The Assay, Purification and Properties of Vaccinia Virus-induced Uncoating Protein

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SUMMARY

Vaccinia virus cores prepared in vitro can be uncoated by incubation with extracts of cells infected with vaccinia virus, as determined by the conversion of the genome to DNase susceptibility. This uncoating activity had all the characteristics of the corresponding in vivo activity and of the agent responsible for non-genetic reactivation. Thus, it was not induced by heat-inactivated virus, nor was it produced in the presence of inhibitors of RNA or protein synthesis. The uncoating protein induced by cowpox virus will uncoat vaccinia virus cores. The uncoating protein was concentrated from infected cell extracts by ultrafiltration and purified by gel filtration and ion-exchange and affinity chromatography. It was characterized as a trypsin-like protease with a mol. wt. of 23050. Cores treated with the purified uncoating protein had an altered sedimentation rate but no differences between treated and untreated cores were detected by electron microscopy or polyacrylamide gel electrophoresis.

INTRODUCTION

The uncoating of poxviruses has been studied by electron microscopy (Dales, 1963, 1965; Dales & Kajikia, 1964), monitored biochemically (Joklik, 1964a, b) and by analysis on sucrose gradients (Sarov & Joklik, 1972; Holowczak, 1972). These approaches have complemented each other and divided the process into two well defined stages. The first stage of uncoating involves the rapid hydrolysis of some virion protein and all the virion phospholipid. This stage is insensitive to the addition of inhibitors of protein synthesis and produces subviral cores in which the genome is still protected from DNase attack. After a lag period, the disruption of viral cores commences. This second stage of uncoating is characterized by its dependence on protein synthesis and by the conversion of viral DNA to a DNase-sensitive form. The production of a virus-coded uncoating protein is thought to occur during this lag period. Abel (1963) claimed to have identified a 'stripping factor' in extracts of infected cells which uncoats whole virus in vitro. In re-examining this phenomenon we have been unable to confirm Abel's (1963) results, but we have established an in vitro system for studying vaccinia virus uncoating protein.

METHODS

Cells and virus. HeLa S3 cells (Gibco-Biocult) were used throughout this work. Most of the experiments involved vaccinia virus strain WR but in some cases the Brighton strain of cowpox virus was used. The latter was kindly provided by Dr J. Williamson of St Mary's Hospital Medical School, London, U.K. The growth and maintenance of cell cultures, the production and titration of virus stocks, the heat inactivation of virus and the infection of suspension cultures have all been described in a previous publication (Pedley & Cooper, 1984).

Preparation of radiolabelled vaccinia virus cores. Vaccinia virus was grown in the presence of 2 μCi/ml [3H]thymidine and the labelled virus was purified by the method of Joklik (1962). This purified virus was used to prepare cores as described by Kates & Beeson (1970). Briefly, the virus was incubated with 0·5% (v/v) Nonidet P40 and 50 mM-2-mercaptoethanol at 37 °C for 30 min. The cores where then centrifuged at 30000 g for 30 min in the

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SW50.1 rotor of the Beckman L2 ultracentrifuge. The pellet of cores was resuspended in 50 mM-Tris–HCl pH 8.5 at 37 °C containing 10 mM-2-mercaptoethanol and stored in aliquots at −70 °C.

Preparation of cell extracts. Suspension cultures were infected at a multiplicity of 40 pock-forming units/ml. At various times after infection a sample of cells was removed and centrifuged at 350 g for 10 min. The cell pellet was resuspended at 2 × 10^7 cells/ml in imidazole buffer (300 mM-NaCl, 50 mM-imidazole pH 7.0) and disrupted by ultrasonic vibration in a Megason bath (Schuco International Ltd, London, U.K.). Extracts from mock-infected cells were prepared in the same way and cell samples were stored in aliquots at −70 °C.

Cytoplasmic extracts of infected cells for the purification of the uncoating activity were prepared by washing harvested cells in isotonic buffer (150 mM-NaCl, 5 mM-EDTA, 50 mM-Tris–HCl pH 8.0 at 4 °C) and resuspending them in hypotonic buffer (10 mM-KCl, 5 mM-EDTA, 10 mM-Tris–HCl pH 8.0 at 4 °C) at 2 × 10^7/ml. The cells were allowed to swell for 10 min and then disrupted in a homogenizer calibrated to produce maximum cell breakage with the minimum loss of nuclear material. The nuclei were removed by centrifugation at 400 g for 2 min and the cytoplasmic fraction was collected.

