The Nonstructural Glycoprotein of Rotavirus Affects Intracellular Calcium Levels

PENG TIAN,¹ YANFANG HU,² WILLIAM P. SCHILLING,² DAVID A. LINDSAY,³ JOSEPH EIDEN,³
AND MARY K. ESTES^{1*}

Division of Molecular Virology¹ and Department of Molecular Physiology and Biophysics,² Baylor College of Medicine, Houston, Texas 77030, and Department of Pediatrics, The Johns Hopkins University, Baltimore, Maryland 21205³

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Rotavirus infection of monkey kidney cells has been reported to result in a significant increase in the concentration of intracellular calcium. This increase in intracellular calcium was associated with viral protein synthesis and cytopathic effects in infected cells. We tested the effect of individual rotavirus proteins on intracellular calcium concentrations in insect *Spodoptera frugiperda* (Sf9) cells. Insect cells were infected with wild-type baculovirus or baculovirus recombinants that contained an individual rotavirus gene. The cells were harvested at different times postinfection, and the intracellular calcium concentration was measured by using fura-2 as a fluorescent calcium indicator. We found that the concentration of intracellular calcium was increased nearly fivefold in infected Sf9 cells that expressed the nonstructural glycoprotein (NSP4) of group A rotavirus, and this increase in intracellular calcium concentration coincided with NSP4 expression. A similar result was observed in insect cells expressing NSP4 from a group B rotavirus, suggesting the conservation of this function among rotavirus groups. Expression of the other 10 rotavirus proteins or of wild-type baculovirus proteins in Sf9 cells did not significantly increase intracellular calcium levels. These results suggest that the nonstructural glycoprotein NSP4 is responsible for the increase in cytosolic calcium observed in rotavirus-infected cells.

Rotaviruses are recognized as the most important cause of severe viral gastroenteritis in humans and animals. Rotaviruses are nonenveloped, triple-layered viruses with a genome of 11 segments of double-stranded RNA. Rotaviruses have a unique morphogenesis in which particles obtain a transient membrane envelope that is formed by the budding of newly made subviral particles into the endoplasmic reticulum (ER) (14, 34). This process is mediated by a viral nonstructural glycoprotein called NSP4 (formerly called NS28 [27]). NSP4, a transmembrane protein, is localized to the ER of infected monkey kidney (MA104) cells and insect Sf9 cells. NSP4 functions as a receptor in the ER membrane and binds both newly made subviral particles and the spike protein VP4 (2, 3, 29). As particles mature, they lose the transient membrane and a thin layer of the glycoprotein VP7 forms the virion outer capsid. The nonenveloped particles are liberated from infected cells by cell lysis.

Calcium is very important for rotavirus replication. The physical integrity of rotavirus particles requires calcium. Treatment of infectious triple-layered particles with chelating agents results in the loss of infectivity and the removal of the two outer capsid proteins, VP4 and VP7 (9, 17, 38). Use of the calcium ionophore A23187 to increase the intracellular calcium concentration during the early stages of replication blocks virus uncoating (23). Calcium also plays a major role in the acquisition of specific conformations necessary for correct association of proteins during virus maturation in the ER. For example, calcium is required for the oligomerization of the virus-encoded proteins VP4, VP7, and NSP4; the stability of

VP7 itself; and the conformation of some neutralization epitopes on VP7 (12, 35, 37).

A role for calcium in cytopathic effect (CPE) and cell death has been proposed for many pathological processes induced by viruses (32). Increased intracellular calcium levels have been reported in MA104 cells infected with group A rotavirus (30). This increased intracellular calcium was blocked by cycloheximide but not by actinomycin D, indicating that this effect is dependent on rotaviral protein synthesis (30).

