Nonstructural Proteins NS2 of Minute Virus of Mice Associate In Vivo with 14-3-3 Protein Family Members

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The nonstructural NS2 proteins of the prototype strain of minute virus of mice (MVMp) were previously shown to be involved in parvoviral DNA amplification as well as in efficient virus production in a host cell-specific manner (L. K. Naeger, N. Salomé, and D. J. Pintel, *J. Virol.* 67:1034–1043, 1993). NS2 polypeptides were also reported to participate in the cytotoxic activity of parvoviruses (C. Legrand, J. Rommelaere, and P. Caillet-Fauquet, *Virology* 195:149–155, 1993), for which transformed cells are preferential targets. To identify cellular partners of NS2 proteins, coimmunoprecipitation experiments were performed with various antibodies directed against the parvoviral products. Two cellular proteins with molecular masses of 30 and 32 kDa were found to associate in vivo with the NS2 polypeptides. From amino acid sequence homology, these NS2 partners were assigned to the 14-3-3 family of cellular proteins, showing at least partial identity with the epsilon and beta or zeta 14-3-3 isoforms. In agreement with this assignment, NS2–30/32-kDa protein immune complexes displayed an activating function for exoenzyme S in vitro, a hallmark of 14-3-3 polypeptides. Interactions with 14-3-3 proteins did not appear sufficient for NS2 functions, since they were not disrupted by NS2 C-terminal modifications that impaired virus replication. Binding of NS2 to 14-3-3 proteins was detected in various cells of mouse, rat, hamster, monkey, and human origin, irrespective of NS2 dispensability and host cell transformation or permissiveness. The ubiquitous 14-3-3 proteins were recently reported to associate with several other cellular or viral polypeptides involved in signal transduction and/or cell cycle regulation pathways (A. Aitken, *Trends Biochem. Sci.* 20:95–97, 1995). The NS2 products may connect with one of these pathways through their interaction with specific 14-3-3 polypeptides.

Autonomous parvoviruses are characterized by their dependence for growth on cellular factors that are expressed as a function of cell proliferation and differentiation (for a review, see reference 12). These requirements result in the restriction of productive parvovirus infections to proliferating tissues (in vivo or in culture) and are likely to account for the remarkable oncotropism exhibited by some of these viruses (44, 45, 55). Besides cellular factors, virus-encoded regulatory proteins take part in the control of parvovirus replication. These nonstructural (NS) viral products are the object of intense investigations conducted, in particular, with MVMp, the prototype strain of parvovirus minute virus of mice (MVM), and the closely related rat virus H-1. The genomes of these agents consist of an essentially single-stranded, linear DNA molecule that is about 5,000 nucleotides (nt) long and contains two overlapping transcription units (12). The capsid proteins VP1 and VP2 are generated from the right-hand part of the genome under the control of the P38 promoter. The left-hand transcription unit is directed by the P4 promoter and encodes at least four NS proteins that are implicated in various steps of parvovirus growth. The best characterized of these products, NS1, is a 83- to 88-kDa phosphoprotein that is mostly localized in the nucleus and is required for viral DNA replication and

gene expression. The multiple functions of NS1 include DNA helicase, ATPase, and site-specific endonuclease activities, such as positive and negative *trans* activation of both P4 and P38 viral promoters (for a review, see reference 54). The role of the other nonstructural products, designated NS2, is more elusive. The 23- to 28-kDa NS2 phosphoproteins are mainly cytoplasmic and consist of two (H-1) to three (MVM) isoforms that differ at their carboxy termini as a result of the alternate splicing of a small intron located at map units 44 to 46 (9, 13, 29). All NS2 isoforms share their first 85 N-terminal amino acids (aa) with NS1 (12). Although their mode of action is not known, NS2 proteins are somehow involved in efficient translation of viral mRNAs and in accumulation of viral DNA replication intermediates and mature virions (8, 11, 29, 38, 39).

A close interconnection appears to exist between parvovirus NS proteins and cellular factors. The NS products were found to interfere with a series of nonparvovirus processes, including gene expression from heterologous promoters, replication of extrachromosomal DNA, in vitro neoplastic transformation, and stable cell transfection with exogenous DNA (for a review, see reference 54). These effects may account, at least in part, for the cytotoxic activity of NS proteins, which requires the NS1 product and may also be modulated by NS2 in some cells (4, 7, 9, 28). Conversely, cell transformation with oncogenes proved to exacerbate the cytotoxic function of NS1 (37). Another feature of this cross talk between cell and virus products is reflected in the species dependence of the role of NS2 in the parvovirus life cycle. Indeed, functional NS2 products are absolutely required for MVM and H-1 replication in cells from

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the natural host (mouse and rat, respectively), while this requirement is less pronounced in various cell lines of other origins (29, 38). Together with the diversity of the viral and cellular processes in which NS polypeptides interfere, these observations led us to speculate that the NS proteins need to interact with specific cellular products to achieve some of their functions.

