Cell Surface Expression and Orientation in Membranes of the 44-Amino-Acid SH Protein of Simian Virus 5

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Antiserum was raised against a synthetic peptide containing the N-terminal hydrophilic domain of the small hydrophobic protein (SH) of simian virus 5 (SV5) and used to characterize properties of the SH protein. SH demonstrated properties of an integral membrane protein. Indirect immunofluorescence experiments showed that the protein is involved in the exocytotic pathway, and isolation of plasma membranes from SV5-infected cells showed an enrichment of SH, indicating that SH is transported to the infected-cell surface. Biochemical analysis of the orientation of SH in membranes by proteolysis of intact SV5-infected cell surfaces and intracellular microsomal vesicles indicated that SH is oriented in membranes with its N-terminal hydrophilic domain exposed on the cytoplasmic face of the plasma membrane and the C terminus of approximately five amino acid residues exposed at the cell surface. These data are discussed with respect to positive-acting signals being necessary in the ectodomain of SH for cell surface expression.

The parainfluenza virus simian virus 5 (SV5) is a prototype of the paramyxovirus family of negative-strand RNA viruses and has an RNA genome of approximately 15,000 nucleotides. SV5 encodes two integral membrane glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), which are expressed at the infected-cell surface and form spikelike projections on the outer surface of the virion (4, 5, 31, 32). It has been demonstrated by purification of the glycoproteins, together with biological expression of cloned cDNA of the HN and F mRNAs, that HN has receptor-binding activity (hemagglutination) and neuraminidase activity, while the F protein mediates fusion of the viral envelope with the plasma membrane (24, 31, 32).

In addition to F and HN, we have described an additional gene (for SH) between F and HN on the virion 50S RNA and have identified its ~292-nucleotide mRNA and its encoded protein (SH), which is very small and hydrophobic (12, 13). The SH protein can be divided into two domains, an N-terminal hydrophilic region of ~16 amino acid residues and an extensively hydrophobic region of 23 amino acid residues located near the C terminus. Examination of the relative hydrophobicity of this region, compared with that of other known proteins, suggests that it is sufficiently hydrophobic to interact in a stable manner with membranes and that it could be a third SV5-encoded integral membrane protein (13).

Several small proteins with extensively hydrophobic domains have been identified as encoded by negative-strand RNA viruses, e.g., M₂ protein of influenza A virus (18), NB of influenza B virus (41), 1A protein of respiratory syncytial virus (RS virus) (7), and SH of SV5 (13), but their roles in the replicative cycle of these viruses remain to be investigated. Although the subcellular localization and membrane orientation of the influenza virus proteins M₂ and NB have been determined (18, 41, 44), little is known about RS virus 1A or SV5 SH. The M₂ protein is an integral membrane protein expressed at the influenza virus-infected cell surface and is orientated in membranes with an extracellular N-terminal domain of 18 to 23 residues and a C-terminal cytoplasmic

domain of ~56 residues (18, 44). The NB protein of influenza B virus (34) is also an integral membrane protein which is expressed at the infected cell surface (41). NB is anchored in membranes in the same orientation as M₂; however, unlike M₂, NB is a glycoprotein and contains two N-linked carbohydrate chains (41). These oligosaccharides are capable of being further modified by the addition of polylactosaminoglycan as NB is transported to the cell surface (42).

We describe here experiments designed to characterize properties of the SH protein of SV5. The SH protein is shown to have properties of an integral membrane protein and is expressed at the infected-cell surface. The protein is orientated in membranes with its N-terminal hydrophilic domain exposed on the cytoplasmic face of the plasma membrane, suggesting that only approximately five amino acid residues of the C terminus of SH are exposed at the cell surface.

MATERIALS AND METHODS

Preparation of antisera. Synthesis of peptides and immunization of rabbits were performed as described elsewhere (29). The SH peptide (NH₂-L-P-D-P-E-D-P-E-S-K-K-A-T-R-R-A-G-COOH) was coupled to keyhole limpet hemocyanin in 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. The reaction was terminated by using 0.2 M lysine.

Rabbits were injected subcutaneously with 1 mg of conjugated peptide (1 mg/ml) emulsified with 1 ml of Freund complete adjuvant. After 3 weeks, the rabbits were injected with 1 mg of conjugated peptide (1 mg/ml) emulsified with 1 ml of Freund incomplete adjuvant; this injection was repeated at week 5. At week 7, the rabbits were bled and sera were tested. At week 8, rabbits were boosted by intravenous injection of 2 mg of free peptide in 1 ml of PBS. Further boosting with free peptide was performed at 3-week intervals.

