VIROLOGY 244, 20–26 (1998)
ARTICLE NO. VY989103

RAPID COMMUNICATION

Role for the Vaccinia Virus A36R Outer Envelope Protein in the Formation of Virus-Tipped Actin-Containing Microvilli and Cell-to-Cell Virus Spread

Elizabeth J. Wolffe, Andrea S. Weisberg, and Bernard Moss[1]

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Drive, Bethesda, Maryland 20892-0445

Received January 27, 1998; accepted February 20, 1998

A small-plaque-forming vaccinia virus mutant with a deletion in the A36R gene encoding an outer envelope protein (Parkinson and Smith, *Virology,* 204, 376–390, 1994) was shown to assemble wrapped forms of intra- and extracellular virus particles and to mediate acid-induced polykaryon formation. The intracellular virions, however, did not acquire actin tails and those on the cell surface were not associated with specialized microvilli. This phenotype is similar to that of the A34R (E. J. Wolffe, E. Katz, A. Weisberg, and B. Moss, *J. Virol.* 71, 3904–3915, 1997) and A33R (R. Roper, E. J. Wolffe, A. Weisberg, and B. Moss, *J. Virol.,* in press) deletion mutants. Taken together, these data support a model in which the envelope proteins encoded by the A33R, A34R, and A36R genes are all required for nucleation of actin tails, which facilitate dissemination rather than egress of virus particles.

Investigation of the cell-to-cell spread of vaccinia virus is complicated by the existence of multiple, related, intra- and extracellular viral particles (*12*). The potentially infectious intracellular mature virions (IMV) are wrapped by cisternae derived from the *trans*-Golgi network to form intracellular enveloped virions (IEV; *9, 17*). Some IEV are propelled by actin tails to the periphery of the cell, where long, thickened microvilli form (*3, 18*). Fusion of IEV with the plasma membrane generates extracellular enveloped virions (EEV), some of which remain cell associated (CEV; *2, 14*). Information regarding the roles of individual viral membrane proteins in morphogenesis has come largely from analyses of mutant viruses. Repression or deletion of the gene encoding the A27L IMV (*15*), F13L (*1*), or B5R (*6, 19*) EEV membrane protein has no effect on the production of infectious IMV, but prevents their wrapping to form IEV, CEV, and EEV, resulting in a small plaque phenotype. While those studies indicated that the IMV do not mediate efficient virus spread in cell monolayers, they did not distinguish between the roles of the various wrapped virus particles in this process. Deletion of the gene encoding the A36R (*13*) or A34R (*11*) EEV membrane protein also caused a small plaque phenotype without diminishing infectious IMV formation. The phenotype was attributed to a 2.5- to 5-fold reduction in the amount of fully infectious EEV in the case of an A36R deletion mutant (vΔA36R; *13*) and to a 5- to 6-fold decrease in specific infectivity of greater than usual amounts of EEV for an A34R deletion mutant (*11*).

Renewed interest in the role of the virus-tipped actin containing microvilli in virus spread (*3*) led us to further investigate the basis for the small plaque phenotypes of A34R and A36R deletion mutants. We confirmed the data of McIntosh and Smith (*11*) regarding the A34R deletion mutant, but also found that neither actin tails nor specialized microvilli formed, providing an alternative explanation for the small plaque phenotype (*20*). Here we show that vΔA36R has a similar defect and elsewhere we show that an A33R deletion mutant has a related phenotype (*16*), indicating that the A33R, A34R, and A36R proteins are all required for nucleation of actin tails, which facilitate the dissemination rather than the egress of virus particles.

