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Effect of Chemical Modification and Fragmentation on Antigenic Determinants of Internal Protein p30 and Surface Glycoprotein gp70 of Type C Retroviruses

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The effects of protein modification on the antigenic determinants of p30 and gp70 of type C retroviruses were investigated by using solid-phase competition radioimmunoassays. Proteins were modified by reduction with 2-mercaptoethanol and subsequent carboxymethylation of SH groups with iodoacetamide or by amidination of α and ϵ amino groups with methylacetimidate. The type-specific determinants of gp70 were found to be conformational in nature, as they were destroyed by these chemical modifications. Group- and interspecies-specific determinants of gp70 antigens, however, appear to be sequential and do not involve residues susceptible to these chemical reagents. Conformation-dependent type-specific determinants of p30 were affected only by methylacetimidate. Group- and interspecies-specific determinants of p30 are similar to those of gp70 in that they also appear to be sequential antigenic sites. Therefore, the broadly reactive group- and interspecies-specific determinants of gp70 and p30 can be followed into small peptides. Accordingly, a cyanogen bromide cleavage fragment derived from the carboxyl-terminal one-third of Rauscher leukemia virus p30 was found to carry group-specific determinants but no detectable interspecies-specific determinants. In contrast, a peptide obtained by limited trypsin cleavage of p30, which was derived from the NH_2-terminal region of the protein, contained at least one of the interspecies determinants shared with feline leukemia virus p27.

The mammalian type C viral proteins gp70 and p30 have been shown to contain type-specific, group-specific, and interspecies-specific determinants (13, 29, 37). We previously investigated the relationship among these determinants in p30 by various immunochemical methods (6-9). These studies demonstrated that mammalian p30's contain multiple antigenic determinants in both species-specific and interspecies-specific categories. The glycosylated envelope protein gp70 is much more complex in structure and function than p30, as it is important both in the recognition and attachment of viruses to specific surface receptors and in the primary immunological response of hosts to viral infection (11, 21, 36, 39). Vaccines have been prepared by using inactivated virus or purified gp70, and passive immunization with anti-gp70 IgG has been shown to protect against viral infection and the development of leukemia (10, 11, 17-19, 31, 34).

Chemical modification of proteins combined with other approaches, including fragmentation, has been shown to provide an extremely effective method for the immunological characterization of protein antigens. Such studies have recently led to the determination of the entire antigenic structure of myoglobin and lysozyme (1, 2). In this communication, we present the results of studies of the antigenic changes brought about by chemical modification of disulfide bonds, sulfhydryl groups, and amino groups of two viral structural proteins, p30 and gp70. We also report the findings of partial antigenic mapping of Rauscher leukemia virus p30. These studies are a first step in an attempt to elucidate in detail the relationship between the primary structure and the nature and location of the antigenic determinants of the type C viral proteins. This is essential to the development of specific synthetic viral antigens (immunogens) for eventual use as vaccines to stimulate the immune responses of hosts.

For the present studies in which competition assays, were used, a solid-phase radioimmunoassay (RIA) was developed, which used purified protein A covalently attached to Sepharose 4B.

MATERIALS AND METHODS

Virus. Sucrose gradient-purified virus samples obtained from the Viral Resources Laboratory, Frederick Cancer Research Center, Frederick, Md., were as follows. Rauscher murine leukemia virus (R-MuLV) was grown in monolayer cultures of chronically infected BALB/c mouse bone marrow, JLS-V9 cells; AKR-MuLV was obtained from virus-producing NIH Swiss embryo cells originally provided by Janet Hartley,

National Cancer Institute, Bethesda, Md.; and feline leukemia virus (FeLV) (Theilen strain) was obtained from a chronically infected cat lymphocytic cell suspension culture (FL74). Total viral protein was determined by the method of Lowry et al. (25).

Protein purification. R-MuLV p30 and gp70 were purified as previously described (26, 28, 38) by using either isoelectric focusing or NaCl solubilization followed by chromatography on Whatman P11 ion-exchange cellulose (Whatman, Clifton, N.J.) and gel filtration on Bio-Gel P100 (Bio-Rad Laboratories, Richmond, Calif.). Purified proteins were quantitated and assayed for purity by amino acid analysis (16, 30) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

Iodination. Proteins were iodinated by using 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (IODO-GEN; Pierce Chemical Co., Rockford, Ill.), according to the original method of Speck et al. (12; J. C. Speck, P. J. Fraker, and J. J. O'Donnel, Fed. Proc. **35**:1450, 1976), as previously described (4). After the addition to IODO-GEN of up to 10 μg of gp70 or p30 and 0.5 mCi of ^{125}I (New England Nuclear Corp., Boston, Mass.), the reaction was allowed to proceed for 1.5 min at room temperature. Labeled protein was separated from free iodine by gel filtration on Bio-Gel P10 (Bio-Rad Laboratories). Samples were counted with a Beckman Gamma 300 system gamma counter. The percentage of protein-bound iodine for each probe was determined by ascending paper chromatography on Whatman SG 81 paper developed with butanol-acetone-water-ammonium hydroxide (20:65:15:0.1, vol/vol) (A. M. Schultz, Frederick Cancer Research Center, unpublished data) and found to be at least 95%. Iodinated proteins were analyzed for purity by autoradiography (23) of sodium dodecyl sulfate slab gels (22).

