Identification and Characterization of a Baboon Reovirus-Specific Nonstructural Protein Encoded by the Bicistronic S4 Genome Segment

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All characterized orthoreoviruses encode a characteristic spike-like protein on their polycistronic S1 genome segments that mediates virus cell attachment. In the case of baboon reovirus (BRV), the polycistronic S-class genome segment corresponds to the smallest S4 segment. We recently determined that the 5'-proximal open reading frame (ORF) of the bicistronic S4 segment encodes a nonstructural protein responsible for virus-induced syncytium formation. Current analysis indicates that the p16 protein encoded by the 3'-proximal ORF of the BRV S4 genome segment shows no sequence similarity to any other protein encoded by the orthoreoviruses, including the well-characterized α1/αC reovirus cell attachment protein. Results indicate that p16 is a BRV-specific nonstructural protein that is not required for virus infection in cell culture and is not involved in viral cell attachment. In conjunction with previous studies of the BRV S1, S2, and S3 genome segments, the current results indicate that, unlike all other orthoreoviruses, BRV does not encode a cell attachment protein in its S-class genome segments. Furthermore, cell binding and infectivity studies suggested BRV may not utilize a functional homolog of the prototypical reovirus α1/αC cell receptor-binding protein to mediate endocytic uptake by cells.

INTRODUCTION

Receptor binding represents an early essential event in the virus replication cycle. The specificity and avidity of such virus–cell interactions have profound effects on virus tissue tropism, host range, and pathogenicity. In the case of the nonenveloped orthoreoviruses, a minor outer capsid protein located at the vertices of the icosahedral virus particle facilitates virus cell attachment (Lee et al., 1981; Pacitti and Gentsch, 1987; Masri et al., 1986; Lee and Leone, 1994). Genetic studies indicate that the S1 genome segment of mammalian reovirus (MRV), which is bicistronic and encodes the α1 cell attachment protein, plays a significant role in the pathogenic potential and tissue tropism of the different MRV serotypes (Kaufman et al., 1983; Sharpe and Fields, 1985; Tyler et al., 1986; Nibert et al., 1995; Morin et al., 1996).

Recently, a novel reovirus was isolated from brain tissue of a baboon suffering from meningoencephalomyelitis (Duncan et al., 1995; Leland et al., 2000). Extensive sequence divergence and limited antigenic similarity between baboon reovirus (BRV) and the prototype MRVs contributed to the designation of BRV as a distinct species of orthoreovirus (Duncan, 1999). Unlike MRV, BRV is potentially pathogenic in nonhuman primates (Leland et al., 2000). The identification of the BRV cell attachment protein and the association of this protein with BRV pathogenesis has not been determined.

The orthoreoviruses are an unusual example of nonenveloped viruses that utilize a spike protein to mediate cell attachment (Lee et al., 1981; Furlong et al., 1988). The trimeric MRV α1 protein contains a long fibrous tail topped by a compact globular head (Furlong et al., 1988; Strong et al., 1991; Duncan et al., 1991; Lee and Gilmore, 1998). An extended heptad repeat region that encompasses the amino-terminal one-third of the protein is conserved in the α1 proteins of all mammalian reoviruses (Duncan et al., 1990; Nibert et al., 1990) and in the homologous αC proteins of avian reovirus (AVR) and Nelson Bay reovirus (NBV) (Shapouri et al., 1995, 1996; Martinez-Costas et al., 1997; Grande et al., 2000; Shmulevitz et al., 2002). This heptad repeat is a hallmark feature of the reovirus cell attachment proteins and contributes to the formation and stability of a coiled coil responsible for the generation of the functional trimeric form of the α1 protein (Leone et al., 1991a,b, 1992; Gilmore et al., 1996).

Previous analysis detected clear sequence conservation between the BRV S1, S2, and S3 genome segment-encoded polypeptides and the MRV major α-class core, outer capsid, and nonstructural proteins, respectively, indicating the homologous nature of these proteins (Duncan, 1999). Consequently, unlike all previously characterized reoviruses, BRV does not encode a homolog of α1/αC in its S1 genome segment. The S4 genome segment of BRV was recently shown to be functionally bicistronic, encoding a p15 protein that is responsible for
the unusual syncytium-inducing ability of this nonenveloped virus, and an uncharacterized p16 protein from a second 3′-proximal open reading frame (ORF) (Dawe and Duncan, 2002). Since the cell attachment proteins of the other orthoreoviruses are all encoded by polycistronic S-class genome segments, and since none of the other S-class genome segments of BRV encode a cell attachment protein homolog, we investigated whether the only uncharacterized BRV α-class protein, the p16 gene product of the second S4 ORF, was responsible for viral cell attachment. Results indicate that p16 is a BRV-specific nonstructural protein that is not required for virus infection in cell culture. It would appear that the means by which BRV interacts with receptors to promote endocytic uptake may be quite distinct from the α/αC paradigm established for all other reoviruses.

