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A 165 kd PROTEIN OF THE HERPES SIMPLEX VIRION SHARES A COMMON EPITOPE WITH THE REGULATORY PROTEIN, ICP4

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We have investigated the possibility that immediate-early (IE) protein ICP4 could be a part of herpes simplex virus type 1 (HSV-1) virion particle. Immunodetection with a monoclonal antibody against ICP4 reveals that a component of the virion, migrating at 165 kd, shares a common epitope with this immediate-early protein. Immunolocalization studies on purified virions indicate that the antigen can be detected only in virions without membranes, and is located outside the capsid, most probably in the tegument. Ultrastructural localizations on HSV-1 infected BHK cells extracted with a nonionic detergent confirm that the protein immunoreacting with anti-ICP4 is present in virions.

Viral protein IE 175K, or infected-cell protein ICP4, is one of the five polypeptides synthesized early in herpes simplex virus type 1 (HSV-1) infected cells [1,2]. Genetic and biochemical studies have demonstrated that ICP4 is required in a functional form throughout the HSV infectious cycle, for expression of early and late genes [3,4]. ICP4 acts as a positive regulator of early gene transcription and a negative regulator of ICP4 gene transcription [3-8]. This polypeptide of 175 kd undergoes post-translational modifications [8] and is phosphorylated [9,10]. Immunolocalization studies have detected ICP4 mainly in nuclei of cells infected with wild-type virus [11-15].

The total number of virion polypeptides has been estimated at 24 to 33 [16]. Certain component(s) of the infecting HSV virus particle have been found responsible for regulatory functions: a virion-associated polypeptide, Vmw65, activates immediate-early genes IEs 0, 4, 22, 27 and 47 [17,18]. Conflicting results have been published on whether or not ICP4 could be a part of the virion. A polypeptide with an electrophoretic mobility identical to that of ICP4, VP4, reproducibly copurifies with virions [9,16,19]. However Cabral *et al.* [12], in their study on localization of ICP4 at the electron microscopic level, failed to detect an association of ICP4 with nucleocapsids or enveloped virion particles. Using a different monoclonal antibody to ICP4 [20], and immunodetection with protein A-gold [21,22], we now present evidence that a protein with the same immunological determinant as ICP4, is associated with the virus particle.

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Materials and Methods

Cells and Virus infection

Baby hamster kidney cells (BHK-21) were grown at 37°C in α-medium (Gibco) supplemented with 10% foetal calf serum (Flow Laboratories, mycoplasma free). For viral infection, BHK cells were infected with HSV-1 strain KOS, and incubated for different lengths of time at 34°C in α-medium supplemented with 2% foetal calf serum.

Virion purification

For virions production, BHK cells were infected at a multiplicity of infection (m.o.i.) of 1 p.f.u per cell with HSV-1 and incubated for 36-48 h at 34°C. Infected cell were scraped off in medium with a rubber policeman and centrifuged. Pellet was resuspended in a small volume of ice-cold hypotonic buffer (10 mM Tris-HCl pH 7.8, 3 mM $CaCl_2$, 1 mM EDTA, 10 mM KCl). Nuclei were isolated [23] and removed by low-speed centrifugation.

Enveloped virus particles were purified from cytoplasmic extracts, using Dextran T10 gradients [16].

Immunolocalization on isolated virus particles

All treatments were carried out in microtiter plate wells [21]. Purified virions were resuspended in PBS containing 1% egg albumin. A 25-µl volume of suspension was mixed with 25 µl of monoclonal antibody 58S against ICP4 [20] diluted 1:10 in PBS containing 1 mM phenylmethylsulphonide fluoride (PMSF) as a protease inhibitor. The mixture was incubated for 45 min at 37°C in a humidified environment. A 25-µl volume of protein A-gold preparation was added directly to the antigen-antibody mixture and incubated for an additional 45 min. A 50-µl volume of the antigen/antibody/protein A-gold suspension was then transferred to another microtiter well containing a Formvar/carbon EM grid placed on top of 1% agar, and allowed to diffuse for 30 min. Grids were washed with PBS then with water, before negative staining with 1% phosphotungstic acid pH 7.2, for 1 min.

