C). Fractions corresponding to the F185−Brij36T complex peak (Figure 7A) were collected, concentrated, and treated with thermolysin to remove the fusion peptide. Analysis of the thermolysin cleavage products on gel filtration chromatography (Figure 7B) showed a discrete peak at the expected position of trimeric HA_2 (residues 24−185) (9.1 mL) and two peaks corresponding to peptides. This result indicates that the fusion peptide was responsible for binding of F185 to detergent, as previously established for the low-pH-treated BHA (*3*). Thus, the FLAG peptide prevents protein−protein micelle formation by F185 in the absence of detergent, but nevertheless, the fusion peptide in F185 appears to be capable of interacting with detergent.

F185 Has a Higher Affinity for Lipid Vesicles in the Absence of the FLAG Peptide. In the absence of enterokinase treatment, analysis of a mixture of F185 and lipid vesicles by sucrose gradients and Western blotting showed that F185 remains in the 30% sucrose fraction (lanes 1−5 of Figure 8) while lipid vesicles floated to the top of the sucrose gradients. This result indicates the F185 did not bind to the lipid vesicles containing 80% POPC and 20% POPG. Incubation of F185 with lipid vesicles in the presence of enterokinase results in partial cleavage of the FLAG peptide

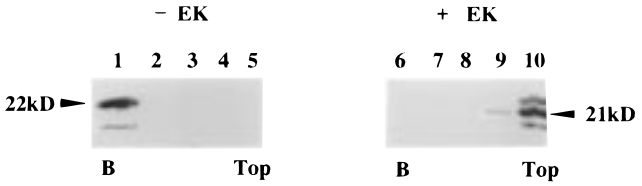

FIGURE 8: Binding of F185 to lipid vesicles. F185/lipid mixtures were incubated at 37 °C for 30 h in the absence or presence of EK, and then made 30% in sucrose, overlaid with sucrose gradients from 0 to 7.5%, and centrifuged to reach equilibrium. Fractions from the gradients were analyzed by Western blot methods using the anti-HA antibody. In lanes 1−5, mixtures without EK treatment show F185 at the bottom of the gradient. In lanes 6−10, F185 cleaved with EK in the presence of lipid shows F185 with the FLAG octapeptide removed (EKF185) has bound lipid and floated to the top of the gradient with the lipid vesicles.

(~21 kDa band, lane 10 of Figure 8), and the protein was detected in the same fraction of the lipid vesicles (about 5% sucrose) (lanes 6−10 of Figure 8), indicating binding of the protein to the lipid vesicles upon enterokinase removal of the FLAG peptide. We observed that for some lipid compositions (e.g., 1:1:1:1.5 phosphatidylethanolamine/phosphatidylcholine/sphingomyelin/cholesterol) F185 associated with lipid vesicles even in the absence of enterokinase cleavage (data not shown), consistent with the indication that the fusion peptide may be available under some conditions as in the detergent binding result above or that F185 may interact with these liposomes in some other way, perhaps electrostatically.

DISCUSSION

The structure of the native state of the influenza virus HA (*9*, *10*) indicated that the N-terminal nonpolar fusion peptide of the HA_2 subunit was buried in the trimer interface about 100 Å from the distal tip of the spike glycoprotein. The fusion peptide was found at the end of a short α-helix that was part of a helical hairpin; the short helix is followed in the sequence by an unstructured loop and a long α-helix that packed antiparallel to it. Low pH induced two remarkable conformational changes in this helical hairpin. The extended loop and short helix became an extension of the central triple-stranded coiled coil, springing out of the helical hairpin like a blade out of a jackknife, moving the fusion peptide over 100 Å toward the target membrane (*4*, *5*). Equally dramatically, the middle of the original long α-helix unfolded to form a reverse turn, and the second half of the long α-helix jackknifed back to lie antiparallel against the first half of that α-helix (*5*), like the second blade of a jackknife being folded back into the handle, again moving structures more than 100 Å, this time structures preceding the C-terminal anchor.

The proximity of the N-terminal fusion peptide and the C-terminal membrane anchor at one end of a rod-shaped molecule was also observed in the core of the HIV-1 membrane fusion protein, gp41 (*27*−*29*). The fact that HA and gp41 share this structural feature suggests that it may be important in the mechanism of protein-mediated membrane fusion, possibly by facilitating apposition of the viral and host membranes during the fusion process (*28*). A complex of the v-SNARE and t-SNARE molecules involved in the fusion of synaptic vesicles to the plasma membrane in neurons has also been shown, by electron microscopy, to have the same characteristics as these viral membrane fusion proteins; the membrane anchors of the proteins in the two membranes to be fused are found at the same end of a rod-shaped complex (*30*). Recent data indicated that the rod-shaped complex of apposing SNARE molecules alone may have a minimal fusion activity (*31*).

The F185 molecule, a solubilized form of the complete HA_2 ectodomain (Figure 1), expressed and studied in this work appears to adopt spontaneously the rod-shaped conformation induced in the native HA by fusion pH. Like $EBHA_2$ (Figure 1), a soluble fragment of HA_2 residues 38−175 expressed in bacteria, F185 spontaneously folds into trimers inside the bacteria, without any low-pH treatment (*12*). This was indicated by chemical cross-linking and size exclusion chromatography (Figure 3). The oligomeric state, high α-helical content, thermal stability (Figure 4), and antibody binding specificity of the F185 are all similar to the characteristics of both $TBHA_2$ prepared by low-pH treatment of viral HA (*8*) and $EBHA_2$ expressed in bacteria (*12*). A further indication of the similarity of the conformation of F185 and both $TBHA_2$ and $EBHA_2$ is that it was cleaved by thermolysin treatment at the same places as in those two molecules (Figure 6), and the sites of cleavage were flexible loops in the $TBHA_2$ X-ray structure (*5*, *12*). Both $TBHA_2$ and $EBHA_2$ lack the N-terminal fusion peptide, which is present in F185.

