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Overexpression of the Vaccinia Virus A38L Integral Membrane Protein Promotes Ca^{2+} Influx into Infected Cells

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The vaccinia virus Western Reserve A38L protein is a hydrophobic integral membrane glycoprotein with amino acid similarity to mammalian integrin-associated protein. The protein has an N-terminal immunoglobulin superfamily domain, followed by five membrane-spanning domains and a short cytoplasmic tail. Deletion of the protein reduces virus plaque size but does not affect virus virulence (J. E. Parkinson, C. M. Sanderson, and G. L. Smith, Virology, in press). In this study, we have used a recombinant vaccinia virus in which the A38L gene may be inducibly overexpressed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG), to demonstrate that overexpression of the vaccinia virus A38L gene produces drastic changes in the morphology, permeability, and adhesion of infected cells. In particular, A38L overexpression caused swelling of cells, marginalization of nuclear chromatin, and vacuolization of the endoplasmic reticulum, features characteristic of cell necrosis. By 18 h postinfection, cells become permeable and lytic as defined by the free entry of propidium iodide and loss of the cytoplasmic enzyme lactate dehydrogenase. Chelation of extracellular Ca^{2+} 22 h postinfection inhibited further release of lactate dehydrogenase, showing that Ca^{2+} influx was required for A38L-induced lysis. Direct measurement of $^{45}Ca^{2+}$ influx showed that the rate of Ca^{2+} uptake was directly related to the period of A38L induction. The A38L protein, therefore, promotes the formation of pores within the plasma membrane of cells, and these pores facilitate Ca^{2+} entry and induce necrosis. Addition of rifampin inhibited virus assembly but not the ability of A38L to induce necrosis, indicating that pore formation is independent of viral morphogenesis. Finally, overexpression of the A38L protein resulted in a reduced plaque size and a threefold decrease in production of infective particles in vitro. The A38L protein represents the first example of a virus protein which directly or indirectly promotes the influx of extracellular Ca^{2+}.

The A38L protein of vaccinia virus (WR strain) encodes a 33-kDa glycoprotein which is expressed at low levels during infection (20). Analyses of the predicted amino acid sequence of the A38L protein revealed 28% identity with the mammalian integrin-associated protein (IAP) (12), which in turn was found to be identical to the ovarian tumor marker OA3 (5) and the Rh^--related antigen CD47 (13). These proteins all contain an N-terminal immunoglobulin variable-like domain connected to a hydrophobic C-terminal region containing five membrane-spanning domains representing 60% of the protein (12). Biochemical analyses of the A38L protein in vitro indicated that the N-terminal immunoglobulin variable-like domain would be extracellular while the hydrophobic C-terminal segment would pass sequentially in and out of the membrane, generating four loops, and terminate in an 11-amino-acid cytoplasmic tail. The membrane topology of the A38L protein of vaccinia virus and mammalian IAP is therefore highly conserved (12, 20).

The A38L open reading frame is highly conserved in the Copenhagen (8) and Western Reserve (26) strains of vaccinia virus and the Harvey (1), Bangladesh-1975 (16), and India-1967 (25) strains of variola major virus, but the function of the A38L protein remains unclear. Although the protein is not essential for vaccinia virus production or virulence, deletion of the gene diminishes the size of plaques produced in vitro (20). Consequently, the A38L protein is functional, but a more detailed analysis is required before its role in infection can be identified. One approach is to analyze the role of the A38L protein by extrapolation from the known properties of the mammalian homologs (IAP, OA3, and CD47). For example, IAP binds to the β_3 subunit of the $\alpha_v\beta_3$ integrin (3) and facilitates a transient influx of extracellular Ca^{2+}, which accompanies integrin occupancy (24). This observation, together with the report that the Rh^--related antigen CD47 may facilitate Ca^{2+} influx into synthetic liposomes (23), led to speculation that the IAP may itself form a Ca^{2+} pore. This idea is supported by the physical structure of IAP, which possesses multiple membrane-spanning domains that could arrange in such a way as to form an ion channel. To see if the A38L protein possesses any of the functional properties described for the mammalian IAP, we analyzed the properties of A38L in vivo by using recombinant vaccinia viruses which either lack the A38L gene or overexpress the gene by approximately fivefold upon addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (20). This enabled selective regulation of A38L expression, allowing the effects of A38L on virus production and host cell pathogenesis to be monitored. The results show that enhanced expression of the A38L gene increased Ca^{2+} influx into cells, which in turn induced cell necrosis and reduced virus production and spread.

MATERIALS AND METHODS

Cells and viruses. BS-C-1 cells were grown in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum. The sources of viruses are as described previously (2). Vaccinia virus WR and recombinants derived from it were grown in BS-C-1 cells. Working stocks of viruses were prepared by Dounce homogenization of infected cell extracts, removal of nuclei by centrifu-

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gation, and sedimentation of virus in the supernatant through a sucrose cushion (14). Virus infectivity was determined by plaque assay on BS-C-1 cells (14). The construction of recombinant viruses which either lack the A38L gene (ΔA38L) or contain a single copy of the gene under control of the IPTG-inducible p4b promoter (22) within the thymidine kinase locus (IndA38L) has been described previously (20).

Cell detachment assay. BS-C-1 cells were infected with either WR virus or IndA38L virus at 5 plaque forming units (PFU)/cell. Cells were incubated in the presence or absence of 10 mM IPTG for either 12, 24, or 36 h before the percentage of detached cells was determined. The culture medium was carefully passed over the cell monolayer four times to free loosely adhering cells, the number of detached cells was counted, and adherent cells were removed from the dish by trypsinization and counted.

DAPI staining of infected cells. BS-C-1 cells grown on glass coverslips were infected with 5 PFU of either WR or IndA38L virus per cell. At 24 h postinfection (p.i.), cells were washed in phosphate-buffered saline (PBS) and then fixed in acetone-methanol (1:1) at −20°C for 10 min. After fixation, coverslips were air dried and mounted in Mowiol–4′,6-diamidino-2-phenylindole (DAPI) mounting medium, which was prepared as follows. Mowiol 4-88 (2.4 g; Nova Biochemicals) was incubated with 3 g of glycerol for 1 h, 6 ml of water was added, and the solution was mixed for a further 2 h. Then 12 ml of 0.2 M Tris-HCl (pH 8.5) was added, and the mixture was incubated at 50°C for 10 min. The resulting solution was centrifuged, and 1 μg of DAPI per μl was added to the supernatant. The completed mounting medium was stored in 200-μl aliquots at −20°C until required.