Reduction and carboxymethylation. Reduction and carboxymethylation of up to 1 mg of total protein was carried out by the method of Chavez and Scheraga (5). Briefly, samples of purified proteins (p30 or gp70) or sucrose gradient-purified virus were taken up in 1 ml of 6 M urea in 0.1 M Tris-hydrochloride buffer, pH 8.3, and gassed with N_2 for 5 min. A 50-μl amount of 2-mercaptoethanol was added, and the samples were kept under N_2 for an additional 5 min. They were then sealed, capped tightly, and incubated in the dark for 3 h at 37°C. Iodoacetamide (Pierce Chemical Co.) in excess was added under N_2, the vials were resealed, and the samples were incubated in the dark at room temperature for 1 h. After completion of the reaction, samples were exhaustively dialyzed in the cold against two changes of Tris buffer (0.025 M Tris-hydrochloride, 0.125 M NaCl, pH 8.2) containing 2 mM iodoacetamide, followed by dialysis against the buffer without idodacetamide.

Amino acid analysis of reduced and carboxymethylated (RCM) p30 showed that carboxymethylcysteine was present in the amount of one residue per molecule of protein. This is in agreement with the published amino acid composition of p30 (30). RCM gp70 was found to contain at least 10 residues of carboxymethylcysteine per molecule of protein, which is in the range of published values for the half-cystine content of gp70 (3, 26).

Reaction with MAI. Methylacetimidate (MAI) (Pierce Chemical Co.) was dissolved in 1 M $NaHCO_3$ (pH 9.3), and the pH was adjusted rapidly back to 9.3. Before reaction with MAI (40), samples containing up to 1 mg of purified protein or viral protein mixture were dissolved in 0.5 M $NaHCO_3$ containing 6 M guanidinium chloride (final pH, 9.3) and incubated at 37°C for 1 h. After the sample was cooled to room temperature, a portion of the freshly made MAI (10 mg/ml) solution was added to give a molar excess of approximately 20-fold over the total number of lysine residues present. For the viral protein mixtures, only a gross estimate of lysine residues could be made on the basis of the known compositions of the individual protein components and their approximate molar proportions in the virus particles. After 2.5 h of reaction at room temperature, the addition of MAI was repeated, and the reaction was allowed to proceed overnight at room temperature. To prepare the samples for use in subsequent experiments, Nonidet P-40 and sodium deoxycholate were added to final concentrations of 0.5%, and the samples were dialyzed for 24 h against 0.02 M Tris-hydrochloride (pH 7.5) containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.05 M NaCl (TND buffer).

Cyanogen bromide cleavage. Cyanogen bromide cleavage at methionine residues (14) of R-MuLV p30 (two methionines per molecule [30]) is used routinely in our laboratory for primary structure analysis by amino acid sequencing. To produce fragments, RCM p30 prepared as described above was dissolved in 70% formic acid (1 mg/ml) and reacted with excess cyanogen bromide at room temperature overnight. After removal of cyanogen bromide and formic acid by repeated lyophilization, the extent of p30 cleavage was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis (L. E. Henderson, unpublished data) to be 80% or higher. As expected, three fragments are produced (27; L. E. Henderson, T. D. Copeland, and S. Oroszlan, Proc. Int. Cong. Biochem. 11th, p. 19, 1979): (i) CB fragment 1, which has an approximate molecular weight of 20,000 and represents the NH_2-terminal region; (ii) CB fragment 2, a peptide of approximately 10,000 daltons which is adjacent to CB fragment 1; and (iii) CB fragment 3, a short peptide corresponding to the C-terminal piece. CB fragment 2 has been purified to homogeneity by gel filtration on Bio-Gel P30 eluted with 0.1 M acetic acid (L. E. Henderson et al., manuscript in preparation). This purified fragment and the unresolved cleavage mixture were used in the competition RIAs described below.

Limited tryptic digestion. Purified p30 was subjected to limited trypsinization, and the resulting trypsin-resistant core peptide (TRCP) was purified by isoelectric focusing as previously described (7). This homogenous fragment of approximately 10,000 daltons is derived from the NH_2-terminal domain (29) of p30 and corresponds to approximately one-half of CB fragment 1.

Antisera. The glycoprotein purified as described above was subjected to an additional purification step by electrofocusing in a 1 to 6 M urea gradient in the presence of 1% Ampholine (pH 3 to 10). The major peak fractions (pH 5.0 to 6.0) were dialyzed against

phosphate-buffered saline and used for immunization. At 10-day intervals, three injections of this immunogen (250 μg/injection) in Freund complete adjuvant were administered to a goat. At 2 weeks after the final injection, the animal was bled. The serum obtained was shown by XC tests to contain neutralizing antibody against R-MuLV, AKR-MuLV, and FeLV (titers, 1:640, 1:160, and 1:80, respectively).

To prepare antisera against chemically modified viral antigens, R-MuLV and FeLV were disrupted, reduced, and carboxymethylated as described above. Antisera against these antigens were prepared in guinea pigs by using standard laboratory protocols for immunization (28). Anti-R-MuLV p30 goat serum and guinea pig serum and anti-R-MuLV gp70 guinea pig serum were available from previous studies.