Assay of uncoating activity. Uncoating was followed by measuring the conversion of labelled DNA in vaccinia virus cores from a DNase-resistant to a DNase-sensitive form. The assay mix contained 50 μl of labelled cores (approx. 2 × 10^3 to 4 × 10^3 c.p.m.; 1 μg total protein) and 200 μl of cell extract. The mix was incubated for 1 h at 37 °C after which one half of each sample was treated with DNase and MgCl2 (100 μg/ml and 10 mM) and the other half with imidazole buffer. Incubation was continued for a further 30 min. The reaction was stopped by the addition of TCA to a final concentration of 5%. After 20 min at 4 °C, the precipitate was collected on Whatman GF/C filters, washed three times with 5% TCA and once with 99% ethanol. The discs were dried and their radioactivity was determined in an Intertechnique SL3000 scintillation spectrometer with an efficiency of counting of 35%. The scintillation fluid contained 0.4% (w/v) PPO and 0.005% (w/v) dimethyl POPOP in toluene.

Techniques used in the purification of the uncoating activity. Ultrafiltration was done with continuous gentle stirring through an Amicon PM10 Diaflo ultrafilter under a constant pressure of 345 kPa. This filter retains molecules with a mol. wt. greater than 10000.

Gel filtration was performed using Sephadex G-75 in a 1.7 cm × 90 cm column in imidazole buffer at a flow rate of 1.6 ml/cm^2·h. The column was calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin and albumin (low molecular weight calibration kit, Pharmacia) for the mol. wt. determination of the uncoating activity.

Ion-exchange chromatography was with DEAE Sephadex A-50 in a 0.9 cm × 15 cm column at a flow rate of 15.6 ml/cm^2·h. The uncoating activity was adsorbed to the column in imidazole buffer and after washing in this buffer, the activity was eluted with a linear salt gradient from 300 mM-NaCl to 400 mM-NaCl in 50 mM-imidazole at pH 7.0.

Affinity chromatography was performed with soybean trypsin inhibitor agarose in a small column made from a Pasteur pipette at a flow rate of 16 ml/cm^2·h. The uncoating activity was adsorbed in imidazole buffer and after washing of the column, the activity was eluted with 11.65 mg/ml lysine in imidazole buffer.

Protein assay. For the estimation of protein concentration the Bennet (1967) modification of the assay of Lowry et al. (1951) was used.

Materials. Trizma base (reagent grade), actinomycin D, cycloheximide, imidazole, sucrose, 2-mercaptoethanol, aprotinin, 4-methylumbelliferyl-p-guanidobenzoate (MUGB) and DNase I were purchased from Sigma. PPO and dimethyl POPOP were from Koch-Light Laboratories. [Me⁻³H]thymidine (60 Ci/mmol) was from Amersham. All other chemicals were of AnalaR grade and were purchased from BDH. DEAE Sephadex A-50 and Sephadex G-75 were from Pharmacia and the soybean trypsin inhibitor agarose was obtained from Bethesda Research Laboratories.

RESULTS

Development of the assay for uncoating protein

An essential requirement for the assay was preparations of viral cores in which, in the absence of uncoating protein, their DNA remained resistant to DNase. Only one of several methods of core preparation investigated consistently produced stable cores, that of Kates & Beeson (1970). However, resuspension of cores by sonication renders their DNA susceptible to DNase. Consequently, core preparation were always resuspended by gentle pipetting. Cores prepared in this way could be stored at −70 °C, but at −20 °C or +4 °C DNA resistance was lost after only a few days.

The action of DNase on the product of core uncoating was relatively slow, a time course indicating that at least 20 min was required to degrade 50% of the DNA. Prolonging the incubation did not increase this level of 50%, the maximum achieved in all assays. In contrast,
**Vaccinia virus uncoating protein**