This report represents a first attempt to identify cellular proteins that become associated with the NS2 products of MVM. These partner proteins were sought on the basis of their ability to be coimmunoprecipitated with NS2 from infected cell extracts in the presence of antibodies directed against the viral products. Using this approach, we found that NS2 binds to at least two cellular polypeptides which both belong to a family of highly conserved proteins termed 14-3-3. This family comprises at least seven isoforms in mammalian cells (2). Some of the 14-3-3 proteins were recently reported to interact with other cellular and viral products, in particular Raf-1 (20), Bcr and Bcr-Abl (5, 41), protein kinase C (PKC) (53), cdc25 (10), or middle T antigen from polyomavirus (40), and so are thought to be connected with signal transduction and cell cycle control pathways (for reviews, see references 1 and 6). The role of these interactions is presently a matter of speculation, as in the case of NS2-14-3-3 complexes. However, NS2 interactions with the 14-3-3 proteins establish a link between NS2 and cellular regulatory cascades, providing a rationale for the apparently multiple levels at which NS2 products affect the viral life cycle and possibly the host cell.

MATERIALS AND METHODS

Cells and viruses. The FREJ4 cell line, a derivative of the FR3T3 established line of Fisher rat cells (48) transformed by the c-Ha-ras oncogene, was described previously (55). These cells were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate and 10% donor calf serum. Mouse A9 fibroblasts and simian virus 40 (SV40)-transformed human NB-324K cells (52) were grown in Eagle's minimum essential medium with 1% nonessential amino acids and 5% fetal calf serum. Baby Syrian hamster BHK cells (3) and their SV40-transformed THK derivatives (kindly provided by D. Ward, Yale University) were grown in Dulbecco's modified Eagle's medium containing 1 mM sodium pyruvate, 1% tryptose phosphate broth, and 10% donor calf serum. *Spodoptera frugiperda* Sf9 insect cells (42) were maintained at 26°C in TNM-FH medium (30) supplemented with 10% fetal calf serum. All medium components were purchased from either Gibco BRL or Sigma Chemical Co.

MVMp was propagated in A9 cells, titrated, and purified as described by Tattersall and Bratton (52). Mutant viruses NS2-2268 and Df18 were previously described (8, 38, 39) and were propagated in NB-324K cells as described by Naeger et al. (38). Wild-type linear baculovirus, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), was obtained from Invitrogen Corp. and propagated as previously described (42).

Cell labeling and protein immunoprecipitation. Subconfluent cultures were mock or MVMp infected at a multiplicity of infection of 5 PFU per cell. At 18 h postinfection, cell cultures (1×10^6 to 2×10^6 cells) were metabolically labeled for 2 h with 200 μ Ci of Tran³⁵S-label (1,000 Ci/mmol; ICN Pharmaceuticals) in methionine- and cysteine-free Eagle's minimum essential medium supplemented with 5% dialyzed fetal calf serum. Cells were lysed with either low-detergent buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml, 1 μ g of leupeptin per ml) or radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml, 1 μ g of leupeptin per ml) for about 30 to 60 min on ice. Cell lysates were harvested, and debris were removed by centrifugation at $8,000 \times g$ and 4°C for 5 min. Equal amounts of extracts (10^7 cpm of trichloroacetic acid-precipitable material) were immunoprecipitated with 5 μ l of either polyclonal rabbit antisera directed against NS1 and/or NS2 proteins or normal rabbit serum (Dianova) prior to addition of 50 μ l of a 50% suspension of protein A-Sepharose beads (Pharmacia) as previously described (31). Immunoprecipitates were washed three times with lysis buffer and resuspended in loading dye buffer. Dissociated immunocomplexes were separated by electrophoresis on SDS-12% polyacrylamide gels and visualized by autoradiography.

Antisera. The polyclonal rabbit antisera SP8 and SP6 are directed against carboxy-terminal peptides of NS1 (NS1C) and NS2 (NS2C), respectively, and are equivalent to the previously described SP7 (NS1C) and SP4 (NS2C) sera (15). Antisera specific for peptides derived from the amino-terminal domain shared by

NS1 and NS2 (NS1/2N) or the internal part of NS1 (NS1int) were prepared as follows. The 68-mer synthetic oligonucleotide,

5'GATCCCGCTTACTCTGATGAAGTTTGGGAGCAACCACTGGTTAAAGGAAAAAGTAACCAAGATA3'
3'GGCGAATGAGACTACTTCAAAACCTCGTTGGTTGACCAATTTCTTTTCATTGGCTCTATTGCA5'

harboring *Bam*HI and *Hind*III linker sites at its 5' and 3' ends, respectively, and coding for the AYSDEVLGATNWLKEKSNQE amino-terminal peptide common to NS1 and NS2, was inserted between the *Bam*HI and *Hind*III sites of the pLC24 vector (46). Using the same vector, a similar construction was made with the *Pst*I-*Hinc*II (nt 1353 to 1840) fragment from the NS1-specific large intron region of the nonstructural transcription unit of MVMp. These constructions gave rise to temperature-inducible chimeric genes encoding 98 aa of the MS2 polymerase fused to either NS1/2N or NS1int peptide. MS2-NS1/2N and MS2-NS1int fusion polypeptides (10 and 32 kDa, respectively) were isolated and used to immunize rabbits as previously described (22). Antisera recognizing both NS1 and NS2 (SP12) or only NS1 (SP10) proteins from MVMp were thus obtained.