Preparation of cells for electron microscopy. BS-C-1 cells were infected with either WR virus or IndA38L virus at 5 PFU per cell and incubated at 37°C for 24 h in the presence or absence of 10 mM IPTG as indicated. The cells were then washed with ice-cold PBS, fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate (pH 7.4) for 30 min, washed in water, and postfixed in 1% osmium tetroxide–1.5% potassium ferrocyanide for 60 min at room temperature. After being washed in water and then incubated in uranyl acetate overnight at 4°C, the cells were washed again in water and then dehydrated in ethanol and flat embedded in Epon. Sections were cut parallel to the surface of the dish, lead citrate was added as a contrast agent, and the sections were examined in a Zeiss Omega EM 912 electron microscope.

PI uptake experiments. Propidium iodide (PI) uptake was analyzed essentially as described previously (11). BS-C-1 cells were grown to near confluence in 6-cm-diameter tissue culture dishes to give approximately 10^6 cells per dish. The cells were then infected with the IndA38L virus at 5 PFU per cell in either the presence or absence of 10 mM IPTG as indicated. At the desired time, the medium was carefully aspirated from the cells and replaced with 1.5 ml of PI buffer containing PBS, 0.1% bovine serum albumin, and 2 μg of PI per ml. The cells were then incubated on ice for 30 min, after which the supernatant was removed and transferred to a 15-ml Falcon tube. The cells remaining in the dish were then washed twice with PBS, and each time the wash was added to the PI buffer. Any cells contained in the combined supernatants were then recovered by sedimentation, and the resulting postsedimentation supernatant was discarded. The cells remaining in the dish were removed by trypsinization, diluted into MEM containing 10% FBS, combined with cells sedimented from washes, and harvested by sedimentation. Finally, sedimented cells were resuspended in 1 ml of PBS containing 4% formaldehyde, and a 200-μl aliquot of this sample was used for fluorescence-activated cell sorting (FACS) analysis.

Quantitation of LDH release from cells. For each assay, the tissue culture medium (1 ml) was removed from 10^5 BS-C-1 cells and the relative lactate dehydrogenase (LDH) activity was determined with the Sigma colorimetric kit (no. 500) as specified by the manufacturer.

Determination of Ca^{2+} influx into cells. The method of analyzing the rate of Ca^{2+} uptake was adapted from the published method (15). For each determination, 10^5 BS-C-1 cells were preloaded with the calcium chelater dimethyl-BAPTA-AM (Molecular Probes) to prolong the linear phase of unidirectional Ca^{2+} uptake as follows. Cells were washed once in PBS and once in basic Na^+ solution (pH 7.25) containing 140 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Then 1 ml of basic Na^+ solution containing 4 μM BAPTA-AM was added to cells, which were then incubated at 37°C for 30 min. The cells were again washed in basic Na^+ solution and then in basic Na^+ solution plus 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Measurement of Ca^{2+} uptake was then initiated by the addition of 1 ml of basic Na^+ solution (plus EGTA) containing $^{45}Ca^{2+}$ (NEN Research Products, Boston, Mass.) at a final concentration of 10 μCi/ml. At 5-min intervals, cells were washed three times in PBS and removed from the dish by trypsinization. Following removal from the dish, the cell suspension was sonicated to disrupt cells, and an aliquot was used directly for scintillation counting.

RESULTS

To investigate the consequence of overexpressing the A38L protein, cells were infected with either WR or IndA38L in the presence or absence of IPTG and photographed at 24 h p.i. Figure 1 shows that cells infected with the IndA38L virus (without IPTG) were similar to cells infected with WR virus in the presence or absence of IPTG (compare Fig. 1A and B with Fig. 1C). In contrast, cells infected with IndA38L virus (with IPTG) had drastically changed morphology and appeared distended, granular, and less firmly attached (Fig. 1D). Quantification of adherent and detached cells showed that at 24 h p.i. with IndA38L in the presence of IPTG, less than 50% of the original cell population remained attached to the tissue culture dish. However, when cells were infected with the IndA38L virus (without IPTG) or WR virus (with or without IPTG), more than 80% of cells remained tightly adhered to the plate even at 36 h p.i. (Fig. 2).

Comparative morphology of cells infected with IndA38L virus in the presence or absence of IPTG. To more closely examine the consequence of A38L overexpression on cell morphology, infected cells were examined by fluorescence and electron microscopy. Initially, BS-C-1 cells were stained with DAPI to facilitate the comparison of nuclear morphology. Nuclei of cells infected with WR contained uncondensed chromatin in the presence or absence of IPTG (Fig. 3A and B), while cells infected with IndA38L in the presence of IPTG were smaller and more intensely stained in a manner consistent with nuclear condensation (Fig. 3D). As condensation of nuclear chromatin is a phenotype common to both apoptotic and necrotic cells (9), the DNA derived from cells infected with WR virus or IndA38L virus in the absence or presence of IPTG was analyzed. No laddering of the DNA into 123-bp internucleosome fragments, characteristic of apoptosis, was found (data not shown). Cell necrosis was therefore the more likely cause of nuclear condensation resulting from A38L induction. This was confirmed by ultrastructural analyses of BS-C-1 cells infected with the IndA38L virus in the presence or absence of IPTG. The results presented in Fig. 4 show that in the absence of IPTG, cells infected with IndA38L have a normal subcellular morphology in that the nuclei have a regular shape, chromatin is uncondensed, and the endoplasmic reticulum appears as fine ribosome-studded cisternae. In addition, it is possible to see characteristic stages of virus morphogenesis in the cytoplasm of infected cells (Fig. 4A and C). In contrast, cells infected with the IndA38L virus in the presence of IPTG (Fig. 4B and D) had highly convoluted nuclei containing condensed and marginalized chromatin. Also, the cytoplasm of these cells contained large vacuolar structures which represent highly distended endoplasmic reticulum-derived vesicles (Fig. 4D). The source of at least some of these distended cytoplasmic vesicles was shown to be the endoplasmic reticulum by the presence of membrane-bound ribosomes and blistering of the ribosome-studded outer nuclear membrane (data not shown). Because all of these morphological features are used to define necrotic cells (9), it follows that increasing A38L protein expression resulted in cell necrosis.