Our initial experiments confirmed the reported small plaque phenotype of vΔA36R (*13*). To extend the analysis, we examined the morphogenesis of the mutant virus by electron microscopy of infected cells. We found all recognizable stages of viral morphogenesis, including crescent membranes, immature virions, IMV, and IEV (Figs. 1A and 1B). The double outer membrane of the IEV was clearly resolved (Fig. 1B). As IEV fuse with the plasma membrane, they lose one of the two wrapping membranes so that the extracellular particles have only one more membrane than IMV. Numerous particles were found on the outside of cells (Fig. 1A) and in intercellular spaces (Fig. 1B) and the single EEV outer membrane

[1] To whom correspondence and reprint requests should be addressed. Fax: (301) 480-1147. E-mail: bmoss@nih.gov.

0042-6822/98

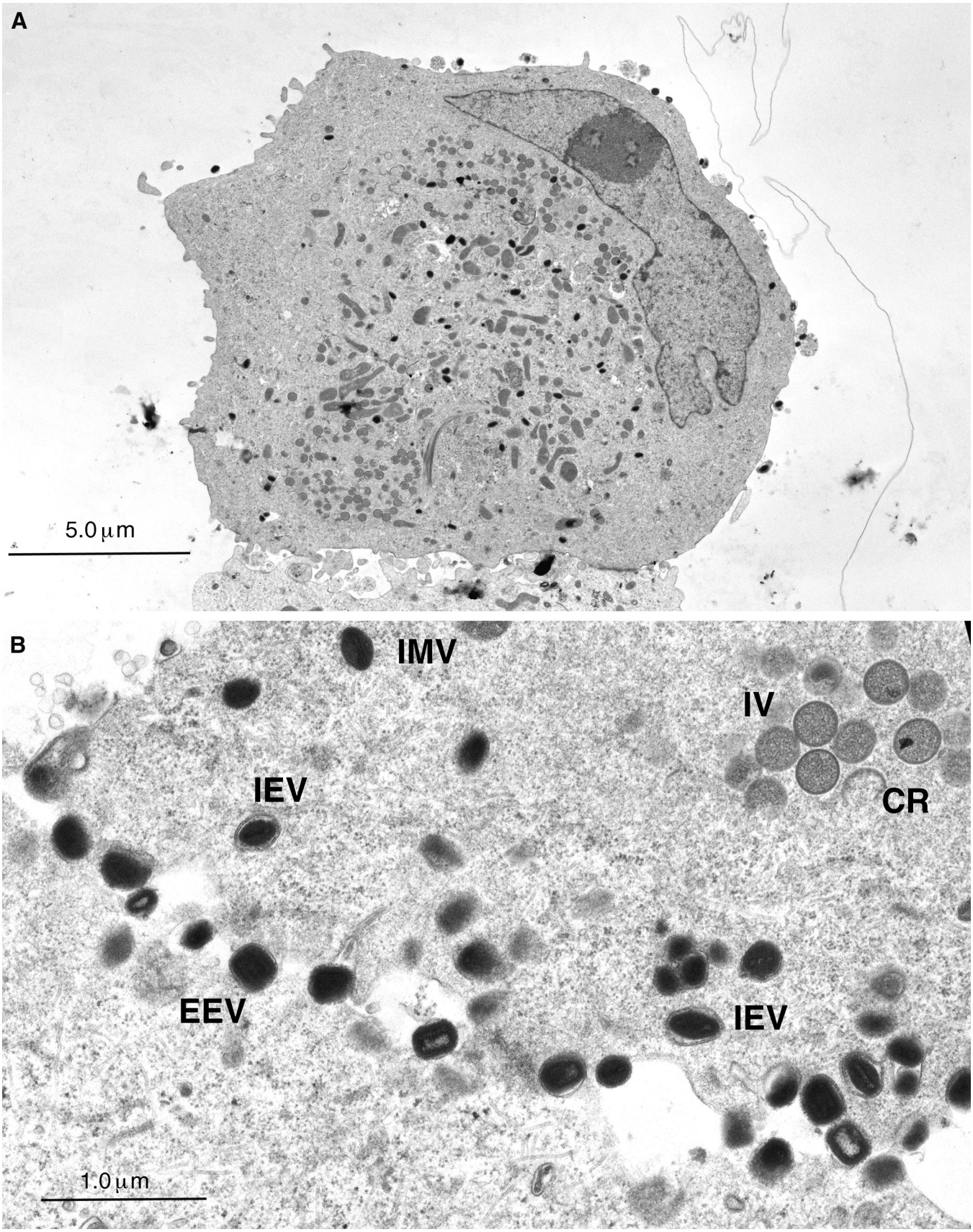

FIG. 1 Transmission electron microscopy of a BS-C-1 cell infected for 24 h with vΔA36R. (A) Low magnification of entire cell with nucleus in upper right quadrant. Numerous intra- and extracellular virus particles are visible. (B) High magnification showing numerous EEV in the space between two cells. Abbreviations: CR, crescents; IV, immature virions; IMV, intracellular mature virions; EEV, extracellular enveloped virions.

was resolved at high magnification (Fig. 1B). These data suggested that the processes of envelopment and virus release were qualitatively normal in cells infected with vΔA36R.

Because IEV and EEV were formed, we looked for actin tails by confocal microscopy. Mock infected cells or cells infected with parental wild-type vaccinia virus (vWTA36R) or vΔA36R for 12 to 16 h were stained with FITC-conjugated phalloidin to visualize F-actin fibers and with polyclonal antiserum against the EEV-specific membrane proteins p37 and gp42 encoded by the F13L and B5R open reading frames, respectively. At late times after infection with wild-type vaccinia virus, the slender actin filaments of uninfected cells became less evident and thickened actin tails attached to virus particles appeared (compare upper and lower panels of Fig. 2). In contrast, thick actin tails were not observed in cells infected with vΔA36R, even though there were many wrapped virus particles that reacted with antibody (Fig. 2, middle panel). The absence of actin tails was due to the deletion of the A36R gene since these structures formed when cells were infected with vRA36R (data not shown), a virus derived from vΔA36R by restoring the A36R gene (*13*).