Production of DHFR-NS2C fusion polypeptide. The prokaryotic expression vector pQE41 (Qiagen) contains a six-His tag-coding sequence in frame with the gene coding for mouse dihydrofolate reductase (DHFR), 5' to a polylinker region. The *Sau*3A-*Stu*I (nt 2201 to 2377) fragment from the right-hand side of the NS2 encoding region of MVMp was inserted between the *Bam*HI and *Hind*III sites of pQE41. This gave rise to an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible chimeric gene encoding a His-tagged mouse DHFR that was fused to the NS2C peptide derived from the carboxy terminus of NS2. Plasmids pQE41 and pQE41-NS2C were propagated in *Escherichia coli* JM109 in LB medium containing 50 μ g of ampicillin per ml.

To produce 6 $\times$ His-DHFR and 6 $\times$ His-DHFR-NS2C polypeptides (apparent molecular masses of 26 and 32 kDa, respectively), *E. coli* M15[pREP4] cells were transformed with either pQE41 or pQE41-NS2C and grown at 37°C in LB medium containing 100 μ g of ampicillin and 50 μ g of kanamycin per ml. Expression was induced by addition of 50 μ M IPTG for 4 to 6 h at 37°C. Bacteria were harvested by centrifugation and lysed in buffer A (6 M guanidinium-HCl, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl [pH 8]). Cellular debris were removed by centrifugation, and the supernatants were loaded onto a Ni-nitrilotriacetic acid (NTA) resin column (Qiagen). The column was washed with buffer A and successive runs of buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris) at pH 8, 7.4, and 6.3. His-tagged proteins were eluted with buffer B at pH 4.5. The purity of eluted proteins was checked by SDS-polyacrylamide gel electrophoresis.

Competition assays. In a first type of assay, bacterially expressed specific (6 $\times$ His-DHFR-NS2C) or control (6 $\times$ His-DHFR) competitor polypeptides were added in various amounts (0.5 to 5 μ g) to labeled cell extracts (10^7 cpm) together with SP6 antiserum (2 μ l). After incubation for 2 h at 4°C, samples were processed for immunoprecipitation as described above. In a second type of assay, extracts from 1×10^5 to 2×10^5 MVMp-infected FREJ4 cells were mixed with extracts from various amounts (2×10^5 to 1×10^7) of uninfected cells, incubated overnight at 4°C, and processed for immunoprecipitation with SP6 antiserum. Depending on the experiment, the infected or uninfected extract was ³⁵S labeled. All competition assays were performed in low-detergent buffer conditions.

Production of His-tagged NS2 protein. Recombinant baculovirus vectors harboring NS2-coding sequences were constructed by using standard methods (46). The MVMp sequence extending from nt 225 to 3453 but lacking both the large (nt 514 to 1990) and small (nt 2280 to 2377) introns, and coding for the major isoform of NS2, was isolated from the MMTV-NS2 (2280/2377) construct (9) and inserted into the *Bam*HI site of the AcMNPV transfer vector pVL941 (3a, 30). The *Bam*HI-*Nco*I fragment of MVM DNA (nt 225 to 261) was then replaced by a 54-nt-long double-stranded oligonucleotide that encodes a row of six histidine residues followed by a factor X cleavage site (a generous gift of R. Roeder, Rockefeller University). Sf9 cultures (2×10^6 cells) were transfected with a mixture of 1 μ g of linear AcMNPV DNA and 3 μ g of purified recombinant pVL941 vector DNA, using the cationic liposome-mediated transfection module supplied by Invitrogen Corp. Recombinant baculoviruses were isolated by selecting nonoccluded plaques in a plaque purification assay and propagated as described elsewhere (42). Baculovirus titers were determined by endpoint dilutions and converted to PFU per milliliter.

For expression of histidine-tagged NS2 (His-NS2), subconfluent Sf9 cultures were infected with the His-NS2 fusion protein-expressing recombinant baculovirus at a multiplicity of infection of 5 PFU per cell. At 40 to 48 h postinfection, cells were harvested, incubated in hypotonic buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 5 mM KCl, 7.5 mM MgCl₂, 0.1 mM dithiothreitol) for 30 min on ice, and subsequently disrupted by 25 strokes in a tight-fitting homogenizer. Lysis of nuclei was achieved by adjusting cell extracts to a final concentration of 300 mM NaCl and incubating them on ice for 30 min. Soluble proteins were cleared from chromosomal DNA and cell debris by centrifugation at $18,000 \times g$ for 30 min at 4°C. Protein extracts were stored at -70°C.

Purification of the NS2-bound 30- and 32-kDa cellular proteins. Extracts prepared from 10^8 FREJ4 cells in low-detergent buffer were loaded onto a

Ni-NTA column (Qiagen) saturated with baculovirus-expressed His-NS2. The column was washed twice with buffer C (10 mM HEPES-KOH [pH 7.5], 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 17% glycerol) and four times with buffer C containing 10 mM imidazole. His-NS2 and bound cellular products were then eluted with buffer C containing 80 mM imidazole. Eluted products were separated by electrophoresis on an SDS-10% polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and reversibly visualized by amido black staining. The bands corresponding to the 30- and 32-kDa NS2-associated proteins were cut from the membrane for further analysis.

