In vitro Cleavage of Pr65\textsuperscript{gag} by the Moloney Murine Leukaemia Virus Proteolytic Activity Yields p30 whose NH\textsubscript{2}-Terminal Sequence Is Identical to Virion p30

By YOSHIYUKI YOSHINAKA\textsuperscript{1,2} ROSE B. SHAMES,\textsuperscript{1} RONALD B. LUFTIG,\textsuperscript{1,*}† GARY W. SMYTHERS\textsuperscript{2} AND STEPHEN OROSZLAN\textsuperscript{2}

\textsuperscript{1}Department of Microbiology and Immunology, University of South Carolina, School of Medicine, Columbus, South Carolina 29208 and \textsuperscript{2}LBI Basic Research Program, National Cancer Institute, Frederick Cancer Research Facility, P.O. Box B, Frederick, Maryland 21701, U.S.A.

(Accepted 19 October 1984)

SUMMARY

In vitro cleavage of Gazdar murine sarcoma virus Pr65\textsuperscript{gag}, which has all of the antigenic determinants of Moloney murine leukaemia virus Pr65\textsuperscript{gag}, i.e. p15, p12, p30 and p10, by the Moloney murine leukaemia virus proteolytic activity yielded a p30 whose partial NH\textsubscript{2}-terminal sequence was identical to Moloney murine leukaemia virus. Both [\textsuperscript{3}H]leucine-labelled and unlabelled Pr65\textsuperscript{gag} were used to generate the cleaved p30.

The process of murine retrovirus morphogenesis appears to encompass three steps: (i) migration of about 2000 polyprotein Pr65\textsuperscript{gag} molecules and two 35S viral RNA moieties per virion, to a site on the plasma membrane; (ii) formation of an 'immature' virion as budding proceeds on the membrane; (iii) maturation of the virion, visualized as a change from type A morphology to type C morphology and detected biochemically by the cleavage of Pr65\textsuperscript{gag} to p15, p12, p30 and p10 (Lu et al., 1979; Witte & Baltimore, 1978; Yoshinaka & Luftig, 1977; R. B. Luftig, unpublished data).

A proteolytic factor with an estimated $M_r$ of 20000 on Sephadex G-75 columns that may be responsible for the cleavage of Pr65\textsuperscript{gag} in vitro has been partially purified in our laboratory from Rauscher (R) and Moloney (Mo) murine leukaemia viruses (MuLV) (Yoshinaka & Luftig, 1980). Recent experiments with the purified factor, together with nucleotide sequence data, suggest that the proteolytic factor is virally coded and has an unhydrated mol. wt. of about 14000 (Y. Yoshinaka & S. Oroszlan, unpublished data). However, due to its instability and low concentration in purified virions, it has been difficult to isolate the factor in sufficient quantities for it to be characterized. Further, the lack of availability of Pr65\textsuperscript{gag} substrates from Moloney and other murine leukaemia viruses has presented another problem which has impeded our studies in determining the mechanism of action of the factor. This latter problem, however, has been remedied by the finding that the uncleaved gag polyprotein of Gazdar murine sarcoma virus (GzMSV), Pr65\textsuperscript{gag}, has all of the antigenic determinants of MoMuLV Pr65\textsuperscript{gag}, i.e. p15, p12, p30 and p10, and constitutes >95% of the protein in virus particles (Maxwell & Arlinghaus, 1981; Yoshinaka & Luftig, 1982). Since GzMSV can be purified directly from culture fluids of HTG-2 cells, it can be readily used as a substrate in studies of the MoMuLV proteolytic factor.

The present report makes use of recent work on nucleic acid and amino acid sequencing of MoMuLV (Oroszlan & Gilden, 1980; Shinnick et al., 1981) to compare the in vitro cleavage site between p12 and p30 with the site recognized by the MoMuLV proteolytic factor in vitro. The in vivo cleavage site between p12 and p30 is:

p12

\[\ldots \text{Thr} \rightarrow \text{Ser} \rightarrow \text{Gln} \rightarrow \text{Ala} \rightarrow \text{Phe} / \text{Pro} \rightarrow \text{Leu} \rightarrow \text{Arg} \rightarrow \text{Ala} \rightarrow \text{Gly} \rightarrow \text{Gly} \rightarrow \text{Asn} \rightarrow \text{Gly} \rightarrow \text{Gln} \rightarrow \text{Leu} \rightarrow \text{Gln} \ldots \]