This paper describes work that was the outcome of several attempts to establish stable cell lines that expressed NSP4. Specifically, plasmids pSV2-neo and pSVT7-G10, which contained rotavirus gene 10, were cotransfected into MA104 cells by using Lipofectin, and cells were selected in medium containing G418. The transfected cells underwent a crisis during subcloning; they became extremely large and filled with cytoplasmic vacuoles and finally died. Therefore, these experiments were uniformly unsuccessful, suggesting that NSP4 was cytotoxic to the transfected cells (unpublished data). We hypothesized that NSP4 might kill infected cells by increasing intracellular calcium levels. This paper reports studies that tested this hypothesis by investigating the effect of NSP4 and other rotaviral proteins on intracellular calcium levels in the recombinant baculovirus insect cell system.

MATERIALS AND METHODS

Cells and viruses. The group A rotavirus genes were inserted into either transfer vector pVL941 (G1, G2, G3, G4, G5, G6, G7, G8, G9, and G11) or pAC461 (G6 and G10). Each of these transfer vectors use the baculovirus polyhedrin promoter to express the gene, but protein expression levels are higher from genes inserted into the pVL941 vector (10, 22, 43). The IDIR strain of group B rotavirus gene 10 was inserted into the pVL941-based pBlueBac transfer vector (Invitrogen Corp., San Diego, Calif.). Baculovirus recombinants containing rota-

* Corresponding author. Mailing address: Division of Molecular Virology, Rm. 923E, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3585. Fax: (713) 798-3586. Electronic mail address: mestes@bcm.tmc.edu.

virus genes were obtained by homologous recombination between the transfer vector and wild-type (wt) baculovirus DNA as previously described (1, 2, 4, 10, 11, 16, 20, 21, 26, 28, 42). wt *Autographa californica* nuclear polyhedrosis virus and recombinant baculoviruses expressing group A simian rotavirus SA11 gene 3 (G3 [21]), gene 4 (G4 [11]), gene 6 (G6 [16]), gene 9 (G9 [11]), gene 7 (G7 [26]), gene 10 (G10 [2]), gene 11 (G11 [28, 42]) and bovine rotavirus Rf gene 1 (G1 [10]), gene 2 (G2 [20]), gene 5 (G5 [4]), and gene 8 (G8 [1]) were used to infect Sf9 insect cells at a multiplicity of infection (MOI) of 10 PFU per cell. Recombinant baculovirus B10 expressing NSP4 from the IDIR strain of group B rotavirus (15) and a control recombinant BK (recombinant between wt baculovirus and transfer vector without insert) were used to infect Sf9 cells at the same MOI. Sf9 cells were grown and maintained in Hink's medium containing 10% fetal bovine serum as previously described (16).

Measurement of intracellular free Ca^{2+} concentration (cytosolic free calcium). Intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, was measured by using the fluorescent indicator fura-2. The $[\text{Ca}^{2+}]_i$ was measured as described previously (36). Briefly, 0.5×10^8 cells were removed from a suspension spinner culture, washed, pelleted, and suspended in 10 ml of extracellular buffer containing 10 mM CaCl_2 , 60 mM KCl, 17 mM MgCl_2 , 10 mM NaCl, 10 mM morpholineethanesulfonic acid (MES), 4 mM glucose, 110 mM sucrose, and 0.1% bovine serum albumin (pH 6.2). One-tenth of the cell suspension was saved as a non-fura-2-loaded control. To load cells with the membrane-permeant acetoxymethyl ester of fura-2 (fura-2/AM; Molecular Probe, Inc., Eugene, Oreg.), the suspended cells (5×10^6 cells per ml) were incubated for 30 min at room temperature with fura-2/AM at a final concentration of 1.25 $\mu\text{g}/\text{ml}$. The cells were pelleted, suspended at $2.5 \times 10^6/\text{ml}$ in extracellular buffer without fura-2/AM, and incubated another 30 min. The cells then were pelleted again and suspended at a concentration of 2.5×10^6 cells per ml in extracellular buffer. Aliquots (2 ml) were subsequently removed, centrifuged, pelleted, and resuspended twice immediately prior to measurement of fluorescence. The aliquots were placed in a quartz cuvette with a magnetic stirrer, and fluorescence was measured in a SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, Ill.), with the excitation wavelength being altered between 340 and 380 nm every 500 ms and emission fluorescence being recorded at 510 nm. An IBM XT computer with SLM software calculated the fluorescence ratio, R , where R equals F_{340}/F_{380} , and F_{340} and F_{380} are the emission intensities at 340 and 380 nm excitation, respectively, corrected for autofluorescence. Autofluorescence also was determined on identically processed cells that were incubated without the fluorescent probe. Fluorescence measured after the sequential addition of 0.1% Triton X-100 and then 50 mM ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) to the cell suspension provided the respective maximum fluorescence ratio (R_{max}) and minimum fluorescence ratio (R_{min}). $[\text{Ca}^{2+}]_i$ was calculated by the following equations:

$$[\text{Ca}^{2+}]_i = K_d (R - R_{\text{min}}) / (R_{\text{max}} - R) \times b$$

$$b = (F_{380\text{E}}/F_{380\text{T}})$$

where $F_{380\text{T}}$ and $F_{380\text{E}}$ are the emission fluorescence values at 380 nm excitation in the presence of Triton X-100 and EGTA, respectively. All measurements were performed at room temperature, and the equilibrium dissociation constant (K_d) for the Ca^{2+} -fura-2 complex was 278 nM (40). Increases in fura-2 fluorescence indicate increased intracellular free calcium lev-

els, because cellular cytosolic esterases cleave the membrane-permeant ester group off the fura-2/AM derivative and leave the membrane-impermeant fura-2 trapped in the cytosol. The binding of Ca^{2+} shifts the absorbance spectra to shorter wavelengths (from 380 to 340 nm).

Radiolabeling and analysis of proteins synthesized in infected Sf9 cells. Sf9 cells infected with recombinant baculoviruses or wt *Autographa californica* nuclear polyhedrosis virus were starved in methionine-free Hink's medium for 30 min between 32 and 36 h postinfection and then radiolabeled for 1 h with 20 μCi of $[\text{S}^{35}]\text{Trans}$ (Amersham Corp., Arlington Heights, Ill.) per ml in methionine-free Grace's medium. The cells were pelleted for 5 min at $1,400 \times g$ at 4°C and suspended in radioimmunoprecipitation assay buffer containing 0.15 M NaCl, 0.01 M Tris hydrochloride (pH 7.2), 1% aprotinin, 1% sodium deoxycholate, 1% Triton X-100, and 0.5% sodium dodecyl sulfate (SDS) (16). Protein products were analyzed by polyacrylamide slab gel electrophoresis, by a modification of the method of Laemmli with 12 to 15% separating and 4% stacking gels as previously described (8). Before electrophoresis, samples were boiled for 3 min in sample buffer containing 1% SDS, 10% 2-mercaptoethanol, 0.5 M urea, 0.05 M Tris hydrochloride (pH 6.8), 10% glycerol, and 0.0025% phenol red. Gels were fixed and then impregnated with Autofluor (National Diagnostics, Atlanta, Ga.) and exposed to X-ray film at -70°C . Radiolabeled proteins were monitored on gels following fluorography as previously described (25).

Western blot analysis of NSP4. A polyclonal antibody against NSP4 was produced by immunizing guinea pigs with a 26K protein isolated from SA11-infected MA104 cell lysates by preparative SDS-polyacrylamide gel electrophoresis (PAGE). The specificity of the polyclonal antibody was confirmed by Western blot (immunoblot) analysis with NSP4 from baculovirus recombinant-infected Sf9 cells as an antigen and monoclonal antibody BA/55 to NSP4 (kindly provided by H. Greenberg) as a standard. Equal aliquots of a sample from baculovirus recombinant-infected Sf9 cells harvested between 16 and 48 h postinfection were analyzed by SDS-PAGE. Proteins were electroblotted onto a nitrocellulose membrane in a transfer buffer containing methanol (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with phosphate-buffered saline (PBS) containing 5% BLOTTO for 30 min at room temperature and incubated overnight at room temperature with the guinea pig polyclonal antiserum against NSP4 (26K). After five washes in PBS, horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin was added and incubated for 2 h at room temperature. Blots were washed five times and developed with 4-chlorol-naphthol (5).