**RESULTS**

The p16 protein is not a α1/αC homolog

Sequence comparisons previously revealed that the BRV S1, S2, and S3 genome segments are monocistronic and do not encode a reovirus cell attachment protein homolog (Duncan, 1999). Recent sequence analysis of BRV S4 identified two partially overlapping ORFs of 140–141 amino acids, both of which are functional (Fig. 1A) (Dawe and Duncan, 2002). Since all characterized orthoreoviruses encode their cell attachment protein on the polycistronic S-class genome segment, we predicted that one of the ORFs on the bicistronic S4 genome segment of BRV would encode a receptor-binding protein. The p15 gene product of the 5′-proximal ORF was shown to be responsible for BRV-induced syncytium formation and as a nonstructural protein, not involved in BRV cell attachment (Dawe and Duncan, 2002). We therefore sought to characterize the p16 gene product of the 3′-proximal S4 ORF and to determine whether it mediates BRV receptor interactions.

The predicted sequence of p16 was examined for sequence similarity to the α1/αC proteins of the other orthoreoviruses. Although p16 is considerably smaller than the typical reovirus cell attachment proteins (molecular mass of 16,854 D), it might represent a truncated α1/αC homolog. However, p16 displayed no significant sequence similarity to α1/αC. More importantly, the heptad repeat structure that is characteristic of the reovirus cell attachment proteins is absent in p16. Correspondingly, the Coils (Lupas et al., 1991), Paircoils (Berger et al., 1995), and MultiCoil (Wolf et al., 1997) algorithms failed to detect a coiled coil motif in p16 (data not shown). These results indicated that p16 is not a homolog of the typical reovirus cell attachment protein.

The BRV p16 protein associates with virus particles

Although p16 did not appear to be a homolog of the α1/αC protein, it was conceivable that it might represent a novel reovirus receptor-binding protein. If so, then p16 should be a structural component of the virus. Radiolabeled BRV particles were isolated from virus-infected cell lysates by differential centrifugation and examined for the presence of p16 using p16-specific antisera. Concentrated virus preparations revealed the characteristic reovirus protein profile with the major λ-, μ-, and α-class proteins in addition to an approximately 15–16 kDa polypeptide (Fig. 1B). Immune precipitation with previously generated antisera specific for p15 or p16 (Dawe and Duncan, 2002) demonstrated that the small virion-associated protein was p16 (Fig. 1B, lane 4). The particle-associated nature of p16 was consistent with a possible role in promoting virus attachment to cells.

Anti-BRV and anti-p16 sera fail to inhibit BRV infection

To further address whether BRV p16 might function as a novel reovirus cell attachment protein, we investigated the ability of antiserum to inhibit the entry of BRV into host cells in a plaque-reduction assay. Prior to infection of Vero cell monolayers, the BRV inoculum was preincubated with either anti-p16 serum or with baboon serum obtained from animals naturally infected with BRV (Le-
land et al., 2000). Compared to normal rabbit serum, neither anti-p16 serum nor anti-BRV serum significantly reduced the number of plaques produced by BRV in Vero cells (Fig. 2A). The ability of these antisera to recognize BRV structural proteins was confirmed by immune precipitation of virus-infected cell lysates. As shown in Fig. 2B, although the baboon sera recognized the major $\lambda$, $\mu$, and $\sigma$-class viral proteins, none of the sera recognized p16. The inability of these sera to neutralize BRV infectivity could not, therefore, be interpreted as evidence against p16 functioning as the BRV receptor-binding protein. Although anti-p16 serum strongly reacts with soluble p16 (Fig. 2B, lane 9), its failure to inhibit BRV infection could have reflected an inability to recognize p16 in the context of the BRV virion.