The defect in actin tail formation led us to examine the surface of infected cells by scanning electron microscopy. Uninfected cells had numerous, slender microvilli, whereas cells infected with vWTA36R or vRA36R displayed thickened microvilli with virus particles at their tips (Fig. 3). In contrast, specialized microvilli were absent from the surface of cells infected with vΔA36R, although virus particles were clearly observed (Fig. 3).

Cells infected with vaccinia virus fuse after brief acid pH treatment (*4, 7*). Polykaryon formation requires the assembly and wrapping of virus particles and is presumably mediated by CEV. Since syncytia did not form in cells infected with an A34R deletion mutant (*20*), it was pertinent to determine whether vΔA36R would induce fusion. Cell monolayers were infected with vWTA36R or vΔA36R for 12 h, incubated for 2 min with a buffer of pH 5.0, 5.5, or 7.4, and then covered with regular medium and incubated for an additional 2 h. Cells infected with vWTA36R formed large syncytia after treatment at pH 5.5 or 5.0, although at the lower pH more polykaryons detached from the plate, but did not form syncytia after treatment at pH 7.4 (Fig. 4). Cells infected with vΔA36R also fused after low pH treatments, but the number and size of the polykaryons were smaller than with wild-type virus, particularly at pH 5.5. No syncytia were formed when mock infected cells or cells treated with an A34R deletion mutant were treated in a similar manner (data not shown).

Our studies with A34R (*20*) and A33R (*16*) deletion mutants indicated that the formation of actin tails and specialized microvilli depends on expression of these two genes, is not required for virus egress, and facilitates cell-to-cell spread. Our present finding, that actin tails do not form when the A36R gene is deleted, suggests that the A33R, A34R, and A36R proteins contribute to the formation of a platform for actin nucleation, although less direct mechanisms can also be envisaged. The present results also support our previous finding that actin tails are not required for virus egress (*20*). Precisely how such IEV move to the periphery of the cell is not understood and a role for actin, that is not organized into tails, has not been excluded by our studies. Importantly, the data presented here further support a model in which the principal role of actin tails is to form the specialized microvilli which permit direct cell-to-cell spread of vaccinia virus.