Solid-phase RIA. A solid-phase RIA was developed by using protein A-Sepharose (Pharmacia, Piscataway, N.J.) to effect the separation of free and bound antigens. In this assay, approximately 5,000 cpm of either ^{125}I-labeled gp70 or p30 was incubated with dilutions of antiserum in 0.5 ml (total volume) of 0.1 M Tris-hydrochloride–0.15 M NaCl (pH 8.2) containing 0.1% bovine serum albumin. Samples were mixed and allowed to stand at 37°C for 30 min. Portions (20 μl) of a protein A-Sepharose bead suspension (145 mg/ml of TND buffer) were added, and the tubes were mixed gently for 2 h at 4°C. After determination of the total counts per sample, the beads were pelleted by centrifugation in a Beckman Microfuge B and washed once with 1.5 ml of 0.1 M Tris-hydrochloride–0.15 M NaCl, pH 8.2. Samples were recounted, and results were expressed as percentage of total counts. Competition assays used ^{125}I-labeled gp70 or p30 and a dilution of appropriate antiserum sufficient to give approximately 50% of maximum precipitation. Competing antigens added in varying amounts were purified protein (untreated or chemically modified) and disrupted virus (untreated or chemically modified) diluted in TND buffer. Results were expressed as percent inhibition of precipitation and were plotted against concentration of competing protein on a semilogarithmic scale. This assay is rapid (it is completed in 3 h), reproducible, and sensitive; the level of detection is 0.5 ng/assay for p30 and 10 ng/assay for gp70.

RESULTS

Purity of ^{125}I-labeled proteins. The specific radioactivities of the iodinated protein probes used throughout this study were in the range of 0.75×10^6 to 1.5×10^6 cpm/μg of protein for gp70 and 1.0×10^6 to 3.0×10^6 cpm/μg of p30. Analysis of ^{125}I-labeled p30 and gp70 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1) showed that both iodinated proteins migrated as one component of the appropriate molecular weight. When the protein A-Sepharose solid-phase immunoassay was used, over 90% of the counts of both p30 and gp70 were uniformly immunoprecipitable.

Titration of antisera. Figure 2A shows the titration curves obtained on precipitation of ^{125}I-labeled R-MuLV gp70 with anti-R-MuLV gp70 goat serum and with guinea pig antisera to RCM R-MuLV and RCM FeLV. The titration curves with ^{125}I-labeled R-MuLV p30 for the two latter sera and anti-R-MuLV p30 goat serum are shown in Fig. 2B. The titer (the dilution required to give 20% of maximum precipitation) was calculated from Fig. 2 for each of the various antisera. For gp70 specific antibody, the values obtained were 1:100,000 for anti-R-MuLV gp70 goat serum, 1:20,000 for anti-RCM R-MuLV guinea pig serum, and 1:5,000 for anti-RCM FeLV guinea pig serum. The titers of p30 specific antibody were 1:60,000 for anti-R-MuLV p30 goat serum, 1:600,000 for anti-RCM R-MuLV guinea pig serum, and 1:2,500 for anti-RCM FeLV guinea pig serum. Although the titration curves of p30 specific antibodies showed that all

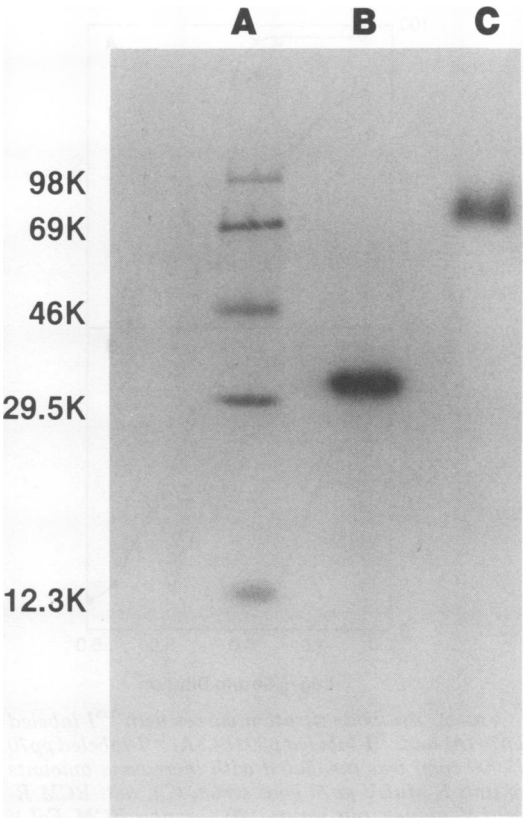

FIG. 1. *Purity of iodinated antigens. Purified proteins were iodinated, electrophoresed on a 7.5 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel, and autoradiographed as described in the text. Track A, Radiolabeled protein molecular weight markers phosphorylase b (98,000 daltons [98K]), bovine serum albumin (69,000 daltons), ovalbumin (46,000 daltons), carbonic anhydrase (29,500 daltons), and cytochrome c (12,300 daltons); track B, R-MuLV p30; track C, R-MuLV gp70.*

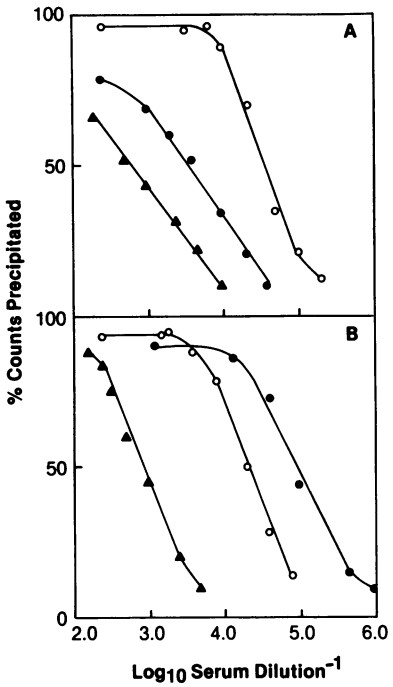

FIG. 2. *Antibody titration curves with ^{125}I-labeled gp70 (A) and ^{125}I-labeled p30 (B). (A) ^{125}I-labeled gp70 (5,000 cpm) was incubated with increasing amounts of anti-R-MuLV gp70 goat serum* (○), *anti-RCM R-MuLV guinea pig serum* (●), *or anti-RCM FeLV guinea pig serum* (▲), *and the percentage of counts precipitated when the solid-phase protein A immunoassay was used was determined as described in the text. (B) Similar antibody titration curves were obtained by using ^{125}I-labeled p30 (5,000 cpm) and anti-R-MuLV p30 goat serum* (○), *anti-RCM R-MuLV guinea pig serum* (●), *or anti-RCM FeLV guinea pig serum* (▲).