**Soluble BRV p16 does not bind cells**

The conserved heptad repeat that serves to stabilize the functional trimeric form of the reovirus $\sigma 1/\sigma C$ cell attachment protein also imparts to the protein its ability to bind to receptors in a virion-independent manner (Yeung et al., 1989; Duncan et al., 1991; Shapouri et al., 1996; Martinez-Costas et al., 1997). We therefore examined whether soluble p16 or any other BRV-encoded gene product could bind to susceptible host cells. As previously demonstrated, soluble MRV-3 $\sigma 1$ derived by in vitro translation exhibited cell-binding activity (Fig. 3, lanes 5 and 6) and served as a positive control for the binding assay. Under similar assay conditions, soluble BRV p16 did not bind to susceptible cell monolayers. This observation applied to p16 derived by in vitro translation (Fig. 3, lanes 7 and 8) and to p16 present in the soluble S100 fraction of BRV-infected cell lysates (Fig. 3, lanes 1 and 2). Binding was noted for an approximately 38-kDa protein present in the BRV S100 fraction (Fig. 3, lane 2). This binding, however, was determined to be nonspecific adherence to cells by the major nonstructural protein $\sigma NS$, as indicated by a similar cell-binding activity for $\sigma NS$ generated by in vitro translation of the BRV S2 genome segment (Fig. 3, lanes 3 and 4). The fact that $\sigma NS$ cell binding is not biologically significant in regard to BRV infection was confirmed using virus particles depleted of $\sigma NS$ (see Figs. 4 and 5).

**BRV p16 is not required for virus infectivity**

The particle-associated nature of p16 lead us to suspect that p16 might be involved in BRV cell attachment. Since BRV particles appear to be unstable in CsCl gradients (data not shown), we used differential centrifugation to obtain partially purified BRV particles. Although p16 was present in such preparations of concentrated BRV, the virus particles were also contaminated with the BRV major nonstructural protein $\sigma NS$ which appears to adhere both to cell monolayers and to virions (Fig. 3). The

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**FIG. 2.** Antisera do not inhibit BRV infectivity. (A) BRV inocula were preincubated with normal rabbit serum (N), anti-BRV serum (BRV), or anti-p16 serum (16) prior to infection of Vero cells. The infectivity of the samples was then assayed in a plaque reduction assay. The results shown are the mean plus/minus the standard error from triplicate samples and are representative of five separate experiments. (B) Radiolabeled MRV-infected cell lysates (INF) were immune precipitated with a panel of baboon sera samples (1–6), normal rabbit serum (N), or anti-p16 serum (16). Precipitates were resolved by SDS–PAGE and detected by fluorography. The positions of the $\lambda$, $\mu$, and $\sigma$-class reovirus proteins are shown at the left and the migration of molecular mass standards are indicated on the right.

**FIG. 3.** Soluble p16 does not bind to Vero cell monolayers. The radiolabeled S100 fraction of a BRV-infected cell lysate, and in vitro translated BRV $\sigma NS$, MRV $\sigma 1$, and BRV p16 were analyzed by SDS–PAGE and fluorography prior to (unbound fraction, U) and following (bound fraction, B) incubation with Vero cell monolayers. The positions of the $\lambda$, $\mu$, and $\sigma$-class reovirus proteins are shown at left and the migration of molecular mass standards are indicated on the right.
contamination of concentrated MRV preparations by αNS has been previously described (Huismans and Joklik, 1976). The presence of a known nonstructural protein in concentrated preparations of BRV particles suggested that p16 may not be a structural protein of the virus but merely particle-associated. We therefore determined purification conditions that would remove p16 and/or αNS from virus particles and assessed the infectivity of such virus particles.

Treatment of MRV preparations with nonionic detergent and RNase A was previously shown to remove contaminating αNS (Huismans and Joklik, 1976). Similar treatment of concentrated BRV particles with 1% Triton X-100 plus 50 µg/mL RNase A followed by recentrifugation of BRV particles through sucrose cushions removed the vast majority (>95%) of αNS, but only approximately 60% of p16 (Fig. 4A, lane 2, and Fig. 4B). Extraction of BRV preparations with freon removed over 98% of BRV-associated αNS and essentially all of the particle-associated p16 (Fig. 4A, lane 3). Since the removal of p16 from BRV did not disrupt the integrity of the BRV virion (freon-extracted virus particles could be harvested after recentrifugation through sucrose), p16 must be only peripherally associated with virus particles.