Overexpression of the A38L protein increases the permeability of infected cells. Necrosis is induced when cells lose the ability to control the ionic balance between the external medium and the cytoplasm due to the formation of pores within the plasma membrane (9). Therefore, we investigated if overexpression of the A38L protein affected the permeability of infected cells. This was determined by infecting cells with either WR or the IndA38L virus in the presence or absence of IPTG and analyzing the relative permeabilities of the cells at increasing times p.i. Permeability was assessed by the uptake of PI, a membrane-impermeable, fluorescent, DNA stain. The percentage of permeable cells was then determined by FACS analyses as described in Materials and Methods. Figure 5 shows that cells infected with WR or the IndA38L virus in the

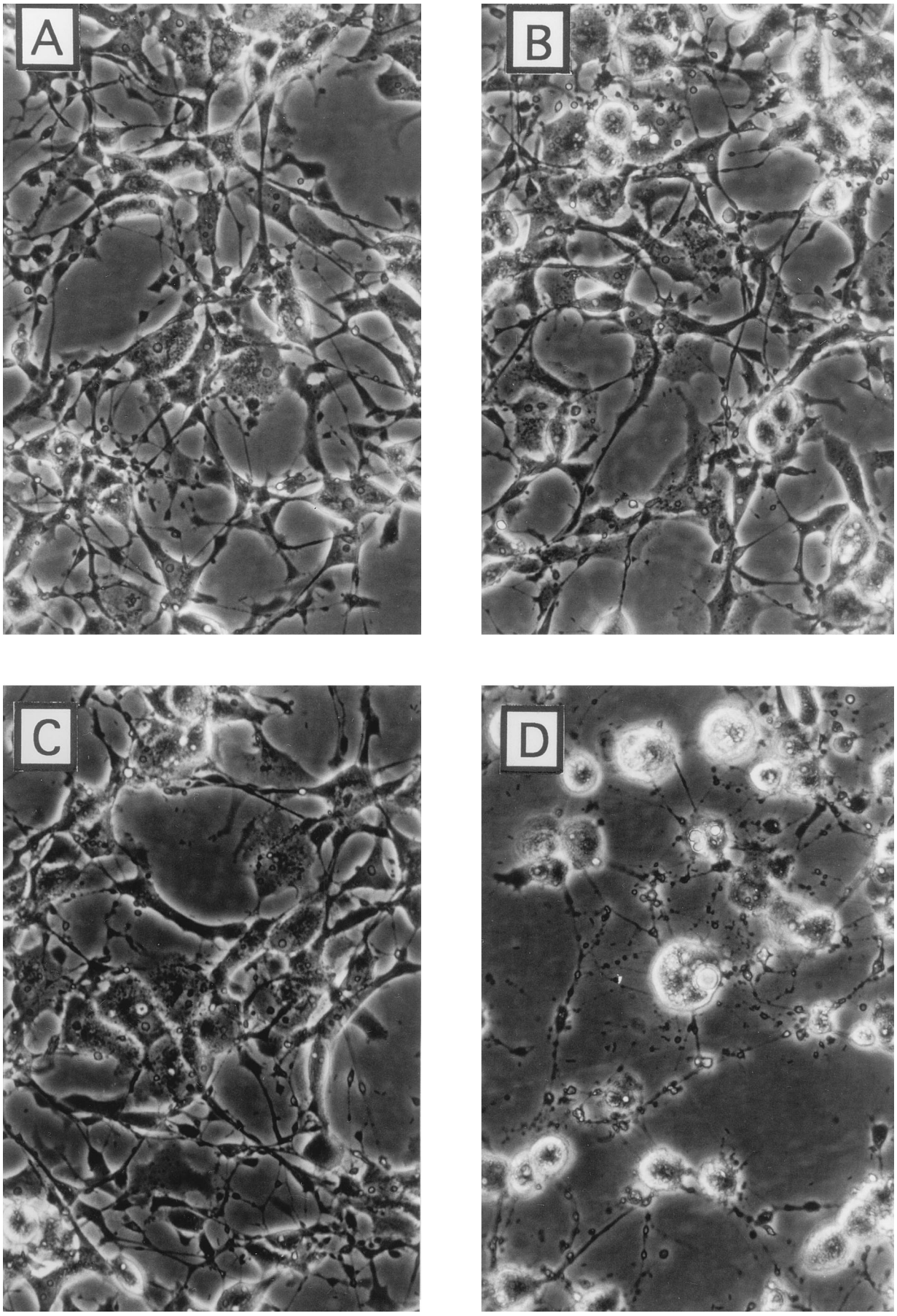

FIG. 1. Overexpression of the A38L gene enhances vaccinia virus-induced cytopathic effect. BS-C-1 cells were infected with WR (A and B) or IndA38L (C and D) at 5 PFU/cell for 24 h in the presence (B and D) or absence (A and C) of 10 mM IPTG. Magnification, ×150.

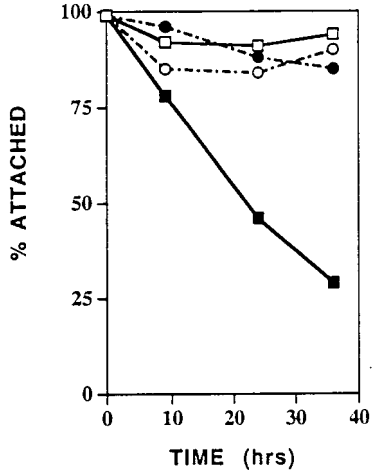

FIG. 2. Overexpression of the A38L gene promotes detachment of adherent cells. BS-C-1 cells grown to (80%) confluence in six-well tissue culture plates were infected with either WR or IndA38L at 5 PFU per cell in the presence (+) or absence (−) of 10 mM IPTG. At the indicated times p.i., the percentage of adherent and detached cells was calculated as described in Materials and Methods. Symbols: □, IndA38L without IPTG; ■, IndA38L with IPTG; ●, WR without IPTG; ○, WR with IPTG.

absence of IPTG remained relatively impermeable to PI throughout the 24-h incubation. However, in the presence of IPTG, IndA38L-infected cells showed a rapid increase in permeability to PI after 18 h p.i., which was not observed when cells were infected with WR virus. Overexpression of the A38L protein therefore enhances the permeability of the plasma membrane of infected cells and establishes a probable cause of the morphological features reported above. Attempts to locate the A38L protein in infected cells by immunofluorescence with the anti-peptide antibody were unsuccessful (data not shown).

