Evidence for a Precursor-Product Relationship
Between Intracytoplasmic A Particles and Mouse Mammary Tumour Virus Cores

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SUMMARY

This report presents evidence which supports a relationship between intracytoplasmic A particles (CAP) and mouse mammary tumour virus (MMTV). Three MMTV-specific antigenic determinants in CAP (MMTV p27, p14 and p10) were detected by immunodiffusion. No structural proteins of comparable mol. wt. were found in CAP; however, exposure of CAP to trypsin resulted in the cleavage of the CAP structural proteins to MMTV-like polypeptides. This process was accompanied by the preservation of MMTV-specific antigenic determinants. Disulphide bonds were necessary for the structural maintenance of CAP. Reducing agents destroyed the organized structure of CAP, whereupon processing of CAP proteins to MMTV-like polypeptides by trypsin was prevented. CAP p82, possessed only MMTV p27 antigenic determinants, while CAP polypeptides p20-18 possessed p10 antigenic determinants. Following processing of CAP structural proteins by trypsin, MMTV-specific p27 antigenic determinants were shifted from CAP p82 to CAP p27; MMTV-p10 antigenic determinants were found with CAP p15-10. These results suggest a model wherein CAP structural proteins are modified by protease during maturation, resulting in the shift of their proteins to sizes consistent with those which have been currently identified as the major internal components of the virion and that this phenomenon is largely predicated on the folding of CAP proteins into the morphologically intact A particle.

Intracytoplasmic A particles (CAP) are morphological entities present in varying numbers in the cytoplasm of cells infected with mouse mammary tumour virus (MMTV). In ML⁺ (a MMTV-specific cell surface antigen) leukaemic cells (Nowinski et al. 1971), plasmacytomas and Leydig cell tumours (Poureau-Schneider, 1967) where MMTV virion production is incomplete, CAP are found in large numbers in the perinuclear cytoplasm. However, in cells where virus production is rapid and complete, e.g. in dexamethasone-stimulated mouse mammary tumour cell cultures (Parks et al. 1975), only a few CAP accumulate in the cytoplasm, most are found included within budding immature virions at the cell surface (Gonda et al. 1976). Comparison of CAP structural polypeptides with those of MMTV by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a complete lack of correlation between their respective gel patterns (Smith & Wivel, 1972; Tanaka, 1977). None of the CAP SDS-polypeptides had electrophoretic mobilities similar to the major internal proteins of the mammary tumour virus, i.e. MMTV p27, p14 and p10 (Cardiff et al. 1974; Parks et al. 1974; Teramoto et al. 1974, 1977). Using exposure to trypsin followed by SDS-PAGE analysis, we demonstrated quantitative conversion of all CAP polypeptides to two bands, one of which had an identical electrophoretic mobility with MMTV p27; the other band had an electrophoretic mobility that overlapped MMTV.
Short communications

p14 and p10 (not shown). In addition, MMTV p27 antigenic specificity could be demonstrated in both mock-digested and trypsin-digested CAP by immunodiffusion (Smith & Lee, 1975). In this paper we extend these observations on the relationship between the polypeptides of CAP and MMTV.

Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Company, Freehold, New Jersey. Intracytoplasmic A particles were purified from transplantable Leydig cell tumours or from primary mammary tumours in C5H/He mice as described (Smith & Wivel, 1973; Smith et al. 1974).

Gel electrophoresis was carried out as described earlier (Maizel, 1971; Smith & Lee, 1975). Briefly, samples containing 50 to 100 μg protein (Lowry et al. 1951) were placed directly on 6-0% polyacrylamide cylindrical gels. The gels were run at 4 mA/gel for 5 to 6 h. The gels were fixed in 25% isopropyl alcohol-10% acetic acid and stained with Coomassie brilliant blue in 7.5% acetic acid. Stained gels were scanned at 550 nm in a Gilford spectrophotometer equipped with a gel scanner.

Immunodiffusion was performed in slides, containing 2% agarose with 0.1% sodium azide and pre-cut wells, purchased from Hyland Laboratories, Costa Mesa, California (pattern C), or by microtechnique in 0.75% agarose containing 0.02% Merthiolate, as described by Crowle (1961). When the Hyland pre-cut slides were used, 0.1% SDS was routinely added to intact CAP preparations to aid solubilization and migration of the antigens. Antiserum prepared against purified MMTV proteins, gp52, p27 and p10 were kindly provided by Dr Robert D. Cardiff, University of California, Davis, California; anti-MMTV p14 and anti-MMTV by Dr Larry B. Arthur, Frederick Cancer Research Center, Frederick, Maryland. Antiserum was raised in rabbits against purified intracytoplasmic A particles as described (Smith & Wivel, 1973; Smith & Lee, 1975). All the antibodies raised against MMTV structural proteins were produced by inoculating SDS-PAGE separated polypeptides into rabbits. The antibodies raised against MMTV gp52, MMTV p27 and MMTV p10 have been shown to precipitate only their respective MMTV polypeptides in radioimmune precipitation tests (Cardiff et al. 1978). Similarly, anti-MMTV p14 from Dr Arthur precipitates only MMTV p14.