† Present address: Department of Microbiology and Immunology, LSU Medical Center, 1901 Perdido Street, New Orleans, Louisiana 70112, U.S.A.
Short communication

Fig. 1

Fig. 2

Fig. 1. Assay of protease activity in purified preparations of MoMuLV and GzMSV. MoMuLV was purified from culture fluids of MJD-54 cells chronically infected with MoMuLV. These cells were obtained from Dr K. Manly (Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.). GzMSV particles were purified from tissue culture fluids of HTG-2 hamster cells generously provided by Dr A. Gazdar (VA Medical Center, Washington, D.C., U.S.A.). Virus purification was done according to the method of Syrewicz et al. (1972). Virus was stored in STE buffer. Protein concentration was determined by the Bio-Rad protein assay. After incubation, the reaction mixtures were analysed by SDS-PAGE (Laemmli, 1970). Protein in the gel was detected by staining with Coomassie Brilliant Blue R [0.25% (w/v) in propanol:glacial acetic acid:water (25:15:60) for 24 h; destaining was in propanol:glacial acetic acid:water (10:10:80)]. The reaction mixtures were: (a) 140 μg MoMuLV 4 °C, 20 h in STE; (b) 140 μg MoMuLV 22 °C, 20 h in 2% NP40, 0.01 M-DTT; (c) 140 μg GzMSV 22 °C, 20 h in 2% NP40, 0.01 M-DTT.

Fig. 2. Cleavage *in vitro* of GzMSV Pr65 gag by the MoMuLV proteolytic activity. The following reaction mixtures were prepared in STE containing 2% NP40 and 0.01 M-DTT: (a) 40 μg GzMSV; (b) 40 μg GzMSV + 10 μg MoMuLV; (c) 40 μg GzMSV + 60 μg MoMuLV; (d) 40 μg GzMSV + 120 μg MoMuLV. Incubation was at 22 °C for 20 h. Protein was analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

To study the *in vitro* cleavage site, we have determined the amino-terminal sequence of p30 generated upon cleavage of GzMSV Pr65 gag by the MoMuLV proteolytic factor. MoMuLV which served as the source of the proteolytic factor was purified from culture fluids of MJD-54 cells and GzMSV, the substrate, was purified from culture fluids of HTG-2 cells. Each preparation was initially checked for proteolytic activity as follows: a sample of virus was disrupted by addition of 2% NP40 and 0.01 m-dithiothreitol (DTT), the lysate was either incubated at 4 °C (control, no activity) or 22 °C (activity) for 20 h and then analysed by SDS-PAGE. Fig. 1 shows what happened when MoMuLV was incubated under these experimental conditions. The small amount of Pr65 gag present in MoMuLV preparations remained intact when incubation was at 4 °C (lane a); however, both Pr65 gag and the intermediate cleavage product Pr40 gag disappeared when the NP40 lysate was incubated at 22 °C (lane b). Further, if GzMSV was incubated alone under these conditions, Pr65 gag remained uncleaved regardless of whether the NP40 extract was incubated at either 4 °C or 22 °C (lane c). This suggested the absence of an active proteolytic factor in GzMSV preparations, and thus we could set up our *in vitro* cleavage system with GzMSV Pr65 gag and MoMuLV as described above.
Table 1. Edman degradation of [3H]leucine-labelled and unlabelled GzMSV p30 generated by cleavage in vitro of GzMSV Pr65\textsubscript{gag} with the MoMuLV proteolytic activity*

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>[3H]Leucine (c.p.m. × 10^{-2})</th>
<th>PTH-amino acid (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Pro 0.55</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>Leu 0.84</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Arg 0.41</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Ala 0.86</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Gly 0.45</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>Gly 0.43</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>Asn 0.39</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>Gly 0.35</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>Gln 0.58</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>Leu 0.51</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>Glu 0.55</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>Tyr 0.30</td>
</tr>
<tr>
<td>13</td>
<td>–</td>
<td>Trp 0.10</td>
</tr>
<tr>
<td>14</td>
<td>–</td>
<td>Pro 0.23</td>
</tr>
<tr>
<td>15</td>
<td>–</td>
<td>Phe 0.27</td>
</tr>
</tbody>
</table>

* Automated Edman degradations were performed with a Beckman Sequenator Model 890C as previously described (Copeland \textit{et al.}, 1980). The PTH derivatives of amino acids were identified by HPLC on a phenyl alkyl column (Henderson \textit{et al.}, 1980).

