CHARACTERIZATION OF RAUSCHER LEUKEMIA VIRUS (RLV) P40-42, AN INTERMEDIATE CLEAVAGE PRODUCT OF THE GROUP SPECIFIC ANTIGEN (gag) PRECURSOR POLYPEPTIDE, P65-70

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Summary: In vitro cleavage of the murine leukemia virus protein P65-70 (MW app. = 65-70,000; the group specific antigen or gag precursor polypeptide) occurs after exposure of virus to 2% nonidet-P40 (NP-40; v/v) at 22°C in 10mM dithiothreitol. The cleavage occurs through an intermediate stage, where a protein P40-42, of MW app. = 40-42,000 is initially formed and then declines. Viral P65-70 contains all four antigenic determinants, while P40-42 contains the determinants for p30 and p10. The results indicate that p30 and p10 are adjacent polypeptides on P65-70 and further suggest that the in vitro proteolytic cleavage of P65-70 is specific.

We have observed that Friend leukemia virus (FLV)[1] harvested from a line of chronically infected mouse embryo cells (Eveline; 1) as well as Rauscher leukemia virus (RLV) harvested from a chronically infected line of mouse bone marrow cells (JLS-V9) contain about 10-15% of the particles in an immature form (2). By immature we mean that they: (i) contain a concentrically-coiled instead of a collapsed internal strand, and (ii) are enriched for in a protein of MW app. 65-70,000 (P65-70), the gag polyprotein precursor (3-6). When virions are disrupted in detergent, incubated at 37°C, and analyzed by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis, a proteolytic activity is observed (4,7) that cleaves P65-70 to a protein of

[1]Abbreviations used in this report: FLV, Friend leukemia virus; RLV, Rauscher leukemia virus; $NaDodSO_4$, sodium dodecyl sulfate; NP-40, nonidet-P40; gag, group specific antigens; P, precursor polypeptide; p, polypeptide; gp, glycoprotein; DTT, dithiothreitol; MW, molecular weight; TNE buffer, 0.01M Tris HCl, 0.13M NaCl, 0.001M EDTA, pH 7.3.

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MW app. = 40-42,000 (P40-42) (7). In the presence of 10mM dithiothreitol (DTT), cleavage is completed and P40-42 is converted to the major virus structural protein, p30. The numbering of polypeptides (p), precursor polypeptides (P), and glycoproteins (gp) by apparent MW is according to convention (8). To understand where the cleavage occurred on P65-70, it was of interest to ask what antigenic determinants were expressed by P40-42. Since both of the proteins p10 and p12 are small enough to be included with p30 in such a complex, it was possible that P40-42 contained polypeptides p30+p10, p30+p12, or a mixture of both classes. Our studies utilizing a novel immunoreplica method (9) indicate that P40-42 has the p30 and p10 antigenic determinants.

Materials and Methods

Viruses and analysis of viral proteins: Rauscher leukemia virus (RLV) grown in chronically infected JLS-V9 mouse bone marrow cells and purified by two stage zonal centrifugation methods was obtained from Resources Division of the National Cancer Institute under the auspices of Dr. Jack Gruber. A typical pattern of the virion polypeptides observed on 12.5% $NaDodSO_4$-containing polyacrylamide gels is shown in Fig. 1. Based on quantitating the Coomassie blue stained bands by densitometry, we found that P65-70 contains about 3%, while P40-42 has <1% of the total staining. As will be shown in Fig. 2, this P65-70 band corresponds to the group specific antigen or gag precursor described by other laboratories as Pr$\overline{4}$ (4,5) or Pr$\overline{6}$5 (3,6). We have called it P65-70 since when marker polypeptides, e.g., bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c, were analyzed in parallel on 12.5% $NaDodSO_4$-containing polyacrylamide gels, the band migrated at positions corresponding to apparent MW's of 65-70,000, depending on the method used to prepare the samples. Agarose was obtained from Sigma Chemical Co., St. Louis, Mo.; NP-40 from Bethesda Res. Labs., Bethesda, Md.; all other chemicals were analytical grade.

