PARTIAL CHARACTERIZATION OF A P70 PROTEOLYTIC FACTOR THAT IS PRESENT IN PURIFIED VIRIONS OF RAUSCHER LEUKEMIA VIRUS (RLV)

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Summary: Incubation of RLV at 22°C in the presence of 2% NP40 (v/v) induces the activity of a proteolytic factor which cleaves P70, the major precursor protein of P30. The cleavage apparently occurs in two steps: first to a protein of MW app. 40-42,000 daltons (P40-42), and then to P30. In the absence of DTT (10mM), the latter step is blocked. In vitro incubation of labeled immature core subparticles, enriched in P70, with a partially purified proteolytic factor fraction shows an enrichment in both P40-42 and P30. This activity is inhibited by TLCK at 1-10mM, but not TPCK nor ZPCK at concentrations ≤2mM, suggesting the factor is more trypsin than chymotrypsin-like.

Proteolytic cleavages have been shown to be of importance in many viral systems, e.g., the assembly of picornaviruses (5,9); processing of Sendai virus glycoproteins (14); and the maturation of the bacteriophage T4 capsid (11,16). The protein P70 (MW app. = 70,000 daltons) is a murine leukemia virus polyprotein found both in infected cells (1,3,6) and isolated virions (8,15). It is the precursor to the major group specific antigens (gag) of the virus. Incubation of whole virus in the presence of NP40 at 37°C causes the activation of a proteolytic factor which cleaves P70 (8). We have extended this observation, and characterized the activity, as well as isolated the factor which appears to be a protease.

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Materials and Methods

Viruses: Unlabeled Rauscher leukemia virus used in this study was kindly supplied by the Frederick Cancer Research Center under the auspices of Dr. Jack Gruber of the N.C.I. The virus had been purified from supernatant fluids of JLS-V9 cells chronically infected with RLV, using differential zonal centrifugation procedures similar to the one described below.

Labeling of Friend leukemia virus (FLV) with ^{3}H-amino acids (New England Nuclear: avg.sp.act. of 27 Ci/mM) was done with 100μCi/ml in MEM containing 1/10th the amount of amino acids. A subconfluent layer of Eveline cells (13) was grown for 20 hrs. at 37°C in several T-75 flasks in a CO_2 incubator, and the virus was harvested from culture fluids by (i) low speed centrifugation in a Sorvall RC-2B SS-34 rotor at 10,000 rev/min for 10 min. at 4°C to remove cellular debris; (ii) centrifugation of the supernatant fluid at 27,000 rev/min for 120 min. at 4°C in a Spinco L5-50 using a 30 rotor to pellet the virus; and (iii) mixing the virus pellet, which had been resuspended in 0.3 ml of TNE (0.01M Tris-HCl, 0.13M NaCl, 0.001M EDTA, pH 7.3) with 100-500 μg of purified RLV, layering the mixture onto a 10-60% (w/v) sucrose gradient in TNE, and centrifuging at 40,000 rev/min for 60 min. with a Spinco SW50.1 rotor. The visible band was collected, and subjected to SDS-PAGE, followed by radioautography or counting of gel slices treated with Aquasol in a liquid scintillation counter. The resultant band pattern was virtually identical to the Coomassie blue stained bands of RLV.

Inhibitors: TPCK, ZPCK, and TLCK are respectively, tosylsulfonyl-phenylalanyl, carbobenzyloxy-phenylalanyl, and tosylsulfonyl-lysyl chloromethyl ketone. They were obtained from Sigma. Solutions were made in 20% DMSO. Experiments were done at 1:1 ratios of inhibitor:virus.

Isolation of immature cores after Nonidet P40 (NP40) treatment of virus: Virus (1.8 mg; Lowry assay (12)) in TNE was treated with 1-4 mg of NP40 in 0.3 ml at 4°C for 15 min., layered onto 10 to 40% (w/v) sucrose gradients in TNE and centrifuged at 30,000 rev/min for 40 min. at 4°C in a Spinco SW50.1 rotor. About 20 fractions were then collected, using a tube piercing device (Hoefer Scientific). Immature cores banded in the lower one third of the gradient, at 350s (18).

