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Alphavirus Spike-Nucleocapsid Interaction and Network Antibodies

MAARIT SUOMALAINEN AND HENRIK GAROFF*

Department of Molecular Biology, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden

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Vaux et al. (D. J. T. Vaux, A. Helenius, and I. Mellman, Nature (London) 336:36–42, 1988) recently reported the production of network antibodies that were suggested to have reconstructed a specific interaction between the nucleocapsid of Semliki Forest virus and the cytoplasmic tail of the viral E2 spike protein. The F13 anti-idiotype antibody, which was raised against anti-E2 tail antibodies, was claimed to recognize the virus nucleocapsid. In this report, we have used recombinant SFV viruses to demonstrate that the F13 antibody is not nucleocapsid specific but instead most likely recognizes some component of the viral replication machinery.

Budding of alphaviruses is a highly selective membrane sorting event: only virus-encoded spike glycoproteins, the E2-E1 heterodimers, are incorporated into the virion envelope, whereas host membrane proteins are efficiently excluded from this structure (12). It has been suggested that the basis for this high fidelity is a specific interaction of virus nucleocapsid with the internal domains of spike heterodimers (3, 11). The internal spike domain is composed of the 31 carboxy-terminal residues of E2 and the two carboxy-terminal residues of E1, both of which are arginines (4). In accordance with this model, Vaux et al. (15) recently reported that the nucleocapsid of Semliki Forest virus (SFV) contains a receptor region for the internal domain of E2. The experimental support for this finding was obtained by an anti-idiotype approach. A synthetic peptide corresponding to the cytoplasmic tail of E2 was used as an immunogen for the production of polyclonal anti-E2 tail antibodies. These polyclonal antibodies were then used as immunogens in a second round of immunization. This resulted in the production of a monoclonal anti-idiotype antibody designated F13, which was claimed to specifically immunoprecipitate the SFV nucleocapsid protein (the C protein). Immunofluorescence staining of SFV-infected cells with this antibody gave a very characteristic punctate cytoplasmic staining pattern (15; see also Fig. 1A in this report). However, this punctate staining pattern was clearly different from that obtained with anti-C antibodies (15; see also Fig. 1G in this report). A monoclonal anti-anti-idiotype antibody raised against F13 (3G10) recognized both the original antigen (the E2 tail peptide) and the F13 immunoglobulin M, confirming that F13 was an authentic internal image anti-idiotype antibody. Therefore, these network antibodies were suggested to represent the first conclusive proof for a specific interaction between the nucleocapsid and the E2 tail.

In this study, we have used recombinant SFV viruses to show that the F13 anti-idiotype antibody is not nucleocapsid specific but instead recognizes some as-yet-unidentified component of the SFV RNA replication machinery.