Electrophoresis of $NaDodSO_4$-containing polyacrylamide gels was performed according to Laemmli (10) using a Hoefer SE-500 slab gel apparatus. 5-30 µg protein samples were loaded and run for five hours at 100 V. Quantitation of the percent staining under the bands in arbitrary Coomassie blue staining units was performed by calculating the areas under the gel band peaks made from Helena Quick Scan tracings.

Immunoreplication technique: The immunoreplicate electrophoresis method of Showe et al. (9) was performed essentially as described. Briefly, after the sample was electrophoresed, the slab gel was cut into five pieces; each piece containing the same amount of protein sample. One piece was stained with Coomassie blue in the

usual manner; the remaining four pieces were overlaid with 0.6% agarose in Dulbecco's phosphate buffered saline (Grand Island Biol. Co., Grand Island, N.Y.) containing 1/10th concentration of monospecific antiserum and developed for 4-5 hours in a moistened chamber. A hole was then punched in the visible precipitates with a small pasteur pipette, so that these regions could be compared with the bands observed after staining of both the agarose and acrylamide gels. The overlay was removed by floating onto a dish containing buffered saline. Excess serum proteins were removed by changing the buffered saline 10 times over a 2-3 day interval. The agarose overlay was then stained with Coomassie blue to localize the precipitin bands. By this method, <1 μg of RLV antigen could make a detectable precipitin line against 1/10 diluted antiserum.

Antisera lots #6S-385(gp69/71), 6S-492(p30), 5S-716(p15), 4S-455 (p12), and 6S-169(p10), were obtained from Dr. R. Wilsnack through the N.C.I. Cancer Resources Division. The antisera titers determined by radioimmunoassay were >3,000 for each specific antigen. The cross-reactivity titers were <50 among different components.

P40-42 isolation: The virion protein of MW app. = 40-42,000 daltons was partially purified by use of a combination column chromatographic and preparative-slab gel electrophoresis procedure. Purified virus (10 mg) was disrupted with 5% nonidet-P40 (NP-40; w/v) and the immature core subparticle fraction, which is enriched in P40-42, was isolated on a sucrose gradient [10-40% in TNE (0.01M Tris-HCl, 0.13M NaCl, 0.001M EDTA, pH 7.3), 30,000 rpm for 40 minutes at 4°C (2)]. This core fraction was then dissolved with 1% $NaDodSO_4$ and loaded onto a Sephadex G-200 column equilibrated with 0.1% $NaDodSO_4$ in TNE. The P40-42 rich fraction (as determined by monitoring each fraction on 12.5% gels) was collected, concentrated and loaded onto a 7% polyacrylamide gel containing $NaDodSO_4$ (see Results and Discussion). The P40-42 rich fraction was then collected and concentrated.

Results and Discussion

We have previously confirmed the observation of Jamjoom et al. (4) that cleavage of P65-70, the gag precursor polypeptide of RLV, occurs after exposure of virus to the non-ionic detergent NP-40 (7). Further, we had noted that the cleavage was initially followed by an increase in P40-42 at low (0.5-2% v/v) detergent concentrations, suggesting that P40-42 might be an intermediate cleavage product of P65-70. This suggestion was confirmed and extended when we examined the detailed kinetics of P70 cleavage (Fig. 1). We incubated the virus in 2% NP-40 (v/v) for various times (0-16 hours), stopped the reaction, and examined the pattern of polypeptides as they appeared by $NaDodSO_4$-poly-