Preparation of monospecific IgG. Immunoglobulin G (IgG) antibodies were purified by affinity chromatography by using synthetic peptide coupled to Affigel-10 resin (Bio-Rad Laboratories, Richmond, Calif.). Antibodies were eluted from the peptide by using 0.1 M citrate buffer (pH 2.5) containing

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10% dioxane. Monospecific antisera to HN and F were those described previously (23). The monoclonal antibodies to HN (28) used in the immunofluorescence experiments were kindly provided by Rick Randall of St. Andrews University, St. Andrews, Scotland, United Kingdom.

Viruses and cells. SV5 W3A was used except where stated. SV5 2WR was obtained from the American Tissue Culture Collection. Monolayer cultures of the TC7 clone of CV1 cells were grown in Dulbecco modified Eagle medium (DME) containing 10% fetal bovine serum.

Metabolic labeling of polypeptides, immunoprecipitation, and polyacrylamide gel analysis. Cells were labeled with ^{35}S -TranS-label (ICN Pharmaceuticals Inc., Irvine, Calif.), [^{35}S]methionine, [^3H]phenylalanine plus [^3H]isoleucine, or [^3H]phenylalanine plus [^3H]leucine as described previously (18). Immunoprecipitations were done as described previously (2). Briefly, cells were lysed in 1% sodium dodecyl sulfate (SDS), and the lysates were boiled for 5 min and diluted with 4 volumes of dilution buffer prior to the addition of antisera. The lysates were incubated with antisera for 2 to 6 h before the addition of agarose beads containing *Staphylococcus aureus* protein A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described elsewhere (13, 16) or, where indicated, on 9% highly cross-linked polyacrylamide gels containing 8 M urea (36).

Cell fractionation. The procedures used were essentially those described previously (8, 17). Confluent monolayers of CV1 cells were infected with SV5 (10 PFU per cell) and incubated in DME containing 5 μg of actinomycin D per ml (22). At 16 h postinfection, infected cells were labeled with 100 μCi of ^{35}S -TranS-label per plate in 2 ml of DME lacking methionine and cysteine for 15 min at 37°C. Cells were washed with 5 ml of DME containing 2 mM methionine; cells from five plates were harvested in ice-cold PBS (pulse), and five plates were incubated in DME for 1 h at 37°C before the cells were harvested in ice-cold PBS (pulse-chase). The cells were pelleted (1,000 $\times g$, 5 min), suspended in 6 ml of ice-cold reticulocyte standard buffer (RSB; 0.01 M KCl, 0.0015 M MgCl_2 , 0.01 M Tris hydrochloride [pH 7.4]), held on ice for 15 min, and disrupted with 20 strokes of a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation (1,000 $\times g$, 5 min), and the supernatant was mixed with an equal volume of 60% (wt/wt) sucrose in RSB. The resulting extract in 30% sucrose was included in a discontinuous gradient of 0, 25, 30, 40, 45, and 60% (wt/wt) sucrose-RSB solutions and was centrifuged at 22,000 rpm for 19 h in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Visible bands were collected with a syringe, diluted with RSB, and pelleted at 45,000 rpm for 30 min in a Ti60 rotor (Beckman). Pellets were resuspended in 0.5 ml of RSB, and aliquots were adjusted to a concentration of 10% in trichloroacetic acid and held on ice for 30 min. Proteins were recovered by centrifugation (14,000 rpm, 15 min), and the pellets were washed in ethanol and solubilized in gel lysis buffer for SDS-PAGE.

Cell surface trypsinization. Confluent monolayers of SV5-infected CV1 cells were labeled with ^{35}S -TranS-label at 12 h postinfection for 1 h, the label was removed, and the cells were incubated in DME for a further 3 h. Cells were harvested with a silicon rubber policeman, resuspended in PBS, and treated with 200 μg of tosylamide-phenylethyl chloromethyl ketone-treated trypsin (Organon Teknika, Malvern, Pa.) per ml for 40 min at 37°C. The reaction was terminated by the addition of 1,000 U of trypsin inhibitor

(Sigma Chemical Co., St. Louis, Mo.), followed by boiling in 1% SDS.

Trypsin treatment of microsomes. Microsomes were prepared essentially by the method of Adams and Rose (1). SV40 recombinant virus-infected cells were labeled with [^{35}S]methionine or [^3H]phenylalanine plus [^3H]leucine, homogenized, and fractionated by centrifugation through a 0.7-ml 10% sucrose cushion at 45,000 rpm at 4°C for 40 min by using a TLS-55 swinging bucket rotor in a TLA-100 tabletop ultracentrifuge (Beckman). The soluble and microsomal fractions were treated with 200 μg of tosylamide-phenylethyl chloromethyl ketone-treated trypsin for 60 min at 37°C. The digestion was terminated by the addition of 1,000 U of trypsin inhibitor and by boiling in 1% SDS. Protected products were analyzed by immunoprecipitation and SDS-PAGE.