Expression of recombinant SFV genomes that lack the C gene. pSFV-tr contains a cDNA copy of a recombinant SFV genome from which the genes for the structural proteins have been deleted and replaced with a cDNA encoding the human transferrin receptor (7). This SFV cDNA can be used as a template for in vitro transcription of the corresponding RNA genome. When this SFV-tr RNA is transfected into host cells, the SFV nonstructural region is translated into proteins which both replicate the RNA genome and produce subgenomic mRNAs encoding the transferrin receptor. In vitro-synthesized SFV-tr RNA can also be packaged into infectious virus particles by cotransfection with a packaging-deficient helper RNA genome that provides the structural proteins (7). When we infected BHK-21 cells with these recombinant SFV-tr viruses (10 infectious units per cell) and at 8 h postinfection stained the cells with F13 as described in reference 15 (F13 antibody provided by David Vaux, European Molecular Biology Laboratory, Heidelberg, Germany), we found that all cells exhibited the characteristic punctate F13 staining pattern (Fig. 1B), even though the C protein was not expressed in these cells. However, a possibility existed that this F13 staining was due to contamination of the SFV-tr recombinant virus stock with virus particles that carried both the SFV-tr and the helper genomes or with particles that carried a recombinant of these two genomes. We therefore analyzed in a pulse-chase experiment which proteins were encoded by the SFV-tr genome. BHK-21 cells were infected with SFV-tr (10 infectious units per cell), and at 6 h after infection, cells were metabolically labeled with [^{35}S]methionine (50 μCi/ml) for 15 min as previously described (16). Label was chased for 15 min, and cell monolayers were solubilized with a Nonidet P-40 buffer (13). Transferrin receptor, C, and E1 were immunoprecipitated from equal volumes of cell lysates with monoclonal anti-transferrin receptor, anti-C, and anti-E1 antibodies, respectively, using goat anti-mouse antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands) as secondary antibody and protein A-Sepharose to collect the immunocomplexes. The precipitated proteins were then solubilized in sodium dodecyl sulfate (SDS)-sample buffer (16), heated to 70°C for 5 min, and subjected to electrophoresis on nonreducing SDS–10% polyacrylamide gels. As shown in Fig. 2, cell lysates contained high levels of transferrin receptor but no C or E1, although all cells expressed an F13-reactive epitope, as indicated by indirect immunofluorescence (Fig. 1B). To further verify that the staining we obtained with F13 was not due to expression of C protein, BHK-21 cells were transfected with in vitro-synthesized SFV-tr RNA by electroporation (8) and directly assayed for F13 reactivity. At 16 h after electroporation, the characteristic punctate F13 staining pattern was clearly identifiable in

* Corresponding author.

