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Shope Fibroma Virus RING Finger Protein N1R Binds DNA and Inhibits Apoptosis

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Shope fibroma virus (SFV) N1R gene encodes a RING finger protein that localizes to virus factories within the cytoplasm of infected cells. Altered proteins, with deletions and site-specific mutations, were transiently expressed in vaccinia virus-infected cells to discern regions of the protein that are required for localization. We have determined that at least part of the RING finger region is necessary for localization but that the RING motif alone is not sufficient. A chimeric protein, however, in which the RING finger region of the herpes simplex virus-1 ICP0 protein replaces the SFV N1R RING motif does localize to virus factories. A region of five highly conserved amino acids at the amino terminus of SFV N1R is also critical for localization. We report that the SFV N1R protein binds double- and single-stranded DNA, suggesting a mechanism for localization, and that overexpression of this protein in vaccinia virus-infected cells reduces apoptosis-associated fragmentation of nuclear DNA.

INTRODUCTION

Poxviruses are large, complex, and relatively autonomous eukaryotic double-stranded (ds)DNA viruses that are unusual because they complete their replication cycle within the cytoplasm of infected cells (Moss, 1996). On infection, a virus factory is established in the cytoplasm, often appearing close to the nuclear membrane, and represents the site of viral transcription and DNA replication. Electron and confocal microscopy has elucidated some of the steps in poxvirus maturation, but the process is still not well understood at the molecular level (Sodeik *et al.,* 1993; VanSlyke and Hruby, 1994). The prototypic poxvirus, vaccinia virus (VV), has a genome of 191 kb and encodes ~200 proteins (Goebel *et al.,* 1990; Johnson *et al.,* 1993). Poxviruses are ubiquitous, infecting mammals, birds, reptiles, and invertebrates, although after the eradication of smallpox [variola virus (VAR)], the only widespread human pathogen is molluscum contagiosum virus (MCV) (Porter and Archard, 1994).

There appears to be a minimum set of poxvirus genes that are required for viral transcription, genome replication, and assembly of progeny virions. These conserved genes are clustered within the central region of the linear genome. A second group of viral genes are not essential for growth *in vitro* but are important for the replication of the viruses in their natural hosts. A number of these have been shown to effect host range, tissue specificity, and virulence of poxviruses; examples include the secreted cytokine binding proteins (Smith *et al.,* 1991; Upton *et al.,* 1992; Spriggs *et al.,* 1992; Spriggs, 1994), the epidermal growth factor-like protein (Opgenorth *et al.*, 1992), and several of the poxviral serpins (Macen *et al.,* 1993; Thompson *et al.,* 1993; Brooks *et al.,* 1995). Approximately 60 of the nonessential VV genes are absent from the distantly related MCV, which has a similar number of genes that are apparently unique to MCV (Senkevich *et al.*, 1996). The examination of such genes and their gene products can provide valuable information about the antiviral response of the host and of the immune system in general.

The focus of this report is another member of this group of nonessential poxvirus virulence proteins. The product of the Shope fibroma virus (SFV) gene N1R has been previously shown to be a 28-kDa protein that localizes to the virus factory and binds zinc, presumably due to the presence of a RING finger motif (C3HC4) at its carboxyl terminus (Upton *et al.,* 1994). The complete homolog of this gene is present in a number of other poxviruses, including myxoma virus (MYX), ectromelia virus (EV) (Senkevich *et al.,* 1994), VAR, and VV strain IHD-W, but it is absent from the VV strain Copenhagen and is truncated in VV strain WR (Upton *et al.,* 1994). It is interesting that inactivation of the gene encoding the EV homolog, p28, had a large effect on the virulence of this virus in mice, its natural host (Senkevich *et al.,* 1994). Mice infected through the footpad with wild-type EV (5 pfu) showed a uniform mortality with a mean day of death of 9.7 days p.i., whereas all mice infected with the p28 mutant EV survived the challenges (Senkevich *et al.,* 1994). The expression of p28 is required for replication of EV in murine resident peritoneal macrophages *in vitro* (Senkevich *et al.,* 1995), and it was proposed that this attenuation of EV pathogenicity was due to failure of the

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FIG. 1. SFV N1R protein modifications. The complete amino acid sequence is shown for the SFV N1R protein. Carboxyl-terminal deletions are indicated by the black bars (ZD1–5, respectively). Internal deletions (boxed) of amino acids 24–28 and 45–95, the end of the amino-terminal deletion of 154 amino acids (right arrow), and positions of site specific mutations (vertical arrows) are indicated. Shown above the SFV sequence are the amino acid sequences of (1) the RING motif from HSV-1 used to create the SFV-HSV fusion protein and (2) the RING motif from EHV.

virus to replicate in macrophages at successive steps in its spread from the skin to target organs.

The RING finger motif is a conserved cysteine-rich amino acid sequence that has been found in >80 proteins of diverse evolutionary origin (Saurin *et al.,* 1996; Borden and Freemont, 1996). The RING finger motif has been defined as C-x(2)-C-x(9–39)-C-x(1–3)-H-x(2–3)-C-x(2)-C-x(4–48)-C-x(2)-C (PROSITE: PS00518, PDOC00449) and is unusual because of the zinc ligation scheme (a cross-brace) in which the first pair of cysteines coordinate a zinc atom with the third pair and a second zinc atom is coordinated by a cysteine and histidine (second pair) and the fourth pair of cysteines (Everett *et al.*, 1993; Schwabe and Klug, 1994; Borden *et al.*, 1995). The RING finger motif has been found in proteins that function in oncogenesis, development, signal transduction, and apoptosis, but there is little evidence of its specific role in these proteins (Freemont, 1993; Borden and Freemont, 1996).