The small plaque-forming EEV membrane protein deletion mutants can be grouped into two classes. The first class, consisting of F13L and B5R mutants, have defects in the wrapping process and do not form substantial amounts of IEV, EEV, or CEV and consequently lack actin tails and specialized microvilli (*1, 6, 19*). The second class, consisting of A33R, A34R, and A36R mutants, form wrapped virus particles but not actin tails or specialized microvilli (*16, 20*; present data). While the phenotypes of the latter three mutants are similar, they are not precisely the same. Thus, the A33R mutant formed the smallest plaques (*16*), the A34R mutant produced the most EEV (*11*), and completely wrapped IEV appeared to be most numerous in cells infected with the A36R mutant (present data).

The A34R mutant also differs from the A33R and A36R mutants with regard to its inability to mediate acid-induced fusion of infected cells (*16, 20*). Vaccinia virus-infected cells form syncytia when briefly exposed to low pH (*4, 7*), which may mimic fusion between virus particles and endosomal membranes (*10*). The inability of cells infected with an A34R deletion mutant to form syncytia, when exposed to low pH, initially raised the possibility that actin tails or specialized microvilli were required in some unknown way for fusion to occur. Since A33R and A36R mutants form syncytia but not actin tails, we suggest that the fusion defect of A34R deletion mutants may be due to a relatively low number of surface particles or altered properties of the outer viral membrane.

Further studies are needed to determine whether the A33R, A34R, and A36R proteins interact with each other and with additional viral and cellular proteins to enable actin tail formation.

Methods used

HeLa cells, BS-C-1 cells, and virus stocks were prepared as described (*5*). Infections with vaccinia virus were carried out at 10 plaque forming units per cell in minimal essential medium containing 2.5% fetal bovine serum. vWA36R, vΔA36R, and vRA36R were previously described (*13*).