HPLC purification and sequencing of tryptic peptides from 30- and 32-kDa proteins. In situ tryptic digestion of destained immobilized 30- and 32-kDa proteins was performed by overnight incubation at 37°C, using sequencing-grade modified trypsin (Promega) at a protein/trypsin ratio of 1:10, essentially as described by Fernandez et al. (17). Tryptic peptides eluted from the membrane during the enzymatic digestion were separated by high-pressure liquid chromatography (HPLC), with 0.12% trifluoroacetic acid (TFA) as solvent A and 0.08% TFA and 80% acetonitrile as solvent B, using a Brownlee C_{18} column and a 130A HPLC separation system (Applied Biosystems). Peptide elution was monitored at 210 and 277 nm, and individual peaks were collected manually and stored at -20°C. The HPLC-separated peptide samples were applied onto TFA-treated filters (Beckman) and placed in the reaction cartridge of a 477A protein sequencer apparatus (Applied Biosystems) for automated Edman degradation analysis. HPLC-separated peptides were also analyzed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS), using a time-of-flight mass analyzer (Vision 2000; Finnigan MAT, San Jose, Calif.) as described by Hillenkamp et al. (23). Data obtained from both internal sequencing and MALDI-MS were compared with entries in the Swissprot sequence databank, using the HUSAR (Heidelberg Unix Sequence Analysis Resources) system (14, 49).

FAS assay. Lysates from 10^5 MVMP-infected or uninfected FREJ4 cells in low-detergent buffer were immunoprecipitated as described above, using a rabbit polyclonal anti-NS protein antibody (SP6 or SP8) or normal rabbit serum. Immunoprecipitates were tested for the ability to activate the ADP-ribosyltransferase function of exoenzyme S from *Pseudomonas aeruginosa* (a reaction termed FAS, for factor activating exoenzyme S) as described previously (19). Briefly, 5 μ l of immunocomplexes, 10 ng of recombinant exoenzyme S (Upstate Biotechnology, Inc.), 6.25 μ Ci of [32 P]adenylate-labeled NAD^+ (1,000 Ci/mmol; Amersham), and 1 μ g of soybean trypsin inhibitor (Sigma) as an artificial substrate were mixed in a final reaction volume of 25 μ l. The assay was performed at room temperature for 30 min. Aliquots were spotted onto P81 paper (Whatman), washed twice with 0.75% phosphoric acid, and washed once with acetone. [32 P]ADP-ribose transfer to the substrate was determined by scintillation counting of the filters. Background values obtained with normal rabbit serum were subtracted.

RESULTS

Coimmunoprecipitation of NS2 with two proteins from MVMP-infected cells. To identify cellular protein partners of NS2, immunoprecipitation reactions were performed with lysates of MVMP- and mock-infected FREJ4 cells metabolically labeled with [35 S]methionine-cysteine. We used various antibody preparations directed against the NS domains indicated in Fig. 1, i.e., the NS2-specific C terminus (SP6), the NS1-specific internal (SP10) and C-terminal (SP8) regions, or the N terminus shared by NS1 and NS2 (SP12). When infected cell extracts were prepared under low-detergent conditions (no SDS), the anti-NS2 sera SP6 and SP12 brought down, besides NS2, two products with apparent molecular masses of 30 and 32 kDa (Fig. 2A, lanes 2 and 5). This reaction was specific for anti-NS2 sera since it was not observed with the SP8 and SP10 antibodies that recognize only NS1 (Fig. 2A, lanes 7 to 12). The specificity of these coimmunoprecipitation complexes was ascertained by conducting competition experiments using a DHFR protein fused to the NS2C peptide that carries the epitopes recognized by antiserum SP6. As shown in Fig. 2B (lanes 2 to 6), the amounts of NS2 and 30- and 32-kDa products in the SP6 immunoprecipitate were reduced in a dose-dependent fashion by the DHFR-NS2 polypeptide, while the DHFR component alone had no detectable effect. These results suggest that the observed precipitation of the 30- and 32-kDa proteins is specific for conditions allowing the formation of NS2 immune complexes. When similar coimmunoprecipitation assays were performed with uninfected cell extracts,

FIG. 1. Mapping of the NS epitopes recognized by the antibodies used in this study. In the scheme of the genome organization of MVMP shown at the top, the viral promoters at map units 4 (P4) and 38 (P38), transcription start sites (arrows), and terminal palindromic sequences (boxes) are indicated. Transcripts R1, R2M, R2tr, and R2r are aligned below, with introns (hyphens), polyadenylation sites (AAA), and open reading frames (ORF) encoding NS1, NS2P (major isoform), NS2Y (minor isoform), and NS2L (rare isoform) proteins indicated. Bars below open reading frames indicate the peptides used to raise antibodies. The NS1-specific antisera SP8 and SP10 recognize NS1C (aa 602 to 672) and NS1int (aa 384 to 525) peptides, respectively. The NS2-specific antiserum SP6 is directed against NS2C (aa 156 to 182) peptide, while antiserum SP12 recognizes NS1/2N (aa 5 to 25) peptide from the amino-terminal region shared by NS1 and NS2.

no 30- and 32-kDa species could be immunoprecipitated by anti-NS2 sera (Fig. 2A, lanes 1 and 4; Fig. 2B, lane 1).

