The punctate sites of accumulation of vaccinia virus early proteins are precursors of sites of viral DNA synthesis

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Several vaccinia virus early proteins (encoded by genes B1R, H5R and I3L) synthesized in the presence of an inhibitor of DNA synthesis localize, at least in part, to punctate inclusions that are visible by immunofluorescence in the cytoplasm of poxvirus-infected cells. It is shown that these inclusions contain DNA (visualized by DAPI staining of the infected cells) and that the number of inclusions is proportional to the amount of input virus. Their mean diameter (about 680 nm) was larger than that of purified vaccinia virus particles. When the inhibition of DNA synthesis was reversed, incorporation of BrdU into the B1R particles was demonstrated after labelling for 30 min, suggesting that these cytoplasmic focal sites correspond to viral DNA replication complexes that have initiated normally but are inhibited at the step of DNA chain elongation. These experiments suggest strongly that these inclusions are the precursors of the virosomes.

Vaccinia virus (VV), a member of the Poxviridae, is a large virus that contains a DNA genome that contains about 200 open reading frames and replicates in the cytoplasm of infected cells (Moss, 1996). The early proteins are translated from mRNA transcribed from input viral cores, which are injected into the cytoplasm and contain all the enzymes and protein factors required for the transcription of early genes. In contrast, the synthesis of the intermediate and late proteins requires viral DNA replication, which occurs in the virosomes, granular sites formed in the cytoplasm of VV-infected cells. Each DNA replication centre originates in an infecting particle (Cairns, 1960). Several early proteins involved in viral DNA synthesis have been identified (reviewed by Traktman, 1996). Notably, they include the serine/threonine protein kinase encoded by the B1R gene and the single-stranded DNA-binding protein I3L (also synthesized at the intermediate time of infection). Both proteins localize in the virosomes (Banham & Smith, 1992; Rochester & Traktman, 1998). The H5R phosphoprotein is the major early virosome component (Beaud et al., 1995; Murcia-Nicolas et al., 1999), but the physiological function of its association with the virosomes remains unknown. Recently, it has been shown that several early proteins synthesized in the presence of an inhibitor of DNA synthesis localize, at least in part, to punctate inclusions in the cytoplasm of poxvirus-infected cells. They include the B1R and H5R proteins (Beaud & Beaud, 1997), the single-stranded DNA-binding protein I3L (Rochester & Traktman, 1998) and an ectromelia virus-encoded RING protein essential for DNA replication in macrophages (Senkevich et al., 1995). However, these focal sites were not characterized. In this paper, we have investigated whether they are the precursors of the virosomes.

Because it has been shown previously that the DNA present in isolated vaccinia virion particles can be visualized by epifluorescence after DAPI staining (Vanderplaschen & Smith, 1997), we used the same technique to investigate whether the particles to which the B1R, I3L and H5R proteins localized also contained DNA. Firstly, we confirmed that the DNA present in the purified VV particles could be stained with DAPI (data not shown). Next, cells infected with VV in the presence of 5 mM hydroxyurea (HU) were stained with both DAPI and an anti-B1R antibody (Banham & Smith, 1992). About a dozen particles present in the field of the microscope (and not located in the cell nucleus) were stained both with the antibody against the B1R protein and with DAPI, as shown by the arrows in Fig. 1a, b. A similar result was obtained with particles synthesized in VV-infected cells stained with an antibody prepared against an N-terminal H5R peptide (a kind gift of G. Griffiths, EMBL, Heidelberg, Germany) or an antibody that we prepared against recombinant I3L protein (data not shown) in the presence of HU (Fig. 1). As expected, these particles were not detected when the primary antibody was a pre-immune serum or was omitted (data not shown). It was therefore concluded that most if not all of the particles to which the early B1R, I3L and H5R proteins located did contain DNA.

It is known that, in the presence of HU, VV development proceeds up to the second stage of uncoating, when DNA replication is inhibited, and it is thus very likely that this DNA was derived from the input virion particles. In order to confirm this hypothesis, we counted the number of particles that were stained with the anti-B1R antibody when the cells were
infected at different m.o.i. in the presence of 50 μM cytosine arabinoside (araC). The number of particles formed per cell appeared to be proportional to the input virus up to 12 p.f.u. per cell and continued to increase for higher m.o.i. (Fig. 2). This experiment suggested strongly that the B1R particles were derived from the input VV particles when the cells were infected in the presence of an inhibitor of DNA replication. It is known that HU and araC inhibit viral DNA synthesis at the elongation step, because 5 mM HU is known to deplete the deoxyribonucleotide pool, primarily dATP (Slabaugh et al., 1991), and araC inhibits the incorporation of deoxyribonucleotides into DNA (Cozzarelli, 1977). Therefore, it was likely that the DNA-containing particles present in cells infected with VV in the presence of either inhibitor represented viral DNA replication complexes inhibited at the step of DNA chain elongation.