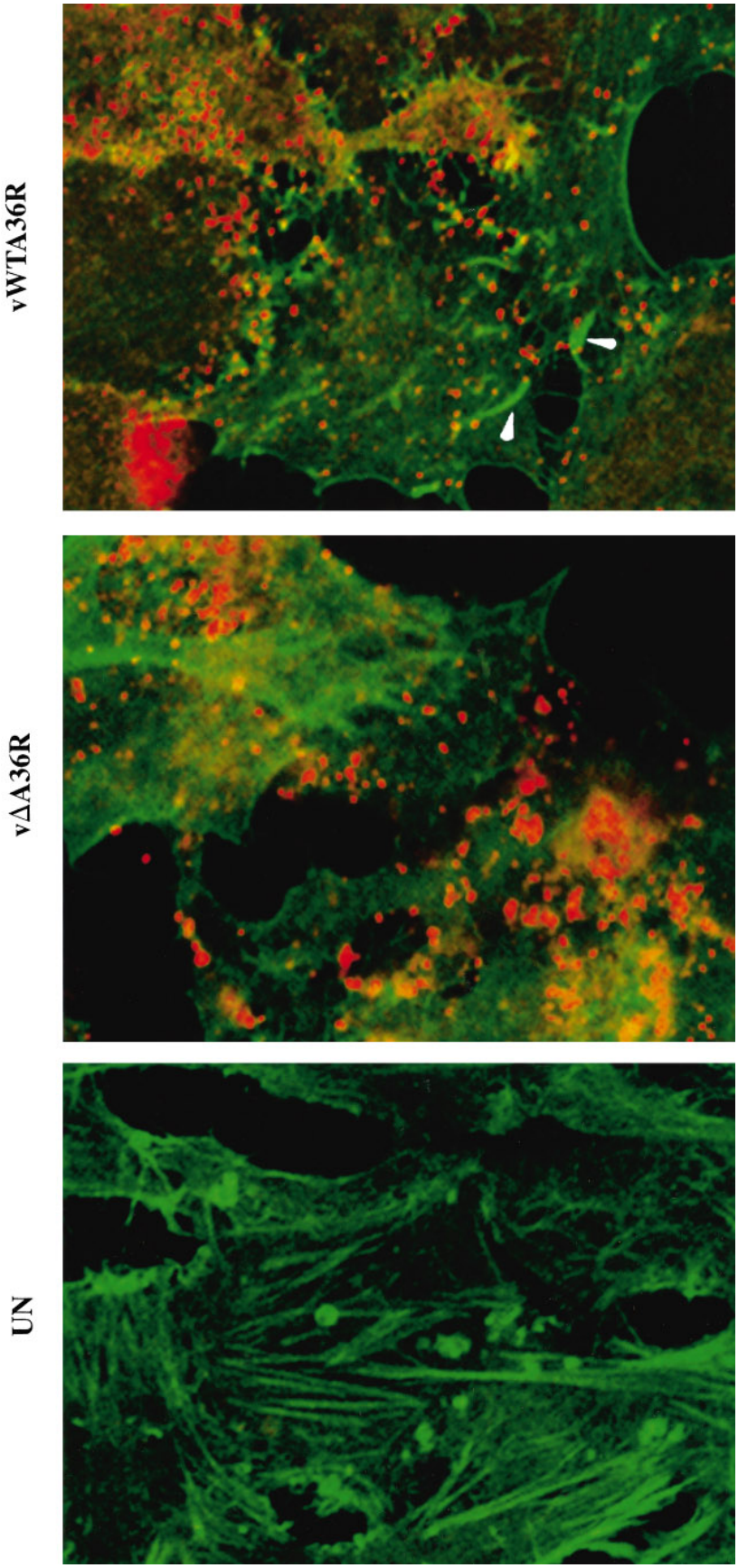

FIG. 2. Confocal microscopy of uninfected HeLa cells (UN) or cells infected with vΔA36R or vWTA36R. After 16 h, the cells were stained with FITC–phalloidin and rabbit antiserum to the EEV-specific proteins encoded by B5R and F13L genes followed by rhodamine-conjugated swine antirabbit antibody. The arrowheads point to virus-tipped actin tails in cells infected with vWTA36R.