Release of LDH following A38L overexpression. Because necrosis is a lytic process, it was possible that A38L overexpression would result in the release of cytoplasmic enzymes such as LDH. To examine this, BS-C-1 cells were infected with either WR or IndA38L virus in the presence or absence of IPTG. At 24 h p.i., the culture medium was collected and the level of LDH in the medium was determined (Fig. 6A). The LDH activity in the medium from mock-infected cells contained 260 Berger-Broida (B-B) units of LDH activity per ml, which was taken as the basal level of LDH release. In comparison, cells infected with WR showed very similar levels of LDH release. However, medium collected from cells infected with the IndA38L virus in the presence of IPTG contained over 1,500 B-B units of LDH activity per ml, approximately six times higher than that released from either mock-infected cells or WR-infected cells in the presence of IPTG. Medium from cells infected with the IndA38L virus (without IPTG) contained slightly elevated levels of LDH (350 B-B units/ml), which is attributable to leakiness of the inducible promoter in the absence of IPTG, as previously reported (22). To determine the kinetics of cell lysis following A38L induction, BS-C-1 cells were infected with the IndA38L virus with or without IPTG and the LDH content of these media was determined at 14 to 24 h p.i. (Fig. 6B). Up to 18 h p.i., both induced and uninduced cells released similar levels of LDH. However, after this time, cells incubated with IPTG released increasing amounts of LDH while cells incubated without IPTG maintained a basal level of LDH release. To determine if A38L-induced LDH release was dependent on the influx of extracellular Ca^{2+}, we analyzed the effect of adding EGTA to the culture medium of cells infected with the A38L virus in the presence of IPTG. Extended incubation with 10 mM EGTA induced cell lysis; therefore, EGTA was added only at 22 h p.i. for 2 h. Figure 6C shows that addition of EGTA arrested further release of LDH. This suggested that influx of extracellular Ca^{2+} was required for A38L-induced cell lysis.

A38L overexpression increases the Ca^{2+} permeability of infected cells. To confirm that A38L facilitates the influx of extracellular Ca^{2+}, we directly measured the rate of Ca^{2+} uptake into cells following A38L induction. BS-C-1 cells were infected with A38L in the presence or absence of IPTG; at 8, 12, 14, 15, or 16 h p.i., the rate of $^{45}Ca^{2+}$ influx was determined over a 30-min period as described in Materials and Methods. Data are expressed as a ratio of the amount of $^{45}Ca^{2+}$ entering test cells to that entering uninfected cells. At 8 h p.i., the influx of $^{45}Ca^{2+}$ into cells infected with IndA38L was strictly regulated and did not differ significantly from that recorded for uninfected cells (Fig. 7A). However, at increasing times p.i., the rate of Ca^{2+} influx into cells infected with IndA38L increased dramatically above that observed for either uninfected cells or cells infected with IndA38L without IPTG (Fig. 7A to D). Importantly, the time of Ca^{2+} influx determination (12, 14, and 16 h p.i.) was well before the onset of membrane permeability as measured by PI uptake and LDH release. Thus, the A38L protein enhances Ca^{2+} entry into cells before lysis, and this leads to cell permeability and necrosis. To confirm this sequence of events, LDH release and Ca^{2+} influx were examined during a 5-min incubation 15 h p.i. within the same cells (Fig. 7E and F). Although LDH is not released above background levels at this time, the uptake of radiolabelled Ca^{2+} was five times higher than that observed in uninfected cells. This shows definitively that the A38L protein promotes Ca^{2+} influx before cells become permeable to LDH.

In view of the enhanced Ca^{2+} influx observed when the A38L protein was overexpressed, the entry of Ca^{2+} into cells infected with WR or ΔA38L viruses was examined at 8, 16, or 24 h p.i. (Fig. 8). The level of Ca^{2+} influx was unaffected by the presence or absence of the A38L protein. Also, in contrast to the situation when A38L was overexpressed (Fig. 7), the level of Ca^{2+} entering WR- or ΔA38L-infected cells was similar to that entering uninfected cells (data not shown). Thus, following vaccinia virus infection, Ca^{2+} continues to enter cells in a manner independent of the A38L protein, and this phenomenon is distinct from the much larger influx of Ca^{2+} entering cells when the A38L protein is overexpressed.

A38L-induced permeability of the plasma membrane is independent of virus particle production. As vaccinia virus particles have been shown to modify the plasma membrane cytoskeleton (10) and IAP is known to interact with integrins (3), it was possible that virus morphogenesis directly influenced A38L function. To address this question, the drug rifampin was added to cells infected with IndA38L in the presence of IPTG and the released LDH and the infectious virus titer were determined at 24 h p.i. (Fig. 9). Inclusion of rifampin inhibited virus particle production by 2 log units; however, the amount of LDH released into the medium in the presence of rifampin was 90% of that released in its absence (Fig. 9). Consequently, the A38L-mediated Ca^{2+} influx is independent of virus production.

Overexpression of the A38L gene decreases the production of infectious particles. Previously, it was demonstrated that deletion of the A38L gene resulted in a reduced-plaque-size phenotype (20). Therefore, the effect of A38L overexpression on virus production and spread in BS-C-1 cells were examined. After infection with IndA38L at 5 PFU per cell, the amount of