Analysis of viral proteins: To 25 μl of each sucrose density gradient fraction an equal amount of 2 times concentrated electrophoresis sample buffer containing 2% SDS and 10% 2-mercaptoethanol was added. The combined sample was boiled for 1 min. and analyzed on 12.5% or 7% acrylamide slab gels made by the procedure of Laemmli [SDS-PAGE, (10)]. The gels were run on a Hoefer SE500 vertical slab gel system for 5 hrs. at 15 ma. Apparent molecular weights of known markers, i.e., bovine albumin, egg albumin,

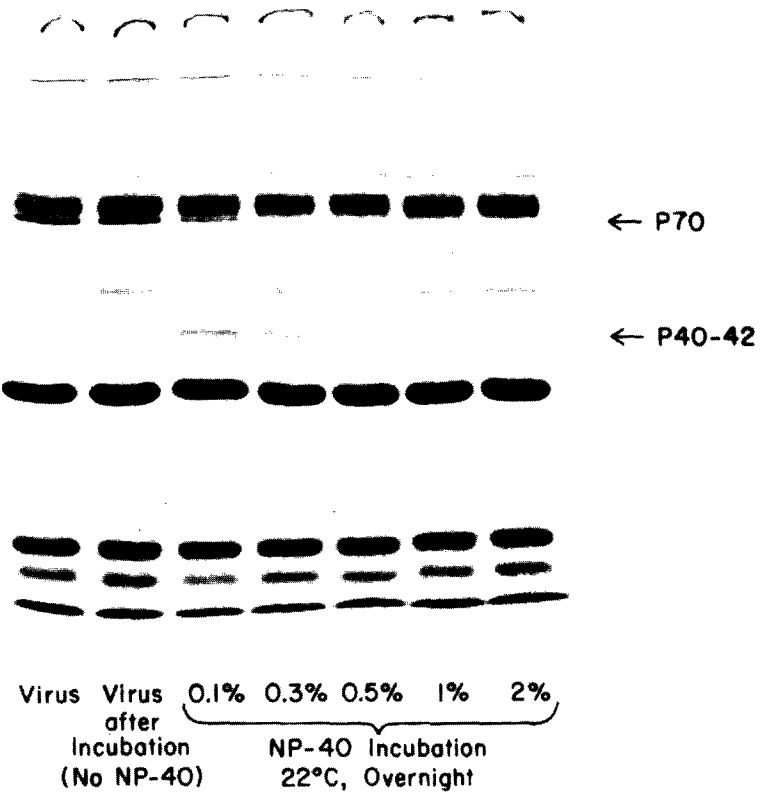

Figure 1: Cleavage of RLV P70 after exposure to (0-2% NP40;v/v) at 22°C for 16 hrs.

chymotrypsinogen, and cytochrome C. The numbering of polypeptides (P) and glycoproteins (gp) by apparent MW is according to convention (2).

Isolation of the P70 proteolytic factor from RLV: Purified RLV (10-20 mg) was disrupted with 50-100 mg of NP40 in the presence of 10mM EDTA and 10mM DTT, layered onto a sucrose gradient and centrifuged as described above. After centrifugation, the top 1/6th of the gradient was removed and passed through Sephadex G-75 column (1.5 x 90 cm) equilibrated with TN (0.01M Tris-HCl, 0.13M NaCl, pH 7.2) containing 0.2% NP40 and 10mM DTT. Each fraction was analyzed on SDS-PAGE to determine its protein composition. The P70 proteolytic activity was assayed by addition of 25 µl of each fraction to an immature core or P70 enriched fraction (25 µl; 10mM DTT and 2% NP40) followed by 16 hrs. incubation at 22°C and SDS-PAGE analysis.

