Cleavage of Rauscher Leukaemia Virus (R-MuLV) Pr65\textsuperscript{gag} by the Moloney Leukaemia Virus (M-MuLV) Proteolytic Activity Produces the R-MuLV-specific but not the M-MuLV-specific 40 000 Dalton Intermediate Polypeptide

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(Accepted 27 November 1980)

SUMMARY

Although the Pr65\textsuperscript{gag} precursor polyproteins of Moloney murine leukaemia virus (M-MuLV) and of Rauscher murine leukaemia virus (R-MuLV) have the same apparent mol. wt. by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), their initial ~40000 dalton intermediate cleavage products differ in mol. wt., i.e. the M-MuLV product (Pr41.5\textsuperscript{gag}) is 1500 daltons larger than the R-MuLV product (Pr40\textsuperscript{gag}). We took advantage of this difference to show that in vitro cleavage of R-MuLV Pr65\textsuperscript{gag} by the M-MuLV proteolytic activity gives rise to R-MuLV Pr40\textsuperscript{gag} and not M-MuLV Pr41.5\textsuperscript{gag}. This result suggests that the specificity for cleavage of the MuLV Pr65\textsuperscript{gag} is built into the substrate.

INTRODUCTION

Partially purified Rauscher murine leukaemia virus (R-MuLV) and Moloney murine leukaemia virus (M-MuLV) preparations (Bolognesi et al., 1973) contain small amounts of the uncleaved core precursor polyprotein Pr65\textsuperscript{gag}, its intermediate cleavage product of about 40000 daltons (Jamjoom et al., 1976; Yoshinaka & Luftig, 1977a, b; Oroszlan & Gilden, 1980) and an MuLV Pr65\textsuperscript{gag} proteolytic factor (MuLV-PF) (Yoshinaka & Luftig, 1977c). We have shown recently that the partially purified MuLV-PF isolated from M-MuLV is similar to that of R-MuLV both in its elution position on Sephadex G-75 columns (mol. wt. 20000 to 22000) and in its ability to cleave Pr65\textsuperscript{gag} to a product of about 40000 daltons (Yoshinaka & Luftig, 1980). During our analysis of this process, we noticed that there was a small but clear difference in the mol. wt. of the M-MuLV and R-MuLV 40000 dalton intermediate products. We decided to take advantage of this difference and see whether it was preserved after in vitro cleavage of R-MuLV Pr65\textsuperscript{gag} by the proteolytic activity present in M-MuLV extracts. The details of this analysis are presented below.

METHODS

Viruses. R-MuLV was obtained from the Resources Division of the National Cancer Institute (Dr J. Cole). M-MuLV was purified as described before from MJD-54 cells (a high virus-producing line of JLSV-9 mouse cells chronically infected with M-MuLV obtained from Dr K. Manly, Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.) (Yoshinaka et al., 1980). \textsuperscript{35}S-methionine-labelled (450 Ci/mol, Amersham Corporation, Arlington Heights, Ill.,

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U.S.A.) R-MuLV was obtained from JLSV-9 mouse cells chronically infected with R-MuLV. The cells had been cultured in methionine-free modified Eagle's medium (MEM) containing 10 μCi/ml 35S-methionine (Gibco) plus 5% dialysed calf serum (Flow Laboratories) for 16 h.

Analysis of viral protein. SDS–PAGE was performed as described previously (Yoshinaka & Luftig, 1977c) based on a modification of the method of Laemmli (1970).

Immunoreplica electrophoresis technique. In the immunoreplica electrophoresis method used (Yoshinaka & Luftig, 1977b), a layer of agarose containing monospecific R-MuLV antisera to p30 (lot 6S-492) or p10 (lot 6S-169) (kindly provided by Dr Wilsnack, Huntington Laboratories, through the Resources Program of the National Cancer Institute) was layered over the SDS–polyacrylamide gel.

Measurement of proteolytic activity. Appropriate amounts of 35S-labelled or unlabelled Pr65 gag-rich cores were incubated with an extract containing the proteolytic factor. In this study Nonidet P-40 (NP-40)-disrupted whole virus was used as the major source of the MuLV proteolytic activity. Incubation was either in 0.1 ml 0.02 M-PIPES [piperazine-N,N’-bis(2-ethanesulphonic acid)] pH 7.0, 0.01 N-dithiothreitol (DTT) (PD), and 1% NP-40, or in 0.01 N-tris-HCl pH 7.2, 0.13 N-NaCl, 0.001 M-EDTA, 0.01 M-DTT (TNED), and 1% NP-40. After incubation at 22 °C for 16 h, we added 2 x concentrated electrophoresis buffer (Laemmli, 1970) to the mixture and analysed the proteins on SDS–PAGE. A decrease in the amount of Pr65 gag was taken as an indication of proteolytic activity.