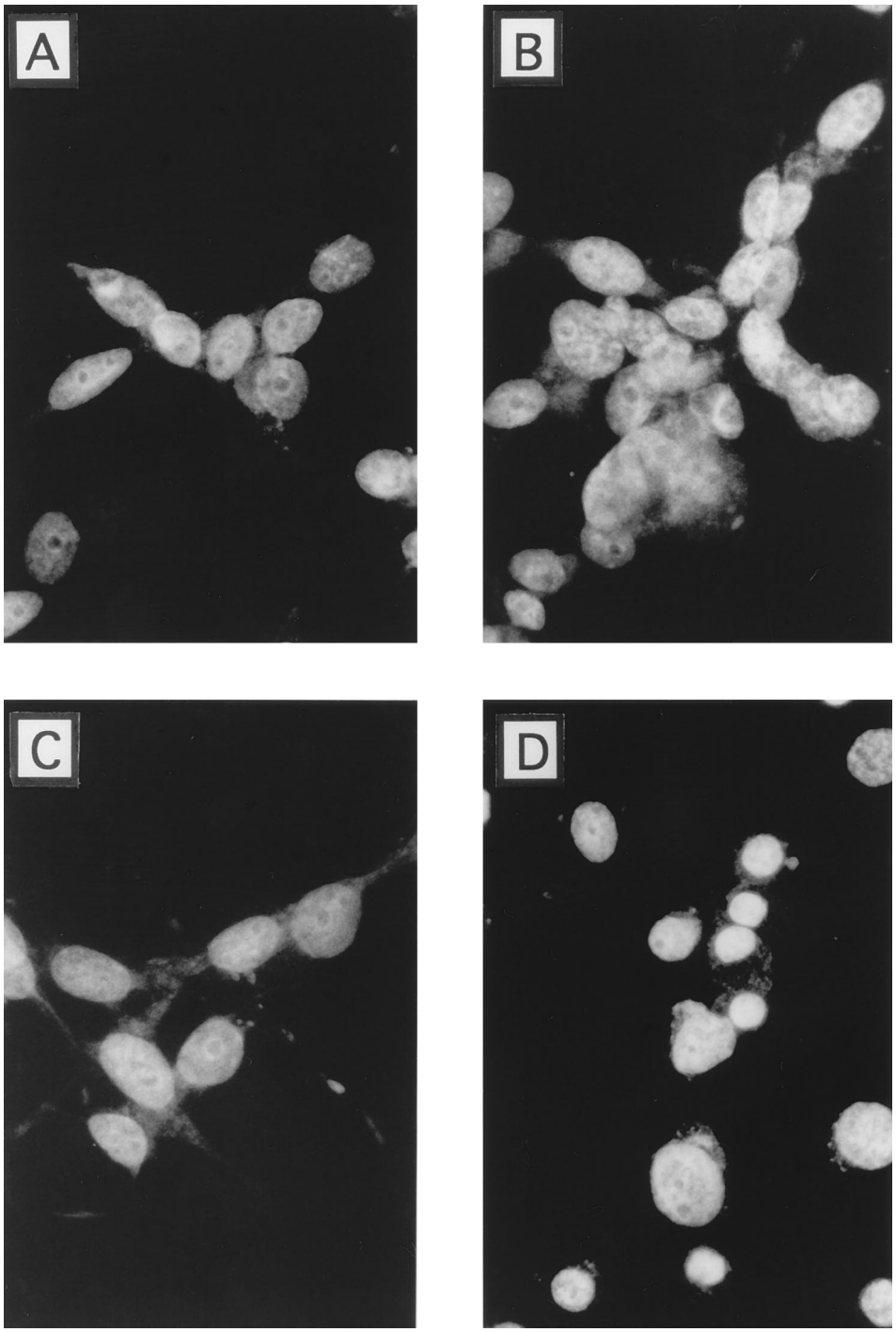

FIG. 3. Overexpression of the A38L gene causes condensation of nuclear chromatin. BS-C-1 cells were infected with either WR (A and B) or IndA38L (C and D) at 5 PFU per cell. At 24 h p.i., cells were fixed in acetone-methanol (1:1) at −20°C for 10 min and stained with DAPI as described in Materials and Methods. Magnification, ×150.

virus produced at 24 h p.i. was reduced approximately threefold when A38L was induced compared with when it was uninduced. This was not due to the presence of IPTG, since production of WT virus was unaltered under these conditions (Fig. 10A). At 36 and 48 h p.i., the plaques produced when A38L was overproduced were significantly smaller than those produced by IndA38L in the absence of IPTG or WR (Fig. 10A and B). At 48 h p.i., the sizes of plaques produced by IndA38L (plus IPTG) and ΔA38L were comparable; however, at 36 h p.i., plaques produced by ΔA38L were slightly smaller than those produced by IndA38L (plus IPTG). Consequently, maximum plaque size required a precise level of A38L protein, since no expression and overexpression are both detrimental to plaque size.

