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Expression of the Rotavirus SA11 Protein VP7 in the Simple Eukaryote *Dictyostelium discoideum*

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The outer capsid protein of rotavirus, VP7, is a major neutralization antigen and is considered a necessary component of any subunit vaccine developed against rotavirus infection. For this reason, significant effort has been directed towards producing recombinant VP7 that maintains the antigenic characteristics of the native molecule. Using a relatively new expression system, the simple eukaryote *Dictyostelium discoideum*, we have cloned the portion of simian rotavirus SA11 genome segment 9, encoding the mature VP7 protein, downstream of a native *D. discoideum* secretion signal sequence in a high-copy-number extrachromosomal vector. The majority of the recombinant VP7 expressed by transformants was intracellular and was detected by Western immunoblot following gel electrophoresis as two or three bands with an apparent molecular mass of 35.5 to 37.5 kDa. A small amount of VP7 having an apparent molecular mass of 37.5 kDa was secreted. Both the intracellular VP7 and the secreted VP7 were N glycosylated and sensitive to endoglycosidase H digestion. Under nonreducing electrophoresis conditions, over half the intracellular VP7 migrated as a monomer while the remainder migrated with an apparent molecular mass approximately twice that of the monomeric form. In an enzyme-linked immunosorbent assay, intracellular VP7 reacted with both nonneutralizing and neutralizing antibodies. The monoclonal antibody recognition pattern paralleled that found with VP7 expressed in either vaccinia virus or herpes simplex virus type 1 and confirms that *D. discoideum*-expressed VP7 is able to form the major neutralization domains present on viral VP7. Because *D. discoideum* cells are easy and cheap to grow, this expression system provides a valuable alternative for the large-scale production of recombinant VP7 protein.

Rotavirus is a member of the *Reoviridae* family and is the major causative agent of viral gastroenteritis in humans and animals (15). Several studies have shown that an initial infection with rotavirus will protect against subsequent rotavirus challenge and decrease the severity of disease associated with reinfection (3, 7), implying that a vaccine against rotavirus could be developed. Two major approaches have been used in the development of a rotavirus vaccine. Firstly, several vaccine trials with asymptomatic rotavirus strains (47), attenuated animal strains (38), and rhesus-human reassortant strains (46) have been conducted. The results from these studies and others (15) have, in general, been disappointing and have highlighted the difficulties in providing protection against multiple rotavirus serotypes.

The second approach is to develop subunit or recombinant vaccines by expressing one or more rotavirus proteins which retain the neutralizing epitopes necessary for effective recognition by the host cell. Both VP7 and VP4, the two protein components of the outer capsid, react with neutralizing antibodies, and monoclonal antibodies (MAbs) directed at either of these proteins are capable of neutralizing rotavirus (28). VP7 is the major outer capsid protein and is primarily responsible for determining the viral serotype. As such, it is a primary candidate for inclusion in a subunit vaccine.

Recombinant VP7 has been expressed in several systems. VP7 expressed in *Escherichia coli* proved toxic to the cell (29), while a β-galactosidase–VP7 fusion protein induced weak production of neutralizing antibodies in mice (2). Similarly, vaccinia virus constructs of the wild-type VP7 gene and a modified construct resulting in partial secretion of VP7 (1) induced only a small increase in neutralizing antibody when injected intradermally into rabbits. Rotavirus proteins expressed in a baculovirus-insect cell system have produced mixed results (9, 30). Cell-mediated clearance of rotavirus was demonstrated in SCID mice receiving $CD8^+$ T cells from BALB/c mice immunized with a crude cell sonicate containing recombinant VP7. However, the BALB/c mice did not produce antibody which could immunoprecipitate viral VP7 (9). McGonigal et al. showed that antiserum raised against baculovirus-expressed VP7 neutralized SA11 rotavirus infectivity in a fluorescent focus assay at a 1/1,000 titer. However, under similar conditions, the neutralizing titer with double-shelled rotavirus was much higher (1/100,000) (30).

Studies using neutralization escape mutants or competition binding have clearly demonstrated an interaction between the A region (amino acids 93 to 99) and the C region (amino acids 211 to 223) of SA11 VP7, implying that the neutralization domains of VP7 are complex and conformationally dependent (28). MAb recognition studies have shown that the membrane-bound form of VP7 found in the endoplasmic reticulum (ER) prior to virus maturation is conformationally distinct from VP7

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found on the assembled virus (23). A successful vaccine which will induce adequate neutralizing antibody will need to present VP7 in a conformation similar to that seen on the mature virus.

In support of this, herpes simplex virus type 1-expressed SA11 VP7 is recognized by only one neutralizing antibody, whereas coinfection of cells with herpes simplex virus type 1 expressing SA11 VP7 and the heterologous rotavirus Nebraska calf diarrhea virus resulted in reconstitution of additional neutralizing epitopes to SA11 VP7 (14). Presumably, these neutralizing epitopes were acquired as the recombinant VP7 associated with the Nebraska calf diarrhea virus particles. Subsequent studies have indicated that, on exposure to low levels of calcium, VP7 undergoes a conformational change which destabilizes complexes between the neutralizing antibody and expressed VP7 (13). However, complexes of neutralizing antibody with cell surface-anchored VP7 expressed in a vaccinia virus system are more stable in a low-calcium environment (12), which may account for the increased immunogenicity of the vaccinia virus-expressed VP7 when modified for expression on the cell surface rather than for secretion or retention within the cell (4). An alternative approach to the maintenance of conformational epitopes involves the production of virus-like particles in a baculovirus system following simultaneous expression of bovine rotavirus strain C486 VP7 with VP6 (the major component of the inner capsid) (37). These particles reacted with antiserum specific for double-shelled C486 rotavirus. However, they have not been tested in vivo for their ability to produce neutralizing antibodies.