Given these observations, a number of different hypotheses can be proposed concerning the 30- and 32-kDa polypeptides, including that they are: (i) cellular proteins that become associated with NS2, leading to their NS2-mediated immunoprecipitation; (ii) modified NS2 products; or (iii) MVMP-induced cellular proteins which share an epitope with NS2, allowing their direct recognition by anti-NS2 antibodies. The latter possibility is not very likely, considering that the 30- and 32-kDa polypeptides were revealed by both antisera SP6 and SP12 that are directed against distant regions of NS2 (Fig. 1). In addition, these products were brought down by a third type of antibodies specific for the carboxy terminus of the major NS2 isoform (13), which is distinct from the carboxy end of the minor NS2 isoform detected by antiserum SP6 (data not shown). Furthermore, anti-NS2 sera failed to immunoprecipitate the 30- and 32-kDa proteins in the presence of SDS, although immunoprecipitation of NS2 was efficient under these conditions (Fig. 2A, lanes 3 and 6). These observations argue for the first hypothesis (ionic detergent-sensitive NS2-30/32-kDa protein complexes) but do not rule out the other possibilities. This prompted us to determine whether uninfected cells contain 30- and 32-kDa polypeptides that can be detected by means of anti-NS2 sera provided that NS2 is added to the cell extracts.

Cellular origin of NS2 partner proteins. To test whether the 30- and 32-kDa products are of cellular origins and are expressed in the absence of virus infection, two types of competition experiments were carried out. In a first type of assay, increasing amounts of unlabeled noninfected cell extracts were added to a constant amount of 35 S-labeled MVMP-infected cell extract before immunoprecipitation with the antiserum SP6. As illustrated in Fig. 3 (lanes 1 and 7 to 10), the unlabeled noninfected cell extract displaced labeled 30- and 32-kDa products from the NS2 immunoprecipitate, resulting in a 80% reduction at the highest competitor concentration tested (com-

FIG. 2. Specific coimmunoprecipitation of NS2 and the 30- and 32-kDa polypeptides from MVMp-infected FREJ4 cell lysates. (A) Lysates of ^{35}S -labeled FREJ4 cells, infected (i) or not infected (ni) with MVMp at a multiplicity of infection of 5 PFU per cell, were prepared in the presence (+) or absence (-) of SDS and immunoprecipitated with indicated antisera. The autoradiogram shows immunoprecipitated materials after separation by SDS-12% polyacrylamide gel electrophoresis. (B) Immune complexes were prepared without SDS, using antiserum SP6, and processed as for panel A. Immunoprecipitation reactions were carried out in the absence of competitor (-) or in the presence of increasing amounts (0.5 to 5 μg) of DHFR-NS2C polypeptide containing the epitopes that are recognized by SP6. The DHFR polypeptide (5 μg) was used as a negative control. Arrows to the left point at NS1 (83 kDa), NS2 (25 kDa), 30- and 32-kDa proteins. M, molecular sizes (in kilodaltons) of prestained protein standards.

pare lanes 10 and 7). This inhibition was specific, since only a slight (10 to 20%) decrease of the amount of immunoprecipitated NS2 proteins was detected under these conditions, which may be ascribed to a dilution effect of the noninfected cell extract added. These results strongly suggest that the 30- and 32-kDa products both preexist in noninfected cells, although it is still possible that their expression is modulated as a result of virus infection. However, an alternative interpretation of these data needs to be considered; i.e., the apparent competition may actually be due to the preferential degradation of the 30- and 32-kDa polypeptides by factor(s) from the uninfected cell extracts.

To rule out the latter possibility, a second type of competition experiments was conducted, in which increasing amounts of labeled noninfected cell extracts were added to a given extract of unlabeled infected cells. In these assays, NS2 proteins were present in constant amount but could not be detected since they were not labeled. As shown in Fig. 3 (lanes 3 to 5), the addition of labeled noninfected cell extracts resulted in a protein concentration-dependent appearance of radioactive 30- and 32-kDa polypeptides in the immunoprecipitates, confirming the presence of these products in uninfected cells. In the absence of extract from unlabeled infected cells, no 30- and 32-kDa proteins were coimmunoprecipitated (Fig. 3, lanes 2 and 6), indicating that the immunoprecipitation of these products by anti-NS2 serum was mediated by some factor(s) expressed in MVMp-infected cells. Since various antibodies directed against distinct NS2 epitopes were all able to coimmunoprecipitate the 30- and 32-kDa proteins (see above), NS2 is most likely the infected cell component responsible for this reaction.

A final proof of the interaction of NS2 with the 30- and 32-kDa proteins was the capacity to use NS2 as a bait to purify these cellular factors by affinity chromatography.

NS2 interaction with members of the 14-3-3 family of cellular proteins. His-NS2 proteins were produced in insect cells by means of recombinant baculoviruses and bound to Ni-NTA columns that were used to purify NS2 partner proteins from

noninfected FREJ4 cell extracts by affinity chromatography. In agreement with above-mentioned data, 30- and 32-kDa cellular polypeptides were retained on Ni-NTA-His-NS2 columns while passing through in the absence of bound NS2 (data not shown). After elution of NS2-30/32-kDa protein complexes, the cellular components were purified by gel electrophoresis and further characterized by tryptic digestion and microsequencing of HPLC-fractionated peptides. As illustrated in Fig. 4, comparison of these amino acid sequences with data bank entries revealed a strong homology of both 30- and 32-kDa polypeptides with the 14-3-3 family of cellular proteins. Indeed, the 32-kDa product was identical to the 14-3-3 isoform ϵ across both peptides analyzed, while the 30-kDa species dif-

