NOTES

Inhibition of Murine Leukemia Virus Pr65^{gag} Cleavage In Vitro and In Vivo by Hypertonic Medium

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Cleavage of murine leukemia virus Pr65^{gag} is associated with the activity of a labile proteolytic factor found in virions. We have shown that the presence of 80 to 100 mM NaCl inhibits this cleavage activity in vitro by over 90%. Further, the addition of 80 to 100 mM excess NaCl in vivo to chronically infected cultures of MJD-54 mouse fibroblasts also caused inhibition of Pr65^{gag} cleavage. Specifically, the excess salt added to cells: (i) caused a >90% decrease in virus production; (ii) increased the Pr65^{gag}/p30 ratio in virions produced by more than threefold; and (iii) in pulse-chase experiments, showed a 10-fold decrease in the amount of Pr65^{gag} cleaved after 3 h. In contrast, during this chase interval there was only a slight diminution, i.e., about twofold, in the cleavage of the *env* precursor polypeptide Pr80^{env}, suggesting that cleavages of Pr65^{gag} and Pr80^{env} are differently controlled. Additionally, electron microscopic examination of the excess salt-treated cells showed a twofold increase in the number of associated immature particles, consistent with the observed higher than average Pr65^{gag}/p30 ratio. The inhibitory effects were also found if excess KCl or MgCl₂ was used instead of NaCl, suggesting that they are caused by the hypertonic state of the medium and are not dependent on the ionic species used.

Several recent reports have shown that hypertonic medium can serve as a useful probe for analyzing different stages of RNA animal virus replication. For example, Sindbis virus penetration is inhibited (3); host mRNA-directed, but not viral mRNA-directed, protein synthesis in poliovirus-infected cells is selectively blocked (13); and protein synthesis directed by certain classes of vesicular stomatitis virus mRNA, i.e., M and G, are selectively inhibited relative to other classes of vesicular stomatitis virus, i.e., N and NS (12). Our interest in the use of high salt as a probe for studying murine leukemia virus (MuLV) replication came about by accident. When we varied the salt concentration of the buffers used for resuspending immature Pr65^{gag}-rich cores (18-21) to remove contaminating proteins, the presence of 130 mM NaCl in TNE buffer (10 mM Tris-hydrochloride, 130 mM NaCl, 1 mM EDTA, pH 7.2) was found to be inhibitory of Pr65^{gag} cleavage. This is illustrated in Fig. 1. The immature cores had been isolated from Nonidet P-40-treated Moloney MuLV (21), using TNE buffer throughout. They contained, in addition to Pr65^{gag}, a significant amount of p30 and some Pr40^{gag} (Fig. 1A, lane C). In an attempt to remove these latter proteins, we re-

suspended the core pellets in buffers such as 10 mM Tris-hydrochloride (pH 7.2)-1 mM EDTA (TE buffer) or 20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0]. In those cases we observed that Pr65^{gag} was cleaved and that immature cores could not be reisolated. This is seen in Fig. 1A, lane -N. If 130 mM NaCl was present in the buffer used to dissolve the pellet, cleavage of Pr65^{gag} did not occur (Fig. 1A, lane +N) and immature cores could be reisolated. We believe that there is a relatively small amount of the MuLV Pr65^{gag} proteolytic factor still remaining associated with immature core pellets that is capable of cleaving the Pr65^{gag} if excess NaCl is omitted. On the other hand, cleavage is inhibited if 130 mM NaCl is maintained. We also note that, in cases where larger amounts of MuLV proteolytic factor are used in such an assay, e.g., as with concentrated fractions from Sephadex G-75 columns (20, 21), cleavage of Pr65^{gag} can occur despite the presence of 130 mM NaCl. Further investigation into how NaCl inhibits Pr65^{gag} cleavage shows that: (i) the inhibition of cleavage is directly correlated with the NaCl concentration in the assay mixture (viz., inhibition of cleavage was about 40% when 50 mM NaCl was added and 100%

FIG. 1. Inhibition of MuLV Pr65^{gag} cleavage in (A) immature core preparations or (B) purified virus, by addition of NaCl. Moloney MuLV was purified from MJD-54 cells, using the media, growth conditions, and isolation conditions previously described (17). In (A), a preparation of Moloney MuLV Pr65^{gag}-rich immature cores was incubated in the presence of 130 mM NaCl (+N) or 13 mM NaCl (-N). C is an uninoculated control. Both incubations were for 16 h at 22°C with 30 µg of unlabeled Pr65^{gag}-rich substrate in TE buffer plus 10 mM dithiothreitol and 1% (vol/vol) Nonidet P-40. After incubation an equal amount of 2X-concentrated electrophoresis sample buffer was added to the mixture, which was boiled for 1 min, and 50 µl (7.5 µg of protein) of each sample was electrophoresed according to Laemmli (5). In (B), 30 µg of a purified Rauscher leukemia virus preparation (obtained from the National Cancer Institute Resources Program) enriched for Pr65^{gag} (17) was mixed with various concentrations of NaCl in TE buffer plus 10 mM dithiothreitol and 1% (vol/vol) Nonidet P-40 in 100 µl, incubated, and assayed as above. Densitometric scans of the Pr65^{gag} band made with a Helena Quick Scan Jr. gave values of 24, 53, 75, and 196 in arbitrary Coomassie blue units for 10, 30, 50, and 100 mM added NaCl. The density of the Pr65^{gag} band at 100 mM NaCl was identical to that at 130 mM NaCl (data not shown).