† Background with the [3H]leucine label varied from 200 to 300 c.p.m. Only 12 cycles were run with this material.

We found, as expected, that when increasing concentrations of the MoMuLV NP40 extracts were incubated with a constant amount of GzMSV, there was a correlative increased cleavage of Pr65\textsubscript{gag} to Pr40\textsubscript{gag} to p30 (Fig. 2). Because of the addition of a relatively large amount of MoMuLV protein to the mixture, the p30 seen in Fig. 2(d) was obtained both from the cleaved GzMSV, as well as from the added MoMuLV extract. In order to obtain p30 that was derived almost solely from GzMSV Pr65\textsubscript{gag}, so that we could use it to identify the NH\textsubscript{2} terminus, we used two approaches.

First, since it was known that the amino-terminal sequence of MoMuLV p30 had leucine at positions 2 and 10 (Oroszlan & Gilden, 1980), we decided to use [3H]leucine-labelled GzMSV Pr65\textsubscript{gag} in the proteolytic assay mixture, then to purify p30 and determine where [3H]leucine was located. If cleavage occurred with fidelity then it should be present at positions 2 and 10 of p30 cleaved in \textit{vitro}. If not, [3H]leucine would be found elsewhere. [3H]Leucine-labelled GzMSV was prepared from labelled HTG-2 cells as follows. Cells were grown to near confluence in four 850 cm\textsuperscript{2} roller bottles, culture fluids were removed and the cells washed twice with leucine-free MEM. Then cells were labelled using 12.5 ml of leucine-free MEM, 10\% dialysed calf serum and 0.1 mCi/ml (sp. act. 40 Ci/mmol) of L-[4,5-3H]leucine (ICN) followed by incubation at 37 °C for 24 h. Virus was precipitated by centrifugation at 40000 r.p.m. for 60 min at 4 °C using a Beckman type 42.1 rotor, the pellet was resuspended with unlabelled GzMSV which had been previously purified from HTG-2 cell culture fluids, and the mixture was repurified on a 20 to 50\% sucrose gradient. Then, labelled virus (0.625 mg [3H]leucine-labelled GzMSV, containing 10\textsuperscript{6} c.p.m.) was mixed with unlabelled MoMuLV in a 1:7 ratio. After NP40 disruption and incubation under our protease assay conditions, the resulting protein mixture was separated by SDS–PAGE (12.5\% acrylamide). The slab gels were sliced into 80 pieces (1.5 mm thick), each fraction was crushed and protein eluted into STE (0.13 M-NaCl, 0.01 M-Tris–HCl, 0.001 M-EDTA, pH 8.5) with SDS (0.1\%). The peak of [3H]leucine was determined by counting the radioactivity in each fraction from the gel, and as expected it was located in the vicinity of the p30 position. This was further analysed by running a 12.5\% SDS–polyacrylamide gel of the peak material, followed by fluorography (Bonner & Laskey, 1974). We found that the autoradiogram had a band only at p30. The isolated [3H]leucine-labelled p30 (100000 c.p.m.) was then subjected to Edman degradation. An aliquot (10\%) of the fractions collected at each cycle was
Short communication

Fig. 3. Incubation of unlabelled GzMSV Pr65\textsuperscript{gag} with a partially purified preparation of the MoMuLV proteolytic factor. SDS-PAGE was performed as described in Fig. 1, using 25 μl samples from each mixture. (a) Partially purified proteolytic activity obtained from MoMuLV as follows: 50 mg of MoMuLV was disrupted in 2% NP40, 0.05 M-Tris-HCl pH 8.5, 10 mM-DTT and 10% glycerol (total vol. 2 ml), centrifuged at 10,000 r.p.m. for 20 min at 4°C in a Beckman J-21 centrifuge, and the supernatant taken for dialysis against 0.01 M-BES pH 6.5, 10 mM-DTT. The dialysate was layered onto a phosphocellulose column, followed by elution with 0.3 M-KCl in 0.01 M-BES pH 6.5, 10 mM-DTT and the peak of proteolytic activity (15 ml fraction) was concentrated about 10-fold by Sephadex G-200, before dialysis against 0.02 M-Pipes pH 7.0, 10 mM-DTT. The final concentration of protein in this most active fraction is 40 μg/ml. (b) GzMSV (1000 μg/ml) was prepared from HTG cells as described by Yoshinaka & Luftig (1980). The protein bands at 50K to 55K mol. wt. appear to be breakdown products of Pr65\textsuperscript{gag} which occur during storage of virus. A densitometer trace (Helena Quick Scan) showed in arbitrary Coomassie Brilliant Blue units that the p30 band in (a) contained about 5% of the staining of the Pr65\textsuperscript{gag} in (b). (c) Two mg of the HTG virus was mixed with 80 μg of the protease active fraction and incubated at 22°C for 16 h in 0.02 M-PIPES, 10 mM-DTT. (d) Supernatant from the 6 M-guanidine-HCl-solubilized, centrifuged suspension of material in (c). (e) The material of (d) was loaded onto a phosphocellulose column (1.5 x 10 cm), eluted with 1 M-KCl, and the eluate was dialysed against STE. Then the dialysate was concentrated to 1 ml with Sephadex G-200. Finally, the concentrated material was passed through a Sephadex G-75 column, the p30 peak fractions were collected and again concentrated about 10-fold by use of Sephadex G-200, prior to SDS-PAGE.