FIG. 3. Cellular origin of the 30- and 32-kDa cellular protein partners of NS2. Competitive immunoprecipitations were performed as for Fig. 2A, in the absence of SDS and with SP6 anti-NS2 serum. Labeled extracts from noninfected (ni) or MVMp-infected (i) FREJ4 cells were tested alone (-) or after addition of unlabeled extracts from noninfected (ni) or MVMp-infected (i) FREJ4 cultures. The use of increasing amounts of extracts (from 2×10^5 to 1×10^7 cell equivalent extracts) is indicated by profiles on top of corresponding lanes. Arrows to the left point at NS2 (25 kDa) and 30- and 32-kDa proteins. M, molecular sizes (in kilodaltons) of prestained protein standards.

FIG. 4. Amino acid sequence comparison of peptides from the 30- and 32-kDa polypeptides and 14-3-3 proteins. HPLC-fractionated tryptic peptides from purified 30- and 32-kDa proteins were sequenced. Italic letters correspond to amino acids which identities were ascertained by molecular mass analysis. Sequences are aligned with the ϵ , β , and ζ 14-3-3 isoforms. Amino acid identities are indicated by vertical bars. The corresponding consensus 14-3-3 sequence is shown above, with capital letters representing amino acids that are conserved in all isoforms. The positions of considered peptides are shown on the upper schematic representation of a 14-3-3 protein; amino acids are numbered from the acetylated amino terminus (AcN), with reference to the consensus sequence.

ferred from the β and ζ isoforms by only one amino acid at a nonconserved position in one of three peptides sequenced (2). This homology strongly argues for the fact that the 30- and 32-kDa partners of NS2 are 14-3-3 members, although no definite assignment to distinct isoforms can be made from the limited sequences available.

It has been reported that 14-3-3 proteins are required as cofactors for the ADP-ribosyltransferase activity of exoenzyme S from *P. aeruginosa*, hence their designation FAS (for factor activating exoenzyme S) (19). This property provides a convenient in vitro functional test for 14-3-3 proteins. To confirm that the 30- and 32-kDa products belonged to the 14-3-3 family, FAS assays were carried out with NS2 immunoprecipitates from MVMp-infected FREJ4 cells, using the soybean trypsin inhibitor as a standard substrate. As shown in Fig. 5, incorporation of ADP-ribose could not be detected in the presence of exoenzyme S or NS2 immunoprecipitate alone but was markedly increased when both components were supplied, as expected from the contribution of FAS in the latter. This FAS function was specific for NS2-cellular protein complexes since it was not exhibited to a significant extent by immunoprecipitates obtained either from uninfected cells or with anti-NS1 and control sera. Although some role of NS2 in FAS assays cannot be ruled out, these data corroborate the assignment of NS2 partners to the 14-3-3 family of proteins.

Ubiquity of the NS2-30/32-kDa protein association. The above-mentioned interaction of NS2 with the 30- and 32-kDa proteins from rat FREJ4 cells was also demonstrated in extracts prepared from MVMp-infected permissive cells of various origins, namely, mouse (A9) or hamster (BHK and THK) and human (NB-324K) cells (Fig. 6, lanes 1 to 8). It should be stated that NS2 proved to be required for efficient MVMp growth in cells from the natural host (in particular the mouse A9 cell line), while its requirement is less pronounced in cells from other species, including the NB-324K cell line (8, 38, 39). It therefore appears that under the experimental conditions tested, the association of NS2 with the 30- and 32-kDa polypeptides took place irrespective of the dependence of MVMp replication on the viral product NS2. This finding suggests that the nonessential character of NS2 in certain cells does not result from the absence or sequestration of the 30- and 32-kDa partner proteins.

We have previously shown that the nontransformed rat cells (FR3T3) resist MVMp, whereas a number of oncogene-transformed derivatives, including the stable *ras* transfectant FREJ4 used in the present work, sustain a productive MVMp infection (45, 55). Though not fully permissive, FR3T3 cells produce a

detectable level of NS proteins, allowing the formation of NS2-30/32-kDa protein complexes to be compared between normal and transformed lines. As illustrated in Fig. 6 (lanes 9 to 14), NS2 immunoprecipitates from both types of extracts included the 30- and 32-kDa proteins, indicating that their interaction with NS2 is not sensitive to cell transformation in this system. Similarly, NS2-30/32-kDa protein complexes were detected in both nontransformed (BHK) and SV40-transformed (THK) hamster cells (Fig. 6, lanes 1 to 4).

In conclusion, NS2 was found to bind to the 14-3-3 related 30- and 32-kDa proteins in extracts from all MVMp-infected cells tested, regardless of their dependence on NS2 for replication or permissiveness to MVMp toxicity. The functional role of this interaction therefore remains elusive. These results are consistent, however, with the reported expression of 14-3-3 polypeptides in a variety of cells (2). The ubiquity of NS2 complexes with 14-3-3 proteins was confirmed by the detection of FAS activity in NS2 immunoprecipitates prepared from MVMp-infected A9, FR3T3, and FREJ4 cells (Fig. 5 and data not shown).

