A vaccinia serine protease inhibitor which prevents virus-induced cell fusion

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A deletion mutant lacking the non-essential vaccinia virus gene K2L, a member of the serine protease inhibitor superfamily, was constructed. This virus replicates in vitro in all cell types tested and its virulence and immunogenicity in vivo are comparable to those of the parent virus in intranasally inoculated mice. However, in a variety of cell lines the cytopathic effect of the deletion mutant (vKL4) is markedly different from that caused by the parent virus: the absence of K2L in infected cells results in extensive polykaryocytosis. Reinsertion of the K2L gene into vKL4 abolishes this fusion activity, thus confirming that the polykaryocytosis is the result of the deletion of K2L rather than of spontaneous mutations elsewhere in the genome, and that in cells infected with the WR strain of vaccinia virus the K2L gene product prevents fusion. The cell type-specific polykaryocytosis induced by vKL4 is apparent at late times post-infection, occurs from within and requires the synthesis of at least one late virus protein. Other vaccinia virus proteins known to be involved in fusion of infected cells are a 14K membrane protein which is required for fusion, and the haemagglutinin which prevents fusion. The haemadsorption properties of cells infected with the parent virus and the deletion mutant were indistinguishable: both haemadsorbed chicken erythrocytes. A monoclonal antibody against the 14K protein inhibited fusion of vKL4-infected cells, thus demonstrating that in addition to the absence of the K2L gene product, the 14K protein is required for fusion to occur.

Introduction

Open reading frames (ORFs) with sequence homology to members of the serine protease inhibitor (serpin) superfamily have been identified in the leporipox, avipox and orthopox genera of the poxvirus family, e.g. malignant rabbit fibroma virus (MRV) (Upton et al., 1990), fowlpox virus (Tomley et al., 1988) and cowpox virus (Pickup et al., 1986), respectively. Three such ORFs have been identified in vaccinia virus, a member of the orthopox genus and the prototypical poxvirus. ORFs B13R and B22R [previously designated B24R (Smith et al., 1991)] are located at the right end of the genome within the HindIII B fragment (Kotwal & Moss, 1989; Smith et al., 1989), and K2L is at the left end within the HindIII K fragment (Boursnell et al., 1988).

Serine proteases, the targets of serpins, are physiologically important enzymes which are, for example, responsible for the regulation of homeostasis, and the control of the cascade systems of haemostasis and complement activation. Although the exact functions of gene products from the three vaccinia virus serpins remain to be determined, preliminary experiments have shown that deletion of either B13R or B22R increases antibody responses to a foreign antigen expressed from recombinant viruses (Zhou et al., 1990). In other poxviruses, serpins are known to be involved in the pathology and virulence of infection. In cowpox virus, a 38K protein (equivalent to the vaccinia virus B13R) is, at least partly, responsible for the haemorrhagic phenotype of pocks produced following infection of the chorioallantoic membranes of fertile hens’ eggs (Pickup et al., 1986). It also prevents the migration of white cells in vitro (Chua et al., 1990) and to infected lesions in vivo (Palumbo et al., 1989). The SERP1 gene of MRV, which has sequence homology with the vaccinia virus K2L, is involved in eliciting the rapidly fatal, immunosuppressive, systemic infection usually observed in infected rabbits, and its deletion attenuates the virus (Upton et al., 1990). Consequently, it seems likely that vaccinia virus serpins may be involved in pathogenesis.

If vaccinia virus recombinants expressing foreign genes are to be used as live vaccines, a greater understanding of virus–host interactions, and of the virus genes important for pathogenesis and virulence is desirable. The roles of the serpins during infection are therefore of interest. In this report the characterization of a deletion mutant lacking the K2L gene is described. In contrast to the normally non-fusogenic phenotype of the WR strain of vaccinia virus, this mutant is shown to
induce extensive fusion of infected cells, thus demonstrating that the K2L gene product is involved in the inhibition of fusion in WR-infected cells.