Alkali treatment of microsomes. Microsomes were prepared as described above (1) with slight modifications. SV5-infected cells were labeled with ^{35}S -TranS-label or [^3H]phenylalanine plus [^3H]leucine for 1 h and disrupted in a Dounce homogenizer, and microsomes were prepared by centrifugation through a 0.7-ml 10% sucrose cushion (45,000 rpm, 4°C, 40 min) by using a TLS-55 swinging bucket rotor in a TLA-100 tabletop ultracentrifuge (Beckman). Microsomal pellets were suspended in 0.3 ml of 50 mM triethanolamine (pH 7.5) and adjusted to pH 11.0 by the addition of 1 N NaOH (25). After incubation of samples on ice for 10 min, the microsomes were pelleted through a 0.2 M sucrose cushion (adjusted to pH 11.0 in triethanolamine) in a TLS-55 swinging bucket rotor (45,000 rpm, 20 min). Microsomes were resuspended in a volume equal to the supernatant volume, and both samples were boiled in 1% SDS and neutralized with 1 N HCl, followed by immunoprecipitation and SDS-PAGE (25).

Indirect immunofluorescence. Cover slips of SV5-infected CV1 cells were prepared for surface fluorescence by fixation in 0.1% freshly prepared paraformaldehyde for 2 min. For internal staining, the cells were fixed in 0.5% paraformaldehyde and made permeable in 100% acetone for 2 min at -20°C or by the addition of 0.1% saponin. To maintain the permeable state, 0.1% saponin was included in all further wash solutions and antibody-binding steps. Antibody addition and mounting of cover slips were performed as described elsewhere (9, 24). Briefly, cells were treated with 1% bovine serum albumin in PBS to block nonspecific binding of antibodies. After the cells were washed in PBS, they were incubated with either monospecific anti-SH which had been diluted 1:10 in PBS containing 1% bovine serum albumin or with a 1:500 dilution of anti-HN monoclonal antibody (28). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used as the second antibody for the anti-SH IgG, and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG was used for HN ascites fluids.

Preparation of plasma membranes. SV5-infected CV1 cells were labeled with [^{35}S]methionine or [^3H]phenylalanine plus [^3H]leucine for 1 h, the label was removed, and the cells were incubated in DME for a further 3 h. Plasma membrane vesicles (PMVs) were then prepared by the method of Scott (33). Cells were treated with 200 mM paraformaldehyde-1 mM dithiothreitol in DME for 3 h at 37°C. The medium containing PMVs was decanted, and the membranes were pelleted by centrifugation (45,000 rpm, 10 min, 4°C) by using a TLA-100 rotor in a TLA-100 tabletop ultracentrifuge (Beckman). Samples were resuspended in PBS, a sample was removed for enzyme assays, and the remainder was adjusted to 1% in SDS for immunoprecipitation analysis.

Bulk plasma membranes were prepared by the method of Maeda and co-workers (19). Labeled cells were washed in PBS and suspended in 7 ml of homogenization buffer (10 mM NaPO₄ [pH 7.13], 1 mM MgCl₂, 30 mM NaCl, 1 mM dithiothreitol, 0.01 mM phenylmethylsulfonyl fluoride, 0.5 µg of DNase I per ml). After incubation on ice for 10 min, the cells were homogenized with a Dounce homogenizer, layered onto a 2.5-ml cushion of 41% sucrose in homogenization buffer, and centrifuged at $95,000 \times g$ for 1 h by using an SW41 rotor. Membranes were collected from the sucrose cushion interface, diluted to 10 ml with homogenization buffer, and recentrifuged to pellet the membranes. Plasma membranes were resuspended in PBS, some of the sample was assayed for enzymatic activities, and the remainder was adjusted to 1% in SDS for immunoprecipitation analysis.

Enzyme assays and protein quantitation. Plasma membrane preparations were tested for both 5'-nucleotidase and NADH-diaphorase activities. These are marker enzymes specific for the plasma membrane and the endoplasmic reticulum, respectively. 5'-Nucleotidase was assayed by the method of Mitchell and Hawthorne (20), and NADH-diaphorase was assayed by the method of Wallach and Kabat (38). Protein quantitation was performed with the Bio-Rad protein dye.

Quantitation of autoradiograms. Quantitation of autoradiograms was performed by scanning laser densitometry.

RESULTS

Production of N-terminal domain-specific antiserum to SH.