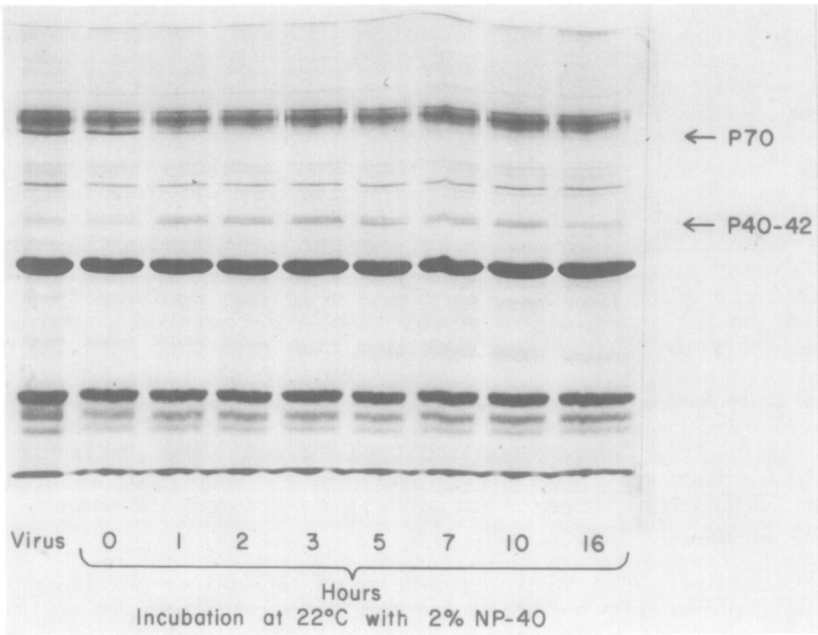

Figure 1: Cleavage of RLV P65-70 after exposure to 2% NP40 (v/v) at 22°C for various times.

acrylamide gel electrophoresis. By two hours all the P65-70 had been cleaved, and there was an increase in P40-42 (from <0.5% to 2% of total staining). With three hours incubation, P40-42 had reached a maximun of ~3% of total staining. Longer incubation times, i.e., 5, 7, 10 or 16 hours lead to an increasingly reduced yield of P40-42, viz., 1%, 1%, <1% and <1%. These data provide support for the contention that P40-42 represents an intermediate state in the cleavage of P65-70. The antigenic composition of P65-70 and P40-42 was then determined. As can be seen in Fig. 2, using the novel immunoreplication technique described by Showe et al. (8) where a dilute solution of antisera is incorporated into an agarose overlay on NaDodSO$_4$-containing polyacrylamide gels, P65-70 has the antigenic determinants of p30, p15, p12, and p10. This confirms our earlier contention that P65-70

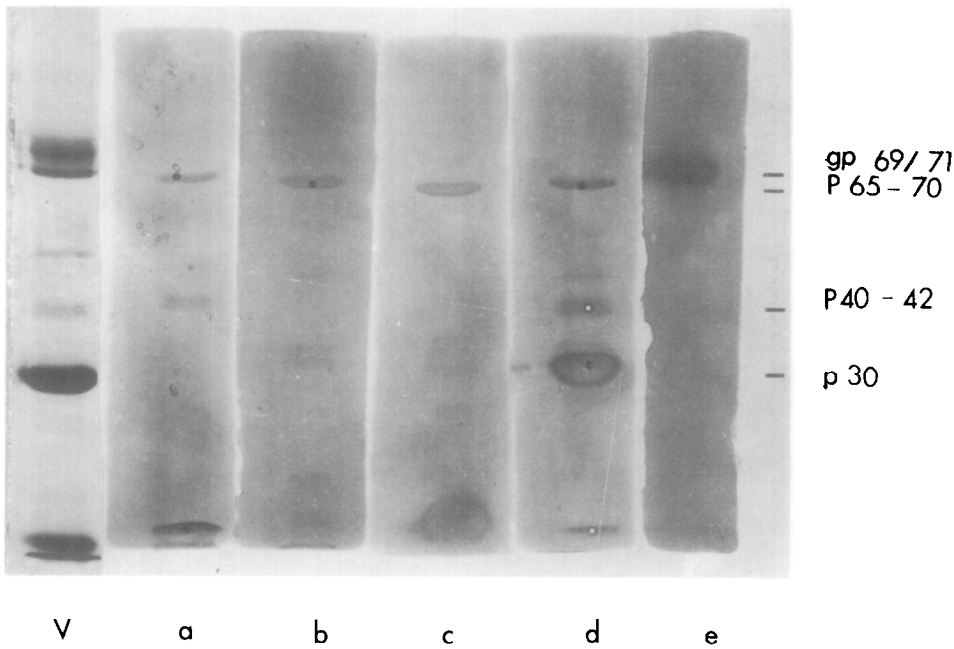

Figure 2: Immunoreplication on 10% gels of RLV reacted against various monospecific antisera. V is the Coomassie blue stained virus pattern. a, b, c, d, and e are the stained immunoprecipitated agarose overlays where antisera to p10, p12, p15, p30, and gp69/71, respectively, had been incorporated into the agarose. The holes in the bands represent regions where an immunoprecipitation band had been observed prior to staining. Note the clear difference in positions for gp69/71, as compared to the P65-70 immunoprecipitation bands.