Two other virus proteins, the 14K protein and the haemagglutinin molecule (HA), regulate polykaryocytosis of vaccinia virus-infected cells. The 14K protein is expressed during the late phase of virus infection, is present on the envelope of intracellular virus particles (Rodriguez et al., 1985), and accumulates in both the cytoplasm and membrane of the infected cell (Rodriguez et al., 1987). This protein is essential for the formation and egress of mature virus particles from the infected cell (Rodriguez & Smith, 1990), and characterization of mutants generated from persistently infected Friend erythroleukaemia cells revealed that it is required for the fusion of vaccinia virus-infected cells (Rodriguez et al., 1987; Rodriguez & Esteban, 1987).

The vaccinia virus HA is a non-essential (Ichihashi & Dales, 1971; Shida & Dales, 1982) glycoprotein (Weintraub & Dales, 1974; Ichihashi, 1977; Shida & Dales, 1981), which is present on both the envelope of extracellular virus (Payne & Norrby, 1976) and the cytoplasmic membrane of infected cells (Weintraub & Dales, 1974; Ichihashi, 1977; Payne, 1979; Shida & Dales, 1981; Payne & Kristensson, 1985) at late times post-infection (Ichihashi et al., 1971). Studies using the IHD-J strain of vaccinia virus have shown that HA is transcribed throughout infection (early and late) and that a 68K form is derived, at late times post-infection, from the mature 85K protein; this process requires synthesis of late virus protein(s) and is inhibited by rifampicin (Brown et al., 1991). The 85K HA molecule on the surface of infected cells is responsible for the haemadsorption of chicken erythrocytes (Blackman & Bubel, 1972; Brown et al., 1991). In addition, HA regulates polykaryocytosis of vaccinia virus-infected cells. Its presence at the cell surface prevents fusion (Ichihashi & Dales, 1971) and mutations in the molecule can eliminate this function, thus inducing extensive cell-cell fusion (Seki et al., 1990).

Unlike the 14K protein, both HA and the K2L gene product inhibit polykaryocytosis. Here we describe investigations to determine whether these three proteins control different aspects of a single mechanism responsible for the fusion which occurs at late times post-infection with fusogenic strains of the virus, and with fusogenic mutants of normally non-fusogenic strains of vaccinia virus.

**Methods**

**Cells and viruses.** CV-1, BS-C-1, RK13, TK-143, BHK-21, D980R (an HPRT-negative HeLa derivative) and Vero cells were cultured in Dulbecco's modification of essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Stocks of vaccinia virus (strain WR) and derived recombinants were propagated in CV-1 or BHK-21 cells, and virus harvested from the infected cells was sedimented through a sucrose cushion (Mackett et al., 1985) and then titrated on BS-C-1 or CV-1 cells. All viruses used in individual experiments were grown and titrated in parallel.

**Plasmid construction**

(i) **pKL20.** The HindIII K fragment of vaccinia virus (strain WR) inserted into the HindIII site of pUC13 was obtained from B. Moss, NIH, Bethesda, Md., U.S.A. To remove the polylinker-derived SalI site, the plasmid was digested with PstI and BamHI, treated with Klenow polymerase and T4 DNA polymerase, and then religated to generate pYC19. The majority of the I-1 kb K2L ORF was deleted from pYC19 by excising a 0.7 kb SmaI–SalI fragment, then treating the digested plasmid with Klenow polymerase and religating to produce pYC14. To obtain the deletion vector pKL20, a 2 kb EcoRI cassette containing the Escherichia coli guanine phosphoribosyl transferase (ECsopor) gene, under the control of the vaccinia virus 7.5K promoter purified from pGpt 07/14 (Boyle & Coupar, 1988), was blunt-ended using Klenow polymerase and then inserted into Smal-linearized pYC14.

(ii) **pYC25.** A 1.5 kb PvuI–NsiI fragment containing the K2L ORF was purified from the cloned HindIII K fragment of the virus genome. After treatment with Klenow polymerase, the blunt-ended fragment was inserted into Smal-linearized pGS20 (Smith et al., 1983). The resulting insertion vector pYC25 contains the K2L ORF flanked by regions of the virus thymidine kinase (TK) gene.

**Generation of recombinant viruses.** vKL4, a deletion mutant lacking the K2L ORF, was generated by transfection of WR-infected cells with pKL20, and selected on CV-1 cells using the transient dominant negative activity of the K2L gene, under the control of the vaccinia virus 7.5K promoter purified from pGpt 07/14 (Boyle & Coupar, 1988). The resulting insertion vector pYC25 contains the K2L ORF flanked by regions of the virus thymidine kinase (TK) gene.