To facilitate experiments on the localization of the SH protein in SV5-infected cells, an antiserum to an SH-specific synthetic oligopeptide (SH residues 2 to 18; 13) was prepared in rabbits. This antiserum was capable of immunoprecipitating the SH protein from lysates of [³H]phenylalanine- and [³H]isoleucine-labeled SV5-infected CV1 cells (Fig. 1, lane 6). This antiserum was also used to immunoprecipitate lysates from CV1 cells infected with the 2WR isolate of SV5. In infected cells, the 2WR strain synthesized a polypeptide of similar mobility to that of SH of the W3A isolate of SV5 (Fig. 1, lanes 5 and 7), and this polypeptide was precipitated by SH peptide antiserum (Fig. 1, lane 8). The strain-specific mobility difference in SH between the W3A and 2WR isolates probably reflects a change in amino acid composition. Thus, the SH protein and gene are conserved between isolates of SV5. These data also provide indirect evidence that the initiator methionyl residue of SH is not removed, since SH can be immunoprecipitated from [³⁵S]methionine-labeled SV5-infected cells (Fig. 1, lane 4).

SH has properties of an integral membrane protein. The predicted amino acid sequence of SH indicates that the C-terminal hydrophobic domain (residues 17 to 39) is of sufficient length and hydrophobicity to span a lipid bilayer (13). To determine whether the SH protein is integrated into membranes in a stable manner, SV5-infected CV1 cells were labeled with either [³⁵S]methionine or [³H]phenylalanine plus [³H]isoleucine and crude microsomal fractions were prepared. These membranes were treated with alkali (pH 11), a strong protein denaturant which has been shown to extract peripheral membrane proteins while integral membrane proteins remain associated with the lipid bilayer (11, 25, 35). As expected for a known and well-characterized integral membrane protein, the majority of the control HN protein was found in the pellet fraction (Fig. 2A). The majority of SH found in microsomes was also resistant to extraction of alkali treatment and was found in the pellet

FIG. 1. Immunoprecipitation of SH with N-terminal domain-specific peptide antiserum. SV5-infected CV1 cells were labeled with [³H]phenylalanine plus [³H]isoleucine or [³⁵S]methionine and lysed in 1% SDS. Appropriate samples were immunoprecipitated with an antiserum raised against an oligopeptide synthesized to residues 2 to 18 of SH by using the amino acid sequence predicted from the nucleotide sequence of the SH gene (13). Samples were subjected to SDS-PAGE by using 9% highly cross-linked polyacrylamide gels containing 8 M urea (36). Lanes 1 and 2: [³H]-labeled lysates analyzed directly; lane 1, uninfected cells; lane 2, SV5 W3A-infected cells. Lanes 3 and 4: [³⁵S]-methionine-labeled lysates immunoprecipitated with the peptide antisera; lane 3, uninfected cells; lane 4, SV5 W3A-infected cells. Lanes 5 to 8: [³H]-labeled samples; lanes 5 and 7, direct lysates; lanes 6 and 8, immunoprecipitated samples using the peptide antisera; lanes 5 and 6, SV5 W3A; lanes 7 and 8, SV5 2WR.

fractions (Fig. 2B and C). Thus, these data indicate that SH has properties of an integral membrane protein.

Cell fractionation. To investigate whether SH in infected cells is inserted into the endoplasmic reticulum (ER) and transported along the exocytic pathway to the Golgi apparatus, biochemical fractionation of the cells was performed by using discontinuous sucrose gradients as described previously (8, 17). Cells were labeled for 15 min with [³⁵S]-Trans-label, the isotope was removed, and cells were fractionated immediately (pulse) or after 1 h of incubation in DME (chase). It has been shown previously by electron microscopic examination and by assaying for marker enzyme activities that by using this procedure, fractions 1, 2, and 3 contain largely PMVs and smooth membrane vesicles, including Golgi vesicles, and fractions 4, 5, and 6 contain rough ER membranes (8, 17). Regardless of the precise origin of each fraction in this separation procedure, it can be seen in Fig. 3A that after a 15-min pulse-label, SH copurifies with the same rough ER membrane fractions as HN and F₀. After a 1-h chase, there is a striking redistribution of SH and HN, since they are now found associated with the smooth membrane vesicle and PMV fractions (Fig. 3B). As expected, after a 1-h chase, F₀ was cleaved and F₁ and F₂ could be identified (e.g., Fig. 3B, lane 2). Thus, SH demonstrates the same exocytic pathway vectorial movement properties from rough ER to smooth membranes as do the well-characterized SV5 integral membrane glycoproteins F and