We used deletion and site-specific mutants of the SFV N1R protein to further characterize requirements for localization to the virus factories and zinc binding. Regions of the RING finger motif and five amino acids close to the amino terminus of the protein were found to be critical for localization. In addition, we have shown that the N1R protein binds DNA cellulose and reduces apoptosis in VV-infected cells.

RESULTS

Carboxyl-terminal deletion analysis of the SFV N1R RING motif

To determine the role of the RING motif in the localization of N1R to virus factories, modified N1R proteins with carboxyl-terminal truncations (Fig. 1) were transiently expressed in VV-infected cells. The vectors were based on pMSN1 (Upton *et al.,* 1994), which uses the strong late promoter of pMJ601 (Davison and Moss, 1990). Localization of the modified proteins was determined by immunofluorescence microscopy. Deletions ZD1, ZD2, ZD3, and ZD4, which remove progressively more of the carboxyl-terminal portion of N1R up to and including the third distal cysteine of the RING motif, had little or no effect on the localization of N1R to the factories (Fig. 2: ZD1, ZD2, and ZD3 not shown). Deletions ZD1 and ZD2 affect only the last pair of coordinating cysteines, but the third distal cysteine is predicted to be involved in coordinating the first zinc atom with the first cysteine pair. The larger deletion, ZD5, which includes the fourth distal cysteine abolished localization to the virus factory (Fig. 2c); thus, the small difference between deletions ZD4 and ZD5 delineates a region of the protein that is required to permit normal localization to virus factories. Each of the mutants created here and others in the following experiments were shown to be expressed at levels comparable to normal N1R and to be the correct size by Western blots of extracts of transfected VV-infected cells (Fig. 3).

In an attempt to correlate localization to virus factories with zinc binding, we tested the deleted proteins in a zinc blot (Fig. 4). All of the proteins, including deletion ZD5, which failed to localize to the virus factory, bound zinc in this assay. Because deletion ZD5 removes both the third and fourth pairs of cysteines, binding of zinc at both sites should be blocked if the SFV N1R is folded in a cross-brace structure (Everett *et al.,* 1993; Borden *et al.,* 1995; Saurin *et al.*, 1996; Borden and Freemont, 1996). Although this result

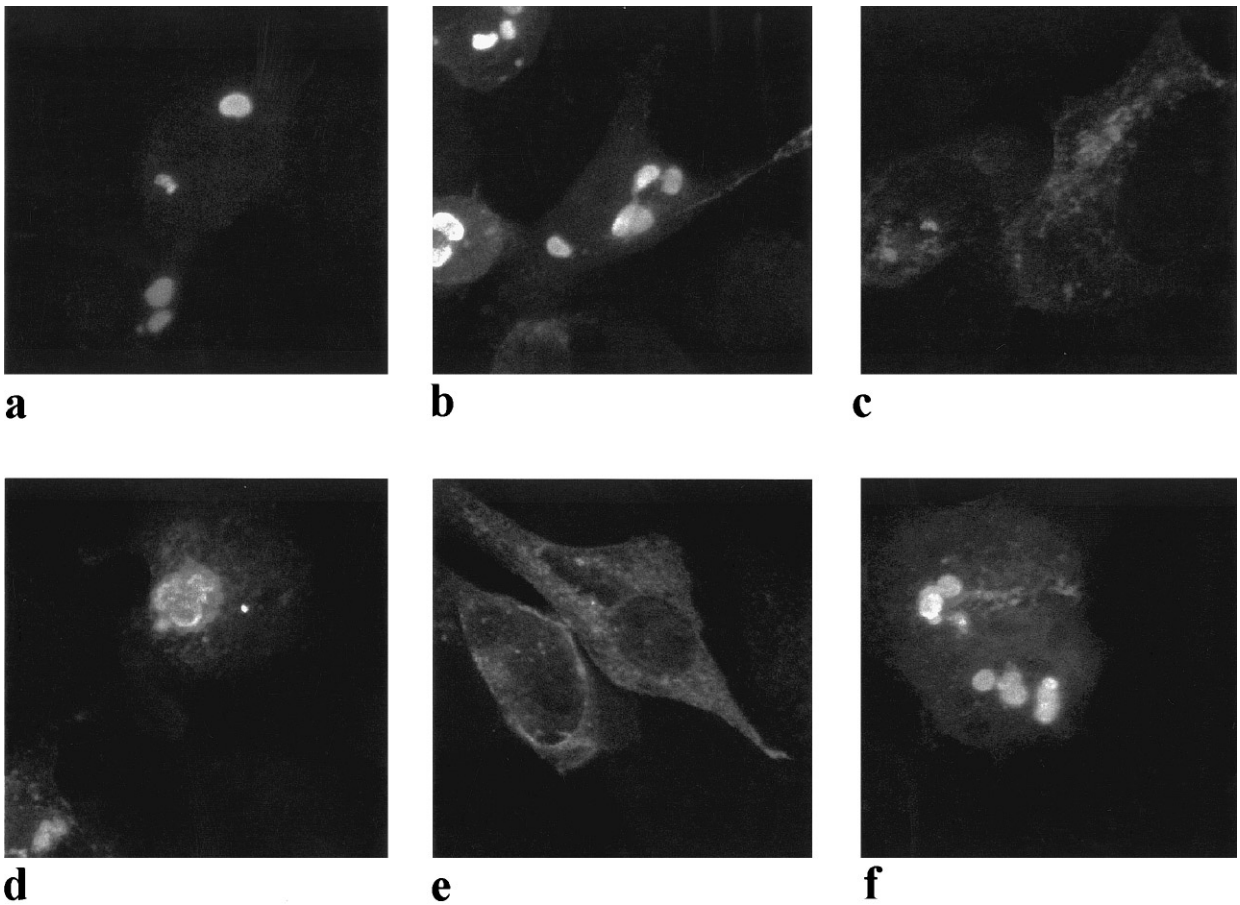

FIG. 2. Localization of SFV N1R protein in VV-infected cells shown by mAb H1119 and confocal microscopy. The proteins expressed from the transfected vectors are as follows: (a) wild-type SFV N1R, (b) N1R-ZD4, (c) N1R-ZD5, (d) N1R-HSV fusion, (e) N1R-d24–28, (f) N1R-mL193N.

suggests that the cross-brace is not used by SFV N1R, the zinc blot assay relies on the renaturation of protein after blotting from an SDS-polyacrylamide gel, and it is therefore possible that the observed zinc binding results from an aberrant folding of the remaining portion of the RING motif region, which still contains three cysteines and a histidine. Molecular modeling (not shown) indicates a cross-brace structure for the poxvirus RING motif is possible, but determination of its structure by NMR or crystallography may be required to resolve this issue.