Fig. 1. Colocalization of VV early proteins and HU particles stained with DAPI. BSC-40 cells were infected with VV (WR strain, 8.5 p.f.u. per cell) and incubated at 37 °C in the presence of 5 mM HU for 5 h (HU was also present during the 1 h adsorption). The cells were then washed with PBS, fixed with 4% paraformaldehyde in PBS (10 min at room temperature) and permeabilized with cold acetone:methanol (1:1) and processed with an anti-B1R protein serum (a, b), an antibody prepared against an N-terminal H5R peptide (c, d) or an antibody that we prepared against recombinant I3L protein (e, f). The second antibody was an FITC conjugate of goat anti-rabbit IgG (Caltag) and the samples were mounted in Mowiol-DAPI (1 μg/ml). Pairs of images (a and b, c and d, e and f) represent the same cells photographed with a conventional fluorescent Leitz microscope with filters for DAPI (a, c, e) and FITC fluorescent emissions (b, d, f). Arrows indicate colocalization of the DAPI and FITC spots.
Localization of vaccinia virus early proteins

It was interesting to compare the size of the B1R particles to that of purified virions. Images of VV particles, stained with an antibody prepared after immunization of a rabbit with VV (Valbiotech), were obtained with a confocal microscope and the diameters of the FITC-labelled virions were measured by using NIH Image 1.62b7 software. The purified VV particles were mostly isolated virions, because about two-thirds of the particles had a diameter of less than 600 nm (data not shown). A mean diameter of $404 \pm 19$ nm $(n = 116)$ was obtained for the virus particles, diameters of which were between 245 and 595 nm, corresponding to the size measured with an electron microscope (approximately 250 by 350 nm) and also in agreement with a previous measurement (Vanderplasschen & Smith, 1997). In contrast, the particles present in the cells infected in the presence of araC and stained with the anti-B1R antibody were two to four times larger than the purified virions, with a bimodal distribution corresponding to mean diameters of $678 \pm 77$ nm $(n = 37)$ and $1579 \pm 72$ nm $(n = 34)$. We could not establish whether the larger B1R particles corresponded to aggregated input particles, but it was clear that the B1R particles were larger than purified virions. This increase in size may result from an increase in the protein

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**Fig. 2.** Numbers of B1R particles present in cells infected with VV at different m.o.i. and in the presence of araC. BSC-40 cells were infected with VV at the m.o.i. indicated in the presence of $50 \mu$g/ml araC and incubated for 3 h in the presence of the inhibitor. Cells were then processed with the anti-B1R antibody (FITC) and stained with DAPI as described in the legend to Fig. 1. At least fifty B1R particles (FITC) and corresponding nuclei (DAPI) present in different fields of the confocal microscope were counted and the ratio of particles per cell is plotted (bars correspond to the SEM).

**Fig. 3.** Incorporation of BrdU into B1R particles after reversal of the blockage of DNA synthesis. Cells were infected for 2 h in the presence of 5 mM HU as described in the legend to Fig. 1 and labelled with 25 $\mu$g/ml BrdU for 30 (a–d) or 60 (e–h) min in the presence of HU (a, b, e, f) or after its removal from the culture medium (c, d, g, h). The cells were then processed as described previously (Girard et al., 1991), first with the anti-B1R serum (and a Cy3 conjugate of goat anti-rabbit IgG) and then with the BrdU antibody and an FITC conjugate of goat anti-rabbit IgG. Pairs of images (a and b, c and d, e and f, g and h) represent the same cells photographed with a TCS4D Leica confocal microscope equipped with an argon–krypton laser operating with the 488 nm line for FITC (b, d, f, h), and with the 568 nm line for Cy3 (a, c, e, g) fluorescent emissions. Images were collected by using an oil immersion lens (63 x, NA 1.4). Focal series of up to 10 sections apart were collected for each specimen and then processed to produce single composite images (extended focus).
content due to newly synthesized protein and/or to unfolding of the viral DNA when viral DNA replication is initiated.

Because it is known that the inhibition of DNA synthesis is relieved when HU is removed from the cell culture medium and that virosomes are then formed (Esteban & Holowczak, 1978), we investigated whether BrdU was incorporated after HU removal into the focal sites visualized by means of the anti-B1R antibody. To this end, cells infected for 2 h in the presence of 5 mM HU were then pulsed with 25 μM BrdU for 30 or 60 min in the absence of HU (and, as a control, in the presence of HU). The cells were then stained by indirect labelling (Girard et al., 1991) with an antibody specific for BrdU (Becton Dickinson) and an FITC-labelled goat anti-rabbit IgG; Caltag) and with the anti-B1R antibody (and a Cy3-labelled goat anti-rabbit IgG; Caltag). Incorporation of BrdU into B1R particles was apparent after 30 min of BrdU labelling when the inhibitor of DNA synthesis had been removed from the medium (Fig. 3 c, d), whereas weak incorporation of BrdU was detected when labelling was carried out in the presence of HU, probably because the inhibition of DNA synthesis was not complete (Fig. 3 a, b). As expected, labelling of virosomes after 60 min incorporation was stronger when the inhibition of DNA synthesis had been reversed (Fig. 3 g, h), but remained low in the presence of HU (Fig. 3 e, f). These experiments suggested strongly that the B1R particles formed in the presence of HU were DNA replication complexes that were inhibited at the DNA elongation step.

We tried to isolate these inclusions from cytoplasmic extracts of cells infected with VV in the presence of araC, but our attempts at biochemical purification failed, presumably because these particles were not stable (A. Murcia-Nicolas, personal communication). However, our experimental approach based on immunofluorescence may be extended to other early proteins when corresponding antibodies are available.

The colocalization of the B1R protein kinase and different early proteins to viral DNA replication complexes suggested that one of the latter might be a physiological substrate of the kinase involved in DNA replication, because temperature-sensitive mutants of the B1R protein kinase are DNA− (Rempel & Traktman, 1992). However, the I3L protein is not a likely substrate of the B1R protein kinase (Rochester & Traktman, 1998) and known functions of the H5R protein imply a role at a late time of infection: H5R protein corresponds to the late transcription elongation factor 4: purification, cloning, and overexpression. Journal of Virology 70, 6796–6802.


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