Immunolocalization in infected cells

BHK cells were infected at 20 p.f.u. per cell and incubated for 2 or 5 h at 34°C. After washing in PBS, cells were resuspended in a lysing buffer containing 10 mM HEPES pH 7.4, 5 mM NaCl, 2.5 mM $MgCl_2$, 0.3 M sucrose and 1 mM PMSF. To prepare cytoskeletons, Triton X-100 (10%) was added dropwise to the cells until a final concentration of 1% [22,24]. After fixation in 1% glutaraldehyde, these residual structures were partially dehydrated in ethanol and embedded in Lowicryl K4M (JBEM Services). This resin is a water compatible medium, and allows particularly good immunostaining with a reasonable degree of ultrastructural preservation [22]. Thin sections mounted on nickel grids were successively incubated for 10 min on a drop of phosphate buffer containing 0.5% Tween 20; for 30 min on a drop of the 58S monoclonal antibody against ICP4 diluted 1:25; and finally for 15 min on a drop of protein A-gold complex containing 0.2 mg/ml of polyethylene glycol. The thin sections were washed thoroughly with buffer, rinsed with distilled water and dried before staining with uranyl acetate and lead citrate .

Western blotting and immunological detection

Proteins (50 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described previously [23]. The nitrocellulose membranes were successively incubated for 4 h with the monoclonal antibody 58S (diluted 1:500 in the buffer), and for 2 h with anti-mouse IgG linked to peroxidase (Boehringer-Mannheim). The complexes were revealed with diaminobenzidine (5 mg/ml) in 50 mM Tris-HCl pH 7.4 containing 0.01% H_2O_2.

Results and Discussion

Detection of ICP4 in virion proteins

To establish whether or not ICP4 was part of the virus particle, proteins from 5-h infected cells or from virion preparations were separated on SDS-PAGE and transferred to nitrocellulose paper. Figure 1 lane C shows the protein profile of virions after SDS-PAGE and amido black staining. This profile was similar to the one described by Spear and Roizman [16] for their purified virions. Proteins from infected cells or virions were then probed for presence of ICP4 with the monoclonal antibody 58S against ICP4 [20]. A polypeptide with the same immunological determinant as ICP4 was detected in virion preparations (Fig. 1, lane B). This polypeptide comigrated with VP4 at 165 kd, but migrated slightly faster than the 170 kd polypeptide detected in infected cells (Fig. 1, lane A).

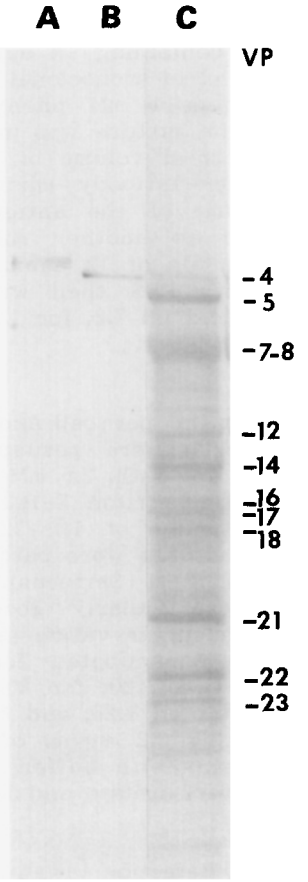

Fig. 1: **Detection of ICP4 in purified virions.** 50 µg of proteins from either 5-h HSV-1 infected BHK cells (lane A) or purified HSV-1 virions (lanes B and C) were separated by SDS-PAGE and transferred to nitrocellulose. (lanes A and B) Filters were probed with a 1:500 dilution of the 58S anti-ICP4, and immunoreactive proteins were detected with anti-mouse IgG labelled with peroxidase. (lane C) Virion proteins (VP) were detected by amido black staining.

Three structural variants of ICP4 designated ICP4a, ICP4b and ICP4c in order of decreasing mobility during SDS-PAGE have been described [9,25], and the 165 kd present in the virion could well correspond to ICP4a or b variants. These variants differ in number and location of phosphate moieties added post-translationally [26].

Immunolocalization of the antigen in virion

To find where the antigen was located, an indirect immunogold staining was used on isolated virions [21], followed by negative staining of the preparations. Figure 2A shows that intact virions did not react with the monoclonal antibody. However after treatment with 1% Triton X-100, the virion particles were decorated with gold (Fig. 2B). Figures 2B and 2C show that labelling occured mainly in the remaining tegument, outside the capsid. As controls, detergent-treated virions were reacted with PBS without any antibody, with polyclonal antibody against nuclear lamins [23], or with the Q1 monoclonal antibody against the viral alkaline exonuclease [27]. In all these cases, reactions were negative. Virion particles were further extracted with either 0.5 M, 1 M or 2 M urea before incubation with anti-ICP4 serum. All these treatments failed to remove the antigen (Fig. 2D and 2E).

