Cleavage Fragments of the Retrovirus Surface Protein gp70 during Virus Entry

By KLAUS B. ANDERSEN

Danmarks farmaceutiske Højskole, The Royal Danish School of Pharmacy, Department of Biology, Universitetsparken 2, DK-2100 København Ø, Denmark

(Accepted 12 May 1987)

SUMMARY

The surface protein gp70 of an ecotropic murine retrovirus was followed during entry of $[3H]$glucosamine-labelled virions into SC-1 mouse fibroblasts. Upon entry, gp70 was cleaved into fragments with molecular weights 35K, 30K and 17K. The 35K and 17K fragments were also observed after trypsin or thermolysin cleavage of the virion, indicating that certain locations on the gp70 molecule are easily accessible from the outside of the virion. The conformation of gp70 on the membrane was shown to have a major effect on the cleavage. This protein is known to be important for early interactions with the cell (binding and membrane fusion). The results indicate that gp70 cleavage may be important for membrane fusion.

INTRODUCTION

Two different routes of entry of retrovirus have been suggested. One route is by endocytosis followed by fusion between the virus membrane and the vesicle membrane (Andersen & Nexø, 1983; Redmond et al., 1984). Another route is by direct entry into the cytoplasm by fusion of the virus membrane and the plasma membrane (Portis et al., 1985). It is agreed that the first step of entry is binding of the virion by its gp70 protein to receptors on the cell surface. The receptors have, however, not been molecularly characterized (Handelin & Kabat, 1985).

Following entry, the virion is internalized, and this has been analysed by a change into a trypsin-resistant form of both bound infectious particles and radioactively labelled particles. The rate of internalization differs widely; a half-time for internalization of 3 to 5 h has been observed for the ecotropic retrovirus C57MC entering SC-1 and BALB/3T3 cells (Andersen & Nexø, 1983), whereas the half-time for entry into a Mus dunnii cell line was 10 min for Friend murine leukaemia virus, but longer for other murine retroviruses (Portis et al., 1985).

In our previous studies of C57MC entering SC-1 and BALB/3T3 cells, the radioactive particles and the infectious activity bound to the cells and were internalized at the same rate, which shows that the results obtained with radioactive particles are valid for the infectious route. All virus proteins were internalized (Andersen, 1985), indicating that the whole virion was internalized. A majority of the virions was degraded in the cell by a mechanism sensitive to lysosomotropic bases (Andersen & Nexø, 1983). Together these results suggest that the virions enter by endocytosis and are degraded in lysosomes.

The route of degradation is presumably abortive, but the infectious particles may, up to a point, follow the same route. Infection can also be inhibited by lysosomotropic bases (Wallbank, 1969; Pazmino et al., 1974; Andersen & Nexø, 1983). From this finding, it was suggested that the infection occurs by endocytosis, and is probably followed by a low-pH-activated fusion between the vesicle membrane and the virus membrane (Andersen & Nexø, 1983) as suggested for a number of other enveloped viruses (for review, see White et al., 1983). The route of endocytosis is supported by the finding that mouse mammary tumour virus induces cell fusion at low pH (Redmond et al., 1984). However, retrovirus-induced cell fusion of the Mus dunnii cell...
line has been shown to occur at neutral pH (Portis et al., 1985), suggesting that entry into these cells occurs through plasma membrane fusion.

Assays of virus-induced cell fusion do not, however, allow firm conclusions to be drawn about the route of entry because they relate to properties of physical virus particles and the number of these is high in comparison to the number of infectious particles (Lonberg-Holm & Philipson, 1974; Andersen & Nexo, 1983); thus the route of the majority of physical particles might not necessarily represent the infectious route.

The proteins gp70 and p15E are located on the viral surface; p15E is anchored in the membrane, and gp70 is attached to p15E by disulphide bonds (Pinter & Honnen, 1983). gp70 is involved in the fusion process, as virus-induced cell fusion can be inhibited by antibodies against gp70 (Zarling & Keshet, 1979). Two monoclonal antibodies, which respectively bind to the N-terminal and C-terminal part of gp70, have been shown to inhibit the virus-induced cell fusion (Pinter et al., 1982, 1986).