RESULTS

Preparations of MuLV analysed by SDS–PAGE contain, in addition to the six major structural polypeptides, small but variable amounts of the precursor polyprotein, Pr65 (1 to 3%) and a polyprotein of mol. wt. about 40000 (approx. 1%). It can be seen in Fig. 1 that M-MuLV and R-MuLV Pr65 migrate to the same position on the gels; however, the M-MuLV protein of mol. wt. about 40000 migrates to a position of approx. 1500 higher mol. wt. than that of R-MuLV Pr40 gag. We have called it M-MuLV Pr41.5 gag. By immunoreplica electrophoresis, we showed that it and R-MuLV Pr40 both contained antigenic determinants of R-MuLV p30 and p10. This is seen in Fig. 1 (b) for anti-p10 serum; an identical immunoreplica staining pattern for R-MuLV Pr40 and M-MuLV Pr41.5 was seen with anti-p30 serum. This is consistent with their derivation from Pr65 (NH2-p15-p12-p30-p10-COOH) via a cleavage between the COOH terminus of p12 and NH2 terminus of p30 (Yoshinaka & Luftig, 1977a, b; Oroszlan & Gilden, 1980). Also, we noted that M-MuLV p10 appears to have a mol. wt. 500 to 1000 higher than R-MuLV p10. This may account in part for the higher mol. wt. of M-MuLV Pr41.5 gag. We wished to take advantage of the observed subspecies size difference between M-MuLV Pr41.5 gag and R-MuLV Pr40 gag to see whether the M-MuLV proteolytic activity, when used to cleave R-MuLV Pr65 gag, yielded an MuLV Pr41.5 gag or Pr40 gag polypeptide. If the latter occurred, this would suggest that the specificity for initial cleavage of Pr65 gag was built into the substrate while, if MuLV Pr41.5 gag was observed, it would suggest that the cleavage specificity was due to the viral proteolytic activity. It can be seen in Fig. 2 that, when immature cores of R-MuLV enriched in R-MuLV Pr65 gag (Fig. 2a) were incubated with increasing amounts of an M-MuLV extract (Fig. 2b; lanes 7 to 3 correspond to a 1/16 to 1/1 dilution) containing the M-MuLV proteolytic activity, R-MuLV Pr65 gag was almost completely cleaved. In quantitative terms, the Pr65 band decreased in intensity from 86 to 12 arbitrary units of Coomassie blue staining. On the other hand, the R-MuLV Pr40 gag band remained relatively constant except at the highest concentration of activity (lane 3) where it, too, was cleaved. At no point during the reaction was any material found in a band position corresponding to M-MuLV Pr41.5 gag (see Fig. 2a, lane M; Fig. 2b, lane 1). If MuLV Pr41.5 gag were present it would have been detected since M-MuLV Pr41.5 gag and R-MuLV Pr40 gag can be clearly
Fig. 1. (a) Whole virus samples of M-MuLV (MJD-54) (M) and R-MuLV (R) run on 10% polyacrylamide gels in the presence of SDS. The putative M-MuLV ~40000 dalton band (M-MuLV Pr41.5 gag) is at a position corresponding to about 1500 daltons higher than the R-MuLV Pr40 gag band. Calibration over several gels was done with known mol. wt. markers (Sigma): bovine serum albumin, 68000; ovalbumin, 45000; soybean trypsin inhibitor, 21000; and cytochrome c, 12400. Based on this calibration, the mol. wt. of M-MuLV Pr41.5 gag was actually 38000 and that of R-MuLV Pr40 gag was 36500. However, we shall continue to refer to these bands as Pr41-5 gag and Pr40 gag since they possess the antigenic determinants of p30 and p10. Regardless of whether M-MuLV from the National Cancer Institute or from MJD-54 cells was used, the Pr41.5 gag position was the same. (b) Immunoreplicas of 12.5% gels for R-MuLV (R) and M-MuLV (M) reacted with monospecific antisera to p10. The holes in the bands represent regions where an immunoprecipitation band had been observed before staining.