The finding that detergent-treatment of virions was necessary in order to detect the ICP4 epitope explains results from Cabral *et al.* [12] who did not detect any association of ICP4 with virions at the electron microscopic level.

Immunolocalization in cytoskeletons

To confirm that results obtained with purified virions were not caused by cellular contamination of virus preparations, HSV-1 infected BHK cells were examined at the electron microscopic level. Since previous experiment had established that the antigen could only be detected in detergent-treated virions, infected cells were extracted with 1% Triton X-100 prior to fixation and embedding. In spite of detergent treatment and reduced fixation needed to preserve immunological reactivity, the ultrastructural morphology was adequately preserved. At early times after infection, virions could be visualized attached to the surface of infected cells. After incubation with the 58S monoclonal antibody and protein A-gold, gold particles were clearly detected on these virions (Fig. 3A and 3B), there was no background outside the cells. Inside the cytoplasm, pockets of virions were also decorated with gold particles (Fig. 3C).

Later during infection, viral nucleocapsids could be observed inside nuclei. These nucleocapsids were not labelled by protein A-gold (results not shown), indicating that the protein reacting with anti-ICP4 is probably not attached to nucleocapsids during their formation and must be acquired by the virion at later stages.

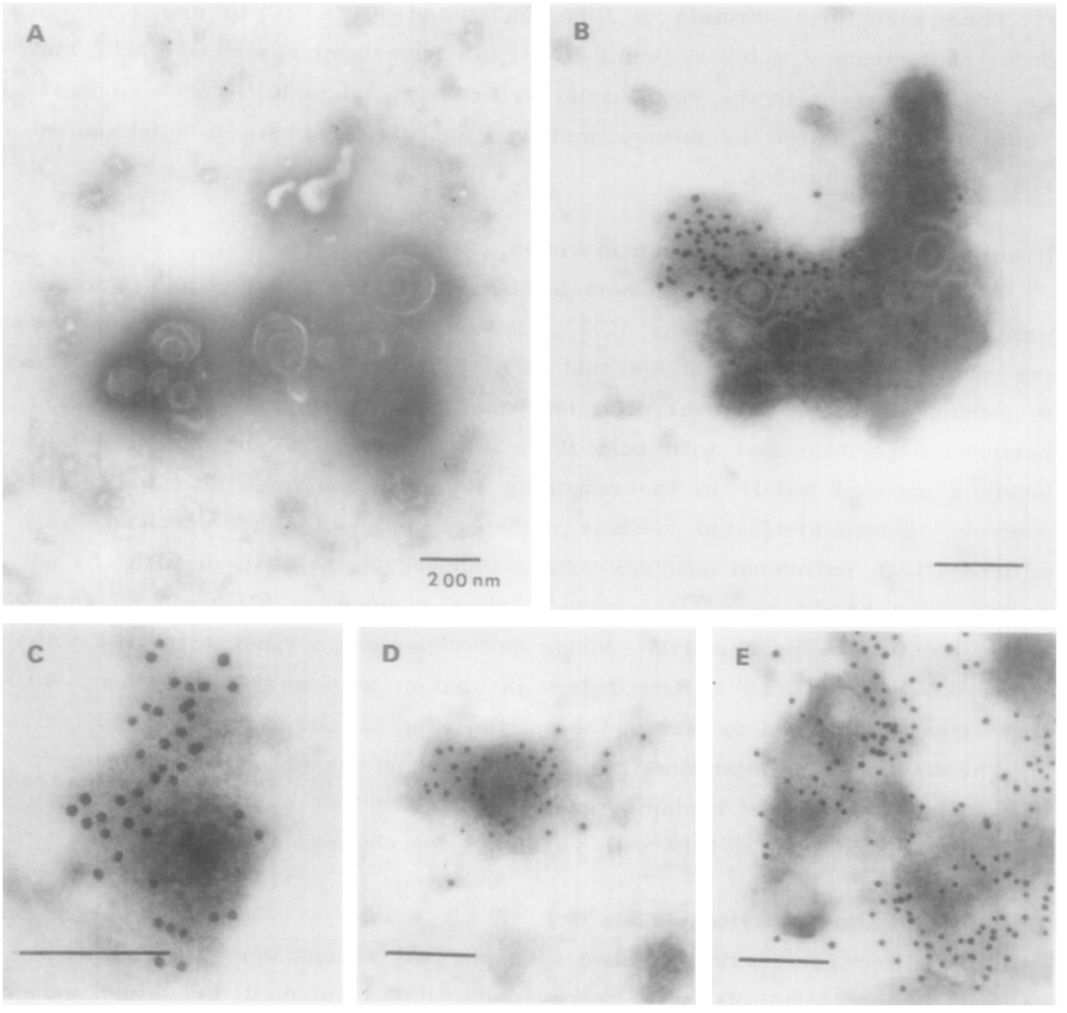

Fig. 2: **Negatively stained HSV-1 virions, labelled with protein A-gold after incubation with the 58S anti-ICP4.** (A) Complete virions. (B and C) Virions pretreated with 1% Triton X-100. (D) Virions pretreated with 1% Triton X-100 and 0.5 M urea. (E) Virions pretreated with 1% Triton X-100 and 2 M urea.

In conclusion, the experiments presented above demonstrate that a virion protein of 165 kd reacts with an monoclonal antibody against ICP4. The similarity between the molecular weight of this protein and ICP4, and the fact that the monoclonal antibody 58S used in this study recognizes uniquely ICP4 in HSV-1 infected cells, make it likely that this virion protein is indeed ICP4. The presence of regulatory protein(s) in the virion does not seem to be an absolute requirement for a productive infection or for expression of viral proteins in the cell [review in 15]. However, tissue trophisms, latent or abortive infections may depend on such factor.