We have developed a new expression system in the simple eukaryote *Dictyostelium discoideum* (11, 42). The single-celled amoebae are robust and grow to a high cell density without the serum factors or special aeration needed by animal cell cultures. There is no cell wall, and the high-copy-number plasmid vectors developed allow the expression of proteins in cell-associated, membrane-attached, or secreted form under the control of regulatable promoters. The major advantages of this system include a very simple and cheap growth medium and the potential for large-scale production of proteins. Recombinant membrane proteins can be purified in a large quantity (50), and their antigen presentation can be improved by incorporation into immune-stimulating complexes or liposomes. The principal aims of this study were to express simian rotavirus strain SA11 VP7 in *D. discoideum*, to develop simple methods for expressing large quantities of the protein, and to partially characterize the recombinant VP7. VP7 is normally retained in the ER prior to viral assembly. The native signal peptide of rotavirus SA11 VP7, H2, is involved in this retention mechanism, as substitution of this peptide with the signal peptide from influenza virus hemagglutinin resulted in the secretion of VP7 (43). In order to express a secreted form of VP7 in the *D. discoideum* expression system, the native H1 and H2 signal peptides were replaced with a *D. discoideum* secretion signal.

MATERIALS AND METHODS

Chemicals and reagents. Restriction endonucleases and polymerases were purchased from Pharmacia and Boehringer Mannheim. Proteinase K, endoglycosidase H (endo H), glycopeptidase F (PNGase F), and deoxynucleotides were purchased from Boehringer Mannheim, and Geneticin (G418), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, 2-mercaptoethanol, dimethyl sulfoxide, RNase, and *o*-phenylenediamine dihydrochloride were purchased from Sigma Chemicals. Protein A-Sepharose CL-4B beads were purchased from Pharmacia, medium 199 (M199) and phosphate-buffered saline (PBS) were purchased from Irvine Scientific, and fetal calf serum was purchased from HyClone Laboratories.

Strains. *E. coli* Sure (Stratagene) was used for all DNA manipulations. *D. discoideum* transformations used the axenic strain NP2 (24), and *D. discoideum* cells were maintained on solid medium (45) with either *Micrococcus luteus* (49) or *Klebsiella aerogenes* for growth with or without G418 selection, respectively.

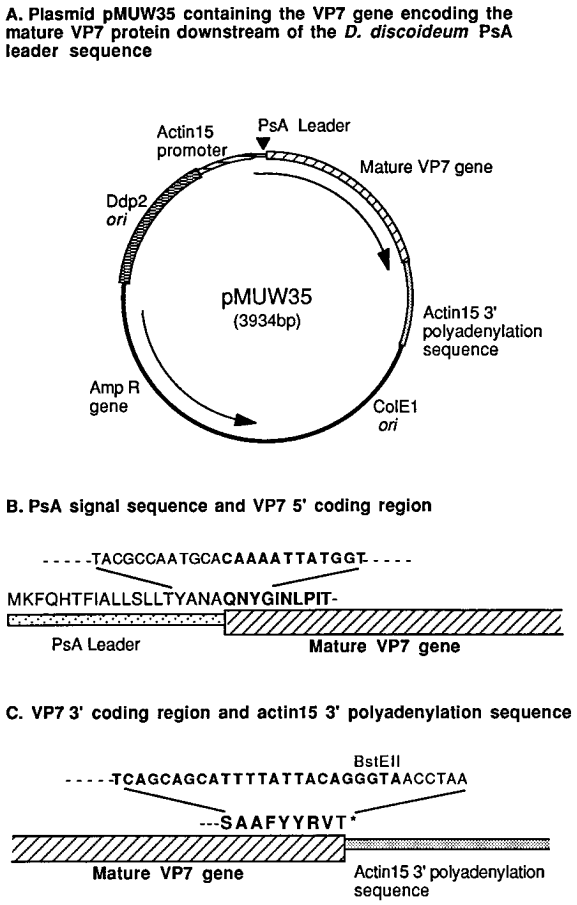

FIG. 1. Construction of a *D. discoideum* secretion vector containing the rotavirus SA11 mature VP7 gene. PCR techniques were used to modify the VP7 gene for insertion into the pMUW1630 secretion vector. (A) Plasmid pMUW35 contains the VP7 gene in frame with the *D. discoideum* PsA secretion signal. The expression of VP7 is under the control of the actin15 promoter. (B) The 5′ end of the gene (in boldface type) was constructed to ligate precisely to the 3′ end of the PsA secretion signal. (C) The 3′ end of the gene (in boldface type) was constructed to contain a *Bst*EII site upstream of the stop codon for subsequent cloning studies. As a result, a single threonine is added to the carboxy terminus of the recombinant VP7 protein. The native stop codon of rotavirus SA11 VP7, TAG, was also altered to TAA, which is a more favored *D. discoideum* stop codon.

Insertion of the SA11 VP7 gene into the secretion vector. The VP7 gene was inserted into the secretion vector, pMUW1630, as follows (Fig. 1). The portion of the rotavirus SA11 VP7 gene encoding the mature protein (amino acids 51 to 326) was amplified by PCR from the rotavirus SA11 VP7 gene (43). The two oligonucleotide primers, GGGGTGCACAAAATTATGGTATTAA and GGG GGTTAGGTTACCCTGTAATAAAATGCTGCT, incorporated an *Asp*HI site at the 5′ end of the mature VP7 gene and a *Bst*EII site 5′ to the stop codon for subsequent modification of the 3′ end of the gene and changed the VP7 gene stop codon from TAG to the more favored *D. discoideum* stop codon TAA. The *Bst*EII site added a single threonine residue to the C terminus (Fig. 1). The PCR product was blunt-ended with T4 DNA polymerase, purified from a 1.0% low-melting-point agarose gel (SeaPlaque) with a NACS Prepac column (Bethesda Research Laboratories), ligated into the *Sma*I site of pGEM7Z (Promega), and then sequenced with a T7 sequencing kit (Pharmacia). An *Asp*HI-*Kpn*I fragment containing the mature VP7 gene was then ligated into the secretion vector, pMUW1630, which had been digested with *Nsi*I and *Kpn*I. The presence of the VP7 gene in the expression plasmid was confirmed by restriction fragment mapping, and the junction of the *D. discoideum* prespore specific antigen (PsA) leader sequence and the 5′ end of the VP7 gene was sequenced. The secretion vector containing the VP7 gene was designated pMUW35.