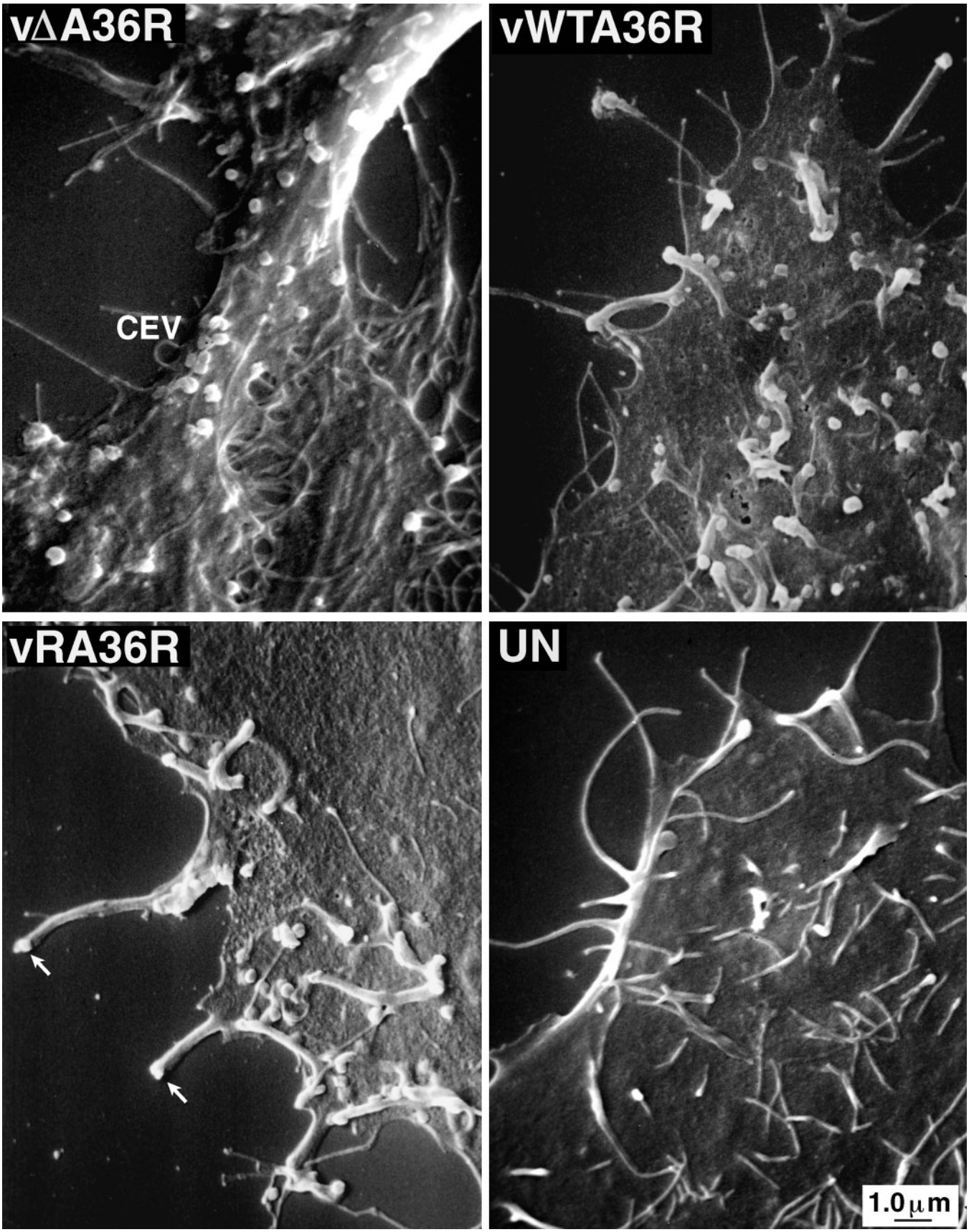

FIG. 3. Scanning electron microscopy of the surface of uninfected HeLa cells (UN) or cells infected with vΔA36R, vRA36R, or vWTA36R. A cluster of CEV is indicated on the surface of cells infected with vΔA36R. The arrows point to virus-tipped specialized microvilli in cells infected with vRA36R.