corresponds to the gag precursor polypeptide reported by other laboratories. For P40-42 it was possible that it could have been composed of the antigens (p30+p10); (p30+p12); (p15+p12+p10), a mixture of any of these, or a non-specific set of cleavage products. In Fig. 2, the results obtained with the small amount of P40-42 present in whole virus suggest that antisera to p30 and p10 only interact with the P40-42 band. However, since the P40-42 band is very faint, this staining could be attributable as well to a cross-contaminating species in the antisera. To eliminate this possibility, we partially purified P40-42 by a

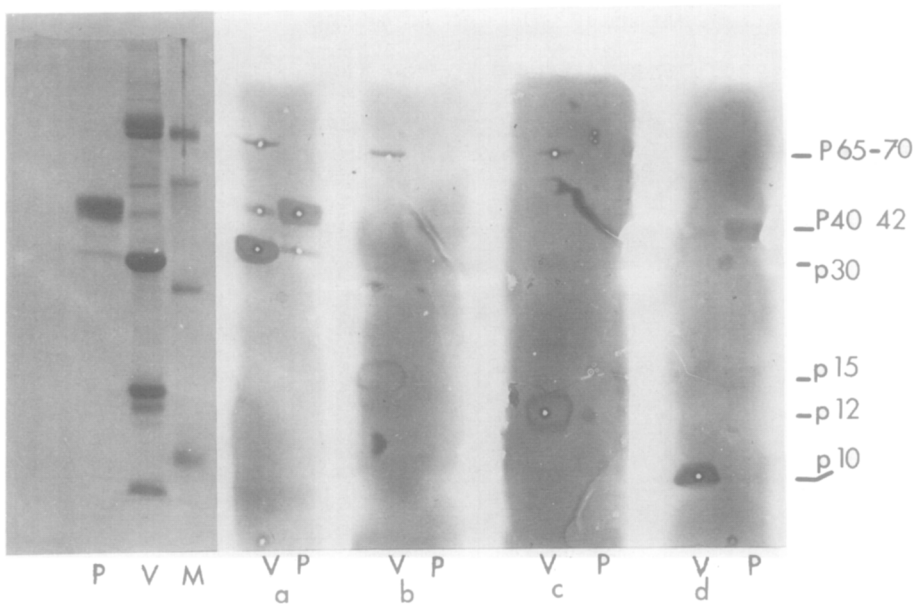

Figure 3: Immunoreplication on 12.5% gels of RLV(V) and the partially purified P40-42(P). M shows the markers of bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c. a, b, c, d are the stained immunoprecipitated overlays where antisera to p30, p15, p12, and p10, respectively, had been incorporated into the agarose. Note the monospecific interactions of p30, p15, p12, and p10 on the V gels, and the precipitation of P40-42 with only p30 and p10 on the P gels.

column chromatographic and preparative $NaDodSO_4$-gel electrophoresis procedure. This gave us larger amounts of material, which were used as follows: 5 µg of the P40-42 rich eluate was electrophoresed on $NaDodSO_4$-containing polyacrylamide gels, then agarose containing the various antisera was layered over the gels, and the immunoreplica prepared. We were now able to observe a strong immunoprecipitin stained band of P40-42 with p30 and p10 exclusively (Fig. 3). As can be seen, p15 and p12 antisera react with either P65-70 or their respective specific proteins bands. These results indicate that P40-42 is composed of (p30+p10), and further suggests that the P65-70 proteolytic factor cleaves P65-70 at a specific location.

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