Contaminating cellular nucleic acids precluded quantification of concentrated BRV virion preparations by spectrophotometry; therefore, radiolabeled virus preparations (untreated, as well as those extracted with either detergent and RNase or freon) were titered by densitometric analysis. Multiple exposures and twofold dilutions of the virus preparations to ensure the fluorograms were in the linear exposure range permitted standardizing BRV particles to within a twofold concentration range. Two-fold dilutions of the standardized BRV particle preparations were used to infect Vero cells and the numbers of infectious foci were quantified by immunostaining. As expected, removal of αNS (detergent and RNase extraction) did not decrease the infectivity of BRV (Fig. 5). In fact, removal of αNS led to a slight, reproducible increase in the per-particle infectivity of BRV as compared to the untreated BRV sample, suggesting that particle-associated αNS actually interferes with BRV infection rather than serving an essential function in BRV cell attachment. More importantly, essentially complete removal of particle-associated p16 by freon extraction had no significant effect on BRV infectivity. These results conclusively demonstrated that p16 does not function to mediate BRV cell attachment and suggested that p16 is a nonstructural protein.
BRV uses an endocytic entry pathway

The absence of a cell attachment protein among the S-class genome segment gene products and the inability to detect a $\sigma 1/\sigma C$ equivalent that functions in a soluble form to bind cell receptors raised the question of whether BRV uses a receptor-mediated endocytosis pathway to infect cells. To address this issue, we examined the effect of the endocytic inhibitor dansylcadaverine on BRV infection (Davies et al., 1984). Treatment of cells with dansylcadaverine during the virus entry phase resulted in the complete inhibition of BRV-induced syncytium formation and led to a >90% inhibition in virus yield (Fig. 6). These results indicated that BRV utilizes an endocytic entry pathway.

DISCUSSION

BRV does not encode a $\sigma 1/\sigma C$ homolog in its S-class genome segments

The role of the MRV $\sigma 1$ receptor-binding protein in serotype-specific differences in tissue tropism and pathogenesis has been amply demonstrated (Kaufman et al., 1983; Tyler et al., 1986; Morin et al., 1996; Nibert et al., 1995; Connolly et al., 2001). Additional studies have revealed the importance of a conserved heptad repeat sequence on protein folding and the formation of the functional trimeric form of the MRV $\sigma 1$ protein (Leone et al., 1991a, 1992; Lee and Leone, 1994; Lee and Gilmore, 1998; Gilmore et al., 1996). This characteristic heptad repeat sequence is conserved in the $\sigma C$ proteins of ARV and NBV despite extensive deletions in the head and neck regions of these smaller reovirus cell attachment proteins (the $\sigma C$ and $\sigma 1$ proteins are approximately 325 versus 455 residues, respectively) (Shmulevitz et al., 2002). We have recently determined that a syncytium-inducing reptilian reovirus represents a fifth species of orthoreovirus that encodes a $\sigma C$ homolog containing the conserved heptad repeat region on its bicistronic S1 genome segment (R. Duncan, unpublished data). This characteristic trimeric cell attachment protein encoded by the polycistronic S1 genome segment is, therefore, a hallmark feature of the orthoreoviruses.

The current data indicate that BRV is the only orthoreovirus that does not encode a $\sigma 1/\sigma C$ homolog on its polycistronic S-class genome segment. The bicistronic S4 genome segment of BRV encodes two nonstructural proteins that are not involved in virus entry. The product of the first ORF, p15, is a nonstructural protein of the virus and is responsible for the unusual syncytium-inducing property of BRV (Dawe and Duncan, 2002). As we have now shown, the p16 product of the second S4 ORF does not contain the characteristic heptad repeat structure of $\sigma 1/\sigma C$, it displays no soluble cell-binding capability, and it is not required for BRV infectivity. Consequently, BRV represents the only example of an orthoreovirus that does not encode a homolog of the $\sigma 1/\sigma C$ cell attachment protein in its S-class genome segments.

It is also possible that none of the BRV-encoded gene products represent a $\sigma 1/\sigma C$ equivalent. Although sequence information is not available for the three L-class and three M-class genome segments of BRV, these segments are in the same size range as the corresponding genome segments of the other orthoreoviruses (Duncan et al., 1995; Duncan, 1999). Furthermore, these genome segments encode $\lambda$ and $\mu$ proteins with similar molecular masses as the corresponding proteins of the other reoviruses. Since the open reading frames encoding the reovirus $\lambda$ and $\mu$ proteins extend for almost the complete length of the genome segment encoding each protein (Bruen et al., 2001), it is unlikely that a homolog of the reovirus $\sigma 1/\sigma C$ cell attachment protein could be encoded by an additional 1000–1600
nucleotide ORF present in any of the L-class or M-class genome segments of BRV. This supposition is supported by the fact that the protein profile of infectious BRV particle preparations contains the standard repertoire of \( \lambda, \mu, \) and \( \alpha \) proteins with no evidence of an additional protein that might represent the \( \alpha1/\alpha C \) cell attachment protein (Fig. 4).