FIG. 5. Analysis of NS2 immunoprecipitates for FAS activity. Immunoprecipitates were prepared from noninfected (ni) or MVMp-infected (i) FREJ4 cells, using normal (N), anti-NS2 (SP6), or anti-NS1 (SP8) serum. Washed immunocomplexes were tested for FAS activity, i.e., ability to activate the ADP-ribosylating function of exoenzyme S (Exo S) from *P. aeruginosa*.

FIG. 6. Association of NS2 with the 30- and 32-kDa proteins from cells of various origins. Immunoprecipitates were obtained in the absence of SDS, using SP6 anti-NS2 serum, and analyzed as for Fig. 2A. Extracts were prepared from noninfected (ni) or MVMP-infected (i) cultures of SV40-transformed (THK; lanes 1 and 2) or nontransformed (BHK; lanes 3 and 4) hamster, mouse (A9; lanes 5 and 6), SV40-transformed human (NB324K; lanes 7 and 8), and EJ-*ras*-transformed (FREJ4; lanes 13 and 14) or nontransformed (FR3T3; lanes 9 to 12) rat cells. ^a, Four-times-higher amounts of cell extracts were used compared with other lanes. Arrows to the left point at NS2 (25 kDa) and 30- and 32-kDa proteins. M, molecular sizes (in kilodaltons) of prestained protein standards.

Association of the 30- and 32-kDa proteins with nonfunctional carboxy-terminally modified NS2 products. It was previously reported that MVMP mutants unable to express NS2 or producing C-terminally modified NS2 proteins were deficient in the accumulation of viral DNA and the production of infectious virions in a cell species-dependent manner (8, 38, 39). These defects were in particular observed with the NS2 mutants Df18 and 2268 that harbor, respectively, a 3-nt insertion at nt 2290, introducing an isoleucine residue into NS2, and a translation termination mutation at nt 2268, truncating NS2 at this position (38). Given the phenotypic alterations of these mutants, it was of interest to determine whether corresponding modified NS2 proteins were still able to associate with the cellular 30- and 32-kDa polypeptides. Since mutated NS2-Df18 and NS2-2268 proteins could not be easily detected in infected cell extracts from the MVMP normal host murine A9 cell line (38), FREJ4 cells infected with wild-type or mutant MVMP were used to address this question. As shown in Fig. 7, the mutated Df18- and 2268-NS2 products were both able to interact with the 30- and 32-kDa proteins as efficiently as wild-type NS2 in coimmunoprecipitation experiments. Similar results were obtained with extracts from the human NB-324K cell line (data not shown). These results allow the NS2 domain involved in the 30- and 32-kDa protein binding to be mapped outside the last C-terminal 11 aa from both the major and minor isoforms of NS2. These results also suggest that the inability of NS2-mutant viruses to efficiently replicate is unlikely due to an incapacity of modified NS2 proteins to associate with the cellular 30- and 32-kDa polypeptides, albeit such complex formation was detected in an environment in which NS2 is not essential.

DISCUSSION

Parvovirus NS2 proteins comprise several isoforms that result from the alternate splicing of a precursor mRNA and differ in their C-terminal regions (9, 13). Three distinct NS2 products have been identified and are termed major, minor, and rare after their relative abundance in parvovirus-infected cells (13, 38). It is not known whether these isoforms have different or redundant functions. The nonstructural proteins NS2 of MVMP and H-1 were reported to take part in the parvovirus life cycle in a cell species-dependent manner (29, 38). In particular, these products are somehow involved in the amplification of parvovirus DNA (8, 29, 38) as well as in the efficient

FIG. 7. Association of modified NS2 products with the 30- and 32-kDa cellular proteins. Immunoprecipitation reactions were performed with SP6 anti-serum in the absence of SDS and analyzed as for Fig. 2A. Extracts were prepared from FREJ4 cells that were either mock treated (–) or infected with wild-type (wt), mutant NS2-Df18 (Df18), or mutant NS2-2268 (2268) MVMP. The NS2-Df18 and NS2-2268 mutants encode NS2 proteins containing a new isoleucine residue immediately after the NS1 termination codon and a stop codon at nt 2268, respectively. Arrows to the left point at the 32- and 30-kDa, wild-type NS2, and mutated NS2-Df18 and NS2-2268 proteins. M, molecular sizes (in kilodaltons) of prestained protein standards.

accumulation of mature viral particles (11, 39). NS2 proteins were also found to reinforce the cytotoxic activity of the parvovirus NS1 product in certain cells (4, 9, 28). As for the replicative function, the toxic activity of NS proteins appears to be modulated by the host cells, being in particular exacerbated as a result of cell transformation by various oncogenes (37). Altogether, these observations suggest that cells differ in the expression of regulators, cofactors, or targets that are required for NS functioning. In a first attempt at identifying these host factors, the present study was conducted to search for cellular proteins that are able to physically interact with the NS2 products of MVMP in a direct or indirect way.

In competitive coimmunoprecipitation experiments, two cellular proteins were found to specifically associate with the nonstructural proteins NS2. These cellular components have apparent molecular masses of 30 and 32 kDa. Antisera that distinguish between the major and minor NS2 products allow us to demonstrate that the 30- and 32-kDa proteins bind to both isoforms. It was not possible to determine whether the 30- and 32-kDa polypeptides were also partners of the rare NS2 product, since antibodies that specifically recognize this isoform were not available. The 30- and 32-kDa cellular proteins failed to interact with the NS1 product of MVMP. These data tentatively map the domain essential for complex formation with the 30- and 32-kDa proteins to the NS2-specific exon 2. The interaction of NS2 with the 30- and 32-kDa polypeptides was confirmed by the ability of NS2 to serve as a bait to trap these factors from cell extracts by affinity chromatography. It should be stated that our data do not indicate whether NS2 binding to either of the 30- and 32-kDa proteins is direct or mediated by other components in the form of a multimeric complex.