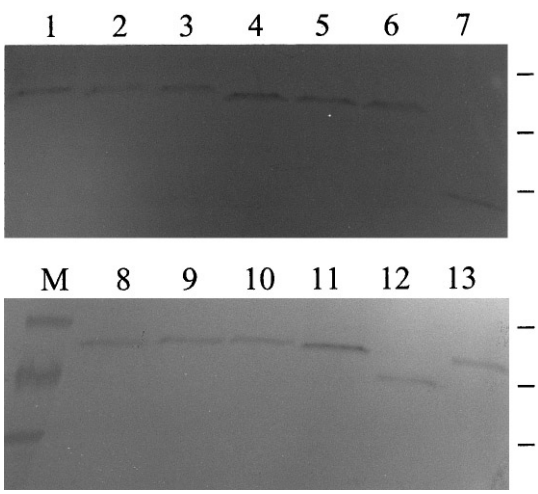

FIG. 3. Transient expression of SFV N1R mutant proteins in VV-infected BGMK cells. (Lanes) 1, SFV N1R (wild type); 2, N1R-ZD1; 3, N1R-ZD2; 4, N1R-ZD3; 5, N1R-ZD4; 6, N1R-ZD5; 7, N1R-ZD8; 8, N1R-mFG190/191LR; 9, N1R-mL193N; 10, N1R-mI202N; 11, N1R-ZD6; 12, N1R-ZD7; 13, N1R-HSV; M, standard proteins (and bars), 32.5, 25, and 16.5 kDa.

Effect of site-specific mutations on localization of the SFV N1R protein

Because carboxyl-terminal deletions up to the third distal cysteine did not affect localization, we made two single-amino-acid changes and one two-amino-acid change at noncysteine positions conserved between the SFV and VV sequences in the central part of the SFV N1R RING motif. Plasmids N1R-mFG190/191LR, N1R-mL193N, and N1R-mI202N replace the following amino acids in the SFV N1R protein: Phe190/Gly191 with leucine/arginine, Leu193 with asparagine, and Ile202 with asparagine, respectively. After transfection into VV-infected cells, these mutant proteins localized to virus factories (Fig. 2f), indicating that although these residues are conserved between SFV and VV, these substitutions are compatible with localization. These results prompted us

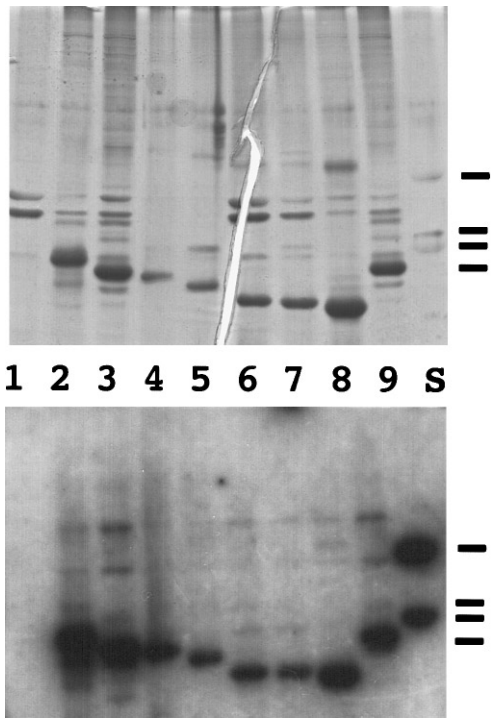

FIG. 4. Zinc binding by SFV N1R proteins with carboxyl-terminal deletions. (Top) Coomassie blue-stained polyacrylamide gel of SFV N1R proteins isolated as inclusion bodies expressed in *E. coli.* Bars indicate (top to bottom) standards (alcohol dehydrogenase, 40 kDa; carboxypeptidase A, 35 kDa; carbonic anhydrase, 29 kDa) and SFV N1R protein. (Bottom) Autoradiogram of a zinc blot from a gel identical to that shown in the top. (Lanes) 1, bacterial vector pET19b; 2, MYX N1R homolog; 3, SFV N1R; 4, SFV N1R-ZD1; 5, SFV N1R-ZD2; 6, SFV NIR-ZD3; 7, SFV N1R-ZD4; 8, SFV N1R-ZD5; 9, SFV N1R; 10, standard proteins.

to question whether other regions of the N1R protein outside of the RING motif were directly involved in localization to virus factories and whether N1R RING motif could be replaced by another from a nonpoxvirus protein.