The isolation of CAP structural proteins was accomplished as follows: at the completion of electrophoresis, one gel was fixed and stained, the remainder were removed from the glass cylinders and frozen at −70 °C. From the Coomassie brilliant blue-stained gel, the positions of CAP polypeptides p82, p64-48, p37 and p20-18 (Smith & Wivel, 1973) were determined and these regions were sliced from the gels and eluted overnight in 0.01 M-tris-HCl, pH 7.8, at 37 °C. The eluates were dialysed against 10−4 M-tris-hydrochloride buffer, pH 7.8, and concentrated by freeze drying. The relative purity of each polypeptide band(s) was confirmed by SDS-PAGE. The identical procedure was used in the isolation of the trypsin-processed CAP polypeptides p27 and p15-10.

A particles were prepared for transmission electron microscopy as described earlier (Smith & Wivel, 1973).

Intracytoplasmic A particles reacted in immunodiffusion plates with anti-MMTV p27, anti-MMTV p14, anti-MMTV p10 and anti-CAP, but not with anti-MMTV gp 52. Thus, CAP possess MMTV-specific antigenic determinants for the internal MMTV proteins, p27, p14 and p10 but not for the MMTV envelope protein gp52. Analyses of CAP structural proteins have shown no polypeptides corresponding in electrophoretic mobility to the low mol. wt. MMTV proteins. However, upon incubation with trypsin, CAP structural proteins were quantitatively converted to two SDS-polypeptide bands, one which co-migrates with MMTV p27 and the other a broad band spanning the MMTV p14 and p10.
Fig. 1. (a) Intracytoplasmic A particles incubated at 37 °C for 20 min then negatively stained with 2 % phosphotungstic acid at pH 6.3. (b) CAP from the same preparation but incubated for 20 min with trypsin (0.2 μg/100 μg protein). (c) CAP sampled immediately after exposure to 0.1 M β-mercaptoethanol. The particles are completely unfolded; however, some of the central 'sub-nucleoids' remain intact (arrows); later these will also be disrupted.
Fig. 1. For legend see opposite.
region (Smith & Lee, 1975). The pattern and relative amounts of trypsin-produced CAP p27 and p15-10 (as measured by total absorbance of Coomassie brilliant blue in each band) remained unchanged with time of incubation and at optimal conditions, although the total recovery of protein in each was diminished. This observation suggested that throughout this time period, the cleavage of A particle protein was orderly.

Fig. 1 (a, b) shows the ultrastructural appearance of CAP after incubation at 37 °C with and without trypsin. The trypsin-cleaved CAP remained intact, possessed a smoother external contour and showed an apparent increase in uniformity and average diameter. In addition, the central 'sub-nucleoid' core of CAP had become much more prominent. Therefore, CAP ultrastructural integrity is preserved in spite of the tryptic cleavage of their structural proteins to sizes comparable to those found in MMTV.

Since A particle proteins were apparently processed by trypsin to MMTV-size polypeptides, we proposed to study which of the major CAP structural proteins were antigenically related to MMTV p27, p14 and p10, and if possible to determine which CAP proteins were cleaved to give the appropriately sized MMTV-like polypeptides. Non-ionic detergents such as 1% Triton X-100 were not sufficient to disrupt A particles. Sodium doceyl sulphate was effective in disrupting the particles, but in the absence of reducing agents [β-mercaptoethanol (β-ME) and dithiothreitol (DTT)] most of the major high mol. wt. (≥ 37000) CAP structural proteins, particularly p82, were unable to enter our 6% acrylamide gels. In contrast, CAP p20-18 seemed to enter the gel readily without reduction of disulphide bonds. CAP in 0.01 M-tris-HCl, pH 7-8, were exposed to relatively high concentrations of β-ME (0.1 M) and DTT (0.1 and 0.2 M) and were then examined by electron microscopy. It was found that all CAP were disrupted under these conditions (Fig. 1c). However, when the reducing agent-disrupted CAP were spun at 30000 g for 15 min a pellet was formed containing the CAP structural proteins with mol. wt. ≥ 37000 while CAP p20-18 remained in the supernatant (Fig. 2b, c). After removal of the reducing agent by dialysis, the respective fractions were concentrated by freeze-drying and resuspended in a volume designed to make the relative protein concentrations equivalent. By immunodiffusion, MMTV p27 antigenic determinants were found only in the pellet (Fig. 2e). The supernatant fraction, largely CAP p20-18, reacted only with anti-CAP. Attempts to locate MMTV p14 and p10 antigenic determinants were unsuccessful although these sera readily reacted with disrupted non-reduced CAP. Tryptic hydrolysates of either fraction resulted in