of these sera were completely capable of precipitating the radiolabeled probe, titrations of gp70 specific antibodies indicated a marked difference between anti-R-MuLV gp70 goat serum and the two guinea pig sera prepared against RCM antigens. Although essentially 100% of the probe was readily precipitable with high dilutions of the first serum, the latter two were not capable of complete precipitation at the highest antibody concentrations used (dilution, 1:250). This is not a host-dependent phenomenon, as guinea pig antiserum to R-MuLV gp70 also gave essentially 100% precipitation of the probe (data not shown). Hence, it indicates that reduction and carboxymethylation affected certain immunogenic determinants of gp70. The effectiveness of a similar chemical treatment was demonstrated with lysozyme, an unrelated protein known to carry conformational determinants dependent on disulfide bonds (2). As expected, antiserum raised in an identical fashion against RCM lysozyme did not show any antigenic cross-reactivity with native lysozyme (data not shown). In subsequent competition assays, each antiserum was used at a dilution giving 50% of maximum precipitation with the radiolabeled probe under study.

Antigenic analysis of untreated and chemically modified gp70's by solid-phase competition RIA. Competition RIAs are capable of detecting subtle differences between related proteins. These assays are valid only when the labeled protein probes are shown to be pure, as was demonstrated in this case (Fig. 1). A panel of antisera prepared against either purified native R-MuLV gp70 or the RCM proteins and glycoproteins of R-MuLV and FeLV was used in competition RIAs that included type- and/or group- and interspecies-specific assays. Competition assays were performed with the following untreated and chemically modified antigens: R-MuLV gp70, R-MuLV, AKR-MuLV, and FeLV. Chemical modification involved reduction of S-S bonds and subsequent carboxymethylation of SH groups or blocking of both α and ϵ amino groups with MAI, as described above.

Competition for antibodies against native R-MuLV gp70. Initial experiments were carried out by using a homologous system composed of a ^{125}I-labeled R-MuLV gp70 probe and anti-R-MuLV gp70 goat serum at the appropriate dilution. Competition assays performed with untreated and chemically modified R-MuLV gp70 (Fig. 3A) demonstrated that, although the native protein competed completely for the probe, giving a profile with a steep slope, competition with RCM R-MuLV gp70 or MAI-treated R-MuLV gp70 was less efficient, substantiating the results of titrations with antisera against RCM antigens (Fig. 2A). The results of a similar experiment in which detergent-disrupted untreated or modified R-MuLV was used as the competing antigen are shown in Fig. 3B. In this case, the untreated virus antigen again competed completely for the probe, giving a profile having the same slope as that obtained with purified native R-MuLV gp70 (Fig. 3A), and chemical modification of the virus proteins by either treatment resulted in less efficient competition.

The results of competition assays in the same system with untreated and modified AKR-MuLV antigens are shown in Fig. 3C. The inhibition profile obtained with untreated AKR-MuLV was less steep than that with untreated R-MuLV gp70 antigen and, in fact, had a slope indistinguishable from that obtained with either RCM or MAI-treated R-MuLV (Fig. 3B). This

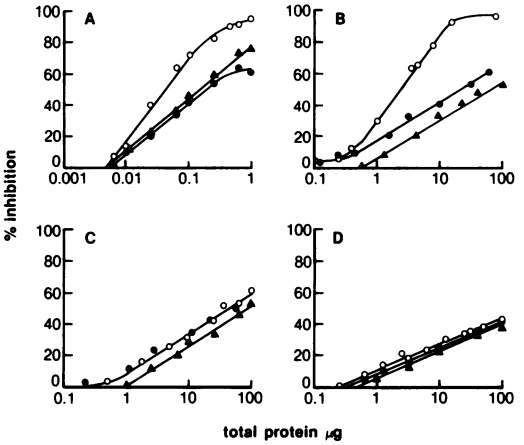

FIG. 3. *Antigenic characterization of R-MuLV gp70. ^{125}I-labeled R-MuLV gp70 (5,000 cpm) was incubated with anti-R-MuLV gp70 goat serum in the presence of increasing amounts of the following. (A) Purified unlabeled R-MuLV gp70* (○), *RCM R-MuLV gp70* (●), *or MAI-treated R-MuLV gp70* (▲). *(B) Unlabeled detergent-disrupted R-MuLV* (○), *RCM R-MuLV* (●), *or MAI-treated R-MuLV* (▲). *(C) Unlabeled detergent-disrupted AKR-MuLV* (○), *RCM AKR-MuLV* (●), *or MAI-treated AKR-MuLV* (▲). *(D) Unlabeled detergent-disrupted FeLV* (○), *RCM FeLV* (●), *or MAI-treated FeLV* (▲). *Assays were performed and results were calculated as described in the text. All data shown are averages of at least two estimations.*

suggested that the gp70 antigenic determinants detected in untreated AKR-MuLV are the same as those in chemically modified R-MuLV. By definition, these would be the group-specific determinants. Furthermore, the slope obtained with either RCM or MAI-treated AKR-MuLV as the competing antigen was unchanged from that obtained with untreated virus, suggesting that the group-specific determinants of gp70 were not affected by either of the chemical treatments performed. This is in contrast to the results of the competition assays shown in Fig. 3A and B, where the shallower slope of the competition curves with chemically modified antigens was evidently due to the loss of R-MuLV gp70 type-specific antigenic determinants.