Deletion of the amino-terminal 154 amino acids of SFV N1R (slightly more than half of the protein; Fig. 1) blocked localization, demonstrating that the RING motif region is not sufficient for association with the virus factory (data not shown). Similarly, deletion of amino acids 45–95 from SFV N1R (Fig. 1) resulted in loss of virus factory localization (data not shown). In an attempt to delineate a smaller region of the amino-terminal region of SFV N1R that might be involved with localization, we examined the alignment of the SFV and VV N1R protein homologs (Upton *et al.*, 1994). There is only 28% amino acid identity between these proteins with the greatest similarity at the carboxyl terminus, in the region of the RING motif. There is, however, one block of five absolutely conserved amino acids close to the amino terminus of SFV N1R (residues 24–28; Fig. 1). This sequence, Tyr-Ile-Asn-Ile-Thr, was deleted from SFV N1R by site-specific mutagenesis in plasmid N1R-d6. The deletion of this small region prevented the SFV N1R protein from localizing to the virus factors in VV-transfected cells (Fig. 2e). Thus, factory localization requires a small region at the amino terminus of the protein and part of the RING motif; however, it is not possible to determine whether this amino-terminal region is critical for a binding event or whether it is required for correct folding of the protein.

Although the RING motif has been observed in a great variety of proteins, its function remains unknown. We set out to test the hypothesis that these motif regions may fulfill similar functions in these proteins by constructing a chimeric gene to express a protein that had the N1R RING motif replaced by the RING motif of herpes simplex virus type-1 (HSV-1) immediate-early protein ICP0. Transfection of this construct (N1R-HSV) into VV-infected cells demonstrated that this chimeric protein did indeed localize to discrete regions within infected cells (Fig. 2d). Because ICP0 is a nuclear protein, we confirmed that the site of localization was the virus factories outside the nucleus by using Hoechst dye 33342 to stain both the viral DNA and the host nuclear DNA (Fig. 5). Thus, the HSV ICP0 RING motif can replace the SFV N1R RING motif in supporting factory localization.

SFV N1R protein binds to both dsDNA and single-stranded DNA cellulose

To differentiate whether the SFV N1R protein is localized to the virus factory because of binding to viral DNA or an affinity for another virus factory protein, we tested its ability to bind to DNA cellulose columns. The recombinant virus VV-N1R was used to produce the SFV N1R protein, all of which appears to localize to virus factories when examined by immunofluorescence (data not shown). Little soluble N1R protein was observed in lysates of infected cells, but the protein was extracted when 0.6 M NaCl was included in the buffer. Desalted protein extracts were loaded onto dsDNA and single-strand (ss)DNA columns and eluted with NaCl step gradients. A Western blot showing SFV N1R protein binding to and eluting from dsDNA (0.5–0.75 M NaCl) is shown in Figure 6. A similar profile was seen for ssDNA (data not shown). When 1 mM EDTA was included in the desalting buffer, the protein did not bind to the dsDNA column, suggesting that zinc and the structural integrity of the RING motif region are required for DNA binding. It is possible that SFV N1R protein localizes to the virosome and binds DNA via an associated viral protein. This appears unlikely, however, because the poxviral N1R protein homologs, which are poorly conserved overall, localize in cells infected by distantly related poxviruses. For example, the SFV, MYX, and EV proteins localize in VV-infected cells, and the VV, EV, and SFV proteins localize in MYX-infected cells (data not shown).

Expression of SFV N1R protein delays apoptosis in VV-infected cells

Apoptosis, often referred to as programmed cell death, is a physiological process by which the cell actively

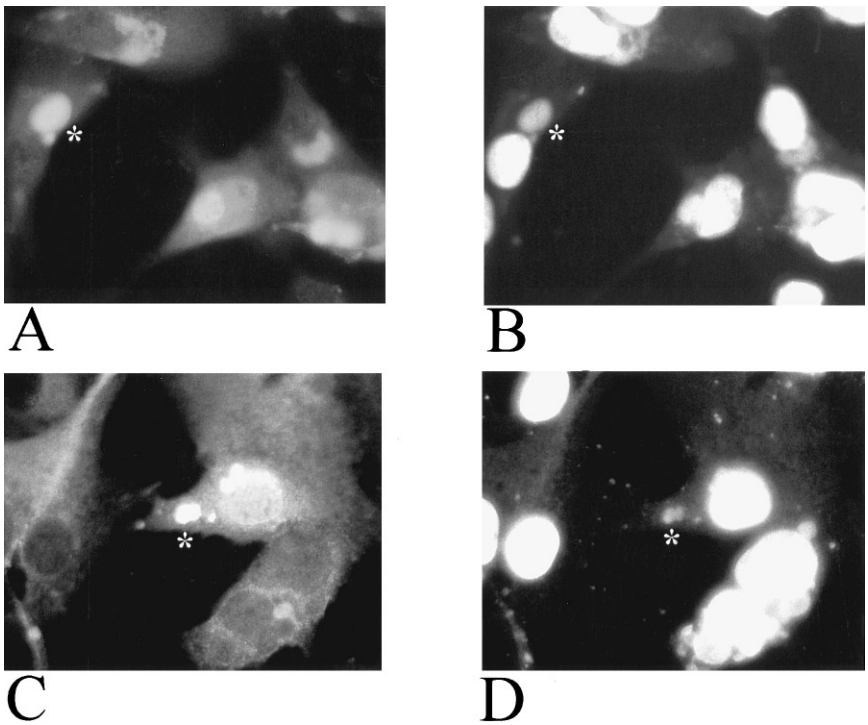

FIG. 5. The HSV-1 ICP0 RING motif can replace the SFV N1R RING motif for virosome localization. Vectors (A and B, SFV N1R; C and D, N1R-HSV) were transfected into VV-infected cells, and localization was determined by immunofluorescence. (A and C) Protein detection by immunofluorescence. (B and D) Identical fields visualized for DNA stained with bisbenzimide. ∗, Virus factories.