Infection of SC-1 and BALB/3T3 cells by C57MC virus is inhibited by the protease inhibitor leupeptin at an early stage after binding (Andersen, 1983), suggesting that proteolysis is necessary for infection. Inhibition of infection by protease inhibitors has also been observed with coronavirus (Appleyard & Tisdale, 1985) and influenza virus (Zhirnov et al., 1984).

As mentioned above, gp70 is active in the early steps of infection (binding and membrane fusion), at which period proteolytic cleavage seems to be important. It is therefore interesting to follow the degradation of gp70 during entry into the virus particle. This was possible since gp70 can be selectively labelled with $[^{3}H]$glucosamine.

**METHODS**

*Cells and virus.* The mouse fibroblast line SC-1 (Hartley & Rowe, 1975) was grown in monolayer cultures in Eagle's minimal essential medium supplemented with 5% foetal calf serum (Gibco), 0.5 mg/ml glutamine, 0.03 mg/ml streptomycin and 250 units/ml penicillin. All cultures were grown at 37 °C in 5% CO$_2$ unless otherwise noted. The murine retrovirus C57MC pool 1636 (Schuh et al., 1976) was metabolically prelabelled in infected SC-1 cultures (in 80 cm$^2$ flasks, Nunc) with 1 mCi $[^{3}H]$glucosamine (30 Ci/mmol) (Amersham) and collected by centrifugation.

*Infection experiments.* Two cm$^2$ cultures were seeded with 50 000 cells. The following day the medium was removed and new medium containing 10 mM-HEPES pH 7.0 and 5 μg/ml polybrene was added. After at least 1 h, radioactive virus was added. After various times of incubation, the cultures were harvested. The medium was removed and the cells were washed with phosphate-buffered saline (PBS) followed either by lysis of the monolayer in 1 mg/ml SDS or 1/4 × sample buffer (see below), or treatment with trypsin (2.5 mg/ml in PBS) for 3 min at 20 °C. The trypsin removes surface-bound material (Andersen & Nexo, 1983) and loosens the cells from the culture dish. Soybean trypsin inhibitor (2 mg/ml, Sigma) was added to stop the action of trypsin. The trypsin-treated cells were collected by centrifugation (200 g for 3 rain), washed with 2 mg/ml trypsin inhibitor and resuspended in 0.5 mg/ml trypsin inhibitor in TEN-8 (10 mM-Tris·HCl, 1 mM-EDTA, 100 mM-NaCl pH 8.0) and lysed by addition of SDS or sample buffer. The cell lysates were sonicated to lower the viscosity.

*Immunoprecipitation.* Antiserum against Rauscher murine leukaemia virus gp70 was obtained as a gift from Bjorn A. Nexo. Cell lysates in 1 mg/ml SDS were added to 10 mg/ml Triton X-100, 5 mg/ml sodium deoxycholate and 1 mg/ml PMSF and were precipitated with antiserum. The precipitates were collected with formaldehyde-fixed *Staphylococcus aureus* (Nexo & Ulrich, 1983).

*SDS-PAGE.* Samples were added to 0.33 vol. sample buffer (40% v/v glycerol, 20% v/v 2-mercaptoethanol, 80 mg/ml SDS and 125 mM-Tris·HCl pH 6.8) and kept on a boiling water bath for 1-5 min. The samples were electrophoresed in SDS-polyacrylamide slab gels (22.5%) according to Laemmli (1970). The gels were treated with the scintillator PPO (Bonner & Laskey, 1974) and fluorographed at −80 °C.

**RESULTS**

*Virus preparations.*

Virus preparations were obtained from cell cultures labelled with $[^{3}H]$glucosamine. As seen in Fig. 1 (lane 1) gp70 was the only viral protein which was labelled. Although glycolipids were also labelled, they electrophoresed at the dye front (not shown), but their radioactivity was negligible in comparison with that of gp70.
We estimated that the specific activity was approximately $1.6 \times 10^{-3}$ c.p.m./virion. This value was based on the specific activity of the glucosamine used, and assuming $10^9$ as the molecular weight of the virion, 2% carbohydrate content and 30% of the carbohydrates being glucosamine (Tooze, 1973). The biological activity of the virus preparations was approximately 1 p.f.u./c.p.m. (as analysed by immunoperoxidase staining; Nexo, 1977).