**Inoculation of animals.** Six- to nine-week old female BALB/c mice, anaesthetized with diethyl ether, were inoculated intranasally (i.n.) with 20 µl virus diluted in 10 mM-Tris-HCl pH 9. Groups of five animals were inoculated with 1 × 105, 1 × 104, 1 × 103 or 5 × 102 p.f.u. of virus per animal. Animals were monitored daily and symptoms were recorded. Bleeds were taken from animals inoculated with 1 × 105 p.f.u. of virus, both prior to and 3 weeks post-infection, and the vaccinia virus-specific antibody responses were determined by ELISA.

**Haemadsorption assay.** At 24 h post-infection with recombinant or wild-type vaccinia virus (at dilutions suitable to produce 100 to 200 plaques per 6 cm dish), cell monolayers were washed once with PBS and overlaid with a 0.5% suspension of chicken erythrocytes (from J. McCauley, AFRC Institute for Animal Health, Compton, Berkshire, U.K.) in PBS. After a 1 min incubation at room temperature, the unadsorbed erythrocytes were aspirated and the monolayers washed twice with PBS. Plaques were scored as haemadsorption-positive or -negative.

**Results**

**Construction of a deletion mutant lacking the K2L gene**

Preliminary analyses have shown that the K2L ORF is transcribed at early times during infection (Smith et al., 1989). To facilitate characterization of the putative gene product, a deletion mutant (vKL4) lacking the majority
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Fig. 1. Genomic structures of vaccinia virus mutants. (a) Schematic diagrams of the genomes. The majority of the K2L gene (stippled box) is removed in vKL4. In vKL6 the K2L gene, with portions of K1L and K3L, is replaced within the TK locus (diagonal stripes). (b, c) Autoradiographs of Southern blots. Virus core DNA was extracted, digested with HindIII, and separated by electrophoresis through a 0.8% agarose gel. After transfer to nitrocellulose membranes the DNA was probed with a SalI-HindIII fragment of HindIII K (b) or the HindIII J fragment of the genome (c). Sizes of DNA molecular size markers are indicated.

Table 1. Comparison of the mortality of BALB/c mice after i.n. inoculated with various titres of WR or recombinants vKL4 or vSAD7

<table>
<thead>
<tr>
<th>Titre (p.f.u.)</th>
<th>WR</th>
<th>vSAD7</th>
<th>vKL4</th>
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<tr>
<td>$5 \times 10^7$</td>
<td>$5/5^*$</td>
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<td>$1 \times 10^7$</td>
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* Results are expressed as no. dead/no. infected.

Virulence of the K2L deletion mutant

Although the K2L ORF is non-essential for the replication of vaccinia virus in vitro, deletion of the similar SERP1 gene from MRV decreases the virulence of infection in rabbits. For this reason, the pathogenicity of the deletion mutant vKL4 was compared with that of the parent virus. Mice inoculated i.n. with $10^5$ to $5 \times 10^7$ p.f.u. of either vKL4, WR or vSAD7 (a TK- recombinant) were examined daily for 2 weeks and their symptoms recorded. The results obtained are summarized in Table 1. Whereas the mice infected with the TK- recombinant remained healthy throughout the experiment, those infected with the higher doses of WR or vKL4 became ill after day 3. All mice infected with $5 \times 10^7$ p.f.u. of either vKL4, WR or vSAD7 or WR had died or were killed in extremis by day 7. These results show that the K2L gene is non-essential for replication in vivo and that, in the model used here, the gene product is not involved directly in the virulence of the WR strain of vaccinia virus. The SERP1 gene of MRV is at least partly responsible for the severe immunosuppression associated with MRV infection of rabbits (Upton et al., 1990). For this reason the antibody responses to vaccinia virus in mice infected with $1 \times 10^5$ p.f.u. of either WR or vKL4 were compared. The virus-specific Ig levels in mice immunized with vKL4 were similar to those obtained with WR-infected animals (results not shown).