participates in a cascade of biomolecular events that result in the death and disposal of the cell. It is important in development, in the regulation of cell numbers, and as a defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes, tumor cells, and virus-infected cells (Nagata, 1997). We decided to test the hypothesis that the SFV N1R protein might be involved in blocking apoptosis induced by VV because a number of cellular proteins with RING motifs have key roles in regulation of apoptosis (Rothe *et al.,* 1994; Uren *et al.,* 1996; Lee *et al.,* 1997; Hu *et al.*, 1994; Thut *et al.*, 1997; Devereaux *et al.,* 1997) and inactivation of the EV homolog (p28) significantly reduces virus virulence (Senkevich *et al.*, 1994). Apoptosis was measured using a standard assay for the fragmentation of host chromosomal DNA into small oligonucleosomes (Koyama and Miwa, 1997). No DNA laddering was seen at 24 h, but VV-infected BGMK cells showed significant laddering by 48 h. In contrast, cells infected with a VV recombinant expressing the SFV N1R protein (VV-N1R) showed little or no laddering at this time or at 54 h (Fig. 7). The sensitivity of this assay (Eastman, 1995; Gavrieli *et al.*, 1992; Salgame *et al.,* 1997), which uses ethidium bromide to visualize DNA separated on agarose gels, is such that we believe the majority of infected cells are undergoing apoptosis and contributing to the observed signal. This is supported by the data from an ELISA measuring cytoplasmic apoptotic nucleosomes. The assay, which is at least 10-fold more sensitive than the laddering assay, shows a similar absence of apoptosis at 24 h and inhibition of nucleosome release by VV-N1R at 48 h. Apoptosis was induced in control uninfected cultures by treatment with cycloheximide (200 μg/ml); microscopic inspection of these cells indicated that all cells had undergone morphological changes. A lower cycloheximide concentration (30 μg/ml) has previously been shown to induce apoptosis in >60% of HeLa cells (Kettle *et al.,* 1997). The extent of nucleosome release in the VV infected cells at 48 h was comparable to that observed in

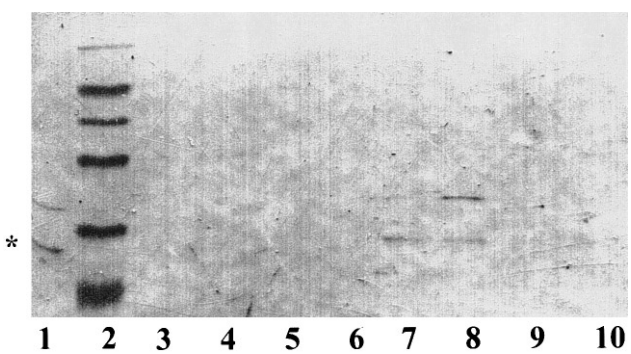

FIG. 6. Western blot showing binding of SFV N1R protein to dsDNA cellulose. Buffer was 50 mM NaCl, 3 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris (pH 8.0). (Lanes) 1, Protein extract loaded onto column; 2, molecular weight standards; 3, recycled flowthrough; 4, wash, no NaCl; 5, 0.1 M NaCl; 6, 0.25 M NaCl; 7, 0.5 M NaCl; 8, 0.75 M NaCl; 9, 1.0 M NaCl; 10, 1.5 M NaCl. ∗, SFV N1R protein. The band above the SFV N1R protein represents a host protein that cross-reacts with mAb H1119.