The cell-binding results (Fig. 3) provided more compelling evidence that BRV does not encode a \( \alpha1/\alpha C \) homolog in its genome. Other than the nonspecific cell adherence displayed by \( \alpha NS \), no soluble protein present in the S100 fraction from BRV-infected cells displayed any cell-binding capability. Even though the ARV and MRV \( \alpha1/\alpha C \) proteins share almost no sequence identity, an N-proximal heptad repeat structure stabilizes the trimeric form of both proteins which function in a soluble form to bind cell receptors. The absence of such cell-binding activity by any of the soluble BRV proteins present in virus-infected cell lysates suggests BRV may not encode a \( \alpha1/\alpha C \) homolog, at least as far as the ability of such a protein to function in a soluble, non-virion-associated form.

The cell-binding capability of \( \alpha NS \) has no biological relevance to virus cell attachment since removal of virion-associated \( \alpha NS \) actually enhanced, rather than inhibited, virus infectivity (Figs. 4 and 5). The MRV \( \alpha NS \) protein associates with cytoskeleton, it binds ssRNA in a sequence-independent manner, and it has been implicated in RNA replication and packaging (Anciazak and Joklik, 1992; Huismans and Joklik, 1976; Mora et al., 1987; Gillian et al., 2000). The BRV \( \alpha NS \) protein also associates with concentrated virus particles (Fig. 4). Apparently, the reovirus \( \alpha NS \) protein possesses the ability to interact with numerous macromolecular structures, a property that may contribute to the nonspecific association of the protein with cell monolayers.

**The p16 protein is a BRV-specific nonstructural protein**

The fact that BRV particles devoid of p16 were fully infectious (Figs. 4 and 5) conclusively demonstrated that p16 is not required for BRV infectivity and implied that p16 is a nonstructural protein. Whether the association of p16 with concentrated BRV preparations is biologically relevant, reflecting an affinity of p16 for capsid proteins, or represents nonspecific protein association is not clear.

Other than virus particle association, no other biological properties are known for the BRV p16 protein that would facilitate speculation as to the role of this protein in the virus-replication cycle. The p16 protein is acidic (pl = 4.53) and lacks any identifiable structural or functional motifs. Direct sequence comparisons revealed no obvious sequence similarity to any other small nonstructural proteins encoded by the orthoreoviruses or by other members of the family *Reoviridae*. This included the small, basic, nonstructural proteins encoded by other orthoreovirus polycistronic S-class genome segments that display no conservation of similar amino acids as indicated by their distinct hydropathy profiles (data not shown). The BRV-specific nature of p16 (i.e., other orthoreoviruses do not encode this protein) suggests this is an accessory protein of the virus that is not essential for virus replication in cell culture, similar to the situation with the \( \alpha1NS \) protein of MRV (Rodgers et al., 1998).

**BRV receptor interactions in the absence of a prototypical reovirus cell attachment protein**

Although BRV appears to utilize a receptor-mediated endocytic entry pathway (as evident by the inhibition of virus infection by dansylcadaverine), the absence of a \( \alpha1/\alpha C \) homolog among the BRV-encoded gene products suggests that BRV has evolved a novel strategy for cell attachment. How BRV mediates cell attachment is not clear. Our inability to inhibit BRV infection using baboon sera obtained from animals exposed to BRV was unexpected, especially since the sera clearly recognized all of the obvious \( \lambda, \mu, \) and \( \alpha \) proteins of the virus (Fig. 2). A recent report also failed to detect neutralizing antibodies against BRV in five acute or convalescent sera samples obtained from baboons infected with BRV (Leland et al., 2000). Whichever viral proteins or capsid structures mediate BRV cell attachment, these receptor-binding entities do not elicit a strong neutralizing antibody response, contrary to the situation with the \( \alpha1/\alpha C \) proteins of other orthoreoviruses. It is conceivable that the inability to trigger a neutralizing humoral response might contribute to the pathogenic potential of BRV. Additional studies of BRV receptor recognition and immune interactions offer promising avenues for investigating the pathogenicity of this novel reovirus in nonhuman primates.