Transformation of *D. discoideum*. Our *D. discoideum* expression system involves the cotransformation of two plasmids, an integrating plasmid (pMUW110)

and the secretion vector. Plasmid pMUW110 contains a G418 resistance marker (32) for the selection of transformants and the *rep* gene derived from the native *D. discoideum* plasmid Ddp2 (6). The product of the *rep* gene enables the secretion vector, pMUW1630, which contains the Ddp2 *ori* region, to replicate extrachromosomally in a high copy number (42).

NP2 cells (10^7) were cotransformed with the integrating plasmid, pMUW110 (17 μg), and the expression vector, pMUW35 (10 μg), by the standard calcium phosphate method (32), with the following modifications. The transformed cells were incubated overnight at 21°C without shaking in 1 ml of liquid medium (48). Following a second overnight incubation in 1 ml of fresh medium containing active G418 (10 μg/ml), the cells were gently resuspended in 1 ml of fresh medium with a Pasteur pipette, and 100-μl aliquots (approximately 10^6 cells) were spread over a thin lawn of *M. luteus* on SM plates (45) containing 10 μg of G418 per ml. The plates were incubated at 21°C. Transformants were detected after 6 to 9 days and were maintained under G418 selection (10 μg/ml) with *M. luteus* on SM/5 plates.

For the preparation of spores, *D. discoideum* was maintained under selection until fruiting bodies had developed. Spores were banged onto the petri dish lid, resuspended in 5% (vol/vol) dimethyl sulfoxide in liquid medium (48) at approximately 1.5×10^7 spores per ml, and stored at −80°C in 200-μl aliquots. Silica gel stocks were prepared by resuspending the spores in inactivated horse serum (CSL Diagnostics), pouring them into a tube containing silica gel, and storing the desiccated, sealed tubes at +4°C.

PCR screening of transformants. Transformants were screened by PCR for the presence of both the secretion vector and the integrating vector. A primer to either the PsA leader (primer set 1) or the actin15 promoter (primer set 2) together with a primer in the actin15 3′ polyadenylation region amplified the region of the secretion vector containing the recombinant gene, while primers which amplify a small region of the *rep* gene confirmed the presence of the integrating vector. Briefly, approximately 10^5 cells were dispensed into 1 ml of sterile distilled H_2O and heated at 95°C for 20 min. RNase (1 μl at 1 mg/ml) and proteinase K (1 μl at 4 mg/ml) were added, and the suspension was heated at 55°C for 15 min and then at 95°C for 10 min (31). Aliquots (15 μl) of this extract were used for PCR together with 2.5 μl of 10× PCR buffer, 2.5 μl of 8 mM deoxynucleoside triphosphates, 0.2 μl of *Taq* polymerase (2.5 U/μl), and 1 μl of primer solution (12.5 mM) in a total volume of 25 μl. The PCR was performed for 35 cycles of 94°C for 1 min, 44°C for 1 min, and 72°C for 2 min. PCR products were analyzed on either 1.0% agarose gels or prepoured gradient gels (Gradipore).

Growth of transformants and production of VP7. For large-scale production of VP7, the transformed *D. discoideum* cell line, HU2767, was grown as follows. Molten SM agar (1 liter) was dispensed into a sterile stainless steel tray (with a lid) (39 by 39 by 2 cm). A 5-ml suspension of 3×10^6 to 6×10^6 *D. discoideum* spores and approximately 10^{11} *K. aerogenes* cells was spread evenly over the cooled agar, and the tray was incubated for 3 to 4 days at 21 ± 1°C. At this stage, *D. discoideum* cells had multiplied by feeding on the *K. aerogenes* cells, and the majority of the *D. discoideum* cells were still single-celled amoebae. The cells and remaining bacteria were scraped from the tray, resuspended in 200 ml of starvation buffer (20 mM potassium phosphate buffer, pH 6.5) with 2.7 mM $CaCl_2$ at an initial cell concentration of 1×10^7 to 2×10^7 cells per ml, and shaken at 150 rpm at 21 ± 1°C for up to 24 h. Samples (1 ml) were taken regularly and centrifuged in an Eppendorf centrifuge at 1,500 rpm for 4 min to collect the cells. The medium was then centrifuged at 14,000 rpm for 4 min to remove any cellular debris. Samples were stored at −20°C. For control studies, NP2 cells were grown under similar conditions.

Detection and characterization of expressed protein. Cell lysates and culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 12% gels, and recombinant VP7 was detected by Western blot (immunoblot). Cells harvested from 1 ml of starvation buffer were lysed in 1 ml of 5× SDS-PAGE running buffer (1.5% [wt/vol] Tris–7.2% [wt/vol] glycine–0.5% [wt/vol] SDS adjusted to pH 8.6 with 1 N NaOH) containing the cysteine proteinase inhibitor E-64 (1 μM). E-64 (final concentration, 1 μM) was also added to medium samples. For the analysis of proteins under reducing and nonreducing conditions, samples (10 μl) were diluted with SDS-PAGE sample buffer (10 μl) with and without 5% (vol/vol) 2-mercaptoethanol, respectively. The samples were boiled for 3 min prior to loading on the gel. After electrophoresis, proteins were transferred by means of a wet transfer system (27) to a nitrocellulose membrane (Sartorius) in 10 mM CAPS buffer, pH 11.0, containing 10% (vol/vol) methanol. Standard Western blot procedures were used, and the VP7 protein was detected with a rabbit polyclonal anti-VP7 serum (1:1,000 dilution; kindly provided by A. R. Bellamy, University of Auckland, Auckland, New Zealand) as the first antibody and with peroxidase-conjugated sheep anti-rabbit immunoglobulin (1:1,000 dilution; Silenus Laboratories) as the second antibody.