Untreated and chemically modified FeLV antigens were also studied in competition assays (Fig. 3D), resulting in inhibition profiles having still shallower slopes than those of AKR-MuLV. These assays detected interspecies-specific determinants on FeLV gp70 shared with R-MuLV gp70. The identical results obtained with untreated and chemically modified FeLV antigens clearly demonstrated that these interspecies-specific determinants were not affected by either reduction and carboxymethylation or MAI treatment, an observation similar to the finding described above for group-specific determinants.

Competition for antibodies against RCM R-MuLV gp70. To further substantiate these findings, experiments were carried out in a system comprising ^{125}I-labeled R-MuLV gp70 and anti-RCM R-MuLV guinea pig serum. The results of competition assays performed in this system with detergent-disrupted untreated and chemically modified R-MuLV are shown in Fig. 4A. All three antigens competed efficiently for the probe, yielding curves with apparently identical slopes. As expected, this slope was shallower than the slope obtained with untreated R-MuLV gp70 antigens in the experiments in which goat antiserum prepared against native gp70 was used (Fig. 3A and B). The results of competition assays with untreated and chemically modified AKR-MuLV antigens are shown

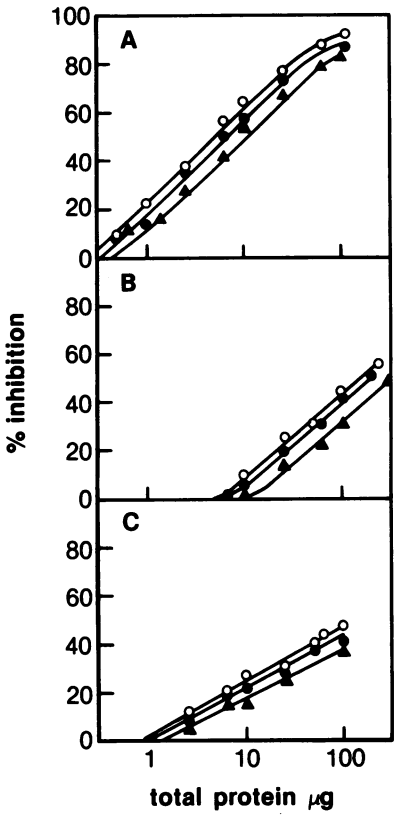

FIG. 4. *Antigenic characterization of R-MuLV gp70. ^{125}I-labeled R-MuLV gp70 was incubated with anti-RCM R-MuLV guinea pig serum in the presence of increasing amounts of the following. (A) Unlabeled detergent-disrupted R-MuLV* (○), *RCM R-MuLV* (●), *or MAI-treated R-MuLV* (▲). *(B) Unlabeled detergent-disrupted AKR-MuLV* (○), *RCM AKR-MuLV* (●), *or MAI-treated AKR-MuLV* (▲). *(C) Unlabeled detergent-disrupted FeLV* (○), *RCM FeLV* (●), *or MAI-treated FeLV* (▲). *All other details were as described in the legend to Fig. 3.*

in Fig. 4B. All three antigens competed equally in this system, indicating that the anti-RCM R-MuLV guinea pig serum detected group-specific gp70 antigenic determinants, which were not affected by the chemical treatments. Moreover, the slopes of these inhibition curves were the same as those obtained with R-MuLV antigens (Fig. 4A). This clearly demonstrates that the anti-RCM R-MuLV guinea pig serum did not contain type-specific antibodies to gp70 at the dilution used (50% of maximum precipitation). However, antiserum raised in guinea pigs against native gp70, when used at similar dilution, readily detected type-specific determinants (data not shown).

When assayed for interspecies determinants, untreated FeLV antigens competed with a shallower slope than AKR-MuLV and R-MuLV (Fig. 4C). In summary, it seems that competition assays in the presence of anti-RCM R-MuLV guinea pig serum detect only group- and interspecies-specific determinants, both of which are unaffected by chemical modification.

Competition for antibodies against RCM FeLV gp70. As the interspecies reaction was relatively weak (maximum inhibition of about 40% at the highest antigen concentration tested) in the two systems described above, a classical interspecies-specific system was set up with ^{125}I-labeled R-MuLV gp70 and anti-RCM FeLV guinea pig serum (Fig. 5). Again, untreated R-MuLV competed well, and the slope of the competition profile was unchanged by chemical modification of the competing antigen (Fig. 5A). AKR-MuLV gave results similar to those with R-MuLV in this system (Fig. 5B), as did FeLV (Fig. 5C). These results fully supported our findings that chemical modification of gp70 by reduction and carboxymethylation or MAI treatment results in a loss of type-specific antigenic determinants without affecting interspecies-specific determinants.