Results

Purified preparations of RLV contain a protein of MW app.=70,000 daltons (P70, Fig. 1; 8,15) as a minor constituent (3% by Coomassie

blue staining). This protein appears to be the same as the polyprotein precursor to P30, P15, P12 and P10 found in infected cells and described by other laboratories (3,6,15). We have partially purified this protein and shown by double immunodiffusion that it contains the immunological determinants of P30 and P15, indicating that it is the P70 polyprotein precursor (18). Most recently we have performed immunoprecipitation studies using ^{35}S-methionine labeled virus, to show that the P70 in immature cores isolated from Friend leukemia virus (FLV) is specifically precipitated by P30 and P15 antisera (unpublished observations), again supporting our contention. We are interested in elucidating the mechanism of P70 cleavage. Extending the original observation of Jamjoom et al. (8), that cleavage of P70 occurs after RLV is incubated in detergent we have observed the following:

1. Cleavage of P70 occurs sequentially, with a protein of MW about 40-42,000 daltons (P40-42) serving as an intermediate.

In Fig. 1 we observe that only P70 and no other polypeptide is cleaved at low detergent concentrations (0.1-0.3%, v/v relative to virus), when RLV is incubated for 16 hrs. A similar cleavage of only P70 occurs at short times (1-5 hrs.) when the NP40 detergent concentration is fixed at 2% (v/v). These cleavages are accompanied by an increase in P40-42. At higher concentrations (0.5-2.0%) or longer times (5-16 hrs.), P40-42 is also cleaved. This suggests complete unfolding of P70 is needed before it can be completely cleaved. In all of these samples, DTT (10mM) was present. In Fig. 2, we observe that the absence of 10mM DTT

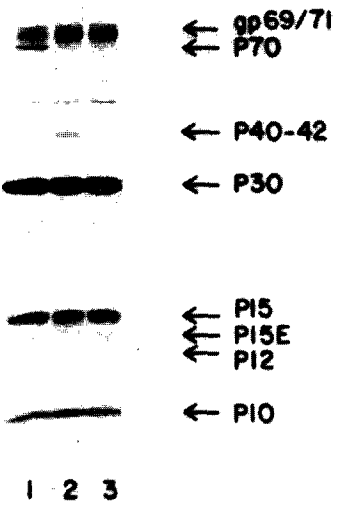

Figure 2: Effect of DTT(10mM) on P70 and P40-42 cleavage. Gel tracks 1, 2, 3 are virus, virus + NP40 (2% v/v), virus + NP40 (2% v/v) + DTT (10mM), respectively. Incubation was for 16 hrs. at 22°C.

leads to an accumulation of P40-42. We have partially purified P40-42 from SDS-gels and intend to examine it for the presence of P30, P10 or P12 antigenic determinants. A similar intermediate cleavage product has been observed in infected cells (3).

2. The proteolytic activity examined in crude virus suspensions has the following properties: a pH optimum of about 7.2; an inhibition by (1-10mM) Mn^{++}; and an inhibition by (1-10mM) concentrations of TLCK (Fig. 3). The activity was assayed by looking for cleavage of P70 on SDS-PAGE after RLV was incubated in 2% NP40 (v/v) for 16 hrs. under the various conditions. Mg^{++} and Ca^{++}, other divalent cations, do not inhibit at up to 10mM

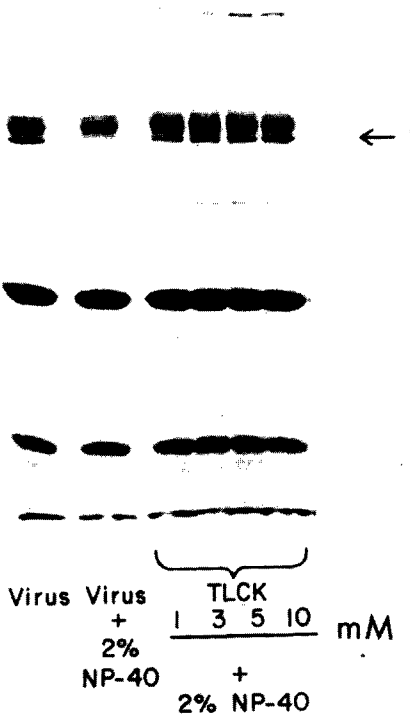

Figure 3: Inhibition of P70 cleavage by TLCK. Concentrations at 1, 3, 5 to 10mM were used.

concentrations, so that the Mn^{++} effect appears specific. Further TPCK and ZPCK do not inhibit the activity at 1 or 2mM in contrast to TLCK; however, at lower concentrations ($\leq$ 0.5mM) TLCK, as well as TPCK and ZPCK, does not inhibit. These protease inhibitor studies suggest that the P70 specific proteolytic activity is controlled by a protease which has some trypsin rather than chymotrypsin-like properties. It is not trypsin, however, since preliminary observations indicate that it has a MW of 10-12,000 on molecular sieve columns, and also it is not inhibited by soybean trypsin inhibitor (1-10 mg/ml).