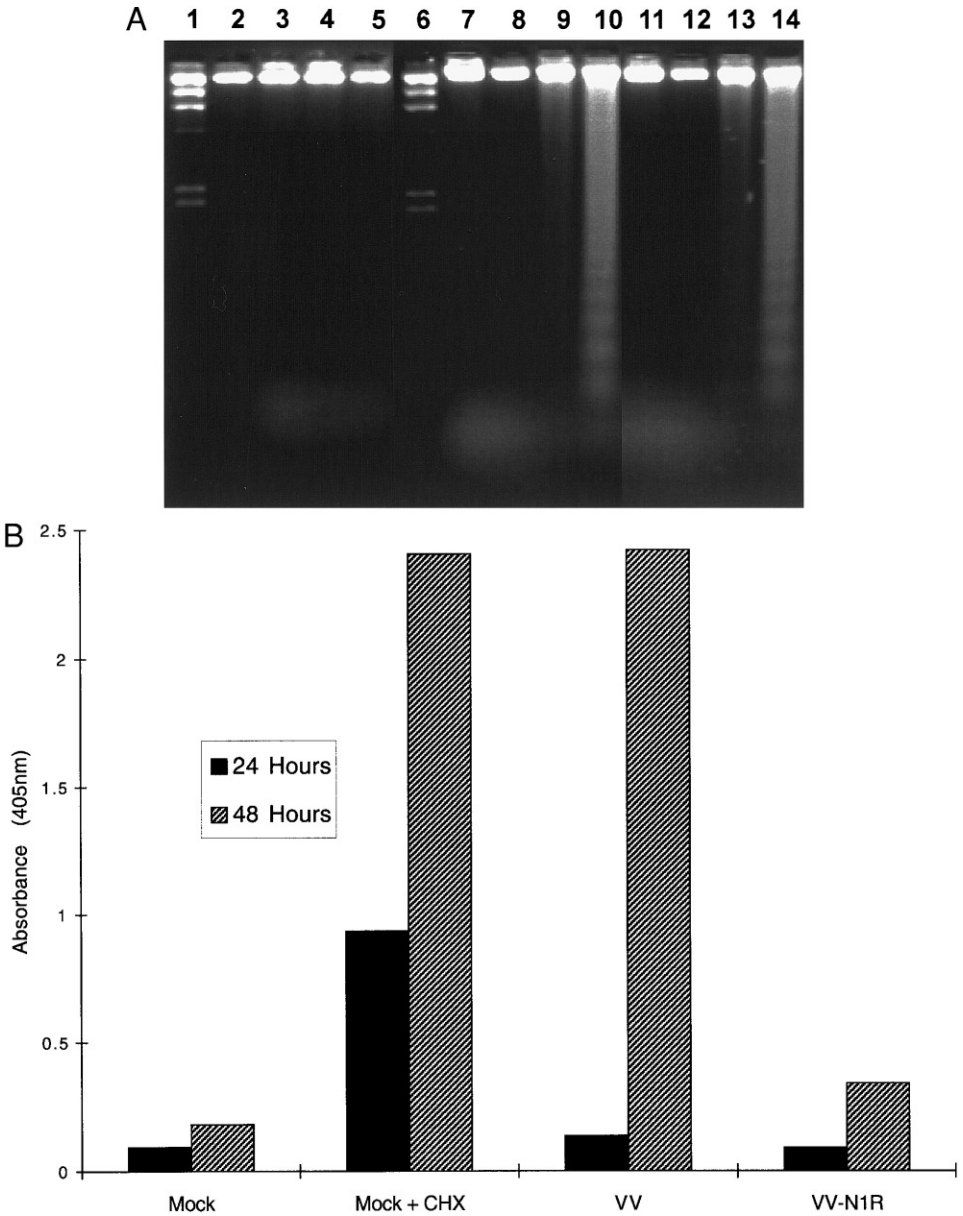

FIG. 7. Protection from apoptosis by expression of SFV N1R in VV-infected BGMK cells. (A) Ethidium bromide-stained agarose gel. (Lanes) 1 and 6, *Hind*III-digested lambda DNA; lanes 2, 7, and 11, mock infected; lanes 3, 8, and 12, mock infected, no serum; lanes 4, 9, and 13, VV-N1R infected; lanes 5, 10, and 14, VV infected. Samples taken at 24 h, lanes 2–5; 48 h, lanes 7–10; 54 h, lanes 11–14. (B) Detection of apoptotic cytoplasmic oligonucleosomes by ELISA. Duplicate samples were collected, and each was assayed in triplicate (ELISA).

a cycloheximide-induced apoptotic culture (Fig. 7B). Thus, the expression of SFV N1R protein in VV-infected cells significantly reduced virus-induced apoptosis. In similar assays, we also observed that EV-infected cells did not produce apoptotic DNA ladders, even at 54 h p.i. (data not shown). It will be interesting to determine whether the EV p28 RING homolog is involved in producing this effect.

DISCUSSION

The SFV N1R RING finger protein and several other poxvirus homologs are known to localize to the virus factory in the cytoplasm of infected cells. We performed deletion and mutational analysis of the SFV N1R RING finger gene and expressed the mutant proteins transiently in VV-infected cells to determine which regions of this protein are required for localization to the virus factory. These experiments have shown that part of the RING motif region is required for localization but that a substantial portion could be deleted with little or no effect. Another deletion mutant demonstrated that the carboxyl-terminal half of the protein, which contains the complete RING motif region, does not localize to virus factories. In addition, we recognized a five-amino-acid region at the amino terminus of the SFV protein (amino acids 24–28) that is highly conserved among the poxvirus homologs and have shown that it is essential

for virus factory localization. It is therefore apparent that the RING motif is not solely responsible for localization, nor is the entire motif required for this effect. This is consistent with a growing body of evidence that suggests the RING motif does not necessarily function as an independent protein domain but rather may be part of a larger domain of those proteins in which it is found (Maul and Everett, 1994; Everett *et al.,* 1995; Bellon *et al.,* 1997; Clem and Miller, 1994). Thus, the structural similarity that the RING motifs appear to share, as inferred by molecular modeling studies, may not be predictive of common functionality. This idea is supported by our result that showed the RING motif of HSV-1 ICP0 could substitute for that of SFV N1R for virus factory localization. Other motif switching experiments, however, have not been as successful. The RING motif of PML does not replace that of HSV-1 ICPO (Everett *et al.* 1995). It will be interesting to determine whether the SFV-HSV-1 fusion we generated retains other characteristics of the wild-type SFV N1R protein.

Although DNA binding has been reported for several proteins containing RING motifs (Hiom and Gellert, 1997; Gong *et al.,* 1997; Bailly *et al.,* 1997; Kanno *et al*., 1995), it does not appear to be a common characteristic of this group of proteins. Attempts to show DNA binding of the EV p28 protein were inconclusive because of protein insolubility problems (Senkevich *et al*., 1994, 1995). After initial difficulties in solubilizing the SFV N1R from VV-N1R-infected cells, we were able to show that this protein binds to both dsDNA and ssDNA cellulose but only when EDTA was omitted from the binding buffer. Two features, the broad range of NaCl concentration over which the SFV N1R eluted from DNA cellulose (0.5–0.75 M NaCl) and its molecular weight, suggest that SFV N1R may be the homolog of a previously described VV 28-kDa virus factory-associated DNA-binding protein (designated FP14) (Nowakowski *et al.,* 1978). However, these experiments were reportedly performed with VV strain WR, and we have shown that VV WR produces a significantly truncated protein that does not localize to the virus factory (Upton *et al.,* 1994). Clarification of this issue must await further experimentation. Studies using the EV p28 mutant indicated that this gene product was required for viral replication in peritoneal resident macrophages because virus factories were not detected by Hoechst dye staining (Senkevich *et al*., 1995). Thus, the DNA-binding activity of this group of poxviral proteins may be required only as an accessory factor for DNA replication or transcription in certain cell types. Alternatively, these proteins may serve to recruit a host protein to the virus factory.