PNGase F and endo H digestion of cell lysates and medium. PNGase F and endo H studies were done on samples collected after the cells were shaken in starvation buffer with 2.7 mM $CaCl_2$ for 6 h. For PNGase F digestion, cells were resuspended in 1 ml of starvation buffer, pH 6.5. Samples of resuspended cells and culture supernatant were adjusted to 10 mM EDTA, 0.2% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 1% (vol/vol) 2-mercaptoethanol, and 10 μM E-64, and 100-μl aliquots were incubated overnight at 37°C with and without 1 U of PNGase F. For endo H digestion, cells were lysed in 1 ml of 0.01 M potassium phosphate buffer, pH 5.8, containing 1% (vol/vol) Triton X-100, 0.2% (wt/vol) SDS, 100 mM mercaptoethanol, and 10 μM E-64. The supernatants were adjusted to 0.02% (wt/vol) SDS, 100 mM mercaptoethanol, and 10 μM E-64. The aliquots were boiled for 3 min and incubated overnight at 37°C with and without endo H at concentrations of 50 and 330 mU/ml for the supernatants and cell lysates, respectively. Reduced samples were then analyzed by SDS-PAGE on a 10% gel, and VP7 was detected by Western blot.

ELISA. A capture enzyme-linked immunosorbent assay (ELISA) established for the detection of epitopes on expressed VP7 was used with the following VP4- or VP7-specific MAbs (20, 41): 2G4, a VP4-specific neutralizing MAb; M60 and M129, nonneutralizing MAbs against VP7; and 159, 4C3, and 4F8, serotype 3-specific neutralizing MAbs recognizing VP7 (12, 14). The SA11 lysate was prepared as previously described (14). Briefly, MA104 cells were infected with trypsin-activated rotavirus SA11 in serum-free medium (M199) at a multiplicity of infection of 0.1. One hour after the infection, the medium was replaced with M199 plus 10% fetal calf serum. The cells were harvested from the cell culture medium 22 h after the infection, freeze-thawed three times, sonicated three times in 10-s pulses, and clarified by centrifugation at 12,000 × *g* for 15 min. The lysate of uninfected MA104 cells was prepared in a similar manner. *D. discoideum* HU2767 and NP2 cells were grown on trays and then shaken at 150 rpm for 3 h at 21 ± 1°C in 200 ml of starvation buffer with 2.7 mM $CaCl_2$. Samples (1 ml) were collected, and the cells and supernatant were separated as described above. The cells were resuspended in 100 μl of Tris buffer, pH 8.5 (50 mM Tris, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 0.01% NaN_3, 1 μM E-64), and subjected to three freeze-thaw cycles. Lysed cells were spun at 1,400 × *g* for 3 min, and the pellet was extracted with either 1 ml of 1% Triton X-100 in Tris buffer, pH 8.5 (Tris-TX-100), or 1 ml of 10 mM Tris-HCl–0.74 M betaine (Sigma)–0.3% octyl β-D-thioglucopyranoside (OTP; Sigma) (Tris-OTP-B), pH 8.0.

ELISAs were done in both the presence and absence of calcium in the wash solutions, which were based on either TNCa buffer (10 mM Tris-HCl, 100 mM NaCl, 1.5 mM $CaCl_2$ [pH 7.5]) or PBS buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgSO_4$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), respectively. The PBS-based ELISA was done as follows. MAbs (50 μl) diluted in PBS containing 0.05% sodium azide (PBS-az) were incubated in ELISA plate wells overnight at 4°C so that the plates would become coated. The plates were washed twice with PBS-az and incubated overnight at 4°C with PBS-az containing 10% fetal calf serum (PBS-az-FCS) (200 μl per well) so that nonspecific protein-binding sites would be blocked. The plates were washed with PBS-az and then with PBS-az-FCS. Antigen (cell lysates diluted 1:5 for SA11 and MA104 samples and cell lysates and medium diluted 1:20 for *D. discoideum* samples) diluted in PBS-az-FCS was added to a final volume of 50 μl per well, and the plates were incubated overnight at 4°C. The plates were washed twice with both PBS and 0.2% Tween 20 in PBS (PBS-Tw) and once with PBS-Tw-FCS. Captured antigen was detected by incubating it with the polyclonal serum GP962 (50 μl per well at a dilution of 1/4,000 in PBS-Tw-FCS) (12) for 2 to 3 h at 37°C. The plates were washed as described before and then incubated with peroxidase-labelled goat anti-guinea pig immunoglobulin G (50 μl per well of 0.5 μg of immunoglobulin G per ml diluted in PBS-Tw-FCS) for 1 h at 37°C. The plates were washed three times with PBS-Tw and PBS. To develop the plates, 100 μl of 0.4 mg of *o*-phenylenediamine dihydrochloride per ml–0.012%–H_2O_2–24 mM citric acid–50 mM $Na_2HPO_4 \cdot 12H_2O$ (pH 5.0) was added to each well. When a visible yellow color developed, the reaction was stopped by adding 50 μl of 1 M H_2SO_4 and the A_{490} was measured. The protocol for the TNCa-based ELISA was identical to that of the PBS-based ELISA, except that the diluent for the antigen, subsequent antibodies, and washing buffers was based on the TNCa buffer. The results are reported as A_{490} values above the mean of values obtained with the five negative control antigen preparations consisting of ELISA buffer alone, the two NP2 cell lysates prepared with Tris-TX-100 and Tris-OTP-B, the NP2 medium sample, and uninfected MA104 cells. The standard deviations determined are those of the five negative control samples. The SA11 antigen contained 4.4 ng of VP7 per well, estimated by methods previously described (12), while the *D. discoideum* cell lysates and culture supernatant contained 3 ng of VP7, estimated as described below.