In the following experiments, up to 5 c.p.m. of $[^3]$Hglucosamine-labelled C57MC bound per cell seeded (corresponding to approximately 3000 virions per cell). To examine whether the high virus to cell ratio had any influence on the entry, binding and internalization were examined after 4 h. At this time, both binding and internalization are still proceeding (Andersen & Nexo, 1983), and the results would therefore show changes in the kinetics of entry. As seen in Table 1, binding and internalization did not decrease until more than 5 c.p.m. of $[^3]$Hglucosamine-labelled C57MC was bound per seeded cell.
Table 1. Binding and internalization at various virus to cell ratios

<table>
<thead>
<tr>
<th>Number of seeded cells*</th>
<th>$^3$H-labelled virus added (c.p.m.)</th>
<th>Bound radioactivity (%)</th>
<th>Internalized radioactivity (%)</th>
<th>Bound radioactivity (c.p.m./cell)</th>
<th>Estimated number of virions bound per cell seeded†</th>
</tr>
</thead>
<tbody>
<tr>
<td>50000</td>
<td>366</td>
<td>48.5</td>
<td>34.9</td>
<td>0.0036</td>
<td>2.2</td>
</tr>
<tr>
<td>50000</td>
<td>3310</td>
<td>49.7</td>
<td>40.7</td>
<td>0.033</td>
<td>21</td>
</tr>
<tr>
<td>50000</td>
<td>35300</td>
<td>46.2</td>
<td>36.8</td>
<td>0.32</td>
<td>200</td>
</tr>
<tr>
<td>50000</td>
<td>496000</td>
<td>45.0</td>
<td>35.3</td>
<td>4.5</td>
<td>2800</td>
</tr>
<tr>
<td>10000</td>
<td>3730</td>
<td>42.5</td>
<td>27.1</td>
<td>0.16</td>
<td>100</td>
</tr>
<tr>
<td>10000</td>
<td>44900</td>
<td>42.4</td>
<td>30.9</td>
<td>1.9</td>
<td>1200</td>
</tr>
<tr>
<td>10000</td>
<td>517000</td>
<td>34.6</td>
<td>16.4</td>
<td>18.0</td>
<td>11000</td>
</tr>
</tbody>
</table>

* SC-1 cells were seeded in 2 cm² (50000 cells) and 0.4 cm² (10000 cells) cell culture wells. $^3$HGlucosamine-labelled virus was added the following day and the cultures were harvested after 4 h.
† The calculation of the number of virions is based on an estimated specific activity of $1.6 \times 10^{-3}$ c.p.m./virion (see text).

In vitro degradation of gp70

Degradation of gp70 in situ was examined by trypsin and thermolysin treatment of the labelled virions (Fig. 1). At high protease concentrations, gp70 was totally degraded (not shown). The cleavage patterns are shown for concentrations giving limited degradation. The most intense fragments obtained after trypsin treatment had molecular weights 35K, 27K, 17K and 8K. The bands were diffuse, as was the gp70 band, probably because of the heterogeneity of the carbohydrate moieties (Witte et al., 1977).

Thermolysin cleaved gp70 into 35K, 32K and 17K fragments. Though trypsin (a serine protease) and thermolysin (a metalloprotease) have different sites of action, it is interesting that they both generated the 35K and 17K fragments. Other fragments, however, were different. Similarity of gp70 fragments generated by different proteases has been noted in earlier studies (Niman & Elder, 1982; Pinter et al., 1982).

The cleavage patterns of gp70 from C57MC and Moloney murine leukaemia virus were compared (results not shown). The patterns were not similar, as would be expected for different gp70 molecules. The cleavage patterns of AKR and Rauscher virus gp70 have previously been analysed (Niman & Elder, 1982; Pinter et al., 1982) and also differed from C57MC virus. Slight variation within the degradation pattern of C57MC gp70 was also observed (compare Fig. 1 and Fig. 4). This may have been due to changes in accessibility of the gp70 molecules in different C57MC preparations.