Solubilization of the fusion peptide containing F185 by the N-terminal FLAG octapeptide was designed to allow manipulation of the rod-shaped conformation in studying its interaction with membranes. As expected, removal of the FLAG octapeptide by enterokinase treatment resulted in aggregation of the HA_2 (residues 1−185) ectodomains (Figure 5). That this aggregation was due to the fusion peptide was demonstrated by the solubilization of these aggregates by thermolytic cleavage which removes the fusion peptide (Figure 6). Gel filtration chromatography in the presence of the detergent Brij36T indicated that F185 binds detergent, even with the FLAG peptide in place (Figure 7). Thermolysin digestion of F185 bound to the detergent led to the release of HA_2 residues 24−185 from the detergent micelles, indicating that F185 binds to nonionic detergent by the fusion peptide (Figure 7). With lipid vesicles of some compositions, F185 would only associate with the vesicles after removal of the FLAG octapeptide with enterokinase, suggesting that removal of the hydrophilic sequence led to increased exposure of the nonpolar fusion peptide (Figure 8). A recombinant HA_2 fragment of residues 1−127, containing the fusion peptide and the coiled-coil region, has been reported to be membrane-bound (*32*). It may be necessary to add larger more polar sequences at the N terminus of F185 to block completely binding of an F185-like molecule to detergent micelles and lipid vesicles of all compositions. By addition of the C-terminal membrane anchor and expression of the F185-like molecules in the membranes of cells, it may be possible to determine whether this defined conformation of the HA can carry out membrane fusion. The current results with F185 provide evidence that the conformation of HA defined in the $TBHA_2$ crystal structure has membrane-binding behavior similar to that of the low-pH-induced conformation of the viral molecules, adding to the evidence that this is the conformation involved in membrane fusion.

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REFERENCES

1. Wiley, D. C., Skehel, J. J., and Waterfield, M. (1977) *Virology 79*, 446−448.
2. Brand, C. M., and Skehel, J. J. (1972) *Nat. New Biol*. *238*, 145−147.
3. Skehel, J. J., et al. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 968−972.
4. Carr, C. M., and Kim, P. S. (1993) *Cell 73*, 823−832.
5. Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) *Nature 371*, 37−43.
6. Harter, C., Bachi, T., Semenza, G., and Brunner, J. (1988) *Biochemistry 27*, 1856−1864.
7. Harter, C., James, P., Bachi, T., Semenza, G., and Brunner, J. (1989) *J. Biol. Chem. 264*, 6459−6464.
8. Ruigrok, R. W., et al. (1988) *J. Gen. Virol. 69*, 2785−2795.
9. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) *Nature 289*, 366−373.
10. Wiley, D. C., Wilson, I. A., and Skehel, J. J. (1981) *Nature 289*, 373−378.
11. Daniels, R. S., Douglas, A. R., Skehel, J. J., and Wiley, D. C. (1983) *J. Gen. Virol. 64*, 1657−1662.
12. Chen, J., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12205−12209.
13. Wharton, S. A., Skehel, J. J., and Wiley, D. C. (1986) *Virology 149*, 27−35.
14. Ruigrok, R. W. H., et al. (1986) *Virology 155*, 484−497.
15. Carr, C. M., Chaudhry, C., and Kim, P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14306−14313.
16. Sueyoshi, T., Park, L., Moore, R., Juvonen, R., and Negishi, M. (1995) *Arch. Biochem. Biophys. 322* (1), 265−271.
17. Chen, G., and Gouaux, J. E. (1996) *Protein Sci. 5*, 456−467.
18. Tomich, J. M., et al. (1998) *Biophys. J. 74*, 256−267.
19. Steinhauer, D. A., Wharton, S. A., Skehel, J. J., Wiley, D. C., and Hay, A. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11525−11529.
20. Garboczi, D. N., Hung, D. T., and Wiley, D. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3429−3433.
21. Rashtchian, A., Thornton, C. G., and Heidecker, G. (1992) *PCR Methods Appl*. *2*, 124−130.
22. Montich, G. G., Montecucco, C., Papini, E., and Marsh, D. (1995) *Biochemistry 34*, 11561−11567.
23. Nilsson, J., Larsson, M., Stahl, S., Nygren, P. A., and Uhlen, M. (1996) *J. Mol. Recognit. 9* (5−6), 585−594.
24. Chubet, R. G., and Brizzard, B. L. (1996) *BioTechniques 20* (1), 136−141.
25. Russ, G., Polakova, K., Kostolansky, F., Styk, B., and Vancikova, M. (1987) *Acta Virol. 31*, 374−386.
26. Wharton, S. A., et al. (1995) *EMBO J. 14*, 240−246.
27. Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997) *Cell 89*, 263−273.
28. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997) *Nature 387*, 426−430.
29. Tan, K., Liu, J., Wang, J., Shen, S., and Lu, M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12303−12308.
30. Hanson, P., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. (1997) *Cell 90*, 523−535.
31. Weber, T., et al. (1998) *Cell 92*, 759−772.
32. Kim, C., Macosko, J., Yu, Y., and Shin, Y. (1996) *Biochemistry 35* (17), 5359−5365.

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