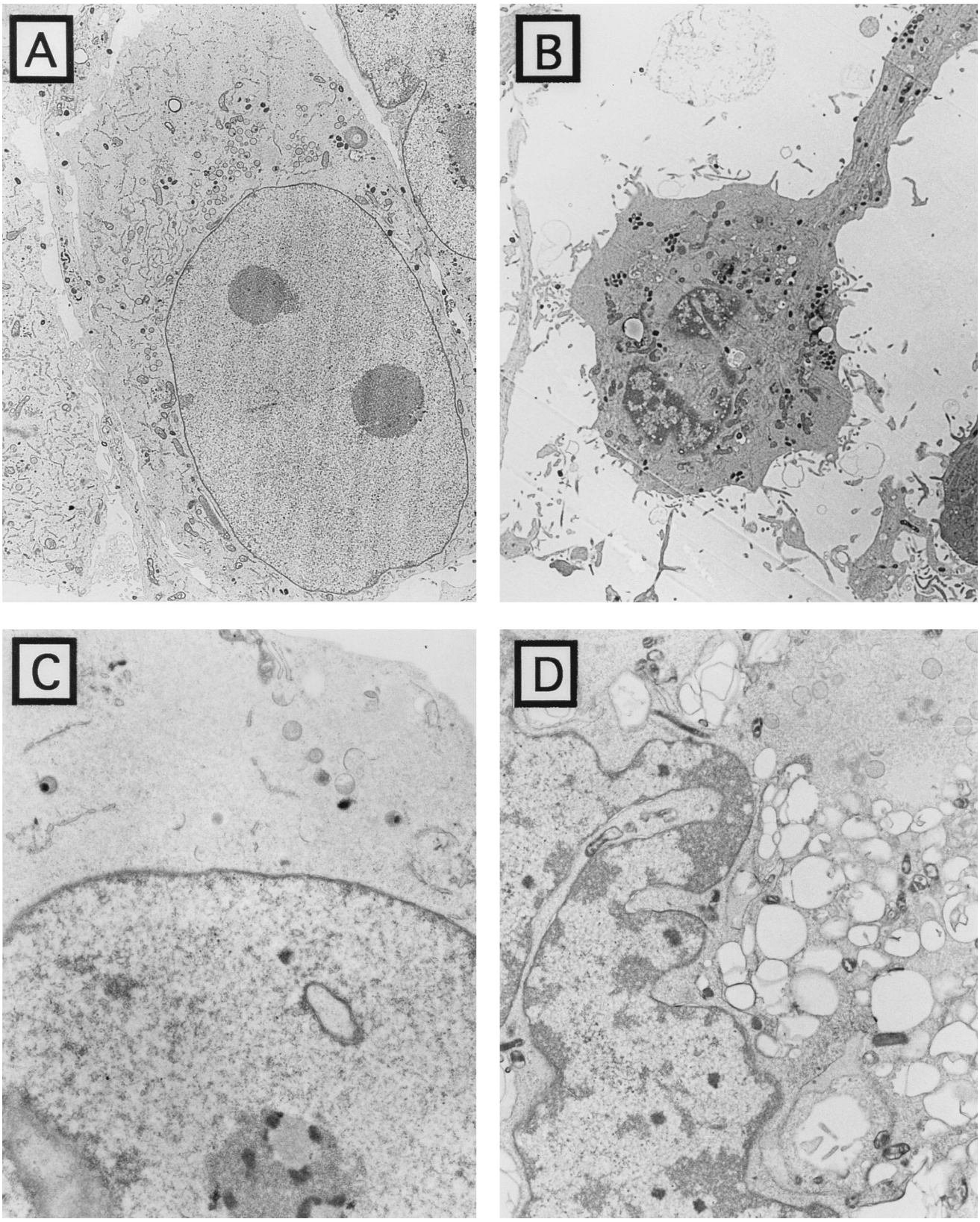

FIG. 4. Electron micrographs of cells infected with IndA38L virus. BS-C-1 cells were infected with IndA38L at 5 PFU per cell in the presence (B and D) or absence (A and C) of IPTG. The cells were then processed for transmission electron microscopy as described in Materials and Methods. (A and B) Magnification, ×2,944. (C and D) Magnification, ×11,040.

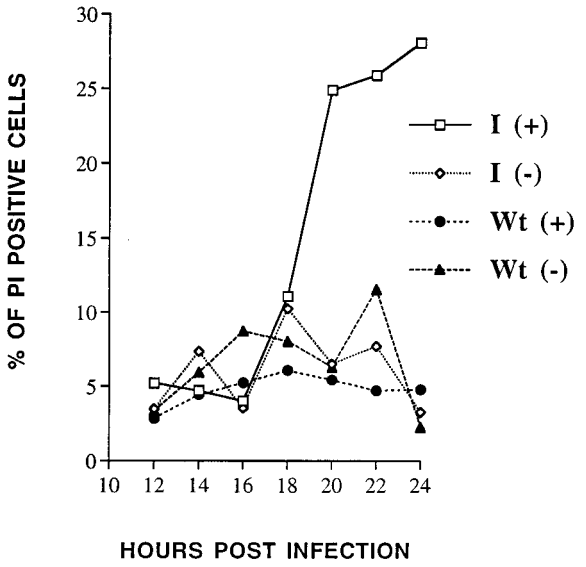

FIG. 5. Overexpression of the A38L gene increases plasma membrane permeability. BS-C-1 cells were infected with either WR virus (Wt) or the IndA38L virus (I) in the presence (+) or absence (−) of 10 mM IPTG. At the indicated times, cells were incubated with PI as described in Materials and Methods, and the number of permeable cells was determined by FACS analyses.

DISCUSSION

In this report, overexpression of the A38L protein of vaccinia virus is shown to promote the influx of extracellular Ca^{2+}, leading to membrane permeability and cell necrosis. To our knowledge, this is the first example of a viral protein which directly or indirectly promotes Ca^{2+} influx into cells. The data also show that the A38L protein has common functional properties with the mammalian IAP, and they provide the first insight into the possible function of the A38L protein in vivo.

Induction of the A38L gene resulted in Ca^{2+} influx and cell necrosis, demonstrating that pores are formed in the plasma membrane of infected cells. However, it is unclear if the A38L protein itself forms pores or if it interacts with other membrane proteins to regulate pore formation. Also, although the data show that these pores can transport Ca^{2+}, they do not indicate if these are unidirectional, voltage dependent, or ion specific. However, it has been shown that IAP induces Ca^{2+} influx via voltage-independent channels, and therefore it may be that A38L-induced Ca^{2+} influx has similar properties.

As A38L-induced necrosis is initiated rapidly after 18 h p.i., it is distinct from permeability changes which accompany viral entry (7) and is not induced by normal virus egress. Also, it appears that cells can tolerate pore formation up to a point at which a threshold is exceeded. This sequence of events is consistent with evidence presented for complement attack (19) and perforin-induced necrosis (11). In each case, cells recovered from the initial formation of pores by internalizing the damaged plasma membrane (11, 19). However, when pore formation exceeded the rate of membrane turnover, cells rapidly became necrotic. The results presented here would suggest that a similar mechanism operates after A38L induction. Indeed, the observed toxicity of A38L overexpression would explain why the protein is expressed at low levels during WR virus infection (20). Furthermore, as both enhanced expression and deletion of A38L reduced virus plaque size, there is an optimal level of A38L synthesis required to maintain efficient virus spread.

The A38L protein and IAP have a common structural topology, and each facilitates Ca^{2+} influx into cells, so that it is tempting to speculate that they are functionally homologous. However, this may well not be the case, because although each protein directly or indirectly mediates Ca^{2+} transport, its function may depend on its ability to interact with other proteins within the membrane, as described for the interaction between IAP and the β_3 integrin (3). In this regard, A38L and IAP share only 28% identity, and the variation in amino acid se-