separated in this gel (12.5% polyacrylamide) system (Fig. 2a, lanes M and R). The failure to see also a significant accumulation of R-MuLV Pr40 gag at decreasing dilutions of proteolytic activity may be due to a simultaneous cleavage of R-MuLV Pr65 gag to R-MuLV Pr40 gag and of R-MuLV Pr40 gag to p30 (Yoshinaka & Luftig, 1977c). One criticism, however, of this interpretation is that the cleavage of R-MuLV Pr65 gag may occur so rapidly that any potential MuLV Pr41.5 gag produced is not seen, i.e. it would be immediately cleaved to p30. The constant R-MuLV Pr40 gag band seen at the varying dilutions of activity could then be taken to represent the underlying Pr40 gag of the immature R-MuLV core preparation. We think this criticism is unwarranted, however, because it has previously been shown that in vitro R-MuLV Pr65 gag cleavage by homologous R-MuLV-PF to R-MuLV Pr40 gag occurs rapidly.
Fig. 2. Specificity of cleavage of R-MuLV Pr65\textsuperscript{ag} to R-MuLV Pr40\textsuperscript{ag} by the M-MuLV proteolytic factor. (a) shows preparations of M-MuLV (M) and R-MuLV (R) immature cores enriched in Pr65\textsuperscript{ag}, and the \textasciitilde40000 dalton intermediate gag cleavage product. (b) shows cleavage of R-MuLV Pr65\textsuperscript{ag} by the proteolytic activity present in a detergent-treated M-MuLV extract. Lane 1 is an unincubated control where 50 \mu l M-MuLV in TNED buffer (50 \mu g) plus 50 \mu l of 2 \times concentrated sample buffer was used. In lane 2, 100 \mu l M-MuLV in TNED buffer was incubated by itself in 1\% NP-40 at 22 °C for 16 h. In both cases, a 50 \mu l sample size was used on the gel. Lanes 3 to 7 represent samples containing 50 \mu l R-MuLV (10 \mu g) immature cores incubated with (1/1, 1/2, 1/4, 1/8 and 1/16) amounts of M-MuLV extract (50 \mu g in 50 \mu l at the highest concentration). Incubation was in TNED buffer plus 1\% NP-40. Densitometric scanning of the bands showed, in arbitrary Coomassie blue units, that Pr65 gag decreased from 86 (lane 7) to 63, 37, 29 and 23 (lanes 6 to 3 respectively) units while Pr40 gag changed from 175 (lane 7) to 212, 205, 163 and 10 (lanes 6 to 3 respectively) units. Scanning was done with a Helena Quick Scan, Jr.

(about 1 h), while further cleavage of the intermediate R-MuLV Pr40\textsuperscript{ag} occurs relatively slowly (about 10 h in the assay used) (Yoshinaka & Luftig, 1977b). However, we decided to definitely rule out the possibility that there may still be a rapid cleavage of R-MuLV Pr65\textsuperscript{ag} to MuLV Pr41.5\textsuperscript{ag} to p30 in the heterologous cleavage assay system by cleaving \textsuperscript{35}S-methionine-labelled R-MuLV Pr65\textsuperscript{ag} with the unlabelled M-MuLV extract. For this experiment, shown in Fig. 3, we calibrated the gels before and after cleavage of the \textsuperscript{35}S-methionine-labelled R-MuLV Pr65\textsuperscript{ag}. We used the distance from the actin band as a measure of position for MuLV Pr41.5\textsuperscript{ag} (6 mm below) and MuLV Pr40\textsuperscript{ag} (8 mm below). As the concentration of unlabelled M-MuLV supplying the proteolytic activity was increased, we found only labelled MuLV Pr40\textsuperscript{ag} and no labelled MuLV Pr41.5\textsuperscript{ag}. Quantitatively, we found at the highest concentration of activity that there was both a concomitant fivefold decrease of R-MuLV Pr65\textsuperscript{ag} and an increase of MuLV Pr40\textsuperscript{ag} (Fig. 3 b). Examination of the unlabelled
Moloney leukaemia virus proteolytic activity