Antigenic analysis of untreated and chemically modified p30's by solid-phase competition RIA. It was of interest to study the effect of chemical modification on the antigenic determinants of p30 by RIAs. In view of the difference in the number of SH groups between gp70 and p30 (gp70 has about 10 times as many SH groups as p30), it was of particular importance to compare the effect of reduction and carboxymethylation on the two proteins.

An assay system with ^{125}I-labeled R-MuLV p30 and goat anti-R-MuLV p30 serum was set up. In this system, purified native p30 competed completely for the probe (Fig. 6A). RCM R-MuLV p30 competed in a similar manner, but MAI-treated R-MuLV p30 competed less efficiently. Similar experiments with unpurified R-

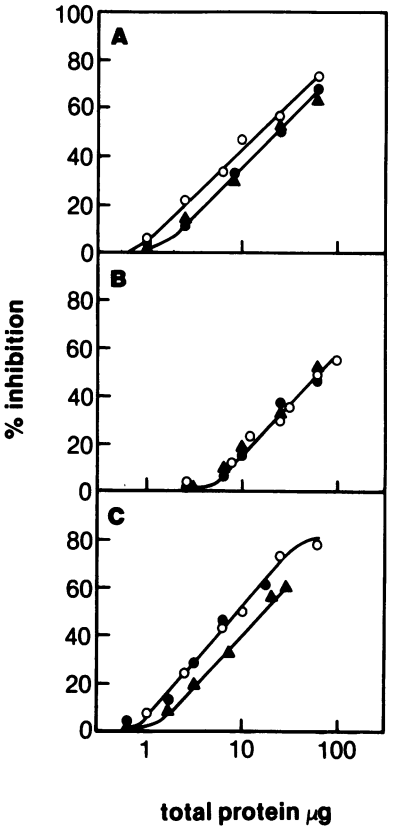

FIG. 5. *Antigenic characterization of R-MuLV gp70. ^{125}I-labeled R-MuLV gp70 was incubated with anti-RCM FeLV guinea pig serum in the presence of increasing amounts of competing antigens, as described in the legend to Fig. 4.*

MuLV antigens (Fig. 6B) paralleled these results. The slope obtained with MAI-treated R-MuLV was the same as the slopes obtained with MAI-treated R-MuLV p30 and AKR-MuLV (Fig. 6C). Chemical modification of AKR-MuLV did not affect the competition profiles observed. FeLV (Fig. 6D) antigens, as expected, competed with a much shallower slope than AKR-MuLV, but were similar to the latter in that the curves were unaffected by chemical modification.

Assays with a p30 probe and anti-RCM R-MuLV guinea pig serum were performed. This serum and guinea pig anti-R-MuLV p30 serum (data not shown) exhibit primarily group-specific activity and only a trace of interspecies-specific activity. Chemical modification of R-MuLV or AKR-MuLV did not affect the group-specific antigenic determinants (Fig. 7A and B). Similarly, in an interspecies system comprised of ^{125}I-labeled R-MuLV p30 and anti-RCM FeLV guinea pig serum, chemical modification did not affect the slopes of the profiles obtained with R-MuLV and FeLV (Fig. 7C and D).

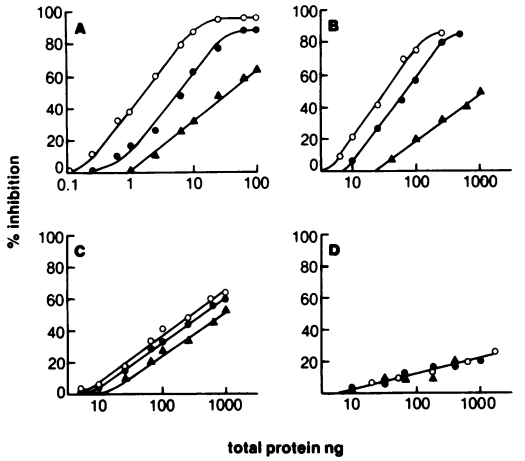

FIG. 6. *Antigenic characterization of R-MuLV p30. ^{125}I-labeled R-MuLV p30 (5,000 cpm) was incubated with anti-R-MuLV p30 goat serum in the presence of the following. (A) Purified unlabeled R-MuLV p30* (○), *RCM R-MuLV p30* (●), *or MAI-treated R-MuLV p30* (▲). *For an explanation of the symbols in (B), (C), and (D) see the legend to Fig. 3.*

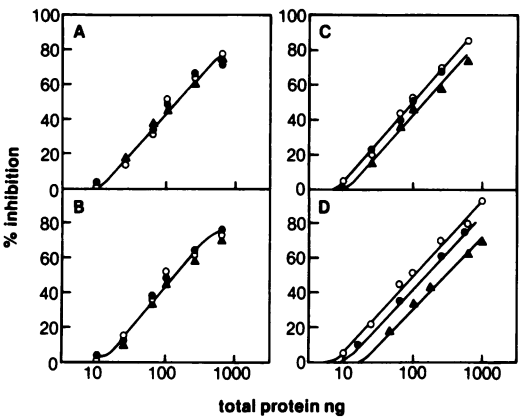

FIG. 7. *RCM ^{125}I-labeled p30 was incubated with anti-RCM R-MuLV guinea pig serum in the presence of (A) unlabeled detergent-disrupted R-MuLV* (○), *RCM R-MuLV* (●), *or MAI-treated R-MuLV* (▲) *and (B) unlabeled detergent-disrupted AKR-MuLV* (○), *RCM AKR-MuLV* (●), *or MAI-treated AKR-MuLV* (▲). *Similar experiments were performed by using ^{125}I-labeled p30 and anti-RCM FeLV guinea pig serum in competition with (C) unlabeled detergent-disrupted R-MuLV* (○), *RCM R-MuLV* (●), *or MAI-treated R-MuLV* (▲) *and (D) unlabeled detergent-disrupted FeLV* (○), *RCM FeLV* (●), *or MAI-treated FeLV* (▲).

