Identification of Immunologically Cross-Reactive Proteins of Sindbis Virus: Evidence for Unique Conformation of E1 Glycoprotein from Infected Cells

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Hyperimmune antisera to purified Sindbis (SIN) or Semliki Forest (SF) virus were used to identify alphavirus-specific and cross-reactive proteins in virions and infected cells. The hyperimmune sera participated in homologous and cross-cytolysis of alphavirus-infected cells, and the use of monospecific antisera to SIN structural proteins suggested that E1 and E2 could serve as target proteins in cytolysis. Proteins from purified virions or infected cells were extracted with Nonidet P-40, denatured by procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose solid supports, and reacted with hyperimmune sera and ¹²⁵I-labeled protein A (immunoblotting on denatured proteins). Alternatively, native proteins extracted by mild Nonidet P-40 treatment were precipitated with hyperimmune sera before denaturation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After immunoblotting, homologous antiserum reacted with the virus structural proteins E1, E2, capsid extracted from purified virions, and the counterparts of these proteins extracted from infected cells. In addition, PE2 and a 92,000-molecular-weight protein from infected cells reacted with homologous antiserum. These proteins were also immunoprecipitated with homologous antiserum. After immunoblotting, the Sindbis capsid protein was shown to be cross-reactive whether derived from purified virions or from infected cells; no cross-reactivity was observed with PE2 or E2 from either source, and the E1 glycoprotein was shown to be cross-reactive only when obtained from virions. However, the E1 glycoprotein could be cross-immunoprecipitated from infected cells (as well as from disrupted virions), and, in addition, capsid and a 92,000-molecular-weight protein were cross-immunoprecipitated from infected cells. These results suggest that a native conformation of the cell-associated E1 glycoproteins may be required for immunological cross-reactivity (immune precipitation), whereas virion but not cell-associated E1 retains immunological cross-reactivity after denaturation (immunoblot technique). The findings extend our previously published evidence which suggested that alphavirus maturation is accompanied by a change in immunological cross-reactivity with respect to E1.

The alphaviruses are small, enveloped RNA viruses which have been well characterized molecularly and immunologically (reviewed in reference 23). The virion contains an icosahedral capsid surrounded by a lipid bilayer and two (in Sindbis [SIN] virus) or three (in Semliki Forest [SF] virus) surface glycoproteins designated E1, E2, and E3. The latter two glycoproteins are derived from the proteolytic cleavage of a common precursor, PE2/p62, found in infected cells (11, 22, 23).

The alphaviruses have been serologically classified into three distinct subgroups by neutralization reactions (23). SIN and SF viruses are representative members of the western and Venezuelan equine encephalitis subgroups, respectively, and as such do not participate in cross-neutralization reactions. However, immunological cross-reactivity between SIN and SF viruses has been demonstrated by antibody-dependent complement-mediated cytolysis (ADCMC; 8, 14, 30) with hyperimmune serum, by hemagglutination-inhibition with hyperimmune serum (5), and by cytolytic T lymphocyte recognition (17, 31). Cytolytic T lymphocyte cross-reactivity does not appear to require hyperimmunization. Through the use of monospecific antiserum (5, 6) and monoclonal antibody (24), the neutralization site has been generally accepted as being localized on the E2

glycoprotein, and the hemagglutination site on the E1 glycoprotein, although certain monoclonal antibodies appear to complicate this distinction presumably by steric inhibition (20). The ADCMC cross-reactive sites between SIN and SF viruses have been reported to involve the E1 glycoprotein in assays with temperature-sensitive mutants (30). ADCMC cross-reactive sites in SIN- and western equine encephalitis-infected cells (members of the same alphavirus subgroup) have also been reported with monoclonal anti-E1 antibody (4).

In this report, we have used our hyperimmune sera in an application of the immunoblot technique and in immune precipitation to identify virus-specific and cross-reactive proteins in virions and infected cells. Cross-reactivity to capsid protein was apparent in both virions and infected cells, regardless of whether the proteins were in native (immune precipitation) or denatured (after immunoblotting) conformation at the time of reaction with antiserum. Cross-reactivity to E1 was apparent only in virions but not in infected cells after denaturation. However, a cross-reactive E1 glycoprotein was detected in infected cells by immune precipitation, suggesting a requirement for native conformation of the cell-associated viral E1 in immune cross-reactivity.