In vivo degradation of gp70

The $^3$Hglucosamine-labelled virus preparations were added to cell cultures for 5 to 6 h. At this time, the majority of the virions are expected to be bound to the cells, and internalization is still expected to occur (half time 3 to 5 h; Andersen & Nexo, 1983). The cells were harvested by lysis in SDS to examine all bound material and by trypsin treatment followed by lysis in SDS, to examine the internalized material. Trypsin removes surface-bound material (Schlessinger et al., 1978).

35K and 17K fragments were observed in the SDS lysate together with gp70 (Fig. 2, lane 4). These fragments were observed with apparently the same intensities in the lysate of the trypsin-treated cells (Fig. 2, lane 5). However, only a low amount of gp70 was observed in the latter lysate. A novel band above the 17K fragment was observed in lane 5. It may have been formed by the trypsin treatment, but it is possible that it was diffuse material displaced by soybean trypsin inhibitor (molecular weight 21K), which was used in the procedure.

To ensure that no degradation of gp70 had occurred during the harvest, the virus preparation was added to uninfected cells just before lysis (compare Fig. 2, lanes 1, 2 and 3); no degradation occurred in the cell lysates. These controls did not show whether the gp70 fragments were...
Fig. 2. Degradation of [3H]glucosamine-labelled virus in SC-1 cultures. Virus (460,000 c.p.m.) was added to two SC-1 cultures at 37 °C and incubated for 5 h. Fifty to 55% of the radioactivity bound to the cells. One culture (D) was lysed in SDS and another (E) was treated with trypsin before lysis (60% of the bound radioactivity was trypsin-resistant). Controls: (A) 120,000 c.p.m. of the virus preparation; B and C, 120,000 c.p.m. of the virus preparation added to cell lysates obtained respectively as for D and E, but without prior addition of virus. One-fifth of each sample was examined directly by SDS-PAGE (lanes 1 to 5, samples A to E respectively). The remaining portions were immunoprecipitated with gp70 antiserum before SDS-PAGE (lanes 1' to 5'). The material released by trypsin from sample E was also immunoprecipitated (lane 6'). Both gels were fluorographed for 29 days.

generated during the trypsin treatment of the cells. However, it was seen that the fragments did not change in size or increase in intensity after the trypsin treatment (lanes 4 and 5). A 35K fragment was also observed in the supernatant of the trypsin-treated cells (lane 6'). Trypsin also generated a 35K fragment (Fig. 1), so the observed fragment was presumably formed from gp70 of surface-bound virions during the trypsin treatment.

The lysates examined were also precipitated with antiserum against gp70 (Fig. 2, lanes 1' to 5'). gp70 and the 35K fragment were clearly precipitated, but not the 17K fragment. A band of molecular weight 30K was also precipitated. This band could be seen faintly in the unprecipitated samples (Fig. 2, lanes 4 and 5). These immunoprecipitation results support the theory that the 35K and 30K fragments originate from gp70.

It is possible that impurities in the virus preparation could bind to and enter the cells. The intensity of the 35K and 17K fragments showed that they could not be accounted for by impurities. The question was, however, examined further by binding at 5 °C (a temperature at which internalization does not occur; Andersen & Nexo, 1983). Fig. 3 shows a comparison of the bands observed after incubation at 5 °C and 37 °C. As seen, the 35K and 17K fragments were only present after incubation at 37 °C, showing that they were formed after binding had occurred.

**Protection of gp70 against proteolysis by its conformation in the virus envelope**

It is interesting that the 35K and 17K fragments were both observed in *vivo* and by cleavage *in vitro* with trypsin and thermolysin (compare Fig. 1 and 2). Different proteases cleave gp70 at
closely located sites (Niman & Elder, 1982; Pinter et al., 1982), probably because only certain areas on the gp70 molecule are easily accessible to the proteases, due to the conformation of the gp70 molecule. The following experiment showed that gp70 was greatly protected against degradation by its location in the virus envelope. Virions were solubilized by mild detergents (Triton X-100 and deoxycholate) and degraded by trypsin (Fig. 4). In contrast to intact virions, only 1/100 of the trypsin concentration was necessary to degrade gp70 from solubilized virions. Furthermore, the cleavage pattern was also altered: very intense fragments with molecular