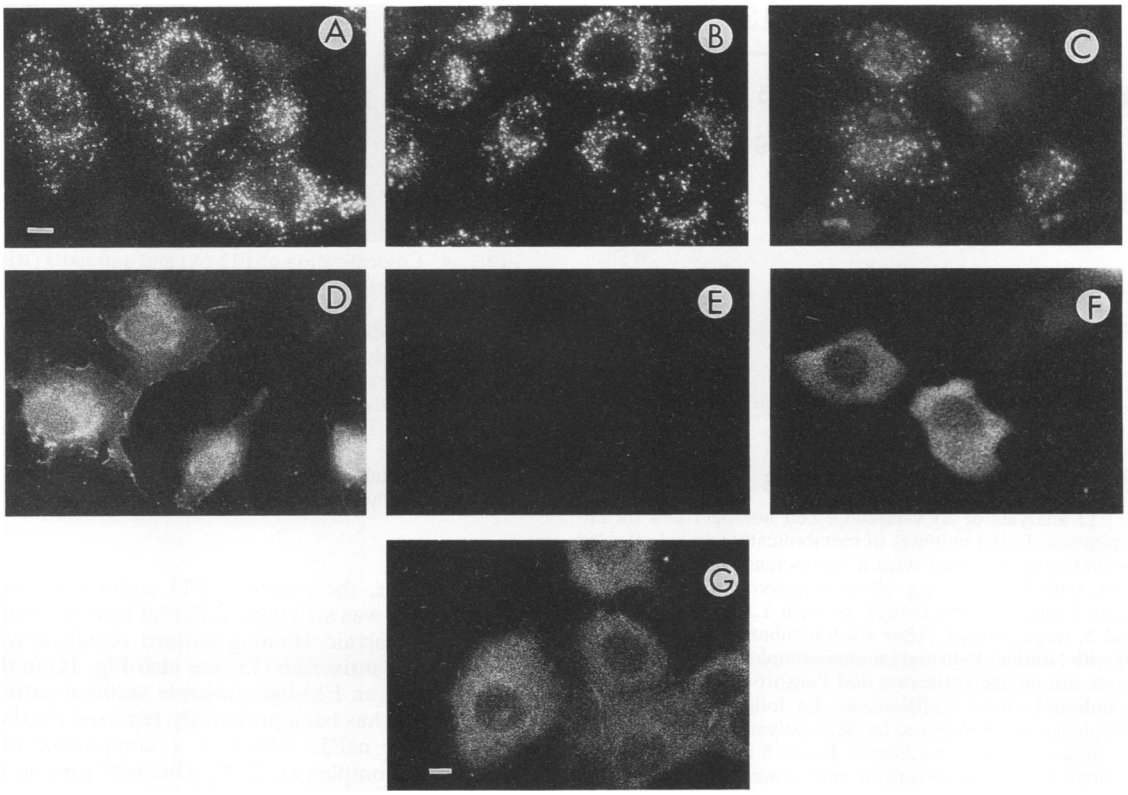

FIG. 1. F13 staining of cells expressing recombinant SFV genomes. Cells were fixed with methanol and prepared for indirect immunofluorescence by using the F13 anti-idiotypic antibody (A to C) or a monoclonal anti-C antibody (F and G) as primary antibody and FITC-conjugated sheep anti-mouse antibodies as secondary antibody or by using polyclonal anti-E2 antiserum and F13 as primary antibodies and rhodamine-conjugated goat anti-rabbit and FITC-conjugated sheep anti-mouse antibodies as secondary antibodies (D and E). Panels show F13 staining of cells infected with wild-type SFV virus (A) or recombinant SFV-tr virus (B) or transfected with in vitro-transcribed SFV-tr RNA (C). pSVS-SFV-transfected cells were double stained with a polyclonal anti-E2 antiserum (D) and F13 (E). The same field was exposed for rhodamine (D) and fluorescein (E). (F) pSVS-SFV-transfected cells stained with monoclonal anti-C antibody; (G) SFV (wild-type)-infected cells stained with monoclonal anti-C antibody. Bar = 8 μm.

these SFV-tr-transfected cells (Fig. 1C), whereas mock-transfected cells were negative (data not shown). Similar efficient expression of the F13-reactive antigen was also found in cells transfected with another recombinant SFV genome expressing only the spike proteins but no C protein (data not shown; the recombinant genome was SFV-spike [14]).

We also tested whether cells expressing only the SFV structural region (but not the nonstructural, polymerase-encoding region) could be specifically stained with F13. For this purpose, we used the pSVS-SFV plasmid (6) that contains the cDNA of the wild-type subgenomic 26S RNA under the control of the simian virus 40 early promoter. The pSVS-SFV was transfected into BHK-21 cells by using Lipofectin as described in reference 14, and at 24 h after transfection, cells were fixed with methanol and stained with a monoclonal anti-C antibody (5) or double stained with F13 and a polyclonal anti-E2 antiserum. Cells transfected with pSVS-SFV expressed large amounts of C protein, as evidenced by the strong signal obtained by the monoclonal anti-C antibody (Fig. 1F). However, no F13 reactivity was visible in transfected cells (compare Fig. 1D and E). Thus, we conclude that the F13 staining pattern is a result of this antibody interacting with some antigen other than the C protein.

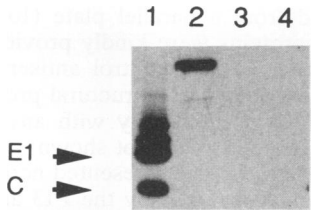

FIG. 2. Immunoprecipitation analysis of SFV-tr-infected cell lysates. Lane 1, SFV virion proteins; lane 2, immunoprecipitation with anti-transferrin receptor; lane 3, immunoprecipitation with anti-C; lane 4, immunoprecipitation with anti-E1. Equal volumes of cell lysates were used for anti-transferrin receptor, anti-C, and anti-E1 immunoprecipitations.

Analysis of homogenates from SFV-infected cells by immunoprecipitation with F13. Immunoprecipitation was originally used to biochemically identify the F13-reactive antigen (15). The primary antigen-antibody reaction was carried out in crude postnuclear supernatant fractions. These were then detergent solubilized, and immunocomplexes were collected with fixed *Staphylococcus aureus* cells. This homogenate-based immunoprecipitation procedure was necessary, since preliminary assays had indicated that the F13-reactive epitope was detergent sensitive. By this assay, F13 was shown to immunoprecipitate the C protein in a concentra-