In summary, the results of the competition assays with p30 indicate that type specificity of this antigen is destroyed by MAI treatment but, in contrast to gp70, it is not affected by reduction and carboxymethylation. The lack of effect with the latter modification is not surprising in view of the fact that there are only one (or two) SH groups in p30. Neither group- nor interspecies-specific determinants of p30 were apparently affected by the chemical modification procedures performed during this study.

Mapping of p30 antigenic determinants. As the preceding competition assays with chemically modified p30 demonstrated that neither carboxymethylation nor the blocking of lysine residues affected the group and interspecies determinants, preliminary studies on the localization of such determinants on the p30 molecule were performed by using cyanogen bromide fragments of RCM p30 and a tryptic fragment, TRCP (see above). Competition assays were set up using a ^{125}I-labeled R-MuLV p30 probe and either anti-RCM R-MuLV guinea pig serum (containing primarily group-specific antibodies) or an interspecies-specific anti-RCM FeLV guinea pig serum.

In the experiments shown in Fig. 8B and D the competing antigens were native p30, purified CB fragment 2, and the mixture of peptides after cyanogen bromide cleavage of R-MuLV p30. The purified fragment and the mixture competed significantly, but not identically, and both competed less efficiently than native p30 in this group-specific assay (Fig. 8B). In the interspecies assays (Fig. 8D) the mixture competed similarly to native p30, but CB fragment 2 appeared to be ineffective. The antigenic reactivity of p30 in either assay was not altered by exposure to 70%

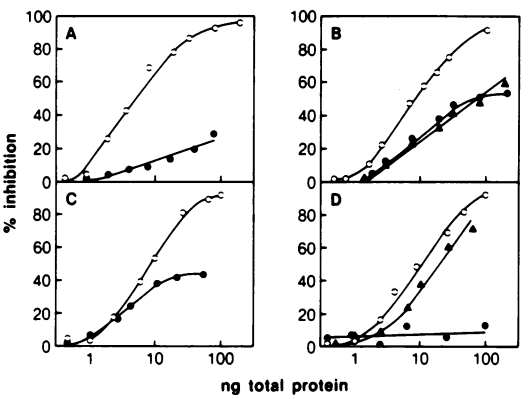

FIG. 8. *Mapping of p30 antigenic determinants. Competition assays with ^{125}I-labeled R-MuLV p30 were performed with purified unlabeled R-MuLV p30* (○) *and TRCP* (●) *in the presence of anti-RCM R-MuLV guinea pig serum (A) or anti-RCM FeLV guinea pig serum (C). Similar assays involving purified unlabeled R-MuLV p30* (○), *R-MuLV p30 CB fragment 2* (●), *or the mixture of cyanogen bromide cleavage fragments from p30* (▲) *were performed in the presence of anti-RCM R-MuLV (B) or anti-RCM FeLV (D) guinea pig antiserum.*

formic acid under the conditions of cleavage (data not shown). This clearly suggests that this fragment, which approximates the C-terminal one-third of the molecule, carries group-specific determinants but none of the interspecies antigenic sites shared with FeLV p27.

In the experiments shown in Fig. 8A and C the competing antigens were native p30 and TRCP. In the group-specific assay (Fig. 8A) the tryptic fragment competed weakly, with a much shallower slope than p30. In the interspecies assay, however, TRCP competed quite efficiently, to a maximum of about 50% inhibition when compared with p30 (Fig. 8C). TRCP, Therefore, must contain interspecies determinants shared with FeLV p27. We have evidence based on structural studies (29; unpublished data) that TRCP is derived from the NH_2-terminal region and represents approximately one-fifth to one-third of the p30 molecule. The present results provide convincing evidence for the localization of at least one interspecies determinant of p30 in this region. A comparison of the inhibition patterns obtained with TRCP (Fig. 8C) and the cyanogen bromide cleavage mixture (Fig. 8D) reveals that the latter contains competing antigenic sites over and above those found in TRCP. It seems likely that these additional determinants are located on CB fragment 1, the 20,000-dalton NH_2-terminal peptide of p30, and not on the much shorter CB fragment 3, which encompasses only a few amino acid residues (L. E. Henderson and S. Oroszlan, unpublished data). Experiments with highly purified CB fragment 1 will be required to elucidate this point.

DISCUSSION

The results presented here provide convincing evidence that conformation-dependent antigenic determinants (discontinuous sites [2]) contribute substantially to the antigenic structure of both gp70 and p30 structural proteins of R-MuLV and other type C viruses of mouse and feline origin. It has also been demonstrated that these antigenic sites represent some of the dominant immunogenic determinants of gp70.