Fig. 3. Degradation of [3H]glucosamine-labelled virus in SC-1 cultures at 5 and 37°C. Virus (147000 c.p.m.) was added to four SC-1 cultures at 5°C (lanes 2 and 3) and 37°C (lanes 4 and 5) and incubated for 6 h. Of the added radioactivity, approximately 15% bound to the cells at both temperatures. Cultures represented in lanes 2 and 4 were lysed in sample buffer and those for lanes 3 and 5 were treated with trypsin before lysis in sample buffer (15% and 46% of the bound radioactivity was trypsin-resistant at 5 and 37°C respectively). Lane 1 shows the virus preparation used (19000 c.p.m.). The samples were run on a SDS-polyacrylamide gel which was fluorographed for 63 days.
Fig. 4. Degradation of [3H]glucosamine-labelled virus by trypsin in the presence and absence of Triton X-100 and deoxycholate. Virus (18,000 c.p.m.) was added to dilutions of trypsin in PBS containing 10 mg/ml Triton X-100 and 5 mg/ml sodium deoxycholate (lanes 1 to 7) or to dilutions of trypsin in PBS without detergents (lanes 8 to 14). The mixtures (30 µl) were incubated for 15 min at 37 °C and the reactions were stopped by addition of sample buffer. The samples were electrophoresed on a SDS-polyacrylamide gel which was fluorographed for 15 days. The virus preparation, with and without detergent, is shown in lanes 1 and 8. Trypsin concentrations: lanes 2 and 9, 0-1 µg/ml; lanes 3 and 10, 1-4 µg/ml; lanes 4 and 11, 10 µg/ml; lanes 6 and 13, 10 µg/ml; lanes 7 and 14, 40 µg/ml.

weights 55K, 40K and 18K appeared together with the 35K, 27K and 17K bands. Several cleavage sites were thus uncovered by the solubilization of gp70 from the envelope. Trypsin cleavage of AKR virus gp70 in intact and solubilized virions has previously been compared (Pinter & Honnen, 1983), also showing a facilitated cleavage of the solubilized gp70.

To examine whether the gp70 fragments remained on the virion, the virions were treated with trypsin and centrifuged through 5% sucrose (Fig. 5a). As seen from lanes 2 and 7 to 10, the 35K, 27K and 8K fragments remained largely on the virions, whereas the 17K fragment was totally released. As expected gp70 of untreated virions sedimented with the virions, though some release was observed (compare lanes 1, 3 to 5 and 6).

The cleavage-sedimentation method was used to determine the sequential trypsin cleavage pattern of gp70. A pellet of trypsin-treated virions was made (Fig. 5b, lane 1); it contained mainly the 35K fragment and little gp70. The pellet was resuspended and treated further with trypsin (lane 2). The 27K, 17K and 8K fragments were then observed, showing that they originated from the 35K fragment.

**DISCUSSION**

It is difficult to draw conclusions about the infectious route from experiments with physical particles alone. We have previously shown that infectious and physical particles have identical
Fig. 5. Centrifugation of trypsin-degraded \(^{3}H\) glucosamine-labelled virus and subsequent degradation. (a) Virus (92000 c.p.m.) was treated with trypsin (lane 2) (0.1 mg/ml for 5 min at 20 °C) or left untreated (lane 1) followed by addition of 0.1 mg/ml soybean trypsin inhibitor. One-fifth of each sample was run in each lane. The remainder of each sample was loaded on top of a 5% sucrose cushion and centrifuged at 16000 g for 1 h. Thirty μl fractions from the upper, middle and lower parts of the sucrose cushions (lanes 3 to 5 and lanes 7 to 9, respectively) and the pellets (lanes 6 and 10) were run on a SDS–polyacrylamide gel. The gel was fluorographed for 4 days (lanes 1 and 2) and for 14 days (all other lanes). (b) A pellet of trypsin-treated virus was prepared as above from 70000 c.p.m. virus. The pellet was resuspended in PBS and one-quarter was run in lane 1. The remaining material was treated again with trypsin (125 μg/ml for 10 min at 20 °C) and run on a SDS–polyacrylamide gel (lane 2). The gel was fluorographed for 69 days (lane 1) and 14 days (lane 2).

kinetics with respect to binding, internalization and passage through a step sensitive to lysosomotropic bases (Andersen & Nexo, 1983), which justifies the present study on physical particles.