Table 1. *Uncoating activity of various cell extracts prepared 3 h after infection*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cell extract</th>
<th>−DNase (c.p.m.)*</th>
<th>+DNase (c.p.m.)</th>
<th>Uncoating (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cores</td>
<td>Mock-infected</td>
<td>2508</td>
<td>2458</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Vaccinia virus</td>
<td>2567</td>
<td>1335</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Cowpox virus</td>
<td>2592</td>
<td>1503</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Vaccinia virus + actinomycin D (10 μg/ml)</td>
<td>2604</td>
<td>2526</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Vaccinia virus + cycloheximide (100 μg/ml)</td>
<td>2626</td>
<td>2630</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Heat-inactivated virus</td>
<td>2573</td>
<td>2470</td>
<td>4</td>
</tr>
<tr>
<td>Whole virus</td>
<td>Mock-infected</td>
<td>10779</td>
<td>10563</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Vaccinia virus</td>
<td>11125</td>
<td>10791</td>
<td>3</td>
</tr>
</tbody>
</table>

* C.p.m. are average of duplicate samples.

under the same conditions, *Escherichia coli* chromosomal DNA was fully degraded within 5 min of the addition of DNase. In view of these findings, after treatment of cores with cell extracts, the incubation with DNase was standardized at 30 min.

The uncoating activity was assayed by finding the dilution that resulted in an uncoating level of about 25%. The activity could then be expressed as the total c.p.m. solubilized using the following formula:

\[
\text{total c.p.m. solubilized} = \text{c.p.m. at approx. } 25\% \text{ uncoating} \times \text{dilution factor} \times \left(\frac{\text{total vol. of sample}}{\text{vol. of sample analysed}}\right).
\]

**Uncoating of virus cores in vitro**

Incubation of vaccinia virus cores with infected cell extracts consistently produced structures within which the viral DNA became susceptible to DNase (Table 1). We interpret this as core uncoating which never occurred when cores were incubated with mock-infected cell extracts. Previous work (Joklik, 1964a, b) shows that, in vivo, uncoating activity depends on de novo protein synthesis, is not induced by heat-inactivated virions and is active on poxvirus strains other than that which cause its production. The results in Table 1 demonstrate that the in vitro uncoating activity displayed those characteristics. Thus, it was not induced in infected cells in the presence of actinomycin D or cycloheximide, nor in cells infected with heat-inactivated virus. However, the activity was induced in cowpox virus-infected cells, as extracts prepared from these cells uncoated vaccinia virus cores. None of the cell extracts were able to uncoat whole virus particles.

**Uncoating of intracellular cores**

In the presence of actinomycin D second stage uncoating is inhibited and viral cores accumulate in the cytoplasm of vaccinia virus-infected cells (Magee & Miller, 1968). Accordingly, cytoplasmic extracts prepared 2 h after infection with [3H]thymidine-labelled virus in the presence or absence of actinomycin D were centrifuged on sucrose gradients. Fig. 1 shows that in the absence of actinomycin D, the bulk of the radioactivity remained at the top of the tube, was completely sensitive to DNase and therefore represented fully uncoated viral DNA. There was also a small peak (2a) which was DNase-resistant and presumably represented viral cores yet to be uncoated. In contrast, in the presence of actinomycin D, three peaks (2, 3 and 4) were resolved, all of which were completely resistant to DNase. When the DNase-resistant peaks were treated with infected cell extracts containing uncoating activity, all but peak 4 were uncoated (Table 2). From its position in the gradient, it is probable that peak 4 represents whole virus, hence its resistance to the uncoating activity, whereas peaks 3 and 2 may represent cores before and after the removal of lateral bodies.
Fig. 1. Fractionation of intracellular viral particles from cells treated with 10 μg/ml actinomycin D (●) and untreated cells (○). Cytoplasmic extracts were centrifuged at 23000 g for 45 min on 25 to 40% (w/w) sucrose gradients.

Fig. 2. Induction of uncoating protein in vaccinia virus-infected cells.

Table 2. Effect of uncoating protein on intracellular particles isolated 2 h post-infection

<table>
<thead>
<tr>
<th>Peak number*</th>
<th>-DNase (c.p.m.)†</th>
<th>+DNase (c.p.m.)</th>
<th>Uncoating (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>706</td>
<td>381</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>3750</td>
<td>1950</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>1875</td>
<td>900</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>556</td>
<td>549</td>
<td>1</td>
</tr>
</tbody>
</table>

* Numbers refer to peaks in Fig. 1.
† C.p.m. are an average of duplicate samples.

Synthesis of the uncoating activity

Cell extracts collected at various times after infection were assayed for uncoating activity. As shown in Fig. 2 uncoating activity was detected within 20 min of infection, reached a maximum at 2 h post-infection and then remained constant up to 24 h.