Quantitation of expressed VP7. The levels of intracellular and secreted VP7 were quantified by estimating the amount of VP7 protein in a silver-stained gel following SDS-PAGE of VP7 immunoprecipitated from cell lysates and the culture supernatant. Cells collected from the culture medium were sonicated in lysing buffer A (100 μl of 1% [wt/vol] deoxycholate–1% [vol/vol] Triton X-100–0.15 M NaCl–20 mM KH_2PO_4–0.5% [wt/vol] SDS–10 μM E-64) for 7 min. Lysing buffer B (900 μl of 0.5% [vol/vol] Nonidet P-40–0.5% [wt/vol] deoxycholate–6 mM EDTA–10 mM KH_2PO_4–10 μM E-64) was added, and the lysate was vortexed and then centrifuged at 14,000 × *g* for 5 min. The culture supernatant was prepared similarly. Rabbit polyclonal anti-SA11 serum (kindly provided by A. R. Bellamy) was added to aliquots of cell lysate and culture supernatant, and the samples were rotated for 3 h. Preswollen protein A–Sepharose CL-4B beads were added, and the samples were mixed for a further 2 h. The pelleted beads were washed extensively with 1% (wt/vol) deoxycholate–1% (vol/vol) Triton X-100–0.15 M NaCl–20 mM KH_2PO_4–0.1% (wt/vol) SDS–5 mM EDTA and then with 0.15 M NaCl–5 mM EDTA–20 mM KH_2PO_4. The antibody-VP7 complex was released from the beads by boiling the beads in 5× SDS-PAGE running buffer with E-64 (10 μM), and the amount of VP7 was estimated by analysis on an SDS–10% PAGE gel. The total cell protein of the lysate was estimated with a Bio-Rad protein assay kit.

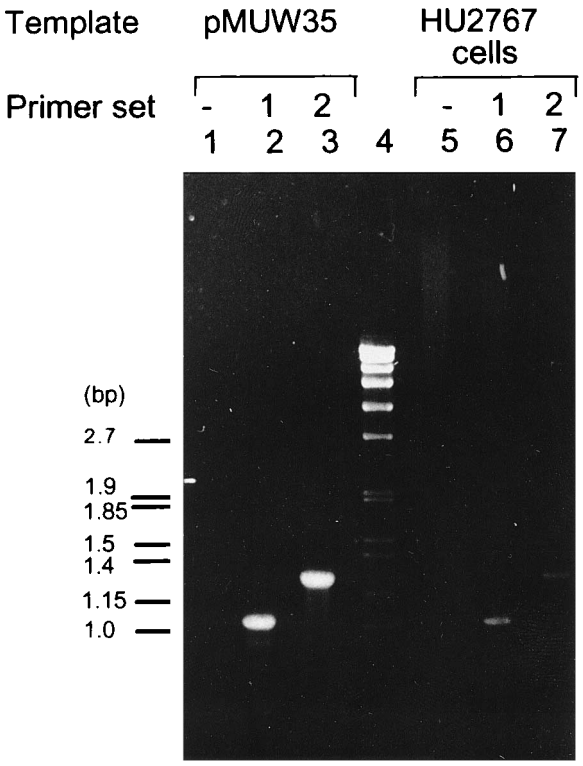

FIG. 2. PCR screening of *D. discoideum* transformants. Transformants were screened for the presence of the VP7 gene with two sets of primers which amplified either the region from the PsA signal sequence into the actin15 3′ polyadenylation sequence (primer set 1) or the region from the start of the actin15 promoter into the actin15 3′ polyadenylation sequence (primer set 2). Lanes: 1 to 3, control PCR with 0.1 ng of pMUW35: 4, molecular weight markers (SPP-1 phage DNA digested with *Eco*RI); 5 to 7, PCR with HU2767 cells. Lanes 1 and 5, no primers; lanes 2 and 6, primer set 1; lanes 3 and 7, primer set 2.

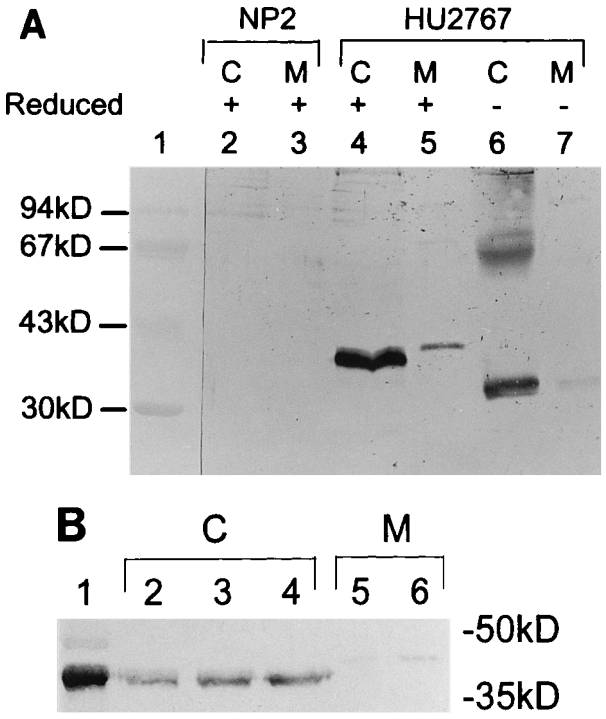

FIG. 3. Western blot analysis of recombinant VP7 expression. *D. discoideum* cells were shaken in starvation buffer for 6 h (A) or 3 h (B). Samples (10 μl) of medium (M) and cell lysate (C) were prepared for SDS-PAGE and run under reducing (lanes 1 to 5 in panel A and all lanes in panel B) and nonreducing (lanes 6 and 7 in panel A) conditions on a 12% gel. (A) Lanes: 1, molecular weight markers stained with Ponceau Red; 2 and 3, control NP2 (cell lysate and medium, respectively); 4 and 6, transformed HU2767 (cell lysate); 5 and 7, transformed HU2767 (medium). (B) Lanes: 1, rotavirus SA11; 2 to 4, cell lysates from three independent experiments; 5 and 6, medium from two independent experiments.

RESULTS

Detection of secretion vector in transformed cells by PCR screening. Since the G418 selective marker for cotransformation of pMUW110 and pMUW35 is carried on the integrating vector, pMUW110, it was necessary to confirm that transformed cells resistant to G418 were also carrying the extrachromosomal expression vector since this plasmid carries no selective marker. NP2 cells and the transformed cells were screened by PCR with primer sets (1 and 2) that recognize the secretion vector (Fig. 2). A third set of primers was used to confirm the presence of the integrating vector (data not shown). PCR products of the expected size were detected following amplification of the transformed cells, and these correlated in terms of molecular mass to the PCR product obtained by amplification of the relevant plasmid controls (Fig. 2). While it is possible to get G418-resistant transformants which only carry the integrating vector, the rate of cotransformation is very high, as all nine separate transformants screened by PCR carried both the integrating vector and the secretion vector (data not shown). One of these transformants, named HU2767, was chosen for further study.