In the present experiments, up to an estimated 3000 virions were bound per cell. Saturation effects were therefore possible. However, the binding and internalization did not show signs of saturation until a ratio of an estimated 11000 virions per cell was reached. In a variety of cell lines, the number of gp70 receptors has been found to be \(7 \times 10^5\) to \(9 \times 10^5\) per cell (Ganguly et al., 1983) which, at 11000 virions per cell, represents approximately 70 receptors per virion. It should be noted that the fragments observed in the present study have also been observed at lower virus to cell ratios (results not shown).

It is interesting that the 35K and 17K fragments were found both in the cells and after limited cleavage with trypsin or thermolysin. As mentioned in Results, the most plausible explanation is that certain areas on the gp70 molecule are less protected by the carbohydrate groups and by the conformation on the virus membrane. The importance of the conformation is illustrated by the result that solubilized gp70 was degraded more easily and at more sites than gp70 in situ (Fig. 4).

The gp70 fragments were not found until after binding and were found inside the cells, indicating that cleavage occurred during or after internalization. We have previously shown that the viral proteins are degraded in acid vesicles in the cell (endosomes or lysosomes). Basically all gp70 was shown to be totally degraded after internalization, but whereas the amino acids were expelled from the cell, the carbohydrate moieties remained inside the cells (Andersen & Nexo,
1983; Andersen, 1985). The cleavage fragments observed in this study are therefore assumed to be intermediates in the full degradation of gp70.

The significance for infection of the cleavage fragments is difficult to assess. The infection is inhibited by the protease inhibitor leupeptin (Andersen, 1983). Leupeptin decreases the degradation of the virions and is believed to block passage of the virions through acid vesicles of the cell. Leupeptin inhibits a broad range of proteases, especially thiol proteases (Umezawa & Aoyagi, 1972), to which group many lysosomal proteases belong (Barrett, 1980). It can be speculated that leupeptin inhibits infection by inhibiting or altering the cleavage of gp70. The effect of leupeptin on the gp70 cleavage in the cells was tested. Leupeptin reduced the degradation of gp70 (Andersen, 1985), but caused only minor changes of the cleavage pattern (results not shown).

It appears, however, that proteolytic cleavage has some biological effect. Virus-induced cell fusion of C57MC-infected SC-1 cells with uninfected cells was increased by trypsin treatment of the cells (H. Skov & K. B. Andersen, unpublished results). Cell fusion induced by virus particles or virus-infected cells is often used as a model of the fusion event leading to infection. But as discussed, it is not certain that the results on virus-induced cell fusion are relevant for the infection process, though they show that a fusion event can certainly occur. As noted in the introduction, gp70 appears necessary for virus-induced cell fusion. The cleavage of gp70 may expose parts of gp70 which possess the fusion activity. In this respect, it should be noted that some of the trypsin-generated fragments remain on the envelope after cleavage (Fig. 5).

Certain changes of the virus or cell envelope have been shown to stimulate virus-induced cell fusion in other retrovirus–cell systems: low pH treatment (Redmond et al., 1984) and addition of amphotericin B (Pinter et al., 1986), a polyene antibiotic which binds to membranes. These results indicate that changes of the viral envelope are necessary for fusion, changes which the cleavage of the viral envelope may possibly generate.

It has previously been suggested that lysosomotropic bases inhibit infection of retrovirus by a low-pH-dependent membrane fusion between the virus membrane and the endosome/lysosome membrane (Andersen & Nexø, 1983; Redmond et al., 1984). They also inhibit degradation of viral proteins (Andersen & Nexø, 1983; Andersen, 1985). Thus in the proposed route of endocytosis and membrane fusion in the vesicles, it is not clear whether low pH or cleavage of the viral envelope, or both, are responsible for fusion.

The protease cleavage of gp70 differs widely between different retroviruses. Thus a common mechanism for cleavage does not seem to exist. To evaluate the biological importance of gp70 cleavage further work is necessary.

The author is indebted to Anne-Merete Mathiesen and Lotte Reinholt Hansen for excellent technical assistance. The work was supported by the Danish Cancer Society, grant 81-074, and by the Danish Medical Research Council, grants 12-4544 and 12-5205.

REFERENCES


(Received 29 October 1987)