MATERIALS AND METHODS

Viruses and virus purification. SIN virus AR339 and SF virus were grown in primary chicken embryo fibroblasts

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(CEF) at a multiplicity of infection (MOI) of 0.01. Virus was purified as described previously (8, 30) by low-speed clarification ($5,000 \times g$), polyethylene glycol precipitation (10% in 0.5 M NaCl), and isopycnic density centrifugation for 16.5 h at $25,000 \times g$ in a 20 to 60% linear sucrose gradient in TNE (0.01 M Tris, 0.05 M NaCl, 5 mM EDTA, pH 7.4). The virus-containing band was collected, pelleted at $25,000 \times g$ for 2 h, and suspended in TNE for storage at -70°C . Protein content was determined by the method of Lowry et al. (16).

Production of hyperimmune antiserum to alphaviruses. Immunization of rabbits with alphaviruses has been previously described (8). Briefly, 250 μg of purified SIN or purified SF virus (ca. 10^{10} PFU) was emulsified in Freund complete adjuvant and injected into the gastrocnemius. Secondary immunizations with 250 μg of purified virus were given intraperitoneally in incomplete adjuvant 1 week later. Additional immunizations over a period of several weeks were given intravenously with 250 μg of purified virus alone. Antisera contained homologous virus-neutralizing titers of $>10^5$ and nondetectable heterologous virus-neutralizing titers ($<1:5$) when assayed by plaque reduction on CEF monolayers. These antisera were shown to contain cross-reactivity by ADCMC of virus-infected L₉₂₉ and CEF cells (8, 30).

Monospecific antisera to SIN structural proteins. Rabbit monospecific antisera to SIN glycoproteins, purified by acid-urea gel electrophoresis, were a gift from J. S. Manning, University of California, Davis.

ADCMC. The ADCMC assay for microtiter plates has been described in detail previously (30, 31). Briefly, dilutions of rabbit hyperimmune serum or monospecific antiserum were made in McCoy's 5A medium and mixed with an equal volume of a 1:7 dilution of rabbit complement (Low-Tox-M rabbit complement; Cedarlane, Accurate Chemical Corp., Hicksville, N.Y.). The antiserum-complement mixtures were applied to 2×10^4 virus-infected (MOI = 10) or -uninfected mouse L₉₂₉ cells that had been previously labeled with $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear Corp., Boston, Mass.). Portions of triplicate samples of the supernatant fluids were analyzed for ^{51}Cr release in a Beckman 4000 gamma spectrometer. Controls consisted of wells which received medium alone, antiserum alone, a 1:14 dilution of complement, or 3% Triton X-100 (total releasable counts). Percent specific release was calculated as:

$$\frac{(\text{cpm released by Ab} + \text{C}) - (\text{cpm released by C}) \times 100}{(\text{cpm released by Triton X-100}) - (\text{cpm released by C})}$$

where cpm is counts per minute, Ab is antiserum, and C is complement.

Extracts of virus-infected cells or virions. Murine L₉₂₉ cells were used as the major source of infected cell extracts to correlate results with our previously published reports on ADCMC of alphavirus-infected cells (14, 30, 31). In some instances, CEF were used as a source for infected cell extracts. L₉₂₉ cells or CEF were grown to confluency in 100-cm² petri dishes in McCoy's 5A medium or medium 199, respectively (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% bovine calf serum (Sterile Systems, Inc., Logan, Utah). Cell monolayers were rinsed twice in Hanks balanced salt solution (HBSS) and infected with SIN or SF viruses at MOI = 20. After a 60-min adsorption at 37°C , unadsorbed virions were washed from the monolayers with HBSS, and cells were fed with McCoy's 5A medium containing 1% fetal bovine serum (GIBCO). Incubation was continued at 37°C . At the designated times, monolayers were

rinsed twice in 0.01 M phosphate-buffered saline (pH 7.4) and removed from the petri dish with a rubber scraper. Cells were lysed in 1% Nonidet P-40 in 10 mM Tris-10 mM KCl-1.5 mM MgCl_2 (pH 7.4) with 5 μM phenylmethylsulfonyl fluoride (Calbiochem-Behring, San Diego, Calif.) on ice for 10 min, sonicated 60 s, and centrifuged for 2 min in a microfuge (Fisher Scientific Co., Pittsburgh, Pa.; model 235A). Supernatant fluids containing solubilized proteins were stored at -70°C . Purified virions were disrupted by the addition of Nonidet P-40 (20% in TNE) to a final concentration of 2%. The mixture was incubated for 30 min at room temperature, sonicated for 30 s, and then centrifuged, and the soluble proteins were stored as described above.