FIG. 2. Alkali treatment of microsomes containing HN or SH. Microsomal membrane fractions were prepared from SV5-infected CV1 cells labeled with [35 S]methionine (A and B) or [3 H]phenylalanine plus [3 H]isoleucine (C). Microsomes were treated with alkali (pH 11) and fractionated through a 0.2 M alkaline sucrose gradient (Materials and Methods) into soluble (S) and pellet (P) fractions. These fractions plus the postmicrosomal supernatant (C) were immunoprecipitated with HN-specific IgG or SH-specific IgG and subjected to SDS-PAGE on 16% polyacrylamide gels containing 4 M urea (A and B) or 9% highly cross-linked gels (C), as in Fig. 1. (A) Fractions immunoprecipitated with HN monospecific IgG; (B) and (C) fractions immunoprecipitated with anti-SH monospecific IgG.

HN. Further evidence for the insertion of SH into the exocytic pathway in rough ER comes from the finding that the *in vitro* insertion of SH into membranes is dependent on the signal recognition particle (D. Hull, S. W. Hiebert, R. Gilmore, and R. A. Lamb, unpublished observations).

Localization of SH by indirect immunofluorescence. To examine further the subcellular localization of SH, indirect immunofluorescence with affinity-purified SH peptide antiserum was done. SV5-infected cells were fixed in 0.5% paraformaldehyde for 5 min and made permeable with acetone at -20°C for 2 min. The fluorescein staining of the SH antibodies (Fig. 4A) colocalized on the same field of cells as the rhodamine staining of the HN antibodies (Fig. 4B). Both SH and HN showed reticular staining characteristic of ER and intense perinuclear Golgi staining. The SH-specific perinuclear staining also colocalized with that of rhodamine-conjugated wheat germ agglutinin, a lectin that preferentially binds to the Golgi apparatus (37) (data not shown). Permeable uninfected cells were used as controls, and they did not exhibit any fluorescence with either SH or HN antibodies (Fig. 4E and F). Surface staining of SH antibody bound to infected cells was also done as an indicator of expression of SH at the plasma membrane. However, because of the domain-specific nature of the SH antibody, a positive result would be obtained only if SH was oriented in membranes with its N-terminal domain exposed at the cell surface. Whereas bright rhodamine staining was obtained for HN (Fig. 4D), no fluorescein surface staining of SH was found on the same field of cells (Fig. 4C).

To explore the possibility that SH is transported to the cell

surface but in an orientation with its N terminus exposed on the cytoplasmic face of the plasma membrane, cells were made permeable with the cholesterol-like detergent saponin. This procedure allows antibodies to penetrate cells without significantly disrupting the structure of the plasma membrane (43). SV5-infected cells were fixed in 0.5% paraformaldehyde, made permeable with 0.1% saponin, and incubated with rabbit anti-SH IgG, mouse HN-specific ascites fluid, and the appropriate secondary antibodies. The fluorescein staining of SH and the rhodamine staining of HN colocalized in cells. By focusing in a plane above the nucleus, a diffuse punctate staining was observed over the entire cell, characteristic of molecules expressed at the surface; in this focal plane there was also intense perinuclear Golgi staining (SH staining shown in Fig. 5A, HN shown in Fig. 5B; the same cell is shown in panels A, B, and C). By focusing in the plane of the nucleus, the punctate staining of cell surface membrane was not observed and a better resolution of the Golgi staining was obtained (panel C). This colocalization of staining of SH and HN in all focal planes provides suggestive evidence that SH is expressed at the plasma membrane with its N terminus in the cytoplasm.

Localization of SH in plasma membrane fractions. To provide biochemical evidence that SH is present in plasma membranes, the membrane fractions were isolated and the coenrichment of SH with F and HN, as well as the plasma membrane enzyme 5'-nucleotidase, was monitored. SV5-infected CV1 cells were labeled for 1 h with [35 S]methionine, followed by a 3-h incubation in DME to allow for transport of labeled proteins to the cell surface. Plasma membranes were prepared from cells by two methods, i.e., the production of PMVs (33) and the separation of plasma membranes onto a sucrose cushion (19). Plasma membrane fractions prepared by both methods were 70 to 80% free of contaminating ER membranes, as determined by NADH-diaphorase activity, and plasma membranes showed an approximately fivefold enrichment for 5'-nucleotidase activity (Table 1). Analysis of the polypeptides contained in these fractions indicated that SH copurified with HN and F in the plasma membranes prepared by the two methods (Fig. 6A, PMVs; Fig. 6B, plasma membranes). There was an approximately fivefold enrichment from the whole-cell lysate to the PMV and plasma membrane preparations of all three SV5-specific polypeptides (Fig. 6; quantitation in Table 1). Thus, these data suggest the SH protein, like F and HN, is localized in plasma membranes.