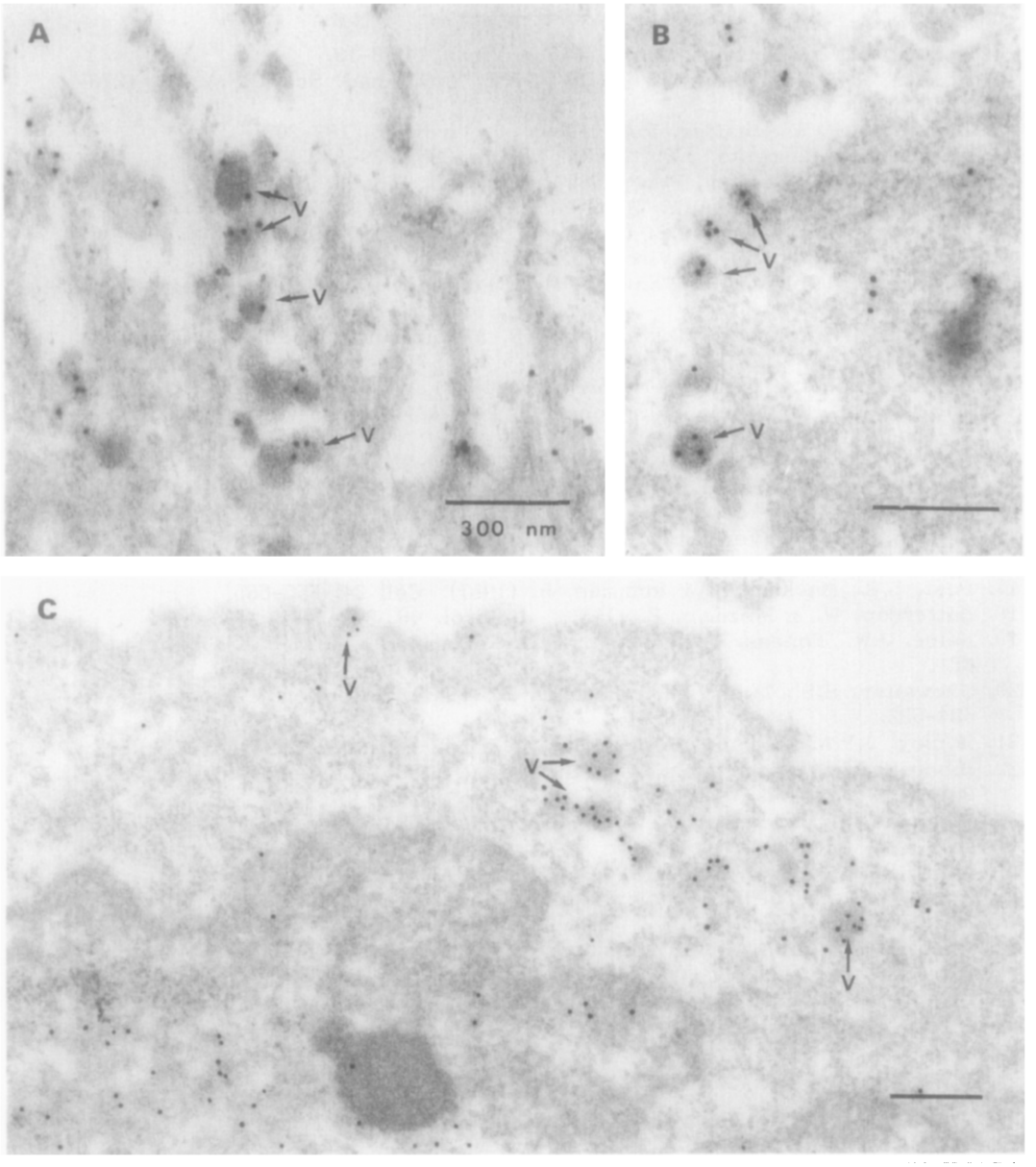

Fig. 3: **Electron micrographs of virions (V) in cytoskeletons.** 2-h infected BHK cells were treated with 1% Triton X-100 before fixation and inclusion in Lowicryl. Thin sections were incubated successively with the 58S anti-ICP4 and with protein A-gold. (A and B) Virions attached to cells are decorated with gold particles. (C) Pockets of virions inside the cytoplasm are also labelled.

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References

1. Honess, R.W. & Roizman, B. (1974) J. Virol. 14, 8-19.
2. Honess, R.W. & Roizman, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1276-1280.
3. Dixon, R.A.F. & Schaffer, P.A. (1980) J. Virol. 36, 189-203.
4. Watson,R. & Clements, J.B. (1980) Nature 285, 329-330.
5. DeLuca, N. & Schaffer, P.A. (1985) Mol. Cell. Biol. 5, 1997-2008.
6. Gelman, I.H. & Silverstein, S. (1987) J. Virol. 61, 3167-3172.
7. O'Hare, P. & Hayward, G.S. (1987) J. Virol. 61, 190-199.
8. Preston, C.M. (1979) J. Virol. 29, 275-284.
9. Pereira, L., Wolff, M.H., Fenwick, M. & Roizman, B. (1977) Virology 77, 733-749.
10. Wilcox, K.W., Kohn, A., Sklyanskaya, E. & Roizman, B. (1980) J. Virol. 37, 431-444
11. Courtney, R.J. & Benyesh-Melnick, M. (1974) Virology 62, 539-551.