Radiolabeling of infected cells with [^{35}S]methionine. Mouse L₉₂₉ cells or CEF were grown to almost confluency in 100-cm² petri dishes in McCoy's 5A medium or medium 199 with 5% bovine calf serum. Monolayers were rinsed twice in HBSS and infected at MOI = 20 with SIN. After a 60-min adsorption at 37°C , cells were washed twice in HBSS and fed with McCoy's 5A medium containing 1% fetal bovine serum. At 1 h postinfection for CEF or 3 h postinfection for L₉₂₉ cells, the medium in cultures that were to receive [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) was diluted 1:4 in methionine-free medium (Flow Laboratories, Inc., McLean, Va.) containing 1% fetal bovine serum to reduce the pool of intracellular, unlabeled methionine. At 3 h postinfection for CEF or 5 h postinfection for L₉₂₉ cells, the medium was replaced with methionine-free medium containing 1% fetal bovine serum and 35 to 50 μCi of [^{35}S]methionine per ml. Incubation at 37°C was continued for 2 h. Radioisotopically labeled cells were rinsed twice in phosphate-buffered saline and harvested as described above for infected cell extracts.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 0.75-cm thick slab gels of 11.2 or 12.3% acrylamide by a modification of the method of Laemmli (15) as described by Smith and Brown (26) for use with an *N,N'*-diallyltartardiamide cross-linking reagent (acrylamide-*N,N'*-diallyltartardiamide = 30:1.6). Stacking gels were 3% acrylamide photopolymerized with riboflavin as described by Powell and Courtney (18). Samples were disrupted by boiling 2 min in an equal volume of SUM (2% SDS, 2% 5 M urea, 2% 2-mercaptoethanol in 150 mM Tris, pH 8.8) and electrophoresed in a discontinuous system at 15 to 20 mA per gel for ca. 2.5 h when the bromophenol blue tracking dye reached the bottom of the gel. Molecular weight markers, labeled with ^{14}C , were obtained from Amersham as a protein mixture of low-molecular-weight standards (14,300 molecular weight [14.3K] to 200K).

Immune precipitation. Extracts of unlabeled or [^{35}S]methionine-labeled virus-infected cells were incubated for 60 min at 37°C with rabbit antiserum in 0.1% Triton X-100 in TNE buffer (0.05 M Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing 5 μM phenylmethylsulfonyl fluoride at a ratio of 1:1:4 for extract, antiserum, and buffer, respectively. The mixtures were incubated for 4 to 6 h at 4°C . The immune complexes were mixed with Pansorbin (1:1; Pansorbin-antiserum [vol/vol]; Calbiochem-Behring, La Jolla, Calif.) overnight at 4°C . Complexes were pelleted in a microfuge and washed 5 to 6 times in TNE with 5 μM phenylmethylsulfonyl fluoride. The final pellet was suspended in sample disruption buffer (SUM) at 37°C for 40 min and boiled for 2 min. The sample was freed from Pansorbin by centrifugation in a microfuge, and the supernatant fluid was electrophoresed as described above for SDS-PAGE. Gels containing

radiolabeled immune precipitates were dried onto Whatman 3MM paper and autoradiographed on Kodak X-Omat AR X-ray film. Unlabeled immune precipitates were subjected to immunoblotting and autoradiographed as described below.

Immunoblotting. The immunoblot method was performed as described by Towbin et al. (28) and Burnette (3) except that electrophoretic transfer was carried out at 15 V/cm for 2 to 2.5 h at 4°C. Briefly, filter papers, polyethylene sheets, and nitrocellulose papers (Schleicher and Schuell, Inc., Keene, N.H.) were wetted in transfer buffer (10% Tris-glycine, at 20 and 150 mM, respectively, plus 20% reagent grade methanol). Immediately after electrophoresis, the SDS gels were placed in direct contact with the nitrocellulose, and these were sandwiched between the filter papers and the polyethylene sheets. The gels were placed in a Bio-Rad electroblot unit filled with cold transfer buffer such that the nitrocellulose was oriented toward the anode. Electrophoretic transfer of the proteins from the gel to the nitrocellulose sheet was accomplished by using a Transphor power supply (model TE-15; Hoefer Scientific Instruments, San Francisco, Calif.).