Detection of recombinant VP7. Expression of VP7 is under the control of the actin15 promoter, which is induced at the onset of starvation. To detect recombinant VP7, cells were grown on a bacterial lawn and then shaken in starvation buffer for 6 h. Up to three forms of VP7 were expressed by HU2767 cells (Fig. 3A, lanes 4 and 5). Under reducing conditions, a strong broad band (35.5 to 36.5 kDa) which was frequently resolved into two distinct bands was detected in HU2767 cells. A third very faint band which was occasionally seen in the cell lysate at around 37.5 kDa was clearly detected in the culture medium from these cells (Fig. 3A, lane 5). Since the lower-molecular-mass forms of VP7 observed in the cell lysates were not detected in unconcentrated medium, we concluded that the presence of extracellular VP7 was due to genuine secretion of a portion of the VP7 and not to cell lysis. These bands were not detected in control NP2 cells or culture medium (Fig. 3A, lanes 2 and 3).

Under nonreducing conditions, the secreted VP7 and more than half of the cell-associated VP7 migrated at a faster rate, with an apparent molecular mass of 30 to 32 kDa (Fig. 3A, lanes 6 and 7), indicating that these VP7 molecules were folded in a monomeric form. The remainder of the unreduced VP7 protein migrated at a much slower rate, with an apparent molecular mass of 58.5 to 61 kDa. The migration of this form suggests that it is a dimer.

Recombinant VP7 expressed in three independent experiments was compared with SA11 VP7 from purified rotavirus (kindly provided by G. Both) (Fig. 3B). The major intracellular recombinant product of VP7 comigrated in SDS-PAGE with the major VP7 band in purified rotavirus, while the secreted form of VP7 migrated at a slower rate. Interestingly, two minor forms of viral VP7 with apparent molecular masses slightly higher than that of the recombinant secreted form were visible.

N glycosylation of recombinant VP7. To establish the differ-

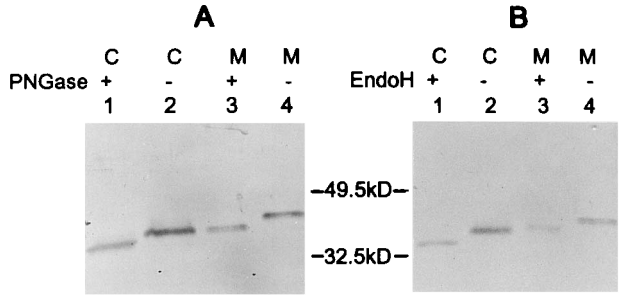

FIG. 4. Analysis of N glycosylation of recombinant VP7. *D. discoideum* HU2767 cells were shaken in starvation buffer for 6 h. Samples of cell lysate (C) and medium (M) were digested with either PNGase F or endo H as described in Materials and Methods. Aliquots (10 μl) were prepared for SDS-PAGE and run under reducing conditions on a 10% gel. VP7 was detected by Western blot. (A) PNGase F digestion. Lanes: 1 and 2, cell lysate incubated with and without PNGase F, respectively; 3 and 4, medium incubated with and without PNGase F, respectively. (B) Endo H digestion. Lanes: 1 and 2, cell lysate incubated with and without endo H, respectively; 3 and 4, medium incubated with and without endo H, respectively.

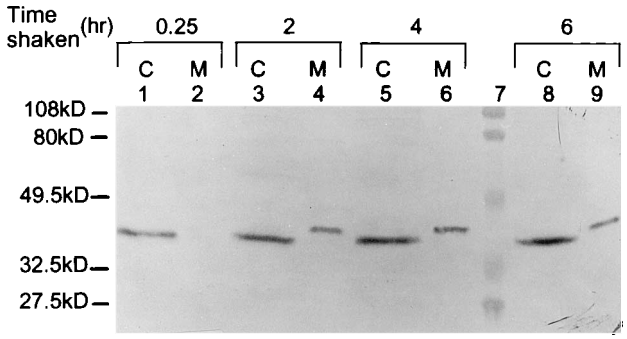

FIG. 5. Time course of detection of recombinant VP7. *D. discoideum* HU2767 cells were shaken in starvation buffer for 6 h, and samples (1 ml) were collected at 0.25, 2, 4, and 6 h. Cells and medium were separated by centrifugation. Aliquots (10 μl) of cell lysate (C) and medium (M) were prepared for SDS-PAGE and run under reducing conditions on a 10% gel. VP7 was detected by Western blot. Lanes: 1, 3, 5, and 8, cell lysate (0.25, 2, 4, and 6 h, respectively); 2, 4, 6, and 9, medium (0.25, 2, 4, and 6 h, respectively); 7, molecular mass markers.

ence between the secreted and intracellular VP7 and to help determine the location of the cell-associated VP7, we examined the N-glycosylation profile of the two forms of VP7.

The VP7 molecule has a single potential N-glycosylation site, NST (Asn-69), which was used to indicate whether the protein had been translocated into the ER. Cell lysates and medium were digested with PNGase F, which cleaves N-glycosylated sugars at the asparagine residue. Both the cellular and secreted forms of VP7 showed an increase in mobility on SDS-PAGE gels after PNGase F digestion, indicating that both forms were originally N glycosylated (Fig. 4A). This confirmed that the *D. discoideum* signal peptide was functioning correctly and had directed the recombinant VP7 across the ER membrane. It was also clear that the difference in mobility between the cellular and secreted forms was not due to a difference in modification of the N-linked sugar since the secreted VP7 still migrated slower than the cellular form after PNGase F digestion. We concluded that the secreted VP7 had undergone additional modifications further along the secretory pathway.

The enzyme endo H was then used to compare the processing of the N-linked carbohydrate in the cellular and secreted forms of VP7. Endo H cleaves the majority of high-mannose sugars but does not cleave complex N-linked sugars. The endo H digestion profile of cellular and secreted VP7 was very similar to that of PNGase F (Fig. 4B), indicating that the N sugars on both recombinant forms of VP7 were of the high-mannose type (Fig. 4B). Hence, passage of the secreted recombinant VP7 through the Golgi bodies had not led to major modifications of the N-linked sugar structure.