Self-destruction of virus-infected cells by induction of apoptosis is an important host defense process, so it should not be surprising that many viruses have evolved mechanisms to avoid or delay its onset (Teodoro and Branton, 1997). Poxviruses block apoptosis at several distinct steps in the induction pathway. Well characterized examples include soluble tumor necrosis factor (TNF) binding proteins that modulate activation of the TNF receptor 1 apoptotic signaling pathway (Sedger and McFadden, 1996) and the serpin encoded by *crmA*, which inhibits cytotoxic T lymphocyte-mediated apoptosis (Fas and TNF induced) by blocking caspase activity (Zhou *et al.,* 1997). MCV possesses two genes (MC159l and MC160l) that contain duplicated death effector domain motifs that are found in proteins in the Fas and TNF signal transmission pathways (Senkevich *et al.,* 1997). Expression of MC159L has been shown to protect cells from apoptosis induced by these ligands (Bertin *et al.,* 1997). Overexpression of SFV N1R gene in a recombinant VV significantly reduced apoptosis of cells infected with this virus compared with cells infected by wild-type VV, suggesting that this protein may be involved in yet another poxviral antiapoptotic process. At early times, induction of apoptosis is expected to inhibit the accumulation of infective virus progeny; at late times, it may serve to interfere with the release and spread of the new virus particles. Our observation is that the SFV N1R protein reduces apoptosis at late times (after 24 h); thus, it may serve to increase the spread of the virus infection in an infected animal host.

A number of other proteins that possess RING motifs have been shown to be involved in the regulation of apoptosis. Baculoviruses encode an inhibitor of apoptosis (IAP) (Crook *et al.,* 1993), and several eukaryotic homologs have been recently cloned and sequenced (Uren *et al.,* 1996). The mammalian IAP homolog (MIH) B has been shown to bind TNF receptor-associated factor TRAF2 (Uren *et al.,* 1996), which also contains a RING motif (Rothe *et al.,* 1994), suggesting that the IAPs may interfere with signaling pathways required for apoptosis. In this respect, a carboxyl-terminal RING motif truncation mutant of MIH-B inhibits nuclear factor-κB induction by TNF and enhances TNF killing (Chu *et al.,* 1997). We have performed immunoprecipitations with monoclonal antibody (mAb) H1119 (which can immunoprecipitate HSV-1 ICP27; S. Rice, personal communication) and VV-N1R-infected cells to detect proteins that interaction with SFV N1R, but we have been unable to immunoprecipitate SFV N1R, although β-galactosidase, which is also expressed by VV-N1R, immunoprecipitated under the conditions tested (data not shown). It appears that the majority of the SFV N1R protein bound tightly in the virus factories, but this, however, does not exclude a role for this protein in binding and possibly sequestering factors that are involved in apoptosis signaling.

Previously, the lack of virulence of the EV p28$^-$ mutant has been attributed to reduced replication in macrophages. Our observation that the SFV N1R protein, a homolog of p28, blocks apoptosis suggests a second function for this protein, which we have shown to localize to the virus factory, possibly due to DNA binding. The relationship between the role of SFV N1R in factory localization and inhibition of apoptosis is presently unknown, but it is not uncommon for viral gene products to have more than one function. Interestingly, in this re-

spect, the baculovirus p35 protein, a broad-spectrum inhibitor of apoptosis, was also found to facilitate viral replication (Hershberger *et al.,* 1992, 1994). Clarification of the role of these poxviral RING finger proteins in blocking of apoptosis or inflammation is especially important because it may have implications for the design of poxvirus-based vaccines.

MATERIALS AND METHODS

Cells and viruses

SFV (strain Kasza), VV (strains WR and IHD-W), and BGMK cells were provided by Dr. G. McFadden. Viruses and cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% newborn bovine serum (GIBCO BRL, Gaithersburg, Maryland), except where indicated.

Antibodies and Western blotting

Mouse mAb H1119 (Goodwin Institute for Cancer Research, Plantation, Florida) recognizes an epitope in a 12-amino-acid sequence of the HSV-1 ICP27 protein. The gene sequence for this epitope is fused to the amino terminus of the N1R gene in plasmid pMSN1, and transfection of pMSN1 and constructed mutants to VV-infected cells was performed as previously described (Upton *et al.,* 1994). Protein samples were separated on 12% SDS-PAGE, transferred to PVDF membrane (Gelman Sciences, Ann Arbor, Michigan), and blocked with 3% bovine serum albumin in Tris-buffered saline overnight before incubation with mAb H1119 (1:2000). Bound antibody was detected by the Immun-Blot alkaline phosphatase assay (BioRad Laboratories, Hercules, California).

Recombinant DNA procedures

Restriction enzymes were obtained from New England Biolabs (Beverly, MA), unless otherwise stated. Plasmid pMSN1 has been described previously and contains an epitope tagged SFV N1R open reading frame under the control of a strong synthetic poxvirus promoter (Upton *et al.*, 1994). Carboxyl-terminal deletion mutants of the SFV N1R gene were constructed by polymerase chain reaction (PCR) (*Pfu* DNA polymerase; Stratagene, La Jolla, California) using a 5′ primer that contains an *Sal*I site immediately upstream of the initiating methionine of the epitope tag and 3′ primers that contained novel stop codons followed by a *Bam*HI site. PCR products were digested with *Sal*I and *Bam*HI and ligated into similarly digested pMSN1. The deletion mutant, which removes 154 amino acids from the amino terminus, was constructed by PCR using a 5′ primer with an *Nco*I site for cloning the PCR product into pMSN1 after the mAb epitope tag. The internal deletion of amino acids 45–95 was constructed by first subcloning the *Sal*I/*Bam*HI gene fragment from pMSN1 into similarly digested pBK-CMV (Stratagene, La Jolla, California). Full-length linear DNA was isolated after a partial *Dra*I digestion, subjected to complete digestion with *Eco*RV, and religated. Clones with the correct deletion were isolated, and the gene fragment was subcloned back into *Sal*I/*Bam*HI-digested pMSN1.