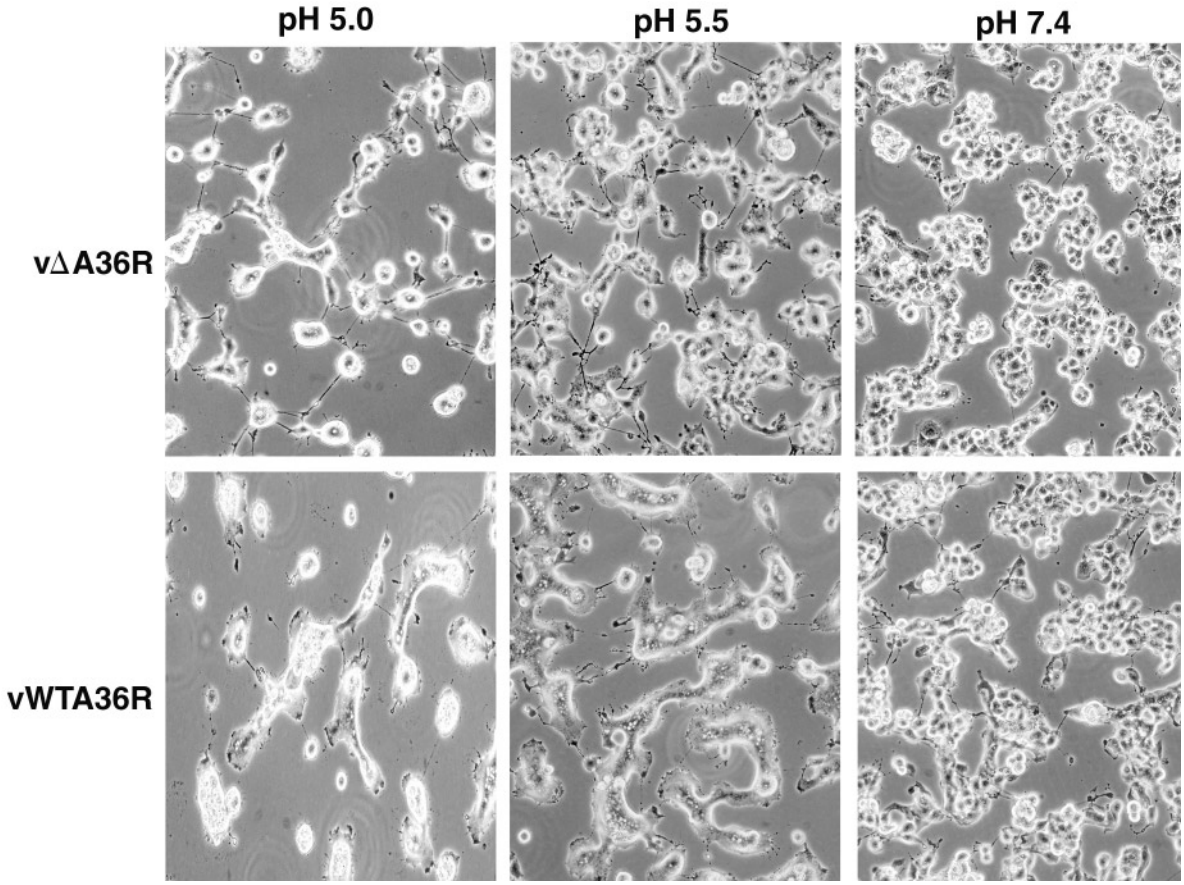

FIG. 4. Acid pH-mediated fusion of vaccinia virus infected cells. BS-C-1 cells were infected with vΔA36R or vWTA36R for 12 h. The cells were briefly exposed to buffer at pH 5.0, 5.5, or 7.4 and then incubated at 37°C in regular medium for 2 h. Cells were viewed by phase-contrast microscopy and photographed at a magnification of 36×.

For transmission electron microscopy, BS-C-1 cells in 60-mm dishes were infected with vaccinia virus at a multiplicity of 10 for 24 h, fixed in 2% gluteraldehyde, embedded in Embed-812, sectioned, and examined with a Philips CM100 microscope as previously described (*20*). For scanning electron microscopy, HeLa cells were grown on coverslips, infected with vaccinia virus at a multiplicity of 10, fixed, and prepared for viewing on an Amray 1820D microscope at an accelerating voltage of 15 kV as described (*20*).

HeLa cells were grown on coverslips, infected with vaccinia virus at a multiplicity of 10, fixed with 3% paraformaldehyde, and permeabilized with saponin. Actin filaments and wrapped virions were visualized as described previously (*20*) with FITC-conjugated phalloidin (Molecular Probes, Eugene, OR) and rabbit antiserum against EEV-specific proteins p37 and gp42 (*8*) followed by rhodamine-conjugated swine-antirabbit antiserum, respectively. Cells were viewed and images collected using an MRC 1024 laser-scanning confocal microscope (Bio-Rad, CA).