Interestingly, despite conspicuous similarities between the orthoreoviruses and aquareoviruses, there is no \( \alpha1/\alpha C \) equivalent present in the gene products of aquareovirus (Attoui et al., 2002). In addition, the density associated with \( \alpha1 \) at the vertices of the MRV virion is absent in image reconstructions of aquareovirus (Shaw et al., 1996). It may be that both BRV and aquareovirus have evolved a cell attachment strategy that is independent of the need for a \( \alpha1/\alpha C \) equivalent. Further investigation of BRV cell attachment may offer additional insights into a diversity of reovirus receptor interactions.

**MATERIALS AND METHODS**

**Viruses and cells**

BRV was isolated from the brain tissue of a baboon with meningoencephalomyelitis (Duncan et al., 1995) and was obtained from Julia Hilliard (Southwest Foundation for Biomedical Research, San Antonio, TX). The virus was plaque purified and high titer stocks were grown in African green monkey (Vero) cells as previously described (Duncan et al., 1995). Mammalian reovirus serotype 3 strain Dearing was obtained from Patrick Lee.
(University of Calgary). The Vero cells were purchased from the ATCC (CCL-81) and maintained at 37°C in a 5% CO₂ atmosphere, grown in medium 199 with Earle’s salts containing 5% heat-inactivated fetal bovine serum (FBS) and 100 units of penicillin and streptomycin per milliliter.

Antibodies

Serum from six baboons exhibiting reactivity with BRV-specific antigens, including a serum sample from a convalescent BRV-infected encephalitic baboon, was kindly donated by Julia Hilliard (Southwest Foundation for Biomedical Research). The generation and specificity of the rabbit polyclonal antisera raised against the BRV p16 and p15 proteins has been described previously (Dawe and Duncan, 2002).

Cloning

The cDNA cloning and sequencing of the BRV S-class dsRNA genome segments have been described previously (Duncan, 1999), as has the subcloning of the individual p15 and p16 ORFs of the S4 genome segment into the pcDNA3 vector (Dawe and Duncan, 2002).

Radiolabeled cell lysates

Vero cells were infected with BRV at a multiplicity of infection of 5 PFU/cell in medium containing 1% FBS. To prepare radiolabeled cell lysates, uninfected or BRV-infected monolayers were labeled 12 h postinfection for 1 h with 100 μCi [³H]leucine (Amersham Pharmacia Biotech) per milliliter leucine-free medium, prepared from the MEM Select-Amine kit (Life Technologies) according to the manufacturer’s instructions. Radiolabeled monolayers were then rinsed three times with phosphate-buffered saline (PBS; 140 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄) and lysed in cold RIPA buffer containing protease inhibitors, harvested, and passed through a 26.5-gauge syringe needle 10 times. Since BRV is unstable in CsCl, virus was purified by extraction and differential centrifugation. Briefly, cellular debris was removed by centrifugation (20 min at 10,000 g) and the virus-containing supernatant was pelleted through a 2-mL sucrose cushion (30% sucrose in medium) for 1 h at 27,000 rpm in an SW 40 rotor (Beckman). Concentrated BRV pellets were resuspended in medium and used as is, or further extracted with freon or 1% Triton-X 100 plus RNase A (50 μg/mL) before pelleting through a sucrose cushion.

To establish the relative concentrations of BRV virus particles and the extent of removal of p16 and αNS from these particles following extraction with freon or Triton X-100 plus RNase, the protein profiles of the various radiolabeled virus preparations were examined by SDS–PAGE and fluorography. The intensities of the three λ-class proteins (number of pixels in a constant area) that comigrated as a single band on a 15% acrylamide gel were scanned and quantified using the Molecular Analyst software (v.2.1.2) and the GelDoc 1000 system (Bio-Rad). Twofold dilutions of the various BRV particle preparations and multiple exposures were used to ensure that the fluorograms were in the linear exposure range of the X-ray film. The BRV particle preparations were then diluted to equivalent particle concentrations based on the relative concentrations of the λ-class proteins.