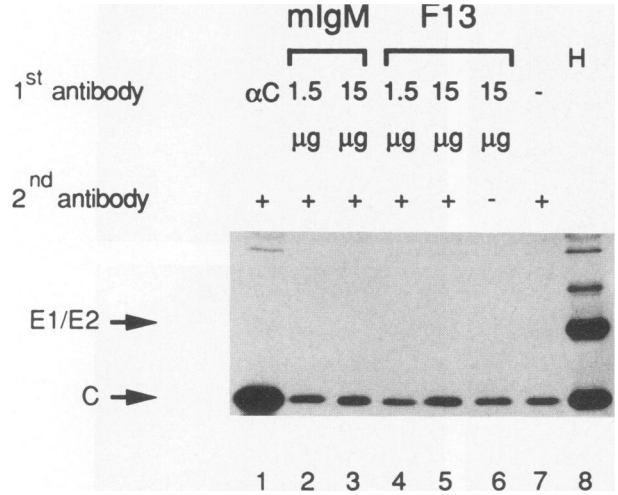

FIG. 3. F13 analysis of SFV-infected cell homogenates by immunoprecipitation. Equal volumes of metabolically labeled infected cell homogenates were mixed with a monoclonal anti-C antibody (αC, lane 1), with 1.5 or 15 μg of the negative control antibody (mIgM; lanes 2 and 3, respectively), or with 1.5 or 15 μg of F13 (lanes 4 and 5, respectively). After a 2-h incubation, samples were solubilized with Nonidet P-40 and immunocomplexes were collected by using goat anti-mouse antiserum and Pansorbin (10% [wt/vol] in phosphate-buffered saline; CalBiochem, La Jolla, Calif.). Isolated immunocomplexes were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Lanes 6 and 7 show immunoprecipitations when the second or first antibody, respectively, was omitted. Lane 8 shows the nonimmunoprecipitated homogenate.

tion-dependent manner (15). However, the proportion of the total C protein that was immunoprecipitable with the F13 antibody was not determined. Because of the results we obtained by immunofluorescence, we attempted to repeat the specific immunoprecipitation of C with F13. We used otherwise the same protocol as Vaux et al. (15), except that goat anti-mouse antiserum (Nordic Immunological Laboratories) was used as the secondary antibody. However, we were repeatedly unsuccessful in specifically immunoprecipitating the C protein with F13. As shown in Fig. 3, there was at most only a minor increase in the amount of immunoprecipitated C protein with 15 μg of F13 instead of 1.5 μg. Similarly, the differences between negative control immunoglobulin M (purified mouse immunoglobulin M myeloma from the Binding Site Ltd., Birmingham, England; Fig. 3, lanes 2 and 3) and F13 (lanes 4 and 5) immunoprecipitations were insignificant. Furthermore, by comparing the monoclonal anti-C and F13 immunoprecipitations (lanes 1 and 4, respectively), it is obvious that the amount of C immunoprecipitated by F13 is only a minor fraction of the total C protein. The inability of F13 to specifically recognize the C protein in our immunoprecipitation assays was not due to an inefficient secondary antibody, since the goat anti-mouse antiserum clearly reacted with the F13 antibody in an Ouchterlony double-diffusion assay (data not shown). Furthermore, increasing the amount of the secondary antibody did not increase the amount of C immunoprecipitated by F13 (data not shown). Taken together, these results strongly suggest that the small amount of C immunoprecipitated with F13 is a result of a nonspecific reaction.