vKL4-induced cell fusion

Although K2L is non-essential for replication in tissue culture, the phenotype of CV-1 cells infected with vKL4
was markedly different from the normal cytopathic effect observed in cells infected with the parent virus (Fig. 2b, c). Extensive polykaryocyte formation was observed in the vKL4-infected CV-1 cells. To ensure that this phenotypic change was caused by the absence of the K2L gene product, the K2L gene was reinserted in the TK locus of vKL4, thus generating vKL6. Although the PvuII–NsiI fragment used to create vKL6 extends 182 nucleotides upstream and 262 nucleotides downstream of the K2L gene, K2L is the only complete ORF reinserted. The genomic structure of vKL6 was confirmed by Southern blot analyses (Fig. 1). Reinsertion of the K2L ORF increased the size of the HindIII J fragment from the wild-type 5 kb to 6.5 kb, and resulted in the hybridization of this fragment with the HindIII K probe. Whereas CV-1 cells infected with vKL4 underwent extensive cell–cell fusion, cells infected with either vKL6 or WR were indistinguishable and did not fuse (Fig. 2c, d and b, respectively) confirming that the altered phenotype of vKL4-infected cells was caused by the deletion of the K2L gene, rather than a spontaneous mutation elsewhere in the genome. In CV-1 cells infected at an m.o.i. of 0.5, the polykaryocyte frequency was greater than 90%, and over 60% of the fused cells contained 20 or more nuclei; in some over 60 nuclei were counted.

Initial observations revealed that in vKL4 plaques, polykaryocytosis did not occur until 2 to 3 days post-infection and was first apparent at plaque centres. At higher multiplicities, although fusion occurred after a shorter time, it remained a late phenomenon. This suggested that the fusion occurred from within, i.e. as a result of virus protein synthesis, rather than from without, i.e. as a result of incoming virus particles. To test this, cycloheximide-treated CV-1 cells infected with vKL4 were maintained for 2 days in the presence of the drug (Fig. 2e). Fusion was not observed at multiplicities of up to 200, thus confirming that the cell–cell fusion caused by vKL4 occurs from within. Therefore, in cells infected with the wild-type virus the K2L gene product has a role in the prevention of polykaryocyte formation.

Although cell–cell fusion is a late phenomenon, the K2L gene is transcribed early during infection with the parent virus (Smith et al., 1989). Hence, a late vaccinia virus protein might be necessary to elicit fusion. To examine this possibility, CV-1 cells were infected with vKL4 in the presence of cytosine β-d-arabinofuranoside (ara-C), which inhibits synthesis of virus DNA and thus late proteins. Polykaryocytes did not form in ara-C-treated cells infected with vKL4 (Fig. 2f), thus corroborating the observations that the fusion, which in WR is inhibited by the early K2L gene product, occurs late and involves at least one late vaccinia virus protein.

The predicted K2L gene product has sequence homology to the serpin family of proteins, and consequently may act as a protease inhibitor in WR-infected cells. Perhaps it is this function which is important in preventing cell–cell fusion. If so, a protease must exist which the K2L gene product inhibits, and which if active
might trigger fusion of infected cells. Analysis of the predicted amino acid sequence of K2L revealed a putative N-terminal signal sequence and signal peptidase cleavage site (Smith et al., 1989), suggesting that the encoded protein is membrane-associated or secreted from infected cells. Therefore, the serum used in the tissue culture medium was considered as a possible source of protease(s) which might be inhibited by the K2L gene product. To investigate this, CV-1 cells infected at an m.o.i. of 0.001 with either WR or vKL4 were maintained in DMEM containing 0, 2 or 10% FBS (Fig. 3). Extensive fusion occurred in cells infected with vKL4 which were incubated in medium containing 2 or 10% FBS (Fig. 3d, f), but in the absence of FBS the WR- and vKL4-infected cells were indistinguishable (Fig. 3a, b). This suggested that a constituent of the FBS was involved in the cell-cell fusion which occurs in the absence of the K2L gene product. However, if a serum protease were involved in syncytium formation a similar result would be expected in cells infected with a higher multiplicity of vKL4. This was not found to be the case. At an m.o.i. of 1, polykaryocytes formed in cells infected with vKL4 in the absence or presence of FBS in the maintenance medium (Fig. 4a, b), a result inconsistent with a constituent of the serum being required for fusion of vKL4-infected cells. Although the extent of fusion was slightly less in cells maintained without serum (Fig. 4a), this may be attributable to a reduction in the ability of the cells to fuse after maintenance in such conditions. Fusion was not observed in cells infected with WR at either multiplicity in the presence or absence of FBS (Fig. 4c, d).