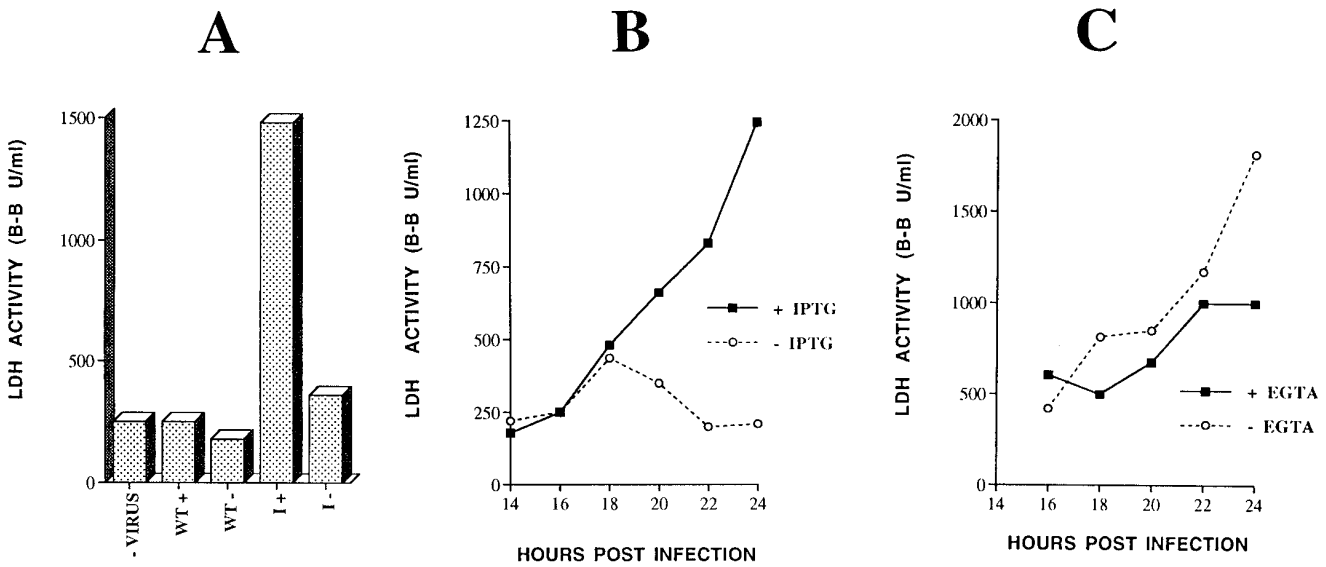

FIG. 6. Overexpression of A38L promotes the release of LDH by a mechanism which is dependent on the influx of extracellular calcium. (A) BS-C-1 cells were mock infected (-VIRUS) or infected with WR virus (WT) or IndA38L (I) in the presence (+) or absence (−) of 10 mM IPTG. At 24 h p.i., the supernatants were removed and the LDH activity in each sample was determined as described in Materials and Methods. (B) BS-C-1 cells were infected with IndA38L virus in the presence or absence of 10 mM IPTG, and supernatants were removed at various times and assayed for LDH activity. (C) BS-C-1 cells were infected with the IndA38L virus in the presence of 10 mM IPTG, and the level of LDH contained within the culture medium at the indicated times was determined. The results obtained from cells incubated in the absence or presence of 5 mM EGTA from 22 h p.i. are shown.

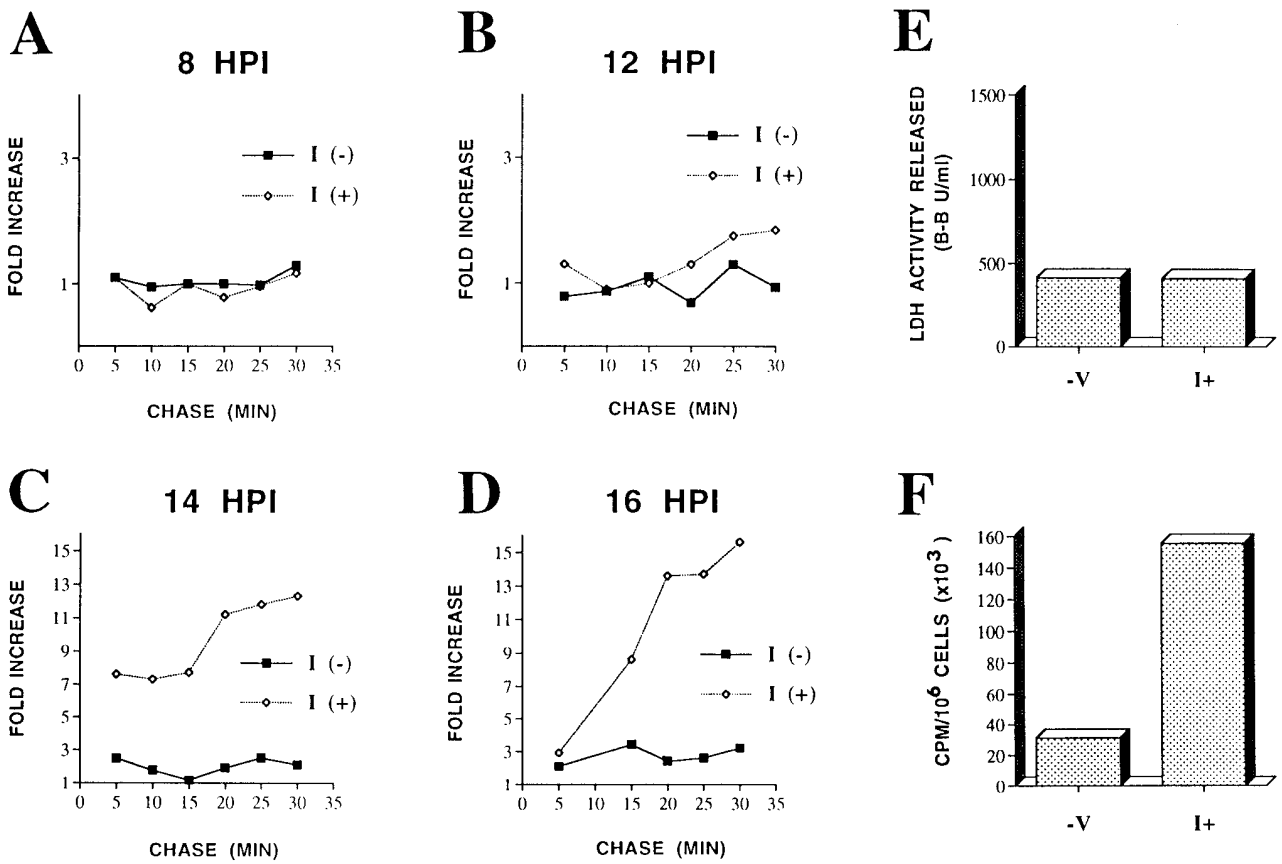

FIG. 7. Overexpression of the A38L protein mediates Ca^{2+} entry into infected cells before the onset of plasma membrane porosity. The rate of entry of $^{45}Ca^{2+}$ into either mock-infected cells or cells infected with the IndA38L virus (I) in the absence or presence of 10 mM IPTG was determined at either 8, 12, 14, 15, or 16 h p.i. as described in Materials and Methods. (A to D) Rate of $^{45}Ca^{2+}$ influx at 8, 12, 14, and 16 h p.i., respectively. (E) Amount of LDH released from cells 15 h after mock infection or infection with IndA38L virus. (F) Amount of $^{45}Ca^{2+}$ entering these cells during a 5-min incubation at 37°C 15 h p.i. −V and I+ denote results obtained from uninfected cells and cells infected with the IndA38L virus in the presence of IPTG, respectively.

quence throughout the protein could significantly alter the ability of the protein to interact with other membrane components. Consequently, a much more detailed understanding of the functional domains of this class of proteins is required before it can be established whether the vaccinia virus A38L protein and the mammalian IAP are truly homologous. Indeed, if this is the case, why does vaccinia virus (and variola virus) require a functional homolog of a protein which is reportedly ubiquitous in mammalian cells (17)? One possibility is

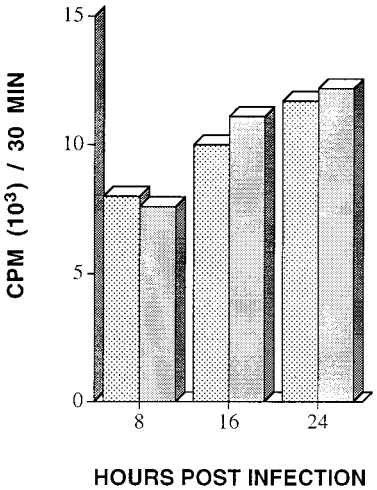

FIG. 8. Influx of extracellular Ca^{2+} into cells infected with either WR (□) or ΔA38L (▨) virus. Entry of $^{45}Ca^{2+}$ into cells infected with WR or ΔA38L viruses was measured after 8, 16, or 24 h p.i. CPM values represent the amount of $^{45}Ca^{2+}$ entering 10^5 cells during 30 min at 37°C.