when 100 mM NaCl was added [Fig. 1B]); and (ii) this inhibition reflects the increase in ionic strength rather than the increase in Na⁺ ions since MgCl₂, MnCl₂, or KCl also inhibited Pr65^{gag} cleavage by 90% or better at concentrations of 130 mM (data not shown).

Once we had observed that cleavage of Pr65^{gag} could be inhibited *in vitro* by excess NaCl or other salts, we wondered whether such an inhibition would also occur *in vivo*. Initially we reasoned that, since cleavage of Pr65^{gag} *in vivo* was linked to maturation (18), after excess salt addition we might find that there was a dimi-

nution in virus production and an accumulation of dense-staining precursor material (16) under the cell surface. We did not find any accumulation of electron-dense material under the surface membrane; however, we did find that virus particle production was diminished by 70 to 90% after exposure of the MJD-54 cells to an excess of 80 to 100 mM NaCl in the culture medium. Virus particle production was assayed by either: (i) measuring the amount of protein (7) remaining in the virus bands partially purified from tissue culture fluids after polyethylene glycol precipitation and sucrose gradient centrifuga-

TABLE 1. Measurement of reverse transcriptase activity in tissue culture fluids after addition of excess salt^a

Expt	Activity (cpm) at given salt concn					
	Control	NaCl (80 mM)	MgCl ₂ (50 mM)	KCl		
				50 mM	80 mM	100 mM
1	619,000	76,500	184,500	308,000	183,000	65,000
2	154,000	12,000	26,500	84,300	20,000	7,500

^a Reverse transcriptase was assayed by using poly(rA)-oligo(dT)₁₂₋₁₈ as a template and measuring incorporation of [³H]TMP (17). Data are presented as averages (±10%) incorporated over two identical runs.

FIG. 2. Protein composition of MuLV particles produced after addition of 0 to 100 mM excess NaCl to MJD-54 cells. The partially purified virus bands obtained after step gradient centrifugation were pelleted and resuspended in TNE buffer (0.3 ml), and 50 µl of each sample was analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels. For the 100 mM sample, the virus band was resuspended in 0.15 ml of TNE buffer since it contained less protein. Densitometry of the bands showed, in arbitrary units, values of 781, 948, 818, and 404 and 224, 359, 595, and 391 for p30 and Pr65^{gag}, respectively, at 20, 40, 60, and 80 mM excess NaCl. For the 0 and 100 mM gels, there was runoff of stained material at the edges, and these gel lanes were not used for quantitation.

tion, or (ii) measuring the amount of reverse transcriptase activity in tissue culture fluids (Table 1). The inhibitory effect also does not appear to be Na^+ specific *in vivo* since addition of excess 80 mM KCl or 50 mM MgCl_2 exhibited a similar 70 to 80% decrease in activity (Table 1).

We next examined the polypeptide composition of the particles produced from MJD-54 cells after exposure of the cells to excess salt. The viral proteins were analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels, and, as can be seen in Fig. 2, there was a clear increase in the amount of Pr65^{gag} relative to p30. Quantitation by densitometry showed that the Pr65^{gag} /p30 ratio was increased from 29 to 97% for the particles produced from cells after treatment with 20 to 80 mM excess salt, respectively. This result is consistent with the *in vitro* results.

A thin-section electron microscopic examination of MJD-54 cells treated with excess salt was also undertaken. Submembranous electron-dense regions which have been seen in several Rauscher leukemia virus temperature-sensitive mutants grown under nonpermissive conditions (16) were not seen, regardless of whether 80 mM

excess NaCl or KCl was used. However, we did note that: (i) the relative number of immature particles (compared with mature particles) was increased nearly twofold after 50 or 80 mM excess NaCl treatment (Fig. 3); and (ii) with 80 mM excess NaCl there was a 30% increase in the number of immature particles with a highly coiled, double-ring morphology (see immature particle with arrow in Fig. 3B). This morphology has previously been associated with particles that possess a high Pr65^{gag} /p30 ratio (10). The rings presumably represent a ribonucleoprotein complex of Pr65^{gag} and 35S or 70S RNA inside the virus particles (8; R. B. Luftig and Y. Yoshinaka, in G. Koch and D. Richter, ed., *Biosynthesis, Modification, and Processing of Cellular and Viral Polypeptides*, in press).