Statistical analysis. Dunnett's multiple comparison test was used to analyze the data. Computational assistance was provided by the CLINFO project at Baylor College of Medicine. Results from at least three independent experiments for each type of baculovirus-infected cell were used to determine the average intracellular calcium concentration. Where indicated, n equals the number of independent infections, with $[\text{Ca}^{2+}]_i$ measurements performed in triplicate for each experiment.

RESULTS

Effect of individual rotavirus proteins on intracellular calcium concentrations in infected Sf9 cells. The intracellular calcium concentration was determined between 32 and 36 h postinfection in insect cells infected with wt baculovirus or a recombinant that expressed 1 of the 11 group A rotavirus genes (Fig. 1). The basal intracellular calcium level in uninfected Sf9 cells was 89 nM ($n = 5$, standard deviation = 14). A significant

FIG. 1. Intracellular calcium concentration in insect cells infected with wt baculovirus or baculovirus recombinants expressing SA11 rotavirus genes. G1 to G11 represent baculovirus recombinants that express SA11 rotavirus gene 1 to gene 11, respectively. Insect cells were infected with each baculovirus at a high MOI (MOI of 10). The intracellular calcium concentration was measured between 32 and 36 h postinfection by using the fluorescent indicator fura-2. Each column shows the mean (■) and standard deviation (□) of three to five independent experiments. $n = 3$ in G3, G7, and G11; $n = 4$ in G2, G4, G5, G6, G8, and G9; and $n = 5$ in G1, G10, wt, and uninfected Sf9 cells. Three measurements were made in each independent experiment.

increase in the intracellular calcium level was observed in the cells infected with baculovirus recombinant G10 (pAC461-SA11-G10) that expressed NSP4 ($P < 0.001$). The intracellular calcium concentration in cells infected with pAC461-SA11-G10 reached values on the order of 412 nM, five times higher than the calcium concentration in uninfected Sf9 cells. No significant intracellular calcium concentration increases were observed between 32 and 36 h postinfection in the cells infected with wt baculovirus or baculovirus recombinants that expressed the other rotavirus proteins ($P > 0.05$).

The expression of individual rotavirus genes in the infected cells was confirmed by SDS-PAGE (Fig. 2). The expressed level of NSP4 was not high, probably because of gene 10 expression being driven from the polyhedrin promoter in the low-level transfer-expression vector pAC461 (22). The expression of NSP4 was confirmed by Western blot analysis (data not shown).

Changes in intracellular calcium levels in infected Sf9 cells correlated with the expression of NSP4 of group A rotavirus. The increase in intracellular calcium levels coincided with the expression of NSP4 in the cells infected with pAC461-SA11-G10 (Fig. 3). By Western blot analysis, trace amounts of NSP4 could be detected as early as 16 h postinfection and reached a peak at 36 h postinfection (Fig. 3a). The accumulated NSP4 decreased after 36 h postinfection, at which time degradation products of NSP4 were observed. There was no difference observed in the intracellular calcium levels at 16 h postinfection in cells infected with wt and pAC461-SA11-G10 (Fig. 3b [$P > 0.05$]). A significant difference in intracellular calcium levels between cells infected with wt and pAC461-SA11-G10 was observed at 20 h postinfection ($P < 0.05$). The increase in intracellular calcium levels reached a peak by 36 h postinfection. At 48 h postinfection, the intracellular calcium level in the cells infected with pAC461-SA11-G10 was significantly lower than at 36 h postinfection but was still significantly higher (288 nM) than the intracellular calcium concentration in the wt baculovirus-infected cells (132 nM). This might be the result of

the degradation of NSP4 at the later stages of infection. There was no significant increase in intracellular calcium level in the cells infected with wt baculovirus until the latest stage of infection (60 h postinfection). This might be a nonspecific effect due to the general inhibition of cellular protein synthesis by baculovirus proteins.