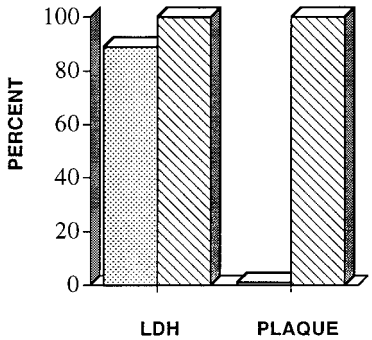

FIG. 9. A38L-induced permeability of infected cells is independent of infectious virus formation. BS-C-1 cells were infected with IndA38L at 5 PFU per cell and were incubated for 24 h at 37°C in the presence of 10 mM IPTG with (□) or without (▨) 100 μM rifampin. The culture supernatant from each sample was then removed, and the LDH activity was determined. The number of infectious particles present was determined (PLAQUE). The amount of LDH released or the number of infectious particles produced in the absence of rifampin was taken to be 100%, and the results obtained in the presence of rifampin are expressed as a percentage of that value.

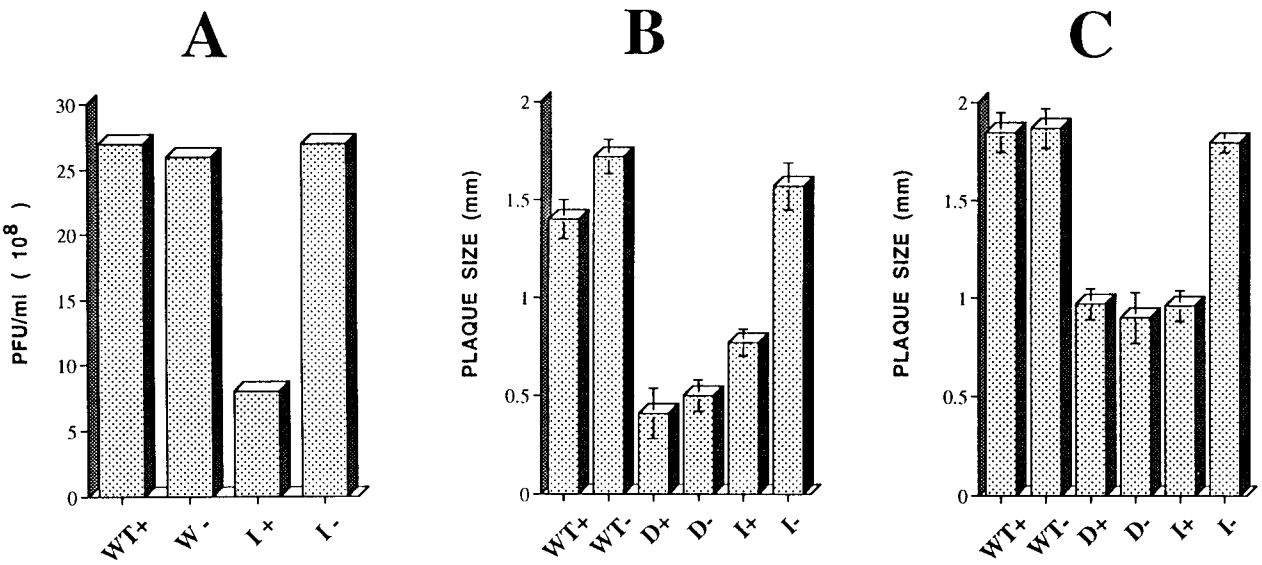

FIG. 10. Effect of A38L induction on particle production and plaque size. (A) BS-C-1 cells infected with either WR (WT) or IndA38L (I) at 5 PFU per cell were incubated at 37°C for 24 h in the presence (+) or absence (−) of 10 mM IPTG. The total number of infectious particles present was then determined by plaque assay. (B and C) Size of plaques at 36 (B) or 48 (C) h p.i. produced by WR (WT), ΔA38L (D), or IndA38L (I) when plaque assays were performed in the presence (+) or absence (−) of 10 mM IPTG.

that the virus-induced inhibition of host protein synthesis leads to IAP depletion and that therefore vaccinia virus needs to encode a functional homolog of IAP to maintain specific cellular functions. Alternatively, the A38L protein may competitively inhibit normal IAP function in order to disrupt cellular responses which would be detrimental to the virus. However, the normal level of A38L protein does not play a major role in controlling total Ca^{2+} influx, since the influx of Ca^{2+} into WR- and ΔA38L-infected cells is similar, and therefore the infected cell maintains other routes of Ca^{2+} entry. It is possible that A38L regulates the influx of Ca^{2+} through a select set of pores which contribute only a small fraction of the total Ca^{2+} entering cells. Additionally, differences in other cells in vitro or during infection in vivo remain possible.

Finally, the A38L-induced detachment of cells is consistent with the observation that increased levels of intracellular calcium stimulate Ca^{2+}-sensitive proteases, one of which is known to degrade talin localized at focal adhesion sites (4).

In summary, the A38L protein is shown to mediate Ca^{2+} influx into cells and, when overexpressed, leads to membrane permeability and cell necrosis. Although many viruses affect the ionic permeability of infected cells (6), there are still very few examples of viral proteins which facilitate ion flux. The best example is the M2 protein of influenza virus, which forms pores that transport H^+ ions (21, 27). As yet, no viral protein has been shown to facilitate Ca^{2+} transport, although synthetic peptides of the human immunodeficiency virus gp41 protein form pores which allow Ca^{2+} influx (18), implying that human immunodeficiency virus may affect Ca^{2+} transport during infection. It is also interesting that both overexpression and no expression of the A38L protein are harmful to vaccinia virus and thus the natural level of expression of the A38L protein is carefully regulated by the virus.

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