Colocalization of F13 and anti-nsP3 immunofluorescence staining patterns in SFV-infected cells. As noted in the original paper, the punctate F13 staining pattern in SFV-infected cells was strikingly different from the rather homogenous cytoplasmic staining pattern obtained with a polyclonal anti-C antiserum (15; see also Fig. 1G in this report). Interestingly, an F13-like punctate staining pattern in SFV-infected cells has been previously reported for the nonstructural protein nsP3, which is a component of the SFV polymerase complex (1, 2, 9). The nsP3 protein localizes to type I cytopathic vacuoles, which are the sites of viral RNA replication in infected cells. To determine whether the F13-reactive antigen could be a component of the viral replication machinery, we performed a double-label indirect immunofluorescence assay in SFV (wild-type)-infected cells by using F13 and affinity-purified rabbit anti-nsP3 antibodies (kindly provided by Johan Peränen and Leevi Kääriäinen, Institute of Biotechnology, University of Helsinki, Helsinki, Finland). BHK-21 cells were infected at 100 PFU per cell, and at 3 h after infection, cells were processed for immunofluorescence. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies (BioSys, Compiegne, France) were used as secondary antibodies. As shown in Fig. 4A (F13) and B (anti-nsP3), both staining patterns were extensively colocalized within the infected cells. This suggested that the F13-reactive antigen could be a part of the viral replication machinery. To determine whether F13 specifically immunoprecipitates any of the viral nonstructural proteins, the F13 immunoprecipitation pattern of SFV-infected cell homogenates was compared with anti-nsP1, anti-nsP2, anti-nsP3, and anti-nsP4 immunoprecipitates of SFV-infected cell lysates prepared from a parallel plate (10; antisera against nonstructural proteins were kindly provided by J. Peränen and L. Kääriäinen). The control antisera immunoprecipitated the corresponding nonstructural protein antigens, but F13 failed to react specifically with any one of the SFV nonstructural proteins (data not shown).

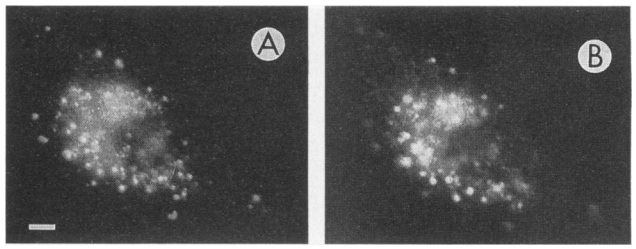

FIG. 4. Colocalization of F13 (A) and anti-nsP3 (B) reactivity in SFV-infected cells. BHK-21 cells were infected with wild-type SFV virus, and at 3 h after infection, cells were fixed with methanol. Double-label indirect immunofluorescence was carried out by using the F13 antibody and affinity-purified anti-nsP3 antibodies as primary antibodies. FITC-conjugated sheep anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies were used as secondary antibodies. The same field was exposed for fluorescein (A) and rhodamine (B). The overall staining intensity produced by the anti-nsP3 antibodies was considerably weaker than that obtained by the F13 antibody. Bar = 4 μm.

Taken together, the data presented here strongly suggest that the antigen recognized by the F13 antibody is not the SFV C protein as originally claimed. Although we were unable to determine the precise identity of the F13-reactive antigen, our results described above suggest that this antigen is a component of the SFV RNA replication machinery. First, the F13 staining pattern extensively colocalized with that obtained with anti-nsP3 antibodies. Second, all SFV

genomes (wild type, SFV-tr, and SFV-spike) that directed expression of the F13-reactive epitope contained an intact nonstructural region, and all these genomes were replication competent. In contrast, the F13 staining pattern did not appear when only the SFV structural region was expressed. If F13 was nucleocapsid specific, pSVS-SFV-transfected cells should also have expressed the F13-reactive antigen, since recent results from our laboratory indicate that SFV C proteins can assemble into budding-competent nucleocapsid structures in the absence of the viral genome (14). Our suggestion as to the identity of the F13-reactive antigen also offers an alternative explanation for the surprising finding that the F13-reactive epitope was also expressed in cells infected with other alphaviruses and flaviviruses (15); this high degree of conservation could be due to F13 recognizing a conserved feature of the RNA replication machinery of these different positive-stranded RNA viruses. Clearly, it would be most interesting to identify the F13-reactive antigen. Another question that remains unanswered is whether the apparent reactivity of F13 with the viral replication sites represents an artifact of the anti-idiotype approach or whether the network antibodies reflect some as-yet-unknown interaction between the cytoplasmic domain of E2 and the viral replication machinery.

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