The nitrocellulose sheets were immersed in 3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in 10 mM Tris-hydrochloride with 0.9% NaCl, pH 7.4, for 60 min at room temperature. Transfers were washed 10 times in distilled water. Antiserum was diluted in 0.05% Nonidet P-40 in NETG buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride, pH 7.4, 0.25% gelatin type III, 225 bloom [Sigma]) and added to the transfers for 2 h at room temperature with shaking. The sheets were then washed 10 times in distilled water. Staphylococcal protein A (Zymed Laboratories, Burlingame, Calif.) was labeled with ^{125}I (Na^{125}I ; Amersham) by the chloramine T method (9). ^{125}I -staphylococcal protein A was diluted to 10^6 cpm/ml in 0.05% Nonidet P-40 in NETG buffer, and 10 ml was incubated with each transfer (10 by 14 cm) for 2 h at room temperature with shaking. The sheets were washed 10 times in NETG buffer containing 0.5% Triton X-100 and 0.1% SDS, washed in distilled water, and air dried. Transfers were autoradiographed on Kodak X-O-Mat AR X-ray film at room temperature or at -70°C with intensifying screens.

RESULTS

Purity of virus. Purified preparations of SIN and SF viruses were subjected to SDS-PAGE and stained with Coomassie blue. These preparations contained the four structural proteins of SF virus (E2, E1, capsid, and E3) and three structural proteins of SIN virus (E1, E2, and capsid) and were shown to be free of detectable nonviral proteins (gels not shown).

Reactivity of antisera by ADCMC. Monospecific and hyperimmune antisera were tested for cytolysis of alphavirus-infected or -uninfected L_{929} cells by ADCMC (Table 1). Monospecific antisera to SIN glycoproteins E1 and E2 participated in cytolysis of SIN-infected cells but not in cytolysis of SF-infected or -uninfected cells. Generation of cross-reactive antiserum required hyperimmunization with whole virus. Hyperimmune serum to purified SIN virus or to purified SF virus lysed cells infected with either SIN or SF viruses but did not lyse uninfected cells. The cross-reactivity of hyperimmune serum preparations is consistent with our previous reports of cross-reactivity (8, 14, 30).

Homologous immune reactivity of viral proteins after SDS-PAGE and immunoblotting. Purified SIN or SF viruses or extracts of virus-infected or -uninfected L_{929} cells harvested at various times postinfection were electrophoresed on SDS-

TABLE 1. ADCMC of alphavirus-infected L_{929} cells

Antiserum	% Cytolysis ^a of cells		
	Infected with SIN virus	Infected with SF virus	Uninfected
Monospecific			
Anti-E1 ^b	22.5	1.5	3.7
Anti-E2 ^b	37.9	3.8	1.8
Hyperimmune			
Anti-SIN	74.1	69.9	3.6
Anti-SF	75.0	67.8	2.3
Normal serum	2.9	4.2	1.7

^a Percent cytolysis is the maximum percent-specific ^{51}Cr release by ADCMC observed over serial twofold dilutions of monospecific antiserum (1:10 to 1:320), hyperimmune serum (1:20 to 1:5120), or normal serum (1:10 to 1:80).

^b Monospecific antisera to SIN glycoproteins were gifts from J. S. Manning.

PAGE, and the proteins were electrophoretically transferred to nitrocellulose. The transferred proteins were reacted with hyperimmune serum prepared against the homologous virus (e.g., SIN anti-SIN or SF anti-SF) followed by reaction with radioiodinated staphylococcal protein A. Extracts of infected cells at 7 or 9 h postinfection were more intensely stained in the immunoblot procedures than were extracts prepared earlier or later in infection (data not shown). Autoradiographs of the proteins in virions and virus-infected or -uninfected cells which react with homologous antiserum are shown in Fig. 1A (SF) and B (SIN). Purified virion preparations contained the proteins E1, E2, and capsid, whereas extracts of virus-infected cells contained PE2 and a 92K protein in addition to E1, E2, and capsid. The high-molecular-weight protein of ca. 92K, also referred to as B protein, has been identified in SIN-infected BHK cells as an unprocessed polypeptide containing E1 and PE2 sequences (22, 23; C. M. Rice, personal communication). The 92K protein observed in alphavirus-infected L_{929} cell extracts may be analogous to the B protein of SIN-infected BHK cells. An additional protein of ca. 44K (seen in Fig. 1A, lane 2), observed in extracts of infected and uninfected L_{929} and CEF cells (the latter not shown), reacted with anti-SF serum but not with anti-SIN serum and is presumed to be of cellular origin. Preparations of SF virions or SF-infected cells often produced a double band in the region of capsid protein after immunoblotting and reacting with antiserum. This observation has been described previously for the alphavirus, western equine encephalitis, in which the additional band was identified as a shortened form of capsid protein (10).