Purification of uncoating protein

The cytoplasmic fraction of cells harvested 3 h after infection was centrifuged at 100000 g for 1 h in the 50 Ti rotor in the Beckman L2 ultracentrifuge. The supernatant fluid was concentrated by ultrafiltration. Attempts to concentrate the uncoating activity by ammonium sulphate precipitation resulted in an almost total loss of activity. Gel filtration of the concentrated material on Sephadex G-75 resulted in the uncoating activity eluting as a single symmetrical peak (Fig. 3a). These peak fractions were pooled and loaded onto a DEAE Sephadex A-50 column. Again, the uncoating activity eluted in a single symmetrical peak at 0.34 M-NaCl (Fig. 3b). Finally, the pooled peak fractions from the ion-exchange column were applied to soybean trypsin inhibitor agarose and the uncoating activity was eluted with 11.65 mg/ml lysine. A
Vaccinia virus uncoating protein

Fig. 3. Purification of uncoating protein (∆) by (a) gel filtration on Sephadex G-75 and (b) ion-exchange chromatography on DEAE Sephadex A-50. The continuous line represents A_{280} and the broken line the NaCl concentration.

Fig. 4. Time course of the uncoating of vaccinia virus cores in vitro by purified uncoating protein.

Table 3. Purification of uncoating protein

<table>
<thead>
<tr>
<th>Stage in purification</th>
<th>Total uncoating activity (c.p.m.)</th>
<th>Yield compared to whole cell homogenate (%)</th>
<th>Total protein (mg)</th>
<th>Specific activity (total c.p.m./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell homogenate</td>
<td>8.4 \times 10^7</td>
<td>100</td>
<td>58</td>
<td>1.4 \times 10^6</td>
</tr>
<tr>
<td>Nuclei</td>
<td>4.2 \times 10^6</td>
<td>5</td>
<td>14</td>
<td>3.0 \times 10^5</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>7.8 \times 10^7</td>
<td>92.8</td>
<td>42</td>
<td>1.9 \times 10^6</td>
</tr>
<tr>
<td>100000 g supernatant fluid</td>
<td>6.9 \times 10^7</td>
<td>82</td>
<td>30</td>
<td>2.3 \times 10^6</td>
</tr>
<tr>
<td>100000 g pellet</td>
<td>4.8 \times 10^6</td>
<td>5.7</td>
<td>11</td>
<td>4.4 \times 10^5</td>
</tr>
<tr>
<td>Ultrafiltration ‘eluate’</td>
<td>2.7 \times 10^6</td>
<td>3.2</td>
<td>7</td>
<td>3.9 \times 10^5</td>
</tr>
<tr>
<td>Ultrafiltration ‘retentate’</td>
<td>4.1 \times 10^7</td>
<td>48.8</td>
<td>20</td>
<td>2.1 \times 10^6</td>
</tr>
<tr>
<td>Gel filtration peak</td>
<td>1.7 \times 10^7</td>
<td>20.2</td>
<td>0.4</td>
<td>4.3 \times 10^7</td>
</tr>
<tr>
<td>Ion exchange peak</td>
<td>8.8 \times 10^6</td>
<td>10.4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Affinity chromatography peak</td>
<td>4.0 \times 10^6</td>
<td>4.7</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* Protein concentration below detectable levels.

A summary of the purification data is presented in Table 3. All the steps were performed at 4 °C and took a total of about 72 h.

Properties of the purified uncoating activity

The purified uncoating activity was virtually abolished by 0·5 units/ml of the general protease inhibitor aprotinin (Steven & Griffin, 1981) and also by 3·3 μM-MUGB, a specific inhibitor of
Fig. 5. Effect of uncoating protein on the sedimentation rate of viral cores. Cores + imidazole buffer (●); cores + imidazole buffer + DNase (○); cores + uncoating protein (■); cores + uncoating protein + DNase (□). Radioactivity in all samples collected in fractions 1 to 7 and 16 to 24 was of background level and for clarity the results are not shown.

trypsin-like enzymes (Coleman et al., 1976). The use of MUGB enabled the uncoating activity to be uncoupled from the subsequent DNase treatment. Thus by adding MUGB at various times to terminate the uncoating reaction and treating each sample with DNase for 30 min, a time course of uncoating was constructed (Fig. 4). There was a distinct lag of about 15 min before uncoating commenced, followed by a linear increase until the maximum 50% level after about 40 min incubation.