Increase in intracellular calcium concentration in insect cells expressing NSP4 from a group B rotavirus. To determine whether the effect of NSP4 on intracellular calcium levels was conserved in different rotaviruses, the intracellular calcium concentration was measured in Sf9 cells infected with a baculovirus recombinant expressing NSP4 from a group B rotavirus (IDIR). There was a significant increase in the intracellular calcium levels of the cells infected with the baculovirus recombinant that expressed NSP4 of IDIR compared with that of a control baculovirus recombinant derived from recombination of wt baculovirus DNA and transfer vector DNA without any insert (Fig. 4). The intracellular calcium concentration began to increase by 28 h postinfection and was maximal at 48 h postinfection. After 48 h postinfection, the intracellular calcium concentration was difficult to measure because of low cell viability. The expression of the IDIR NSP4 in the Sf9 cells was confirmed by SDS-PAGE (data not shown). This result indicates that this function of NSP4 is conserved among rotavirus groups.

DISCUSSION

Calcium plays a significant role in the rotavirus replication cycle. Shahrabadi et al. reported that reduction of extracellular calcium levels decreased bovine rotavirus maturation and CPE (37, 38). Michelangeli et al. reported that MA104 cells infected with the OSU strain of porcine rotavirus showed increased intracellular calcium levels (30); this increased intracellular calcium was dependent on viral protein synthesis and appeared to be related to cell permeabilization and death caused by rotavirus. In the presence of low extracellular calcium, CPE

FIG. 2. The expression of individual rotavirus genes in baculovirus recombinant-infected insect cells. Insect cells were infected at a MOI of 10 with wt baculovirus or baculovirus recombinants that express individual rotavirus genes from SA11 rotavirus. The infected cells were labeled with [35 S]Trans at 36 h postinfection for 1 h. Cells were lysed with radioimmunoprecipitation assay buffer, and the lysate was analyzed by SDS-15% PAGE. Proteins labeled in SA11-infected MA104 cells were used as a marker with an extended exposure time (2 days for SA11-infected cell lysate versus 12 h for baculovirus recombinant-infected cell lysate). Glycosylation of the two glycoproteins (VP7 and NSP4) was inhibited by using tunicamycin (TM). The empty circles show individual rotavirus proteins and the polyhedrin protein expressed in the infected insect cells. The data shown in Fig. 1 and here are from the same experiment.

also was found to be reduced. This might be related to the inability of the virus to induce increased intracellular calcium levels. To determine which viral proteins are involved in this event, we used baculovirus recombinants expressing individual rotavirus genes to infect Sf9 insect cells.

In the baculovirus expression system used, foreign protein synthesis is controlled by the baculovirus polyhedrin promoter, a late promoter. Foreign proteins produced by using this promoter are easily detected 24 h postinfection, and these proteins accumulate to an extremely high level after 36 h postinfection (22). In some cases at late stages of infection, host protein synthesis is reduced by the expressed proteins or by baculovirus proteins (22). Similar kinetics of protein expression have been observed for several rotavirus genes produced by using baculovirus recombinants (10, 11, 16, 20, 24, 26, 42). Such effects might influence the regulation of intracellular calcium levels at late stages of viral infection. To avoid nonspecific effects on intracellular calcium levels occurring at late stages of baculovirus infection, we measured calcium levels between 32 and 36 h postinfection. Each of the rotavirus proteins could be detected in the cells infected with the baculovirus recombinant at this point. In fact, we did observe an increase in the intracellular calcium levels at very late times (after 60 h) postinfection in wt baculovirus-infected cells (data not shown).