The E3 glycoprotein of SF virus in virions or infected cell lysates was not detectable with our antiserum after SDS-PAGE and immunoblotting, although the E3 glycoprotein was present in the purified virion preparations stained with Coomassie blue (data not shown). To determine whether this glycoprotein was transferred from the gel to nitrocellulose, parallel gels of SF virion and SF-infected and -uninfected cell lysates were electrophoresed as described above. One gel was subjected to electroblot transfer of proteins to nitrocellulose and then stained with Coomassie blue to detect nontransferred, residual protein in the gel. The second gel was stained directly with Coomassie blue. A comparison of the protein profiles in these gels suggested that all of

FIG. 1. (A) Immunoblot reactions of SF proteins with anti-SF serum. Lane 1, uninfected cell extract control; lane 2, SF-infected cell extract at 7 h postinfection; lane 3, purified SF virion. (B) Immunoblot reactions of SIN proteins with anti-SIN serum. Lane 1, uninfected cell extract control; lane 2, SIN-infected cell extract at 7 h postinfection; lane 3, purified SIN virion.

the viral proteins, including the E3 glycoprotein as well as numerous cellular proteins, were transferred from the gel (data not shown). Thus, the absence of immunologically detectable E3 after the immunoblot technique is most likely attributable to an inability of the antinative SF serum to bind SDS-denatured E3 glycoprotein. However, we cannot rule out the possibility that the quantity of E3 transferred to or retained by nitrocellulose and the amount remaining in the gel are below the limit of detection.

Immune cross-reactivity of viral proteins after SDS-PAGE and immunoblotting. SIN virion and SIN-infected L₉₂₉ cell extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and reacted with hyperimmune anti-SF serum. Strong cross-reactivity with capsid protein was apparent in virion and infected cell extract preparations (Fig. 2). Weak cross-reactivity was also detected with the E1 glycoprotein (confirmed by Fig. 3) but only in preparations of purified virions and not in the infected cell extracts. Recall that E1 glycoprotein is detected in infected cell extracts as well as in virions in the homologous reaction (anti-SIN plus SIN-infected cell extracts). The immunoblot experiments were repeated with infected cell extracts that were concentrated by acetone precipitation in an effort to detect cross-reactive E1 that might be at a lower concentration than homologous E1. These studies confirmed the absence of cross-reactivity with cell-associated E1, although cross-reactivity with capsid protein remained. Thus, our ability to detect cross-reactive E1 only in virions but not in infected cells did not appear to be based solely on quantitative differences in E1 from these two preparations, although the acetone precipitation experiments do not conclusively eliminate this possibility. Purified SIN virion preparations were electrophoresed on a single gel, transferred to nitrocellulose, and then separated for reaction with homologous (anti-SIN) or cross-reactive (anti-SF) antiserum to compare reactivity with the E1 glycoprotein. Anti-SIN serum reacted with a broad protein band of SIN virion E1, whereas anti-SF serum reacted with a more compact band which appeared to migrate in the lower-, but not lowest-, molecular-weight region of the SIN virion E1 (Fig. 3). These results were consistently reproducible by

simultaneous electrophoresis and immunoblotting with both antisera.

The immunoblot reactions were repeated with extracts of infected CEF, in which we have also demonstrated cross-ADCMC (8, 14, 30), and because the purified virus preparations were derived originally from CEF. Immune reactivity with infected CEF yielded results identical to those observed for infected L₉₂₉ cells with both homologous and heterologous antisera. Other preparations of hyperimmune serum further confirmed that cross-reactivity to SIN glycoprotein E1 was apparent after immunoblot reactions only with purified virions but not with infected cell extracts.