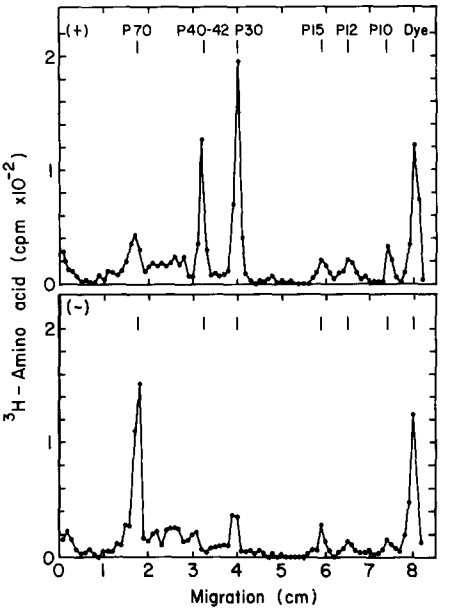

Figure 4: SDS-PAGE profiles after ^{3}H-amino acid labeled FLV cores were exposed to a partially purified fraction of the P70 proteolytic factor (+) or an equivalent amount of TN (-).

3. A partially purified proteolytic factor fraction when added to immature cores (made from ^{3}H-amino acid labeled FLV), exhibited a decline in P70 accompanied by increases in P40-42 and P30. The sucrose gradient, purified 350s band of immature cores is enriched for P70 (17; Fig. 4). After dialysis against TN(pH 7.2), a fraction (50 µl) of immature cores was incubated with 50 µl of the proteolytic factor (+ graph) or 50 µl of TN (- graph). After 16 hrs. the mixtures were subjected to SDS-PAGE, the gels sliced and counted. As seen in Fig. 4, the immature cores treated with proteolytic factor (+) exhibited a 60% decrease of counts in P70 and concomitant increases of about 25% and 35% in P30 and P40-42. Further studies are being undertaken to define

the conditions necessary for this *in vitro* reaction to go to completion, *i.e.*, to P30, P15, P12 and P10. Presumably obtaining a large amount of the proteolytic factor and determining conditions for stabilizing its activity are most important for this effort.

Discussion

We have presented experiments which detail some properties of a polyprotein (P70) proteolytic factor which can be found in virions of RLV. Our goal is to determine whether the proteolytic factor is a protease and define its substrate specificities. These projected studies present a formidable challenge since the activity represents only a very small percent of the virus protein. From 20 mg of starting material, the Sephadex G-75 peak activity fraction shows a faint band (about 1 μg equivalent) at about 10-12,000 daltons (unpublished observations). In addition the activity is lost at a dilution of 1:4. Thus, before we can attempt to purify this factor we have to (a) find methods to increase its yield, and (b) determine what additional factors are needed to stabilize its activity.

In the meantime, our partial characterization of the P70 cleavage has allowed us to distinguish it from the proteolytic activity against *Prla* (~200,000 daltons) described by Jamjoom and Arlinghaus (7). The *Prla* cleavage in contrast to the P70 cleavage is insensitive to TLCK, but sensitive to TPCK and ZPCK. In addition, the processing of the envelope proteins gp70 and p15E (4) seems to occur rapidly in infected cells and apparently differs from the slower P70 cleavage. Thus, several different

proteolytic activities appear to be involved with MuLV assembly at different times, suggesting a host rather than viral origin for all of them since the MW for proteins provided by the genome is essentially accounted for by the known proteins. However, due to its small size, we can't rule out the possibility that the P70 proteolytic activity is viral coded. It is relatively unique in that it is present in isolated virions and its function appears to be specific, viz., cleaving P70 into the four polypeptides p15, p12, p30, and p10. Studies on its temporal appearance in acutely infected cells should assist us in deciding whether the activity is host or viral in origin.

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