In vitro transcription and translation

BRV p16 uncapped transcripts were synthesized from XhoI-linearized pcDNA3 plasmids using bacteriophage T7 RNA polymerase. MRV-3 S1 uncapped transcripts were synthesized from a HindIII-linearized pGem-4Z plasmid using bacteriophage SP6 RNA polymerase. All transcription reactions were performed using 1 μg template DNA, 2.5 mM NTPs, and 125 units polymerase, and incubated 2 h at 37°C. All transcription reagents were obtained from Life Technologies Inc. RNA (250 ng per 50 μL reaction) was translated in the presence of [³H]leucine (1 μCi per 50 μL reaction) using nuclease-treated rabbit reticulocyte lysates (Promega) according to the instructions provided by the manufacturer.

Cell-binding assay

The cell-binding assay was based on the procedure previously described for MRV α1 (Lee et al., 1981). Confluent Vero cell monolayers in six-well plates (Falcon) were washed once with PBS and the S100 fractions from radiolabeled BRV-infected cell lysates, or radiolabeled in vitro translated BRV p16, BRV αNS, and MRV-3 α1, containing 5 mg/mL BSA and 1.2 μg/mL leucine, were added to the wells. The plates were incubated at 4°C with intermittent rocking for 1 h, after which the supernatant was removed and the monolayers were washed five times with PBS. The cells were lysed with cold RIPA buffer containing protease inhibitors, harvested, and mixed with protein sample buffer (Laemmlı, 1970) prior to analysis of the radiolabeled cell-bound proteins by SDS–PAGE using 15% polyacrylamide gels and fluorography (Bonner, 1984).
Immune precipitation

Aliquots of radiolabeled in vitro translated protein (1/10 translation reaction), cell lysates, or BRV (200,000 cpmp) were immune precipitated in RIPA buffer for 1 h using a 1:100 dilution of anti-p15, anti-p16, baboon sera, or normal rabbit sera. Immune complexes were recovered using fixed Staphylococcus aureus cells (Harlow and Lane, 1988), washed extensively with RIPA buffer, and released by boiling in SDS protein sample buffer (Laemmli, 1970) prior to SDS–PAGE using 15% polyacrylamide gels and fluorography (Bonner, 1984).

Plaque reduction

BRV (250 PFU) was preincubated with equal volumes of either anti-BRV serum from a convalescent baboon, anti-p16 serum, or normal rabbit serum. After 2.5 h shaking at room temperature, samples were diluted 10-fold and used to infect Vero cell monolayers in six-well plates for 1 h with intermittent rocking. Infected monolayers were rinsed three times with warm PBS and overlaid with medium containing 5% FBS and 2% agar. Three days postinfection, the cells were fixed with 10% formaldehyde in PBS and stained with crystal violet in 50% ethanol, and the visible plaques counted.

Cell staining

Vero cell monolayers grown in six-well plates were transfected with pcDNA3-encoded BRV genes (S4, S4 ORF1, and S4 ORF2) using Lipofectamine (Life Technologies) and stained 18 h later with Wright-Giemsa stain (Diff-Quik) according to the manufacturer’s instructions (VWR Scientific) to visualize cell nuclei and polykaryon formation. To detect the visible plaques counted.

Quantification of infected foci

The relative infectivities of different BRV preparations (concentrated, extracted, and adjusted to equal particle concentrations as described above) were compared and quantified using a focus forming assay. Serial twofold dilutions of the different standardized virus particle preparations were used to inoculate Vero cell monolayers, and the infected foci were detected by immunostaining as described above. The stained monolayers were observed using Nikon Diaphot inverted microscope at 100× magnification and the average number of infected foci in five random fields were quantified using computer software Image-Pro Plus (v.4.0) as described previously (O’Hara et al., 2001).

Analysis of BRV endocytic uptake

To confirm that BRV enters cells by endocytosis, dansylcadaverine was used to inhibit the endocytic entry pathway, as previously reported for ARV (Duncan, 1996). Vero cells were pretreated for 1 h with 0.1 mM dansylcadaverine prior to infection with BRV. The virus inoculum containing dansylcadaverine was incubated with cells for 3 h at 37°C to permit virus attachment and entry. The residual inoculum and inhibitor were then removed; cells were overlaid with fresh medium, and infected cells were incubated at 37°C. The infected cell lysates were Giemsa stained at 12 h postinfection to detect virus-induced syncytium formation (an indirect assessment of virus infection) or harvested at 72 h postinfection and processed for quantification of infectious progeny virus production by plaque assay, as described above. Titers were compared to parallel infected cell cultures that were mock treated with the inhibitor.

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