Immune precipitation of cross-reactive proteins. Hyperimmune sera were used to precipitate [³⁵S]methionine-labeled viral proteins from extracts of SIN-infected cells, and the precipitates were analyzed by SDS-PAGE and autoradiography. Alternatively, unlabeled SIN-infected cell extracts were precipitated with appropriate antiserum and subjected to immunoblotting with homologous anti-SIN serum. The results obtained through either technique were comparable, except that the latter method proved to be significantly faster, requiring 24 h or less for autoradiography, and used the ¹²⁵I radioisotope. Immunoprecipitation profiles of a typical experiment are shown in Fig. 4. Cross-reactive anti-SF serum precipitated the SIN E1 glycoprotein from infected cell extracts. In addition, capsid and, in most experiments, the 92K were cross-immunoprecipitated from SIN-infected cells. Neither E2 or PE2 could be cross-immunoprecipitated. Homologous anti-SIN serum immunoprecipitated 92K, PE2, E1, E2, and capsid from SIN-infected cell extracts (Fig. 4).

Partial renaturation of proteins from SDS and immunoblotting. The ability to detect cross-reactivity to SIN E1 from infected cell extracts by immunoprecipitation suggested that cross-reactivity with E1 might become detectable by immunoblotting if the protein could be sufficiently renatured before reacting with antiserum. The renaturation of proteins from SDS was performed by the methods previously described for the renaturation of DNA-binding proteins (2),

FIG. 2. Immunoblot reactions of SIN proteins with anti-SF serum. Lanes 1 and 2, SIN-infected cell extracts at 7 and 9 h postinfection, respectively; lane 3, purified SIN virion; lane 4, uninfected cell extract control.

FIG. 3. Immunoblot reactions of purified SIN virion proteins with anti-SIN serum (left) or anti-SF serum (right) after simultaneous electrophoresis. SIN virion proteins cross-reactive with anti-SF serum were identified as capsid and a low-molecular-weight subregion of E1.

enzymes (29), and viral structural proteins (25), except that renaturation was attempted after the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose. The transfers, containing an equal protein concentration of SIN-infected cell extract, were reacted with anti-SIN or anti-SF serum before renaturation procedures, after washing transfers in 4 M urea to facilitate the removal of SDS, or after washing transfers in urea and in Tris-acetate buffer to facilitate the removal of urea. The initial washing steps of renaturation resulted in a slight but reproducible increase in the amount of detectable protein by immunoblotting with either antiserum but was rapidly diminished when immunoblotting was performed after washing in Tris-acetate, presumably a result of the elution of proteins from the nitrocellulose. Although a slight increase in the quantitative reactivity of the antisera was apparent, sufficient renaturation of cell-associated proteins for the detection of cross-reactive E1 was not achieved. The inability to restore the cross-reactivity of cell-associated E1 may represent an innate property of the protein or may be attributable to a technical difficulty with the renaturation procedure.

DISCUSSION

Hyperimmune sera prepared against purified alphaviruses participated in cross-reactive cytolysis of SIN or SF virus-infected cells (Table 1; 7, 13, 30). Viral proteins recognized in cytolysis reactions were determined through the use of a monospecific antiserum made to SIN proteins, and the results suggested that both the E1 and the E2 surface glycoproteins could serve as target antigens in ADCMC (Table 1). However, note that each monospecific antiserum was not cross-reactive by ADCMC. This finding was consistent with our previously published evidence demonstrating a requirement for hyperimmunization in the generation of SIN and SF cross-reactive antisera (8, 14).

The hyperimmune antisera were used to identify virus-specific and cross-reactive proteins in virions and extracts of infected cells by an application of the immunoblot technique (on denatured proteins) and by immune precipitation (of

mildly extracted native proteins). After immunoblotting, the antisera detected the E1, E2, and capsid structural proteins in virions and infected cells as well as the precursor PE2/p62 and a 92K protein in infected cells when used in homologous reactions (e.g., SIN anti-SIN, SF anti-SF). The results demonstrated that the antisera, prepared against native viral proteins, were efficient in recognizing homologous antigenic determinants after the proteins had been denatured in preparation for immunoblotting. These same proteins in disrupted SIN virions and extracts of SIN-infected cells were also reactive in immune precipitation with homologous anti-SIN serum.

After immunoblotting of SIN virion proteins, cross-reactivity with the E1 glycoprotein and with capsid protein was detected. However, in contrast to homologous reactivity, the cross-reactivity of anti-SF serum with SIN virion E1 appeared to be directed primarily toward a subregion of E1. This cross-reactive subregion migrated in the lower-molecular-weight range of the broad glycoprotein band detectable with homologous anti-SIN serum. Although this result was consistently reproducible, the significance of a cross-reactive E1 subregion is unknown.