Fig. 3. Cleavage of 35S-methionine-labelled R-MuLV Pr65\textsuperscript{aag}-rich immature cores by a detergent extract of M-MuLV. Ten μl 35S-methionine Pr65\textsuperscript{aag}-rich R-MuLV immature cores of 20000 ct/min was added to varying concentrations of M-MuLV. The highest concentration was 50 μg M-MuLV in 50 μl PD buffer plus 2% NP-40. All dilutions were made in PD buffer and, as in Fig. 2, a 100 μl sample was used for each incubation, with 50 μl being placed on the gel. (a) is the Coomassie blue-stained counterpart of the autoradiogram; as increasing amounts of M-MuLV extract (fivefold increments from a 1/25 dilution) were added, there was a very slight increase in the amount of R-MuLV Pr40\textsuperscript{aag} produced. An increase in M-MuLV Pr41.5\textsuperscript{aag} produced by cleavage of M-MuLV Pr65\textsuperscript{aag} was also observed. The M-MuLV proteolytic activity in this preparation did not appear sufficiently high to completely cleave M-MuLV Pr65\textsuperscript{aag}. In the 3-day exposed autoradiogram (b), the cleavage product of R-MuLV Pr65\textsuperscript{aag} can be seen more clearly. Increased amounts of the M-MuLV extract lead to the appearance of increased amounts of MuLV Pr40\textsuperscript{aag}. To ensure that the position seen on the autoradiogram is the same as R-MuLV Pr40\textsuperscript{aag}, we calibrated the positions of R-MuLV Pr40\textsuperscript{aag} (8 mm) and M-MuLV Pr41.5\textsuperscript{aag} (6 mm) on the Coomassie blue-stained gel relative to actin. In the same sized autoradiogram the ~40000 dalton band position was measured exactly at 8 mm from the actin band which indicates that it is MuLV Pr40\textsuperscript{aag} and not MuLV Pr41.5\textsuperscript{aag}. Quantification of the autoradiogram in relative densitometric units shows values for Pr65\textsuperscript{aag} of 245, 173, 51 and Pr40\textsuperscript{aag} of 15, 21, 80 for the added amounts of M-MuLV extract (1/25, 1/5, 1/1 respectively).

gels showed, as expected, that both R-MuLV Pr40\textsuperscript{aag} (from substrate R-MuLV Pr65\textsuperscript{aag}) and M-MuLV Pr41.5\textsuperscript{aag} (derived from M-MuLV Pr65\textsuperscript{aag} present in the extract) were increased after cleavage (Fig. 3 a).

In conclusion, we can say from all of our results that: (i) there appears to be a subspecies difference in size between M-MuLV Pr41.5\textsuperscript{aag} and R-MuLV Pr40\textsuperscript{aag} (Fig. 1); and (ii) M-MuLV extracts contain a proteolytic activity which cleaves R-MuLV Pr65\textsuperscript{aag} to R-MuLV Pr40\textsuperscript{aag} and not to M-MuLV Pr41.5\textsuperscript{aag}. If M-MuLV extracts contain only one Pr65\textsuperscript{aag} protease, then these results suggest that the specificity for initial cleavage is built into the cor-
responding Pr65\textsuperscript{ag} precursors and not the corresponding proteolytic factors which supply the activity (Fig. 2 and 3).

**DISCUSSION**

R-MuLV Pr40\textsuperscript{ag}, the intermediate cleavage product of R-MuLV Pr65\textsuperscript{ag}, is present as a minor component both inside infected cells and in virions. Furthermore, both of these R-MuLV Pr40\textsuperscript{ag} species contain only p30 and p10 group-specific antigenic determinants (Yoshinaka & Luftig, 1977\textit{b}; Naso \textit{et al}., 1979). We have now demonstrated that there is a subspecies difference between R-MuLV and M-MuLV 40000 dalton intermediate \textit{gag} cleavage products, i.e. the mol. wt. of the M-MuLV product appears to be about 1500 higher than that of R-MuLV Pr40\textsuperscript{ag}. We have thus called it M-MuLV Pr41.5\textsuperscript{ag}. \textit{In vitro} cleavage of labelled or unlabelled R-MuLV Pr65\textsuperscript{ag} by the M-MuLV proteolytic activity leads to the production of only R-MuLV Pr40\textsuperscript{ag} and no M-MuLV Pr41.5\textsuperscript{ag}. If we assume that M-MuLV extracts contain only one species of Pr65\textsuperscript{ag} protease, then these experiments support a model where the initial cleavage of Pr65\textsuperscript{ag} is determined by the substrate and not the proteolytic activity. Since M-MuLV p10 appears to be 500 to 1000 higher in mol. wt. than R-MuLV p10, it is also possible that the same or a similar site of cleavage is involved in both R-MuLV and M-MuLV Pr65\textsuperscript{ag} cleavage, i.e. a site at the p12–p30 junction (Oroszlan & Gilden, 1980). This would account in part for the higher mol. wt. of M-MuLV Pr41.5\textsuperscript{ag}. A recent study where p10's from these two viruses were compared found that there are indeed differences between the respective p10 tryptic peptides (Ng \textit{et al}., 1980). End-terminal sequence analysis of the respective 40000 dalton \textit{gag} cleavage products is necessary, however, before it can definitely be proved that the size differences between M-MuLV Pr41.5\textsuperscript{ag} and R-MuLV Pr40\textsuperscript{ag} are due solely to differences in p10's.

We thank C. T. Moran for technical assistance. This investigation was supported by USPHS Grant No. CA-28077 awarded by the National Cancer Institute to R.B.L., and by a grant-in-aid from the government of Japan to Y.Y.

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(Received 10 August 1980)