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Fig. 2. (a to d) Colorimetric scans were made of Coomassie brilliant blue-stained gels as described in the text. Direction of migration is left to right. (a) Typical gel pattern obtained with purified CAP solubilized in 1.5% SDS at 100 °C for 5 min with reducing agents. Ten to eleven bands are routinely resolved in 6% gels; major polypeptides are p82, p37, p20-18. Markers: bsa = bovine serum albumin; cyt c = cytochrome c. (b, c) CAP were incubated in 0.2 M-dithiothreitol and centrifuged as described in text; scan and gel (b) indicate the CAP polypeptides associated with the pellet; scan and gel (c) show the DTT-soluble polypeptides. CAP p82 is the major protein remaining in the pellet; CAP p20-18 is completely solubilized. CAP p37 is split approximately equally into each fraction. (c) Immunodiffusion slide; equal amounts of protein from the DTT-soluble (S) and insoluble (P) fractions were reacted against anti-CAP (A) and anti-MMTV p27 (27). Anti-A and anti-p27 formed a common precipitin line against the DTT pellet. Anti-MMTV p27 failed to react with the DTT-soluble proteins; however, anti-A detected an antigen distinct from p27. (d) Gel and scan show the DTT-insoluble proteins following trypsin digestion of intact A particles. The major band after trypsin is p27, some p27 and all CAP p15-10 were found in the supernatant (not shown). (f) Immunodiffusion tests with equal amounts of CAP-trypsin-DTT insoluble (P) and soluble (S) proteins against anti-A (A) and anti-MMTV p27 (27) found p27 antigenic determinants in both fractions in agreement with the appearance of p27 in the corresponding electrophorograms. In each immunodiffusion test, the antigen wells contained 20 to 25 μg protein and 15 μl of undiluted antiserum were placed in each antibody well.
far more extensive hydrolysis of CAP proteins than that produced in the intact particle. As a consequence, the MMTV p27 antigenic determinants formerly detectable in the DTT pellet were destroyed. These results led to our speculation that disulphide bonds were at least in part responsible for the folding of A particle proteins into their well-known concentrically coiled shape, and further that this folding must in some way influence those sites initially sensitive to proteolytic activity. To test this speculation, we treated CAP with trypsin as before, quenched the reaction and then exposed the A particles to 0.1 mM-dithiothreitol. Upon centrifugation of the disrupted particles, a pellet was obtained containing CAP p27. (Fig. 2d). Likewise, MMTV p27 antigenic determinants were demonstrable in this fraction (Fig. 2f). All of the CAP p15-10 was found in the supernatant, as was some CAP p27 and a variety of larger polypeptides. Anti-MMTV p27 also reacted with this fraction (Fig. 2f) in agreement with the presence of CAP p27 in the gel pattern.

These experiments demonstrated that MMTV-specific p27 antigenic determinants seem to be associated with CAP structural proteins with molecular weights greater than 37000 in the intact A particles. The results imply that following cleavage of CAP structural proteins by trypsin, these higher mol. wt. forms are converted to CAP p27, which retains the specific MMTV p27 antigen. By analogy, CAP p20-18 appears to become CAP p15-10 following trypsin treatment. This conclusion seems warranted from results obtained with very short exposures of CAP to trypsin, which showed that CAP p20-18 was the first SDS-polyacrylamide gel band to disappear, with the concomitant appearance of CAP p15-10. Since CAP p20-18 (in the DTT supernatant) did not react with MMTV p27 antiserum, we thought perhaps it would react in immunodiffusion plates with anti-MMTV p14 or p10, but, as mentioned above, we were not able to demonstrate this reaction with the DTT supernatant, probably because of the difficulty in removing the excess reducing agent. Therefore CAP p82 and CAP p20-18 were obtained by elution from unfixed and unstained polyacrylamide gels following electrophoresis. The eluates were dialysed, concentrated by freeze-drying and subjected to immunodiffusion. The purity of each protein fraction was checked by SDS-gel electrophoresis.