To confirm that the serum was not a source of a protease which in WR-infected cells is inhibited by the K2L gene product, but which in the absence of K2L (i.e. in vKL4-infected cells) is involved in syncytium formation, infected CV-1 cells were maintained in a commercially prepared serum-free medium (Serotec CG medium) rather than unsupplemented DMEM (Fig. 5). CG medium is designed to facilitate the culture of cells in the absence of serum. Therefore, the decrease in the extent of cell-cell fusion seen in vKL4-infected cells maintained without FBS should be overcome if, as believed, it is caused by an alteration in the state of the cells rather than the absence of a specific serum factor required for fusion. In cells infected with vKL4 at an m.o.i. of 1, extensive polykaryocyte formation occurred irrespective of the presence or absence of FBS (Fig. 5a, b). Fusion was also obtained with cells infected at an m.o.i. of 0.001 (Fig. 5c, d). This result supports the earlier conclusion that if a protease which is normally inhibited by the K2L gene product is involved in mediating cell-cell fusion, it does not originate from the FBS, but is of intracellular origin. At an m.o.i. of 1, the degree of fusion observed in unsupplemented CG medium routinely exceeded that seen with DMEM containing 2% FBS (compare Fig. 5a with Fig. 4b). Therefore, it is possible that the decreased extent of fusion observed in vKL4-infected CV-1 cells maintained in DMEM without FBS was caused by adverse effects on the fluidity of the cell membranes.
Fig. 4. The effect of FBS on polykaryocytosis of cells infected with a higher m.o.i. CV-1 cells infected at an m.o.i. of 1 with either WR (c, d) or vKL4 (a, b) were maintained in 0 (a, c) or 2% (b, d) FBS for 48 h and then photographed using phase contrast.

Fig. 5. Increased polykaryocytosis in synthetic medium. CV-1 cells infected with vKL4 at an m.o.i. of 1 (a, b) or 0.001 (c, d) were maintained in DMEM or CG medium supplemented with 0 (a, c) or 2% (b, d) FBS for 48 h and then photographed using phase contrast.

If cellular factors are involved in fusion of vKL4-infected cells the ability of the virus to induce fusion may vary according to the cell type infected. To examine this a number of different types of cell, all of which grow as monolayers in vitro, were infected at a multiplicity of 1 with either WR or vKL4. Extensive fusion occurred in vKL4-infected CV-1, BS-C-1 and D980R cells, but was not observed in TK-143, RK13, BHK-21 or Vero cells. In the cell types which did not undergo fusion, the c.p.e. of WR and vKL4 was indistinguishable (results not shown). The finding that polykaryocyte formation is specific to certain cell types suggests the involvement of one or more host factors and is consistent with a K2L-inhibited protease being of cellular origin.

Interactions between K2L and other vaccinia virus fusion factors

We wished to determine whether the three proteins with identified roles in vaccinia virus-induced cell–cell fusion, K2L, 14K and HA, are components of the same fusion mechanism. A monoclonal antibody (MAb), C3, which recognizes the 14K protein (Rodriguez et al., 1985), and which prevents the extensive polykaryocytosis caused by a spontaneous virus mutant 87–4 (Rodriguez et al., 1987) and by the IHD-W strain of vaccinia virus (Gong et al., 1990), was tested for its ability to inhibit fusion of vKL4-infected cells (Fig. 6). Fusion was not observed in vKL4-infected cells in the presence of MAb C3 (Fig. 6), demonstrating that the fusion of infected cells, which is normally prevented by the K2L gene product, is mediated by the 14K protein.