A significant intracellular calcium level increase was observed only in cells infected with baculovirus recombinants expressing NSP4 ($P < 0.001$). The rate of NSP4 synthesis was quite stable at different times postinfection in the infected cells (data not shown). NSP4 expression could be detected by 20 h postinfection and reached a peak at 36 h postinfection. The time course of intracellular calcium level change in cells infected with a baculovirus recombinant that expresses gene 10 was coincident with the accumulation of NSP4 in the infected insect cells. These results indicate that the increase in the intracellular calcium level was directly related to the synthesis of NSP4.

The level of expression of NSP4 in the insect cells was not high because of the use of the pAC461 transfer vector (instead of pVL941) to obtain recombinants containing gene 10. Although both these vectors express genes from the polyhedrin promoter, expression levels of foreign genes in recombinants made from the pAC461 transfer vector are lower compared with expression levels from recombinants from pVL941, because the 5' leader sequences of the polyhedrin promoter are not optimal in the pAC461 vector (10, 22, 43). We were not successful in obtaining gene 10 recombinants by using pVL941, the higher-level expression vector, presumably because of the toxicity of NSP4. The lack of seeing significant intracellular free calcium increases in cells infected with pVL941-G6 and

FIG. 3. (a) NSP4 expression in insect cells at different times after infection with pAC461-G10 baculovirus recombinant. Infected cells harvested at the indicated times were analyzed by SDS-15% PAGE and Western blot analysis. The synthesis of three forms of NSP4 (28K, 26K, and 20K) in infected Sf9 cells was detected by Western blot analysis with a polyclonal antibody against NSP4. The 28K and 26K forms of NSP4 contain two and one glycosylated sites, respectively. A rotavirus SA11-infected cell lysate was loaded into the last lane (M) together with prestained low-molecular-weight range protein markers as a marker for localization of the three forms of NSP4. The band above 28K NSP4 in the marker lane is a cellular protein in MA104 cells. The migration of 28K NSP4 (two sites glycosylated) from insect cells is always a little faster than the 28K NSP4 from MA104 cells. The migration of the 20K form of NSP4 in both systems is identical. The difference in migration of the 28K NSP4 is thought to result from the differences in the glycosylation-trimming processes in the two systems. Molecular weight markers are indicated in parentheses. The data in panels a and b are from the same experiment. (b) Intracellular

pAC461-G6 (data not shown) indicates that the increased intracellular free calcium levels in the cells infected with pAC461-G10 cannot be contributed to the use of this expression vector.

Although NSP4 expression levels were relatively low by using the pAC461-based recombinant, the amounts of the membrane-associated NSP4 (from the detergent phase of Triton X-114-extracted cells) were equal to or higher than the membrane-associated NSP4 from SA11-infected MA104 cells (data not shown). The increased intracellular calcium levels (4.6-fold) in the infected cells expressing NSP4 were close to the increased levels (5-fold) of intracellular calcium concentration in rotavirus-infected MA104 cells. Cells coinfecting with pAC461-G10 together with baculovirus recombinants expressing other viral structural proteins (VP2, VP4, VP6, and VP7) did not show a further increase in the intracellular calcium levels (data not shown). In addition, intracellular free calcium levels were not increased in insect cells infected with baculovirus recombinants that expressed two other ER-specific proteins, hepatitis B surface antigen and presurface antigen (data not shown). These results suggest that NSP4 was the major factor responsible for the increase in intracellular calcium levels in infected insect cells.

The significance of the increase of intracellular calcium in rotavirus-infected cells is not well understood. It is clear that calcium can play a determining role in a variety of pathologic conditions. Disruption of the mechanisms that regulate intracellular calcium homeostasis often is an early event in the development of irreversible cell injury (41). Recent studies have suggested that viruses and viral components can promote CPE and cell death by increasing intracellular calcium levels. Calcium is required for syncytium formation in respiratory syncytial virus-infected HEP-2 cells (39). A significant intracellular calcium level increase is observed in cytomegalovirus-infected cells, and the increased intracellular calcium levels are responsible for the development of cytomegalovirus cytopathology (33). Human immunodeficiency virus type 1 coat protein neurotoxicity can be prevented by calcium channel antagonists (13). Our preliminary results suggest that NSP4 is a cytotoxic protein. This is supported by the fact that SA11-G10-transfected MA104 cells entered a crisis, showed unusual morphologic changes, and subsequently died (unpublished data), and we could not obtain baculovirus recombinants by using the high-level expression vector. This also is supported by the observation that NSP4 is one of the genes that plays a crucial role in the pathogenesis of diarrhea in the gnotobiotic piglet model (18). We hypothesize that the cytotoxicity of NSP4 is related to the increased intracellular calcium levels and the increased intracellular calcium levels are responsible for cell lysis and the release of mature virus particles during the late stage of the virus replication cycle.