The mol. wt. of the uncoating activity was determined as 23050 ± 660 by gel filtration using four separate preparations of the enzyme.

Effect of purified uncoating activity on virus cores

Labelled virus cores treated with the uncoating activity had a slightly slower sedimentation rate compared to untreated cores (Fig. 5). When the fractions were subsequently incubated with DNase then, as expected, the standard cores remained resistant but those pretreated with the uncoating activity showed the 50% solubilization of their DNA. The sedimentation characteristics of cores treated with extracts from mock-infected cells did not vary from the cores incubated only with buffer.

We could not detect any differences between standard core preparations and those treated with the uncoating activity by either electron microscopy or by polyacrylamide gel electrophoresis.

DISCUSSION

The standard criterion for poxvirus uncoating is the conversion of the genome from a DNase-resistant to a DNase-sensitive form (Joklik, 1964a). Using this standard, we have devised an assay which detects uncoating activity in extracts of infected, but not uninfected cells. Subviral cores prepared in vitro from purified virus previously labelled with [3H]thymidine were used as the substrate. The maximum level of uncoating achieved in this assay was just over 50%. This was not due to insufficient levels of uncoating protein because some extracts could be diluted several hundred-fold and still produce the 50% maximum level of uncoating. The most likely explanation is the presence of DNA-binding proteins in the core which provides partial protection for the DNA. Such DNA-binding proteins have been detected by several workers (Pogo et al., 1975; Nowakowski et al., 1978; Soloski et al., 1978; Soloski & Holowczak, 1981).

The uncoating activity detected by our assay has similar characteristics to the corresponding activity revealed in vivo by other workers (Joklik, 1964a, b; Dales, 1965; Moss & Filler, 1970).
Thus it is not induced in cells infected in the presence of inhibitors of RNA or protein synthesis, nor is it induced in cells infected with heat-inactivated virus. These similarities are reinforced by the fact that subviral cores isolated from infected cells are also uncoated up to a maximum of 50%. However, uncoating can only be demonstrated with subviral cores as substrate, not with whole virus. This differs from the results of Abel (1963) who used extracts of infected cells to return heated virus to an infective state and assumed that these extracts contained 'decoating' enzymes.

Uncoating protein is induced very early in infected cells and can be detected within 20 min of addition of virus. This complements the results of Buchmeier et al. (1979) who found that about 20% of the inoculum virus was uncoated after a 30 min adsorption period. Uncoating activity reaches a maximum level 2 h after infection and is very stable, remaining at the same high level for at least 24 h. These results are entirely consistent with the work of Cairns (1960) who showed that a critical initiating event from one infecting particle resulted in the simultaneous initiation of DNA synthesis by any other virus particle in the same cell. Joklik (1964a) showed that the lag period to DNA synthesis is virtually abolished for a superinfecting inoculum that is added after the time interval normally occupied by the lag period. The kinetics of the synthesis of uncoating protein suggest that its appearance may be the critical initiating event. The uncoating protein may also be the factor in the phenomenon of non-genetic reactivation (Berry & Dedrick, 1936; Joklik et al., 1960a, b) particularly as the cowpox-induced uncoating protein will uncoat vaccinia virus cores.

The stability of the uncoating protein for up to 24 h after infection differs from the results of Abel (1963) who found that uncoating activity had disappeared by 10 h after infection. New progeny virions are being formed within 4 h after infection and it might be expected that high levels of uncoating protein would interfere with the assembly of such virions. However, the first stage of vaccinia virus assembly involves the formation of a viral membrane and the differentiation into mature cores and lateral bodies takes place entirely within this closed membrane (Dales, 1963; Dales & Mosbach, 1968; Grimley et al., 1970; Stern & Dales, 1976). It is presumably this viral membrane that protects the maturing cores and mature virus from the action of the uncoating protein.

Studies on the purified uncoating protein reveal it to be a protease with a trypsin-like activity and a mol. wt. of about 23000. Preliminary experiments on the effect of this purified preparation on cores have been inconclusive. Thus a definite shift in sedimentation rate of treated cores was noted but neither electron microscopy nor polyacrylamide gel electrophoresis revealed any differences between treated and untreated cores. Further work in this area is obviously required.

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REFERENCES


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