Time course of VP7 production. HU2767 cells were grown on large trays with *K. aerogenes* and then shaken in starvation buffer with 2.7 mM $CaCl_2$ at 21 ± 1°C for 6 h. Aliquots of cells and medium were collected regularly. Intracellular VP7 was detected in the cells at all time periods examined, while secreted VP7 was detected in 10 μl of medium after 2 h of shaking. Both forms of VP7 increased in concentration throughout the shaking period (Fig. 5).

Since the actin15 promoter controlling VP7 expression is induced during starvation, some patches of cells grown on a large tray are already producing VP7 before being harvested into buffer. Hence, while there is much less VP7 secreted than retained in the cell, the level of intracellular VP7 reflects the total amount accumulated during the period that the cells were on the tray together with the time they were shaken in the buffer. The level of intracellular VP7 following the shaking of the cells for 3 h in starvation buffer with 2.7 mM $CaCl_2$ was estimated at 0.05% total cell protein, which corresponds to a yield of 0.5 mg/liter of SM agar, while secreted VP7 was estimated at 1 mg/liter of phosphate buffer, which corresponds to a yield of 0.2 mg/liter of SM agar.

Reactivity of *D. discoideum*-expressed VP7 against a panel of MAbs to viral VP7 in an ELISA. Since recognition of VP7 by neutralizing antibodies is conformation dependent, we examined the reactivity of *D. discoideum*-expressed VP7 against a panel of nonneutralizing and neutralizing MAbs by means of an ELISA. The standard PBS ELISA and a modified TNCa ELISA performed in the presence of calcium have been used previously to analyze neutralizing epitopes on recombinant vaccinia virus-expressed VP7 (12). *D. discoideum*-expressed intracellular VP7 extracted with either Tris-TX-100 or Tris-OTP-B was recognized by the nonneutralizing MAbs M60 and M129 in both PBS- and TNCa-based ELISAs (Table 1). *D. discoideum*-expressed VP7 was also recognized by the neutralizing MAb 159 in the PBS- and TNCa-based ELISAs after it had been extracted with Tris-TX-100, but this reactivity was eliminated for VP7 extracted with Tris-OTP-B. Recognition of Tris-TX-100-extracted intracellular VP7 by the neutralizing MAbs 4C3 and 4F8 was dependent on calcium in the washing solutions since intracellular VP7 was recognized by a TNCa-based but not a PBS-based ELISA. This calcium dependence of the reactivity of Tris-TX-100-extracted VP7 with 4C3 was also observed for VP7 expressed in vaccinia virus either in a wild-type form or modified for expression on the cell surface (12). Detergent extraction of VP7 with OTP and betaine completely eliminated reactivity with 4C3 and 4F8 by either a PBS- or a TNCa-based ELISA. The *D. discoideum* cell lysate showed no significant reactivity with 2G4, a MAb directed against VP4. Secreted VP7 was not recognized significantly by any of the MAbs studied (data not shown), despite the fact that it was detected by a polyclonal antibody in a Western blot.

DISCUSSION

Recombinant proteins that have been expressed in *D. discoideum* include the *Plasmodium falciparum* circumsporozoite antigen (16), human antithrombin III (10), *Schistosoma japonicum* glutathione *S*-transferase, *E. coli* β-glucuronidase, and a soluble form of the *D. discoideum* surface membrane protein

TABLE 1. Capture ELISA of SA11 virus VP7 and intracellular *D. discoideum*-expressed VP7 with PBS and TNCa buffers

MAb	A_{490}[a]						SD[b]	
	SA11 VP7		*D. discoideum*-expressed VP7 (Tris-TX-100)[c]		*D. discoideum*-expressed VP7 (Tris-OTP-B)[d]			
	PBS	TNCa	PBS	TNCa	PBS	TNCa	PBS	TNCa
M60	**1.35**	**1.93**	**1.09**	**1.58**	**1.21**	**1.40**	0.01	0.01
M129	**1.08**	**2.12**	**1.02**	**1.96**	**0.95**	**1.06**	0.00	0.02
159	**1.71**	**1.62**	**1.23**	**1.23**	0.02	0.05	0.01	0.01
4F8	**1.45**	**1.57**	0.02	**0.36**	0.02	0.00	0.01	0.02
4C3	**1.44**	**1.40**	0.00	**0.38**	0.02	0.01	0.01	0.01
2G4	**0.46**	**0.44**	−0.01	−0.01	0.00	0.00	0.01	0.02

[a] Values are A_{490} readings above mean negative control values. Boldface type indicates significant ELISA reactivity (an A_{490} greater than 0.1 and greater than 3 standard deviations above the mean of negative control A_{490} readings).
[b] Standard deviation of negative controls (ELISA buffer alone, uninfected MA104 cell lysate, two NP2 cell lysates prepared with the two detergent systems, and the extracellular medium from NP2 cells).
[c] The cell lysate was extracted with Tris-TX-100.
[d] The cell lysate was extracted with Tris-OTP-B.

PsA (11). This study on the rotavirus SA11 glycoprotein VP7 demonstrates, for the first time, the expression of a viral protein in *D. discoideum.*

D. discoideum-expressed VP7 is very similar to viral VP7, specifically in its apparent molecular mass, N glycosylation, endo H sensitivity, predominant intracellular location, and reactivity with a number of MAbs to viral VP7. Up to three forms of VP7 ranging in apparent size from 35.5 to 37.5 kDa were detected. The lowest-molecular-mass intracellular form, the major form observed, was comparable in molecular mass to the predominant form of viral SA11 VP7. It has been reported previously that SA11 VP7 migrates on SDS-PAGE gels in up to three distinct bands (5, 39, 44) ranging from 35.3 to 38 kDa (5, 22, 43). Triton X-100-extracted intracellular VP7 was recognized by a panel of MAbs to viral VP7. Recognition by the nonneutralizing antibodies M60 and M129 and the single neutralizing antibody 159 was independent of calcium in washing solutions, while recognition by the neutralizing antibodies 4C3 and 4F8 was calcium dependent. This pattern of recognition paralleled that observed for herpes simplex virus type 1-expressed VP7 (unpublished data) and vaccinia virus-expressed VP7 (12) and clearly indicates that *D. discoideum*-expressed VP7 can fold to create the neutralizing epitopes present on the mature virus. A combination of the nonionic detergent OTP and the zwitterionic detergent betaine has been used previously in our laboratory to solubilize a native *D. discoideum* membrane protein, PsA (50). Recombinant VP7 extracted with these detergents retained reactivity with the nonneutralizing MAbs M60 and M129 but did not react with any of the neutralizing MAbs tested, suggesting that the correct folding of VP7 was not attained under these conditions of extraction.