In double gel immunodiffusion tests, anti-MMTV p27 formed two immunoprecipitin lines with CAP p82 of which each showed identity with two precipitated by anti-CAP serum. No reaction against CAP p82 was obtained with anti-MMTV p14 or p10. The presence of two immunoprecipitin lines with anti-CAP and anti-MMTV p27 was reproducible in several experiments, suggesting that either p82 existed in two forms with different diffusion rates in the absence of SDS or that two distinct antigenic sites are recognized by anti-MMTV p27 and by anti-CAP on p82. In the latter case, these antigenic determinants would necessarily be present in MMTV p27 since anti-MMTV p27 precipitated only MMTV p27 in radioimmune precipitation tests (Cardiff et al. 1978). Purified CAP p82 produced a single major band in SDS-gels, a minor band (~ p90) was also present, perhaps accounting for the second line of identity discussed above. CAP p20-18 was weakly reactive with anti-CAP and anti-MMTV p10, but was unreactive with anti-MMTV p27. The four minor bands migrating at p64-48 (i.e. mol. wt. greater than 37000; Fig. 2a) in CAP SDS-polyacrylamide gels were found to react only with anti-p27 in immunodiffusion, suggesting that one or all represent intermediate cleavage products of CAP p82 and/or larger CAP polypeptides (data not shown). Immunological evaluation of gel-isolated, trypsin-cleaved A particle polypeptides CAP p27 and CAP p15-10 determined that CAP p27 reacted with anti-MMTV p27 and anti-CAP but not with MMTV p10. Trypsin-produced CAP p15-10 reacted with anti-MMTV p10 but not with anti-MMTV p27, thus providing direct evidence that the cleavage of A particle proteins by trypsin produces SDS-polypeptides with com-
parable sizes and antigenic specificities to those found in MMTV (Cardiff et al. 1974; Teramoto et al. 1977; Cardiff et al. 1978).

The foregoing observations give support to the conclusion that CAP possess incompletely processed virion proteins and are intracellular structural precursors to MMTV cores. The structural proteins of CAP are subject to proteolytic cleavage at sites which produce CAP p27 and CAP 15-10 polypeptide bands which possess electrophoretic mobilities virtually identical to already identified MMTV internal proteins. A requirement for the specificity of these cleavage sites is the morphological integrity of the particle. Three separate MMTV-specific (p27, p14, p10) antigenic determinants are associated with CAP. MMTV p27 determinants are found in CAP proteins with mol. wt. \( \geq 37000 \); these proteins remain particulate after dissociation of CAP with reducing agents. Exposure of intact A particles to trypsin apparently converts these proteins to p27 which also remains particulate or aggregated in 0.2 M-DTT and retains MMTV p27 determinants. In contrast, MMTV p10 determinants, not p27, are found associated with CAP p20-18, which is soluble or non-aggregated in 0.2 M-DTT. The CAP polypeptide bands produced by trypsin, CAP p27 and CAP p15-10, react, on isolation, with anti-MMTV p27 and anti-MMTV p10, respectively.

Dickson has demonstrated an intracellular precursor (gp73) to MMTV glycoproteins gp52 and gp35 (Dickson et al. 1976) and has reported the successful identification of a polyprotein precursor to the non-glycosylated MMTV virion proteins (C. Dickson, personal communication). Earlier, a precursor polypeptide to four major internal non-glycosylated structural proteins had been discovered in avian oncovirus infections (Vogt et al. 1975). Our data suggest that MMTV polyprotein precursors may be incorporated into morphologically identifiable structures in MMTV-infected cells which have been designated intracytoplasmic A particles. It is tempting to speculate that the processing of these precursors occurs at the cell membrane during budding which, when complete, results in the morphological shift from concentric coil to 'bull's-eye' nucleoid. This proposal would be in accord with the original hypothesis based on the morphological observations of Bernhard et al. (1955). We have not, however, been able to show a morphological change analogous to this in the presence of trypsin alone.

Although it has been possible to demonstrate virion-specific structural antigens in association with CAP structural polypeptides, a direct kinetic relationship between the incorporation of MMTV-specific gene products into A particles and their subsequent maturation into MMTV virions has not as yet been shown.

Tanaka (1977) has recently published the observation that A particles isolated from DBA mouse lymphoma cells have high mol. wt. polypeptides which are antigenically related to the non-glycosylated internal proteins of MMTV. By incubating the A particles at 37 °C for prolonged periods, he demonstrated that these high mol. wt. proteins were partially processed into MMTV-like polypeptides which possessed the appropriate MMTV antigenic determinants. We have not observed this phenomenon with our A particles in the absence of added protease. Furthermore, phenylmethane-sulphonyl fluoride (PMSF), which apparently prevented the processing of A particle proteins in Tanaka's system, was ineffective in blocking the cleavage of A particle proteins by trypsin. Since chymotrypsin and papain can also cleave CAP proteins to MMTV-size polypeptides without loss in antigenic specificity (data not shown), it would appear that the folding of A particle proteins rather than the primary specificity of the protease is the more important aspect in correct cleavage. In both systems, the A particles remain structurally intact following exposure to processing conditions, in agreement with this tentative conclusion. However, the requirement for a specific virus-associated proteolytic factor for in situ processing cannot be discounted.
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REFERENCES


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