In competition RIAs, it was clearly shown that those conformational determinants of gp70 which are apparently destroyed by reduction of S-S bonds and subsequent carboxymethylation of SH groups and also by acetimido modification of the α and ϵ amino groups are primarily type specific. Both group- and interspecies-specific determinants on the protein molecule are unaffected by these chemical treatments and hence appear to be primarily sequential (continuous sites [2]); they may be specified solely by the primary structure of the peptide chain. Since the competition curves of both RCM and MAI-treated gp70 antigens generated with anti-native gp70 serum had identical slopes, S-S bonds and lysine residues are probably not directly involved in the type-specific antigenic site, and the effect of the chemical reagents which leads to a loss in antigenicity is clearly a secondary effect due to the change in protein conformation. It is known that reduction and carboxymethylation of protein chains containing intramolecular disulfide bonds results in significant alterations in tertiary structure (2). Amidination of lysyl residues, which generally introduces a much more limited perturbation, has also been shown to prevent certain enzymes from maintaining their active conformations (20). Our results provide evidence that amidination of gp70 introduces antigenic changes and thus indicate that the blocking of lysyl residues may also alter the specific conformation of the immunogen required for stimulation of a type-specific response. Moreover, since RCM or MAI-treated gp70 antigens competed similarly in group- and interspecies-specific assays, these antigenic sites involved neither cystine, lysine, nor the N-terminal alanine.

In competition experiments performed but not discussed here, unfolding of the protein with urea in the absence of reducing agent did not lead to an irreversible loss of type-specific determinants since after refolding, essentially all of the antigenic determinants detectable in these assays were recovered. Further experiments should determine whether this would be the case if the amidination reaction was reversed. Similarly, the antigenicity was not investigated in these studies after reduction of the gp70 without subsequent alkylation, but our results routinely obtained in other experiments have shown that reduction of gp70 in the presence of guanidinium chloride or sodium dodecyl sulfate, followed by removal of SH reagents under conditions where disulfide bonds can reform, does not allow complete recovery of antigenicity (unpublished data). Apparently, after reduction of S-S bonds, the glycoprotein does not refold into its proper conformation and hence does not regain its native antigenic structure.

Evidence was obtained that in the nonglycosylated internal structural protein p30 antigenicity is also dependent on the conformation of the protein. Furthermore, these conformational determinants (discontinuous sites), as in gp70, represent primarily type-specific epitopes. In contrast to gp70, the type-specific determinants of p30 were affected only by blocking of amino groups and not by reduction and carboxymethylation. This is not surprising since, as already pointed out, p30 contains only one (or two) SH groups). As was found with gp70, the group-

specific (species-specific) and interspecies-specific antigenic determinants of p30 detectable in these competition assays may also represent continuous sites. After chemical treatment of the protein, it was capable of competing for the antibody binding sites specified by these determinants as efficiently at the native antigen. These findings imply that p30 is similar to gp70 in that neither disulfide bonds, SH groups, lysine, nor the N-terminal proline are involved in the group- or interspecies-specific sites.

It is of interest to note that in previous studies (9) citraconylated p30 was partially deficient in species-specific (type and/or group) determinants and at least one of three interspecies-specific reactivities. The latter of these results appears to be in conflict with the results of the present study obtained by MAI treatment. However, although MAI reacts equally well and indeed more specifically with amino groups than citraconic anhydride, it does not alter the net charge on the protein molecule and has a lesser affect on protein conformation. An alternative (and perhaps more likely) explanation is that citraconylation could have led to the blocking of groups other than amino groups. *O*-acylation of tyrosine residues or other hydroxyamino acids has been shown to occur with this reagent (15). This apparent loss of an interspecies determinant (9) may be due to the modification within that determinant of an amino acid other than lysine. Recent studies with a synthetic undecapeptide (29, 32), representing the N-terminal conserved region of mammalian p30s (27), and with an octacosapeptide (33), corresponding to the entire N-terminal segment of gibbon ape leukemia virus which contains this common region, indicate that the common hydroxyamino acids may be involved in one of the interspecies antigenic sites (D. L. Fine and S. Oroszlan, manuscript in preparation).

In conclusion, it is proposed that the type-specific determinants detected in these assays and destroyed by chemical modification are non-sequential, whereas the group- and interspecies-specific determinants detected are sequential in nature. Hence, in the search for immunologically important peptides from these proteins, the broadly reactive group- and interspecies-specific determinants should be detectable in isolated peptides. The results of partial antigenic mapping of R-MuLV p30 reported here revealed the presence of group-specific but not interspecies-specific determinants in the COOH-terminal one-third of the molecule. In agreement with previous studies (6–9) the tryptic cleavage fragment TRCP, which represents the NH_2-terminal one-third of p30, contains at least one dominant interspecies-specific determinant in addition to group-specific reactivity. The 20,000-dalton cyanogen bromide cleavage fragment which contains the amino acid sequence of TRCP must therefore carry at least those determinants manifested on the tryptic peptide. The results obtained (Fig. 8C and D) may, in fact, suggest that this fragment also encompasses at least one other dominant interspecies-specific antigenic determinant. Hence, the approximate locations of two group-specific and two interspecies-specific sequential antigenic sites can be postulated.

The techniques developed in the course of this work will be of importance in the attempt to prepare synthetic antigens representing common sequences of mammalian gp70's, which could be used as broadly reactive viral vaccines. Our preliminary results using the immunoreplicate technique (35) and solid-phase RIAs as presented here indicate that antigenically active peptides can be isolated from RCM gp70 by chemical and enzymatic fragmentation. Some of these peptides derived from the C-terminal region of R-MuLV gp70 were found to carry interspecies determinants and thus should represent common continuous antigenic sites. The availability of monoclonal antibodies against AKR-MuLV gp70 (24) and R-MuLV gp70 and RCM gp70 (A. M. Schultz and S. Oroszlan, unpublished data) will facilitate further studies directed toward the elucidation of the antigenic structure of type C virus glycoproteins.

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