Like the K2L gene product, the vaccinia virus HA inhibits cell–cell fusion. Although the active sites for fusion inhibition and haemadsorption map to distinct regions of the HA molecule, a number of haemadsorption-negative mutants of the normally non-fusogenic IHD-J strain of vaccinia virus induce fusion of infected cells (Seki et al., 1990). If the K2L gene product inhibits a
Vaccinia virus serpin prevents cell fusion

Fig. 6. Inhibition of vKL4-induced polykaryocytosis with an anti-14K protein MAb. CV-1 cells infected at an m.o.i. of 50 with either WR (a, b) or vKL4 (c, d) were maintained in the presence of a 1:50 dilution of ascitic fluid of an anti-14K MAb (b, d) or of an irrelevant anti-vaccinia virus MAb (a, c) for 48 h and then photographed using phase contrast.

protease which is otherwise able to cleave HA (see Discussion for more detailed consideration of this hypothesis), cells infected with WR should haemadsorb chicken red blood cells whereas those infected with vKL4 may not due to an alteration of HA. This was not found to be the case (data not shown). A number of different cell types were infected with WR or vKL4 and the ability of the plaques to haemadsorb chicken red blood cells was compared. Both viruses were able to adsorb red blood cells in the cell lines tested (BS-C-1, CV-1, D980R, RK13, and TK− cells) irrespective of the ability of the cells to undergo fusion.

Discussion

The function of the vaccinia virus K2L gene was investigated, because the vaccinia virus ORFs (K2L, B13R and B22R) with homology to members of the serpin superfamily may be important in virus pathogenesis. A virus deletion mutant (vKL4) lacking the majority of the K2L ORF was produced. The construction of this virus demonstrated that K2L is non-essential for vaccinia virus (strain WR) replication in vitro (seven different cell lines were tested), and is consistent with the data of Perkus et al. (1986, 1989, 1991) which show this region of the genome to be non-essential in both the WR and Copenhagen strains of vaccinia virus.

Of the three vaccinia virus serpins, K2L is the most closely related to SERP1 of MRV. It shares 28% amino acid identity with SERP1, and has the same P1 residue (arginine) in the putative active loop which indicates that both proteins may inhibit serine proteases that cleave after this amino acid (Upton et al., 1990). Although SERP1 is non-essential for virus replication in vitro, it is involved in the pathogenesis of MRV in vivo: its deletion attenuates the virus and reduces the degree of immunosuppression caused in infected rabbits (Upton et al., 1990). In contrast, K2L is non-essential for virus virulence in vivo: the pathogenicity and immunogenicity of the deletion mutant and the wild-type virus were indistinguishable in i.n. inoculated BALB/c mice. The different roles of SERP1 and K2L in infection is perhaps unsurprising because, although the P1 residues are the same, the other amino acids in the active loop area are different (ARSSP in K2L, and PRNAL in SERP1) which may alter the specificity of the serpin (reviewed by Carrell et al., 1987). Secondly, unlike K2L, the SERP1 gene is not transcribed at early times during infection (Upton et al., 1990).

Surprisingly, although non-essential for replication in vitro, the absence of the K2L gene product in infected cells resulted in extensive cell–cell fusion at late times post-infection, a c.p.e. not normally associated with the WR strain of vaccinia virus. Characterization of this phenomenon revealed that fusion occurred from within (it is prevented by cycloheximide) and that, in addition to the absence of the early K2L gene product, at least one late virus protein was required. The fusion was cell type-specific, showing that a feature of the host cell is involved in polykaryocytosis. However, no consistent characteristic which might be necessary for fusion was identified after consideration of the organ from which the cell line was derived, whether the cells were epithelial or fibroblast-like, or of primate or non-primate origin.

Because the K2L ORF has sequence homology to the serpin family, the gene product may function as a protease inhibitor. The serine proteases secreted by different cell types vary, hence the presence of a K2L-inhibited protease may be a host factor which could influence the ability of a cell line to fuse. Such a protease must be of intracellular origin. A protease of extracellular origin is not an essential component of the fusion mechanism, as fusion occurred in cells infected with the deletion mutant regardless of the presence or absence of serum in the maintenance medium. However, fusion was
less extensive in the cells maintained in the unsupplemented DMEM. This effect was overcome by using a synthetic medium prepared specifically for cell culture in the absence of serum. It is likely that the suboptimal culture conditions (unsupplemented DMEM) limited the extent of fusion by affecting the fluidity of the cell membranes. It is known, for example, that the ability of cells to fuse is altered by the lipid composition of the membrane (Ross et al., 1990). A second factor which limited the degree of fusion was the m.o.i. used: fusion was less extensive at lower multiplicities. In a number of examples of virus-induced polykaryocytosis, fusion depends on the concentration of the fusogenic virus protein (Henis et al., 1989; Ellens et al., 1990; Clague et al., 1991). If the syncytium formation induced by vKL4 is similarly dependent on the concentration in cell membranes of a virus-specified fusogen, it would explain why the extent of fusion observed at lower multiplicities of infection was less.