Orientation of SH in membranes. The inability to detect SH at the cell surface by using the N-terminal antiserum in immunofluorescence and the strong membrane-associated fluorescence with this antiserum after the plasma membrane was made permeable suggest that SH is anchored in membranes with an N-terminal cytoplasmic domain and a small luminal C terminus of about five residues. Although it is very difficult to provide direct data that the C terminus of SH is exposed at the cell surface, biochemical evidence to confirm the orientation of SH in membranes can be obtained by testing the protease sensitivity of the N terminus. If SH expressed at the cell surface is orientated with a cytoplasmic N terminus, protease treatment of the infected-cell surface should not remove the SH determinant recognized by the domain-specific SH antiserum and SH would be protected from digestion (there is no trypsin cleavage site in the five C-terminal residues except the arginine residue, which is thought to be proximal to the hydrophobic membrane-spanning domain). However, trypsin treatment of microsomal vesicles should digest the N-terminal domain by

FIG. 3. Fractionation of SV5-infected cells into smooth membranes and rough ER membranes. SV5-infected CV1 cells were labeled at 16 h postinfection for 15 min with ^{35}S -TranS-label and processed immediately (pulse) or after removal of the label and incubation with DME containing 2 mM methionine for 60 min (chase). Fractions 1, 2, and 3 contain largely smooth membrane vesicles, and fractions 4, 5, and 6 vesicles from rough ER membranes. (A) Pulse-label samples; (B) chase samples. Samples were subjected to electrophoresis on two gel systems: top panels show a 10% gel for the resolution of HN, F_0 , and NP; bottom panels show the relevant region of a 17.5% plus 4 M urea gel to resolve SH. Lane M, SV5-infected cell lysates as a marker; lanes 1 to 6, fractions 1 to 6 described above.

removing the antigenic epitope, and then SH would not be immunoprecipitated.

For cell surface protease treatment, SV5-infected CV1 cells were labeled with ^{35}S -TranS-label at 12 h postinfection for 1 h, the label was removed, and the cells were incubated for a further 3 h in DME to allow for intracellular transport of the newly synthesized proteins prior to surface proteolysis. As expected for the control proteins, the majority of F and HN was removed from the cell surface by protease digestion (Fig. 7). However, protease treatment of the cell surface renders the cells very fragile and susceptible to lysis during the procedure. Therefore, when assays for SH were done, it was necessary to provide an internal marker that was unaffected by trypsin digestion. Cells expressing the cytoplasmic enzyme chicken pyruvate kinase (PK) (S. W. Hiebert and R. A. Lamb, *J. Cell Biol.*, in press) were mixed with SV5-infected cells prior to the addition of trypsin. The amounts of PK and SH were reduced on trypsin treatment as compared with those of the undigested samples (Fig. 7).

However, quantitation of the autoradiograph by scanning densitometry indicated that the PK-to-SH ratio was unchanged, indicating that SH is not directly digested at the cell surface. The prominent band migrating between HN and PK, which can be observed in the SH lanes of Fig. 7 and which did not change intensity on trypsin treatment, was an artifact of the procedure. It is the heavy chain of IgG that frequently becomes exogenously labeled with ^{35}S -cysteine (from the ^{35}S -TranS-label) during immunoprecipitation procedures, particularly when it is necessary to use large amounts of anti-peptide IgG because of its low affinity.

For protease treatment of microsomal vesicles, SV5-infected CV1 cells were labeled with ^{35}S -TranS-label at 12 h postinfection for 1 h and microsomes were prepared as described in Materials and Methods. The microsomal vesicles were treated with trypsin, and the protease-protected protein fragments were analyzed on gels. The ectodomains of HN and F were protected from protease digestion, and there was a slight increase in electrophoretic mobility com-

FIG. 4. Indirect fluorescent staining of SV5-infected cells. Mock-infected or SV5-infected CV1 cells were fixed in 0.5% paraformaldehyde and made permeable with acetone at -20°C for 2 min for intracellular staining. For surface fluorescence, cells were fixed in 0.1% paraformaldehyde. (A, C, and E) Cells stained with rabbit anti-SH IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG; (B, D, and F) staining of a mixture of monoclonal antibodies to HN and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG; (A and B) staining of permeable SV5-infected cells; (C and D) staining of the surface of SV5-infected cells; (E and F) staining of permeable mock-infected cells; Panels A and B, C and D, and E and F show pairs of the same field of cells. The exposure of panel C was manually adjusted to be the same as that of panel D, and the exposures of panels E and F were manually adjusted to be the same as those of panels A and B, respectively.