used for liquid scintillation counting in order to determine the radioactivity in each cycle. Positive identification and quantification of tritiated phenylthiohydantoin (PTH) leucine was achieved by analysing each residue on a high-performance liquid chromatography (HPLC) column. Fractions eluting at the appropriate retention times (as determined with PTH–leucine standard) were collected, lyophilized and counted for radioactivity. The results are shown in Table 1, column A. It is clear that [\textsuperscript{3}H]leucine was present at positions 2 and 10.

The second approach we used was to isolate GzMSV p30 from an \textit{in vitro} cleavage reaction between unlabelled GzMSV Pr65\textsuperscript{gag} and the MoMuLV proteolytic factor which had been partially purified from 50 mg of MoMuLV by phosphocellulose column chromatography. This proteolytic activity had only a minor amount of contaminating p30 (<5% total staining, in arbitrary Coomassie Brilliant Blue units; Fig. 3a) relative to the added GzMSV Pr65\textsuperscript{gag} (100% total staining; Fig. 3b). Two mg of the HTG virus (1 ml) was thus mixed with 80 μg of the protease active fraction (2 ml), incubated at 22°C for 16 h in 0.02 M-PIPES, 10 mM-DTT (pH 7.2), and the product run on an SDS gel. As seen in Fig 3(c), Pr65\textsuperscript{gag} was predominantly cleaved into a large amount of p30 and some other lower mol. wt. products. In order to remove Pr27\textsuperscript{gag}, p15 and other hydrophobic proteins, a partial purification of p30 was undertaken which
involved solubilization of the mixture with 6 M-guanidine-HCl, followed by dialysis against 0.01 M-BES (pH 6.5) and 10 mM-DTT at 4 °C for 40 h, with three changes of buffer, centrifugation at 10000 r.p.m. for 10 min at 4 °C in a Beckman J-21 centrifuge (lane d). The purity of the p30 in lane (d) is evident relative to that seen in lane (c). Two additional steps, i.e. phosphocellulose (mainly to remove p12), followed by Sephadex G-75 chromatography (to remove p10), gave us the final material seen in lane (e), which we estimate by Coomassie Brilliant Blue staining contains > 95% p30. About 20 μg of this protein was then subjected to Edman degradation as done with the [3H]leucine-labelled p30. Table 1, column B shows the results of Edman degradation for this GzMSV p30 cleaved in vitro. The amino-terminal sequence was identical to that of MoMuLV p30 presented above in this paper (Oroszlan & Gilden, 1980). Further, we note that with alanine in position 4, it differs from p30 of RMuLV which has leucine in position 4. Taken together, the results of columns A and B indicate that the proteolytic factor from purified MoMuLV accurately cleaves GzMSV Pr65 gag in vitro, and further lend support to the hypothesis that this factor is a ‘protease’ which acts in vivo to cleave Pr65 during the final stages of retrovirus maturation.

This work was supported by NIH Grant CA 37380 (formerly CA-27707) and NCI Contract NO1-CO-23909.

REFERENCES


YOSHINAKA, Y. & LUFTIG, R. B. (1982). p65 of Gazdar murine sarcoma viruses contains antigenic determinants from all four of the murine leukemia virus (MuLV) gag polypeptides (p15, p12, p30, p10) and can be cleaved in vitro by the MuLV proteolytic activity. Virology 118, 380–388.

(Received 7 August 1984)