An alternative hypothesis is that the increases in intracellular free calcium levels resulted from (rather than caused) decreased cell viability caused by NSP4 cytotoxicity. This seems unlikely for the following reasons. (i) In cells infected with pAC461-G10, the intracellular free calcium levels are not influenced by cell viability because fura-2/AM cannot be

calcium concentration in insect cells at various times after infection with wt baculovirus or a baculovirus recombinant that expresses NSP4 of a group A rotavirus (G10). Insect cells were infected with wt baculovirus or a baculovirus recombinant that expresses NSP4. Mean and standard deviation were calculated from results of three individual experiments. Three measurements were made in each experiment.

FIG. 4. Time course of intracellular calcium concentration in insect cells infected with a baculovirus recombinant that expresses NSP4 of a group B rotavirus (B10) or a control baculovirus recombinant (BK) derived from recombination of a wt baculovirus and a transfer vector without insert. Mean and standard deviation were calculated from the results of three individual experiments. Three measurements were made in each experiment.

loaded into dead cells. (ii) The increased intracellular free calcium levels appeared before any significant decrease in cell viability in pAC461-G10-infected cells (data not shown). (iii) At 36 h postinfection, when $[Ca^{2+}]_i$ were high in NSP4-expressing cells, the viability of baculovirus recombinants expressing any of the 11 rotavirus genes was very similar (75 to 84% of cells were alive, gene 1 to gene 11). (iv) Finally, only slightly increased intracellular calcium levels (160 nM) were observed in wt baculovirus infected cells at very late times after infection, although the viability of these cells (49% alive at 60 h postinfection) was much lower than in the cells infected with the recombinant expressing NSP4 (76% alive at 36 h postinfection).

It is an enigma how VP4, a cytoplasmic viral protein, and VP7, a luminal ER-associated viral protein, form the outer capsid of mature rotaviral particles. Rearrangements of rotavirus proteins in purified membrane-enveloped intermediate particles have been reported (36). Poruchynsky et al. (35) suggested that, in membrane-enveloped intermediates, VP7 is repositioned from its location in the lumen of the ER back across the viral membrane envelope to the exterior of virus particles during the maturation process. We hypothesize the local calcium concentration changes caused by NSP4 might be involved in this protein rearrangement event. This could occur by ER disassociation and reallocation (19) triggered by changes in the intracellular calcium concentration.

The mechanism responsible for the increase in intracellular calcium levels by NSP4 is not known. Eukaryotic cells have many specific mechanisms for regulating intracellular calcium levels. Intracellular calcium homeostasis is controlled by the concerted operation of plasma membrane calcium translocases

and intracellular compartmentalization (6). Ca^{2+} pumps are responsible for pumping calcium from the cytoplasm out of cells or into intracellular calcium pools (7). Calcium channels on the cytoplasmic membrane or the ER membrane are responsible for increasing intracellular calcium levels (31). The ER is an intracellular calcium pool in cells. NSP4 is an ER transmembrane glycoprotein. We hypothesize that the increased intracellular calcium might be released from the intracellular calcium pool (ER). This might be a direct or an indirect effect due to the expression or translocation of NSP4 in the ER membrane. Studies of the potential channel activity and the phospholipase C activity of NSP4 and the significance of the increased intracellular calcium levels in the rotavirus replication cycle are in progress.

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