To this point we have only examined the effect of high salt on the extent of extracellular Pr65^{gag} cleavage. To see whether the inhibitory effect extended to intracellular processing as well, pulse-chase experiments were performed by labeling cultures of MJD-54-infected cells with [^{35}S]methionine for 10 min followed by a 3-h chase with cold medium containing 0 or 80 mM added NaCl. Then viral proteins were immuno-

FIG. 3. Ultrastructure of MJD-54 cells after treatment with (A) 0 or (B) 80 mM excess NaCl. Typical mature particles are seen in (A) (thick arrow), whereas immature particles are seen in (B). Cells were scraped from T-75 flasks into phosphate-buffered saline, pelleted, fixed with 2% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.4), postfixed with 1% OsO_4 , dehydrated, embedded, sectioned, and stained with lead citrate as previously described (9). (A) $\times 24,000$; and (B) $\times 50,000$.

precipitated with p30 antiserum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A similar experiment was done with gp70 antiserum for comparison. By 3 h postchase with 0 mM NaCl, over 90% of the pulse-labeled Pr65^{gag} has presumably been cleaved and incorporated into virus particles (Fig. 4); it is no longer associated with the cell. There is also very little labeled intracellular p30 present at this time, which is consistent with the results of Schultz et al. (15). When 80 mM excess NaCl was added at the time of the chase, about two-thirds of the initial Pr65^{gag} pulse-label persisted inside the cell at 3 h postchase. As an aside we also note that Pr80^{gag}, which is apparently a precursor to glycosylated *gag*, disappears regardless of whether the chase is done with 0 or 80 mM excess NaCl. These results are consistent with those of Edwards and Fan (2), who

FIG. 4. Effect of excess (80 mM) NaCl addition on the processing of Moloney MuLV polyproteins. Pulse-labeling of MJD-54 cells was performed with [³⁵S]-methionine (10 μ Ci/ml; 850 Ci/mmol) for 10 min; the chase was for 3 h, and cell lysates (lysed in 10 mM Tris, pH 7.2, 50 mM NaCl, 1.5 mM MgCl₂, 0.5% sodium deoxycholate, 0.5% Nonidet P-40) were immunoprecipitated (6) with antiserum to p30 (lot 78S-223) or gp70 (lot 78S-225) from the National Cancer Institute Resources Program (final concentration, 1/20). Incubation was for 20 min at 22°C, followed by incubation for 16 h at 4°C. Immunoprecipitates were collected by centrifugation at 10,000 $\times$ g for 15 min, washed twice with lysing buffer, and resuspended in 0.1 ml of water. After mixing with a 2 $\times$ concentration of electrophoresis buffer (7), the mixture was boiled for 1 min and 50 μ l of the sample was analyzed on sodium dodecyl sulfate-polyacrylamide gels. Fluorography was done at -70°C as previously described (1). Densitometry showed, in arbitrary Coomassie blue units, values of 390, 25, and 248 and 0, 43, and 18 for Pr65^{gag} and p30, respectively (anti-p30 serum, first three lanes). Correspondingly, values of 344 and 462, 234 and 133, and 37 and 56 were obtained for Pr80^{env}, gp70, and p15E, respectively, using anti-gp70 serum (last two lanes).

found that glycosylated *gag* was more rapidly released from cells than Pr65^{gag}, and those of Ledbetter et al. (6), who found that glycosylated *gag* was not incorporated into virions.

In contrast to the pattern of Pr65^{gag} cleavage (10-fold inhibition), we found (Fig. 4) that the cleavage of the envelope precursor Pr80^{env} to gp70 and p15E was relatively less inhibited. Quantitation of the autoradiogram showed that the amounts of gp70 present after 0 and 80 mM excess NaCl addition were 38 and 20%, respectively, of the total *env* label on the gel; for p15E it was about 10% in both cases. Quantitation of Pr65^{gag} showed that 64% of the label was present after the chase with 80 mM NaCl, as compared to about 6% with 0 mM NaCl.

The above labeling results, when taken together with the other studies, suggest that production of mature MuLV (i.e., virions in which Pr65^{gag} is cleaved) is inhibited at several levels by increasing the concentration of salt to 80 mM in the culture medium. The labeling experiments also indicate that the protease involved in the cleavage of Pr65^{gag} may be differently controlled than the protease that cleaves the envelope precursor Pr80^{env}.

In support of these suggestions, it has been shown that in the avian tumor virus system the avian myeloblastosis virus-p15-associated protease which cleaves the avian *gag* polyprotein does not cleave the avian *env* polyprotein (11). Thus, at least a second virus-associated proteolytic activity is involved in maturation. We have also reported recently (Luftig and Yoshinaka, in press) that both serine and thiol protease-sensitive sites exist on MuLV-Pr65^{gag}. Thus, the cleavage of Pr65^{gag} to the intermediates Pr40^{gag} (p30-p10) and Pr27^{gag} (p15-p12) appears to involve the serine protease, whereas processing to the final products (p30, p10, p15, and p12) may involve the activity of a thiol-like protease (14; Luftig and Yoshinaka, in press). This thiol protease activity could represent yet another virion-associated protease. In the future we will try to purify sufficient quantities of the different murine proteolytic activities so that they can be better characterized.

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