While VP7 does not contain any established hydrophobic domains which would account for its association with the ER membrane and for intracellular retention prior to viral assembly, the native H2 signal peptide of VP7 and specific amino acids within the first 31 residues of the mature protein are important since VP7 is secreted in COS cells when either the H2 signal is replaced with the influenza virus hemagglutinin signal peptide (35, 43) or any of three adjacent residues at positions 9 to 11 in the mature N terminus is substituted (26).

In the *D. discoideum* expression system, the H2 signal peptide was replaced with the signal peptide from the native *D. discoideum* membrane protein, PsA. However, in contrast to the findings of studies with a hemagglutinin signal peptide, our findings were that the majority of *D. discoideum*-expressed VP7 was retained in the cell. Several lines of evidence suggest that the intracellular VP7 is folded correctly. Firstly, it is recognized by MAbs directed towards the major conformation-dependent neutralizing domains on VP7. Secondly, when electrophoresed under nonreducing conditions, the cellular VP7 migrates predominantly as a monomer, with an apparent molecular mass of 30 to 31 kDa; large aggregated forms indicative of misfolded proteins are not observed. Thirdly, the *D. discoideum*-expressed intracellular VP7 is immunoprecipitated by a polyclonal antibody raised against purified virus. Since the majority of the intracellular VP7 is of the lowest-molecular-mass form, we concluded that, in the absence of the H2 signal peptide, the retention signal within the mature N terminus of VP7 is sufficient to retain the majority of *D. discoideum*-expressed VP7 in association with the ER.

The highest-molecular-mass form of *D. discoideum*-expressed VP7 (37.5 kDa) was predominantly detected in the medium, and since the lower-molecular-mass forms were not present, we concluded this was a result of genuine secretion rather than cell lysis. Despite secretion, this form of VP7 was sensitive to endo H, indicating that the N-glycan was of the high-mannose type. Unlike glycoproteins secreted from animal cells (25, 36), *D. discoideum* glycoproteins do not possess classical complex carbohydrates, although other modifications, such as fucosylation (21), sulfation (17), phosphomethylation (18), and the presence of an unusual bisecting *N*-acetylglucosamine residue (8), have been described. Since both fucosylation and extensive sulfation of mannose residues can render a protein resistant to endo H (17, 21), this indicates that these modifications did not occur in the secreted VP7 glycoprotein during passage through the Golgi bodies.

While both intracellular and secreted VP7 were sensitive to endo H, they still differed in molecular mass after enzyme digestion. Similar results have been observed with the three distinct bands of viral VP7 (44). This confirms that the heterogeneity in apparent molecular mass observed with both viral VP7 and VP7 expressed in *D. discoideum* is not due to incomplete N glycosylation or to differences in the trimming of the N-linked sugar. Variability in the signal sequence cleavage site may account for the multiple forms of viral VP7; however, in the case of *Dictyostelium*-expressed VP7, it is more likely that additional posttranslational modifications not involving the N-glycan occurred during transport through the secretory pathway, thus giving rise to the highest-molecular-mass form. SA11 VP7 has several potential O-glycosylation sites based on motifs we have identified in our laboratory (19, 33, 34), and this could account for the increase in molecular mass observed following secretion of *D. discoideum*-expressed VP7. Of particular interest are the motifs X-Pro-X-X (where any X is a threonine), which is utilized in the *D. discoideum* cell surface protein PsA (19), and Thr-X-X-X (where any X is a threonine). Specifically, these motifs are found in SA11 VP7 at amino acids Thr-87 and Thr-210, which are situated at the edges of the A and C regions, respectively. These two regions interact to form the major conformation-dependent neutralization domain on VP7, and the neutralizing MAbs 159 and 4C3 and MAb 4F8 are directed against amino acids 94 and 96, respectively, in the A region (28). If either Thr-87 or Thr-210 is O glycosylated during transport through the Golgi apparatus in *D. discoideum*, the neutralization domain may be unable to form, and this would explain the loss of reactivity of secreted *D. discoideum*-expressed VP7 with these MAbs. A similar reduction in the ability of antibody to bind VP7 was found in a mutant contain-

ing an N-linked carbohydrate at Asn-211 within the antigenic C region (40), which presumably altered the interaction between the A and C regions. The nonneutralizing antibodies M129 and M60 bind to epitopes distinct from the neutralization domains (41) and may be affected by similar modifications occurring close to their binding domain.

This study has validated the *D. discoideum* expression system as a cheap alternative for the production of recombinant rotavirus VP7. A method has been developed for expressing milligram quantities of VP7 by growing the *D. discoideum* cells with *K. aerogenes* cells on large trays with an agar-based medium costing approximately US$3 per liter. Using these conditions, we can easily prepare kilogram quantities of cells (50). Effectively, cells expressing recombinant protein can be harvested in preparation for protein purification within 4 days of the inoculation of *D. discoideum* spores onto the tray. Since *D. discoideum* spores can be prepared in bulk and stored for prolonged periods at −80°C, the labor requirements for producing large quantities of proteins with this expression system are low. Preliminary results have shown that intracellular VP7 is targeted to a subcellular compartment, and methods are currently being developed to separate these subcellular organelles. Purification of *D. discoideum*-expressed VP7 in a form which is recognized by neutralizing MAbs will allow us to study the folding of the recombinant protein, and this, together with data available on the peptide sequences involved in the neutralizing domains, will lead to further characterization of the structure giving rise to the complex, conformationally dependent neutralization domains of rotavirus VP7.

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