FIG. 5. Indirect immunofluorescence of SV5-infected cells made permeable with saponin. Mock-infected or SV5-infected cells were fixed with 0.5% paraformaldehyde and made permeable with 0.1% saponin. Saponin was maintained in all wash steps and during antibody binding. Cells were stained with SH-specific IgG plus fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or with HN-specific mouse ascites fluid and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG. (A and C) SH staining to show fluorescence of proteins expressed at the cell surface by focusing above the nucleus (A) and in the plane of the nucleus (C); (B) HN staining on the same field of cells as that of A and C, with the focus as in panel A; (D) mock-infected cells photographed by using the filters for rhodamine staining. The exposure for panel D was manually adjusted to be the same as that for panel B.

TABLE 1. Localization of proteins in plasma membrane fractions of SV5-infected cells

Protein	% Total enzyme activity or labeled protein in the following fraction ^a :	
	PMV	PLM
NADH-diaphorase	25	21
5'-Nucleotidase	ND ^b	82
HN	85	73
F	95	87
SH	82	72

^a Enzyme assays were performed in duplicate; the average value is given. PMV (33) and plasma membrane (PLM; 19) fractions were prepared as described elsewhere. The values for HN, F, and SH were determined by densitometric scanning of the autoradiogram shown in Fig. 6.

^b Could not be determined under the assay conditions used.

patible with the removal of their cytoplasmic tails (Fig. 8A). Quantitation by scanning densitometry of the HN and F bands indicated that 80 to 90% of HN and F is protected from proteolysis. In contrast, protease treatment of microsomes caused a complete loss of the SH antigenic determinant recognized by the N-terminal SH antiserum in immunoprecipitation assays (Fig. 8). To show directly that SH was digested by trypsin and to rule out the possibility that a conformational change had occurred in SH on trypsin treatment, making the antibody unable to recognize the protein even though the immunoprecipitates were done with SDS-denatured antigen, microsomes were analyzed on gels directly, without immunoprecipitation. The 44-amino-acid-residue SH protein was no longer detected (Fig. 8). (There was the appearance of a very rapidly migrating band nearest to the bottom of the gel, but it is not known whether it represents the ~28-amino-acid protected fragment.) Thus, together the two proteolysis experiments indicate that the N terminus of SH can be digested by trypsin and that the SH

FIG. 6. Isolation of plasma membranes from SV5-infected cells. (A) Plasma membrane vesicles. SV5-infected cells were labeled with [3 H]phenylalanine and [3 H]leucine and treated with 200 mM paraformaldehyde and 1 mM dithiothreitol for the isolation of PMVs (33). To examine for SH, F, and HN enrichment in the fractions, the proteins were immunoprecipitated from lysates of PMVs or control whole-cell extracts that contained equal amounts of total protein, as measured by the Bio-Rad protein assay. CON, Whole cells. SH, HN, and F indicate the antisera to the respective SV5 integral membranes used for immunoprecipitation. (B) Plasma membrane fractions. Fractions were isolated from SV5-infected [35 S]methionine-labeled CV1 cells by sedimentation onto a 41% sucrose cushion (19; see Materials and Methods). Immunoprecipitation of lysates containing equal amounts of total protein was done with SH-, HN-, or F-specific anti-IgGs. CON, control whole-cell lysates. PLM, plasma membrane fractions.

protein is oriented in membranes with a cytoplasmic N-terminal region.

DISCUSSION

The predicted amino acid sequence of SH indicates that the hydrophobic domain (13) is sufficiently hydrophobic to interact with membranes in a stable manner (15). The data presented here show that SH cannot be extracted from microsomal membranes at alkaline pH and that SH thus has properties of an integral membrane protein.

The determination of the orientation of SH in membranes and its subcellular localization is complicated because there are only 16 N-terminal residues and five C-terminal residues on each side of the 23-residue hydrophobic domain and no sites for asymmetric modifications such as N-linked glycosylation. In determinations of the orientation of other small integral membrane proteins, such as M_2 and NB of influenza A and B viruses, which have N-terminal ectodomains expressed at the cell surface of 18 to 23 amino acids, use has been made of conventional techniques such as the use of site-specific antisera and digestion with proteases (18, 41, 42). With SH, the five C-terminal amino acids encompass a region too small to act as an antigenic epitope, cannot be digested by protease, and cannot be radiolabeled at the cell surface. If a protein tag was to be added to the C terminus of

SH, such as a known sequence that is recognized by a monoclonal antibody, it is possible that both the membrane orientation and transport of SH would be altered.