12. Cabral, G.A., Courtney, R.J., Schaffer, P.A. & Marciano-Cabral, F. (1980) J. Virol. 33, 1192-1198.
13. Hay, R.T. & Hay, J. (1980) Virology 104, 230-234.
14. Ackermann, M., Braun, D.K., Pereira, L. & Roizman, B. (1984) J. Virol. 52, 1089-118.
15. Gelman, I.H. & Silverstein, S. (1986) J. Mol. Biol. 191, 395-409.
16. Spear, P.G. & Roizman, B. (1972) J. Virol. 9, 143-159.
17. Post, L.E., Mackem, S. & Roizman, B. (1981) Cell 24, 555-565.
18. Batterson, W. & Roizman, B. (1983) J. Virol. 46, 371-377.
19. Heine, J.W., Honess, R.W., Cassai, E. & Roizman, B. (1974) J. Virol. 14, 640-651.
20. Showalter, S.D., Zweig, M. & Hampar, B. (1981) Infection & Immunity 34, 684-692.
21. Hopley, J.F.A. & Doane, F.W. (1985) J. Virol. Methods 12, 135-147.
22. Bibor-Hardy, V., Dagenais, A. & Simard, R. (1985) J. Gen. Virol. 66, 897-901.
23. Dagenais, A., Bibor-Hardy, V. & Simard, R. (1984) Exp. Cell Res. 155, 435-447.
24. Lenk, R. & Penman, S. (1979) Cell 16, 289-301.
25. Morse, L.S., Pereira, L., Roizman, B. & Schaffer, P.A. (1978) J. Virol. 26, 389-410.
26. Wilcox, K.W., Kohn, A., Sklyanskaya, E. & Roizman, B. (1980) J. Virol. 33, 167-182
27. Banks, L., Purifoy, D.J.R., Hurst, P.J., Killington, R.A. & Powell, K.L. (1983) J. Gen. Virol. 64, 2249-2260.