Confluent BS-C-1 cells in 6-well plates were infected at a multiplicity of 10 for 1 h, washed, and incubated in minimal essential medium with 2.5% fetal bovine serum for 12 h at 37°C. The medium was then removed and replaced with phosphate-buffered saline containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid and 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] at pH 5.0, 5.5, or 7.4 for 2 min at 37°C. The buffer was replaced with medium and the incubation continued at 37°C for 1 to 2 h, after which the cells were examined by phase-contrast microscopy and photographed.

ACKNOWLEDGMENTS

We thank G. L. Smith for providing vaccinia virus mutants and R. Roper for helpful discussions.

Note added in proof. Defective actin tail formation by vΔA36R has also been noted by C. M. Sanderson, F. Frischknecht, M. Way, M. Hollinshead, and G. L. Smith (1998). Roles of vaccinia virus EEV-specific proteins in intracellular actin tail formation and low pH-induced cell–cell fusion. *J. Gen. Virol.,* in press.

REFERENCES

1. Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000 Dalton outer envelope protein. *J. Virol.* 65, 5910–5920.
2. Blasco, R., and Moss, B. (1992). Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J. Virol.* 66, 4170–4179.

3. Cudmore, S., Cossart, P., Griffiths, G., and Way, M. (1995). Actin-based motility of vaccinia virus. *Nature* 378, 636–638.
4. Doms, R. W., Blumenthal, R., and Moss, B. (1990). Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. *J. Virol.* 64, 4884–4892.
5. Earl, P. L., Cooper, N., and Moss, B. (1991). Preparation of cell cultures and vaccinia virus stocks. *In* 'Current Protocols in Molecular Biology' (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Ed.), pp. 16.16.1–16.16.7. Greene Publishing Associates/Wiley Interscience, New York.
6. Engelstad, M., and Smith, G. L. (1993). The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* 194, 627–637.
7. Gong, S. C., Lai, C. F., and Esteban, M. (1990). Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. *Virology* 178, 81–91.
8. Hiller, G., Jungwirth, C., and Weber, K. (1981). Fluorescence microscopical analysis of the life cycle of vaccinia virus in chick embryo fibroblasts. Virus–cytoskeleton interactions. *Exp. Cell Res.* 132, 81–87.
9. Hiller, G., and Weber, K. (1985). Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J. Virol.* 55, 651–659.
10. Ichihashi, Y. (1996). Extracellular enveloped vaccinia virus escapes neutralization. *Virology* 217, 478–485.
11. McIntosh, A. A. G., and Smith, G. L. (1996). Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus. *J. Virol.* 70, 272–281.
12. Moss, B. (1996). *Poxviridae:* The viruses and their replication. *In* 'Fields Virology' (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 2637–2671. Lippincott-Raven, Philadelphia.
13. Parkinson, J. E., and Smith, G. L. (1994). Vaccinia virus gene A36R encodes a Mr 43-50 K protein on the surface of extacellular enveloped virus. *Virology* 204, 376–390.
14. Payne, L. G. (1980). Significance of extracellular virus in the in vitro and in vivo dissemination of vaccinia virus. *J. Gen. Virol.* 50, 89–100.
15. Rodriguez, J. F., and Smith, G. L. (1990). IPTG-dependent vaccinia virus: Identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res.* 18, 5347–5351.
16. Roper, R., Wolffe, E. J., Weisberg, A., and Moss, B. (1998). The envelope protein encoded by the A33R gene is required for formation of actin-containing microvilli and efficient cell to cell spread of vaccinia virus. *J. Virol.* 72, in press.
17. Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G., and Griffiths, G. (1994). Assembly of vaccinia virus: The second wrapping cisterna is derived from the trans Golgi network. *J. Virol.* 68, 130–147.
18. Stokes, G. V. (1976). High-voltage electron microscope study of the release of vaccinia virus from whole cells. *J. Virol.* 18, 636–643.
19. Wolffe, E. J., Isaacs, S. N., and Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* 67, 4732–4741.
20. Wolffe, E. J., Katz, E., Weisberg, A., and Moss, B. (1997). The A34R glycoprotein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. *J. Virol.* 71, 3904–3915.