The fluorescence data and the finding of specific enrichment of SH in plasma membranes strongly suggest that SH is localized to the plasma membrane. The ability of trypsin to digest the N terminus of SH in microsomes and the resistance of SH to protease digestion at the cell surface lead to the model for the orientation of SH in plasma membranes shown in Fig. 9, with a C-terminal ectodomain and an N-terminal cytoplasmic tail. Thus, SH can be classified as a class II integral membrane protein (10). The data obtained from cell fractionation, fluorescence studies, and plasma membrane preparations indicate that *in vivo* SH is inserted into the exocytic pathway in the ER membrane and is transported through the Golgi apparatus to the plasma membrane. The initial incorporation of SH into membranes probably occurs by insertion of the hydrophobic domain as a loop, leaving the 16 N-terminal amino acids in the cytoplasm, and subsequent translocation of the small ectodomain across the membrane into the lumen of the ER (3, 14). The small size of SH (44 residues) would suggest that the synthesis of SH must be complete before it emerges from the protease-inaccessible space in the ribosome which protects ~40 residues (11, 39). It is thus of great interest to find in

FIG. 7. Trypsin treatment of the surface of cells. SV5-infected CV1 cells were labeled with ^{35}S -TranS-label for 1 h, and the label was removed and incubated in DME for 3 h. Intact cells were either not treated (lanes C) or treated with trypsin (lanes T). To control for cell loss, CV1 cells infected with an SV40 recombinant virus expressing PK were added before the addition of trypsin to provide an internal marker. The specific IgGs used to precipitate the proteins (F, HN, or SH) are indicated above the lanes. PK-specific IgG was added to the SH-specific immunoprecipitation samples. The use of the SV40 vector expressing PK and the use of PK-specific IgG are described elsewhere (Hiebert and Lamb, in press). Samples were subjected to SDS-PAGE on 16% polyacrylamide gels containing 4 M urea.

vitro studies that the efficient insertion of SH into membranes is strictly dependent on the signal recognition particle (D. Hull [our laboratory], unpublished observations).

Several models for the intracellular sorting of proteins to the cell surface have been proposed (e.g., those reviewed by Pfeffer and Rothman [26]), with many emphasizing the role of positive-acting transport signals within the ectodomain or cytoplasmic tail (for membrane-anchored proteins). An alternative viewpoint is that there is a default bulk flow pathway to the cell surface and sorting occurs by selective retention of ER and Golgi proteins (for a review, see reference 30). This proposal is based on the identification of retention signals within resident proteins of the ER (21, 22, 27) and the rapid rate of secretion of a synthetic glycopeptide which is thought unlikely to contain transport signals (40). The identification of very small ectodomains in naturally occurring membrane proteins, e.g., M_2 protein of influenza A virus (23 residues), NB protein of influenza B virus (18 residues), and SH of SV5 (5 residues), suggests that complex ectodomain positive-acting signals may not be needed for intracellular transport (18, 41, 42, 44).

The role of SH in the SV5 replicative cycle is not known. The available evidence suggests that SH is preferentially

FIG. 8. Trypsin treatment of microsomes from SV5-infected cells. SV5-infected cells were labeled with ^{35}S -TranS-label for 1 h, and microsomes were prepared as described in Materials and Methods. Samples were divided into two aliquots which were not treated (C) or were treated with trypsin (T). The samples were then immunoprecipitated with HN-, F-, or SH-specific IgGs and subjected to SDS-PAGE on 16% polyacrylamide gels containing 4 M urea. Lanes SV5, Lysates analyzed directly without immunoprecipitation and subjected to SDS-PAGE on 9% highly cross-linked gels containing 8 M urea.

excluded from virions. However, because of the sensitivity of the assays, it cannot be rigorously determined that there is not a small amount of SH in virions. The absence of a significant ectodomain in SH suggests that the functional domain may be the highly charged N-terminal region exposed at the cytoplasmic face of the plasma membrane. It is

FIG. 9. Schematic diagram indicating orientation of SH in membranes. The relative locations of the acidic (-) and basic (+) amino acid residues in the polypeptide are indicated.

possible that SH has a role in organizing the assembly of viral proteins to form a viral patch at the plasma membrane.

The primary amino acid sequences of influenza A virus M₂, influenza B virus NB, respiratory syncytial virus 1A, and SV5 SH proteins show little direct homology. However, it is interesting to note that the cytoplasmic domains of M₂, NB, 1A (C terminus; R. Olmstead and P. Collins, personal communication), and SH contain a high proportion of charged residues. The distribution of charged residues is similar in all four proteins, with acidic residues concentrated near the terminus of the cytoplasmic tail and basic residues located near the transmembrane region (7, 18, 34, 41). Although this suggests that there is some structural homology between these proteins, there is at present no direct evidence that they do have similar functions.

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