The K2L gene is transcribed at early times post-infection. Hence, if the encoded protein functions as a serpin, it would be available to inhibit a target protease prior to the synthesis of late virus proteins, and thus protect a specific late protein from proteolysis. Depending on the complexity of the mechanism of vaccinia virus-induced cell–cell fusion, this late protein might be either directly or indirectly responsible for polykaryocytosis. In view of this hypothesis, it is predicted that a late virus protein may exist which in its native form, i.e. in WR-infected cells where it is protected by K2L, either is unable to elicit cell–cell fusion or prevents it occurring. In the absence of the K2L gene product, i.e. in cells infected with the deletion mutant, the active protease may effect an alteration in this late virus protein which renders it either able to elicit fusion or no longer able to prevent it. This is consistent with the finding that a late virus protein was required for fusion of vKL4-infected cells.

A late protein which is present on the cell surface, and which is known to be required for cell fusion is the 14K protein. An anti-14K MAb added to cells infected with the deletion mutant blocked fusion (Fig. 6), thus demonstrating that the 14K protein is involved in the polykaryocytosis which occurs in the absence of K2L. However, there is no evidence to suggest that the role of the 14K protein in fusion is associated with conformational changes of this protein.

A second protein, present at late times during infection and also known to affect fusion, is the fusion inhibition factor, HA. This is a more likely candidate for proteolysis in the absence of K2L. The ability of this protein to inhibit fusion can be negated by locational and structural alterations (Shida & Matsumoto, 1983; Payne, 1979; Seki et al., 1990), and some HA-specific MAbs are able to induce fusion of cells infected with non-fusogenic strains of vaccinia virus. In an attempt to establish a link between the fusion mechanisms inhibited by HA and the K2L gene product, haemadsorption assays were used. It was anticipated that the parent virus would haemadsorb, but that if the absence of the K2L gene product permits proteolytic degradation of HA, the deletion mutant would have lost this phenotype. In fact, the haemadsorption properties of both viruses were indistinguishable. Both the parent virus and the deletion mutant haemadsorbed in the cell lines tested. Although this result failed to demonstrate that HA and K2L control the same fusion mechanism, it is not inconsistent with the known properties of HA: independent regions of the molecule are responsible for fusion inhibition and haemadsorption.

The roles of the K2L gene product, the 14K protein and HA can be accommodated into a hypothesis which at least partly explains the results described here and thus the mechanism of fusion in cells infected with the deletion mutant lacking K2L. The 14K protein, which is present on infected cell membranes, is required for fusion to occur. This protein may normally be masked by another virus protein (perhaps HA) and consequently be unable to elicit fusion. A protease which is able to alter the masking protein could expose the 14K protein, thus enabling fusion to occur. However, this would not occur in cells infected with wild-type virus if the K2L gene product inhibits the required protease, but in the absence of K2L the protease would be active and cell–cell fusion could occur.

In summary, the work described here shows that the K2L gene product prevents fusion of cells infected with the WR strain of vaccinia virus. Deletion of the K2L gene from the virus genome causes infected cells to undergo extensive polykaryocytosis, which is mediated by the previously identified 14K fusion protein. If, as seems likely, a single seemingly complex mechanism is responsible for vaccinia virus-induced fusion, both the K2L gene product and the HA molecule inhibit this. The ability of a virus to induce cell–cell fusion in vivo is usually considered advantageous, because it facilitates spread without exposure to circulating neutralizing antibodies. However, the rigorous control of fusion from within in vaccinia virus-infected cells suggests that the limitation or prevention of this phenomenon is advantageous to the later parts of the virus life cycle. Perhaps fusion limits egress or spread of the virus; such possibilities need to be investigated before the role of the K2L gene product in the virus life cycle can be firmly assigned.

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