The combined results of immunoblotting and immune precipitation suggested that antigenic sites which cross-react between members of different alphavirus subgroups, SIN and SF, are located on the capsid protein and on the E1 glycoprotein in both virions and infected cells. Presumably, all molecular forms of E1 should be present in infected cells at the times postinfection shown in these studies. However, there was an intriguing discrepancy in our ability to detect cross-reactivity with the E1 glycoprotein: virion E1 cross-reactivity was apparent both before and after protein denaturation (immunoblotting), whereas, in contrast to homolo-

FIG. 4. Immunoprecipitation of SIN proteins with anti-SIN serum or anti-SF serum. Proteins from nonlabeled SIN virions or SIN-infected cells were precipitated with the appropriate antiserum and subjected to immunoblotting with homologous anti-SIN serum. Disrupted SIN virion proteins were precipitated by anti-SIN serum (lane 1) or by normal rabbit serum (lane 2). SIN-infected cell extracts were precipitated by normal rabbit serum (lane 3), by anti-SF serum (lane 5), or by anti-SIN serum (lane 6). Uninfected cell extracts were precipitated by anti-SIN serum (lane 4).

gous reactivity, cross-reactivity to the E1 from infected cells was demonstrable only before denaturation of the protein (immune precipitation and ADCMC). These results suggest that alphavirus maturation is accompanied by a stabilization of cross-reactive antigenic sites on E1. This stabilization may not occur until virions are released from infected cells or it occurs at such low levels in infected cells that it is undetectable even after acetone concentration.

The alphaviruses undergo a complex maturation sequence which involves extensive viral protein interactions. During morphogenesis, a loosely associated E1-PE2 complex migrates to the infected cell plasma membrane (1, 19) where E1 is readily detected on the outer surface of the cell, whereas determinants of PE2 remain unavailable for reactivity to ferritin-conjugated antibody or lactoperoxidase iodination (26). However, although PE2 cannot be demonstrated on the outer surface of the cell directly, it is highly glycosylated and is fractionated from the cell in the plasma membrane (7, 21), suggesting that it might be externally situated (D. Brown, personal communication). The E1-PE2 unprocessed polypeptide described in SIN-infected BHK cells (19, 23) has an apparent molecular weight comparable to the 92K protein we have observed in infected L₉₂₉ cells. Our ability to cross-immunoprecipitate E1 and 92K (a polypeptide of E1-PE2) from infected cells suggests that cross-reactive antigenic sites may be exposed on E1 only when it is free of association with other viral proteins (E2) or when it is in an unprocessed form with PE2. However, further investigations are required to determine whether the 92K protein in our system is, in fact, an E1-PE2 polypeptide.

The cleavage of PE2 to yield E2 and E3 is a rate-limiting step in alphavirus maturation. This cleavage event results in the expression of E2 on the infected cell surface (26) and, subsequently, the formation of heterodimers of E1-E2 (19) which become incorporated into mature virions. Our ability to detect cross-reactivity after protein denaturation (immunoblotting) with E1 only from virions, but not from infected cells, suggests that cross-reactive antigenic sites on E1 become stabilized against denaturation once incorporated into virions. This stabilization may be attributable to conformational or molecular changes in E1 which result from the cleavage of PE2 and heterodimer formation with E2.

These results lend additional support to our previously published evidence suggesting that viral proteins expressed by infected cells are immunologically distinct from virion proteins recognized in antibody-dependent cross-reactions between SIN and SF viruses (8, 14, 27, 31). The existence of immunologically unique cell-membrane-associated viral antigens is supported by additional evidence from other laboratories working with alphaviruses, although the work has not involved cross-reactivity between distinct alphavirus subgroups. Kaluza (12) and Kaluza et al. (13) have reported antigenic variations in SF glycoproteins during virus morphogenesis and suggested that differential glycosylation contributed to the variation in availability or expression of antigenic sites in infected cells compared with mature virions. A monoclonal antibody to the SIN E1 glycoprotein was shown to bind to SIN-infected cells and to disrupted virions but not to intact virions (24), suggesting that antigenic sites expressed by infected cells become masked in the mature virus particles. Analogous results have been reported for other virus systems (32).

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