The 30- and 32-kDa cellular partners of NS2 were found to be constitutively expressed in the absence of MVMP infection. Competitive immunoprecipitation experiments gave no evidence of a major MVMP-induced modulation of the expression of these cellular proteins. Furthermore, formation of NS2–30/32-kDa complexes took place irrespective of whether NS2 was essential to the parvovirus life cycle (mouse cells versus cells of other species). It therefore appears that the dispensable character of NS2 in certain cells is not a consequence of the absence of available 30- and 32-kDa targets. This argues against the possibility that the 30- and 32-kDa products

suppress parvovirus replication and are prevented from acting in this way when associated with NS2. In addition, the interaction between NS2 and the 30- and 32-kDa polypeptides could be demonstrated in both permissive (*ras*-transformed FREJ4) and nonpermissive (parental FR3T3) cells, indicating that it is not a rate-limiting step for the parvovirus life cycle in normal versus transformed host cells. In conclusion, these data narrow down the possible role of NS2–30/32-kDa complexes, although positive evidence of their physiological function is still lacking. No such evidence could be obtained by the analysis of NS2 mutants whose replication is known to be impaired under conditions in which NS2 is required, i.e., in mouse cells (8, 38, 39). These mutants harbor nucleotide insertion or substitution in the region coding for the C terminus of NS2, creating one-amino-acid-longer or prematurely terminated translated NS2 products. These modified NS2 polypeptides were still able to interact with their cellular partners in permissive cells, suggesting that the association with 30- and 32-kDa proteins is not sufficient for NS2 functions. However, such interaction may still be necessary, assuming that additional carboxy-terminally located NS2 determinant(s) are required for activity.

On the basis of sequence homology and capacity for activating the ADP-ribosyltransferase function of exoenzyme S, the cellular partners of NS2 could be assigned to the family of 14-3-3 proteins. The 32- and 30-kDa polypeptides are more particularly related to the epsilon and beta or zeta 14-3-3 isoforms, respectively. The 14-3-3 family is composed of acidic proteins with molecular masses ranging from 30 to 32 kDa (34). At least seven isoforms have been identified so far, and they can form homo- or heterodimers (27, 53). Though originally thought to be brain specific, 14-3-3 proteins have recently been identified in a wide variety of mammalian tissues as well as in other eukaryotic organisms, including insects, plants, and yeasts (for a review, see reference 2). Numerous functions have been ascribed to 14-3-3 polypeptides, in particular activation of tyrosine and tryptophan hydroxylases (24), regulation of PKC (for a review, see reference 1), and stimulation of calcium-dependent exocytosis (35). These proteins were also found to serve as cofactor for an ADP-ribosyltransferase of *P. aeruginosa*, an activity termed FAS and often used to monitor 14-3-3 polypeptides (19). Furthermore, proteins belonging to the 14-3-3 family were shown to associate with several cellular or viral components, including the PKC (53), the *cdc25* phosphatase (10), and the oncoproteins Raf-1 (20), Bcr and Bcr-Abl (5, 41), and polyomavirus middle T antigen (40). These partner proteins are all involved in signal transduction pathways and cell cycle control, suggesting that 14-3-3 polypeptides may play a key regulatory role in these pathways. Several of the 14-3-3 cognate proteins, in particular Raf-1, Bcr, PKC, and *cdc25*, are known to be translocated to the plasma membrane (5, 18, 21, 33), pointing to a possible interference of 14-3-3 polypeptides at this level. The stability of partner proteins may also be modulated by their binding to 14-3-3 polypeptides (1, 36). However, the functional significance of these interactions is presently elusive. For instance, PKC proteins appear to be either stimulated (26, 51) or inhibited (43, 53) as a result of their binding to different 14-3-3 isoforms. Activation of Raf-1 kinase can occur in association with 14-3-3 proteins (16, 18, 25) but also in the absence of these products (32, 50). Therefore, the role of NS2 interaction with 14-3-3 proteins is a matter of speculation. The NS2 products are mainly located in the cytoplasm (13) and have a short half-life of less than 60 min (13, 47). The intracellular compartmentalization and/or stability of NS2 may conceivably be modified following complex formation with 14-3-3 proteins. Conversely, NS2 binding to 14-3-3 polypeptides may interfere with some of the signaling pathways

that involve the latter proteins, thereby contributing to cell disturbances and/or stimulation of cell-controlled steps of the parvovirus life cycle. A putative crossing of 14-3-3 and parvovirus regulatory circuits may take place at the level of PKC, which interacts with the former proteins (33, 43) and was recently reported to modulate the activities of the major viral replicative protein NS1 (39a). The precise mapping of the 14-3-3-binding domain of NS2 and the generation of NS2 mutants that are deficient in this interaction should help to unravel the functional role of NS2/14-3-3 protein complexes through the analysis of the phenotypes of these mutants.

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