The SFV N1R-HSV ICP0 RING fusion gene was constructed by PCR Gene Soeing (Vallejo *et al.*, 1995). This construct replaces amino acids 172–234 of the N1R protein with amino acids 116–171 of ICP0. Plasmid pSHZ containing the HSV-1 ICP0 gene was generously provided by Dr. S. Rice (University of Alberta, Edmonton, Alberta, Canada).

Site-directed mutagenesis experiments were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were confirmed by DNA sequencing.

Assay of zinc binding by SFV N1R deletion proteins

Each of the carboxyl-terminal deletion genes were cloned into pET19b (Novagen, Madison, Wisconsin) and expressed in *Escherichia coli* BL21 (DE3)LysS as previously described (Upton *et al.,* 1994). Full-length and mutant N1R proteins were recovered as insoluble inclusion bodies, solubilized in loading buffer, separated on 12% SDS-PAGE, electroblotted to nitrocellulose, and probed with $^{65}ZnCl_2$ as described previously (Upton *et al.,* 1994).

Immunofluorescence and confocal microscopy analysis

Cell cultures were grown in eight-chamber slides (SuperCell, Fisher Scientific, Pittsburgh, Pennsylvania), infected with VV strain WR (m.o.i. = 5), and transfected (LipofectAce, GIBCO BRL) with 10 μg of vector purified on QIAGEN columns (Chatsworth, California) at 2 h p.i. After 18 h, cells were fixed (4% paraformaldehyde in PBS) for 30 min at 4°C, and cells were then rinsed in PBS and incubated with primary antibody (H1119) overnight. Preparations were rinsed in PBS and incubated with FITC-conjugated goat anti-mouse antibody (Biodesign International, Kennebunk, Maine) for 2 h at room temperature. For DNA visualization, slides were also stained with bisbenzimide Hoechst 33342 (Sigma Chemical, St. Louis, Missouri) at 500 ng/ml in PBS for 30 min. After rinsing, coverslips were mounted with Slow Fade (Molecular Probes, Eugene, Oregon) and viewed with epifluorescence or with confocal laser scanning microscopy. Confocal images were collected on a Zeiss LSM 410 inverted microscope. Series images were collected with 4× line averaging, scanning at 8 s per frame; images were 512 × 512 pixels. Projections of series or portions of series were used to make images. mAb H1119 cross-reacts weakly with a host protein that appears to distribute evenly within the plasma membrane. This cross-reactivity is not apparent in the confocal images because the series of images chosen to make the figures did not include those of the cell extremities. Untransfected cells serve as negative controls for conventional epifluorescence microscopy (Fig. 5C, left).

Construction of recombinant VV expressing the SFV N1R protein

BGMK cells were infected with VV strain IHD-W (m.o.i. = 2) followed by transfection of plasmid pMSN1 at 2 h p.i. After 48 h, virus was harvested, and TK^- recombinant VV-N1R was selected by two rounds of growth with 5-bromodeoxyuridine and plaque purified three times using agarose overlays with screening for expression of β-galactosidase. Expression of the epitope-tagged SFV N1R protein was confirmed by Western blot analysis of infected cell lysates using mAb H1119.

Isolation and solubilization of N1R-containing extracts

BGMK cells (10^8) were infected with the recombinant VV-N1R (m.o.i. = 3). After 4 h, the infected cells were transferred to 30°C in an attempt to promote correct folding of the recombinant protein and harvested 24 h p.i. Briefly, cells were trypsinized in standard saline citrate (150 mM NaCl, 15 mM Na citrate, pH 7.2), recovered by centrifugation (2000 *g*, 5 min), washed twice in PBS, resuspended in a hypotonic swelling buffer (10 mM Tris, pH 8.0, 2 mM $MgCl_2$) and lysed using a Dounce homogenizer. After centrifugation (800 *g*, 5 min), the N1R protein was extracted from the pellet using a high salt buffer (50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 3 mM 2-mercaptoethanol, 1 mM EDTA, 10 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Soluble N1R was recovered in the supernatant after centrifugation (2500 *g*, 15 min). For DNA cellulose chromatography, the soluble N1R extract was desalted using a 10-ml KwikSep polyacrylamide 6000 desalting column (Pierce Chemical, Rockford, Illinois) into buffer 1 (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 3 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride).

DNA cellulose chromatography

dsDNA and ssDNA cellulose (Pharmacia Biotech, Piscataway, New Jersey) was prepared in buffer 1. Protein samples in the same buffer were applied to 1-ml columns at a flow rate of 1 ml/h and recycled twice through the column. The columns were washed with 6 ml of buffer 1 and then eluted stepwise with buffer 1 containing 0.1, 0.25, 0.5, 0.75, 1.0, and 1.5 M NaCl. Samples were analyzed by Western blotting after SDS-PAGE and electroblotting to nitrocellulose.

Apoptotic DNA isolation and analysis

For the DNA fragmentation assays, samples of 10^6 BGMK cells, at 80% confluence, were infected with VV (m.o.i. = 3), infected with VV-N1R, mock infected, and mock infected with no serum. Adherent and floating cells were harvested at various times. Apoptotic DNA fragments were recovered (Koyama and Miwa, 1997), separated in a 1.5% agarose gel run at 2 V/cm for 12 h, and visualized by UV after ethidium bromide staining.

For ELISA quantification of apoptosis, a sandwich assay was performed using a pair of mAbs specific for two nucleosomal epitopes to capture and detect cytoplasmic nucleosomes (Salgame *et al.,* 1997). Hybridoma cell lines (LG11–2 and PL2–3) were generously provided by Dr. Marc Monestier (Temple University School of Medicine, Philadelphia, Pennsylvania). mAbs were prepared by Immuno-Precise Antibodies (Victoria, British Columbia, Canada). VV infections of BGMK cells were performed as described for the DNA fragmentation assays, and cycloheximide was used at 200 μg/ml. The ELISA was performed using 2.5×10^4 cell equivalents per well.

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