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Evidence That the 32,000-Dalton Protein Encoded by Bottom-Component RNA of Cowpea Mosaic Virus is a Proteolytic Processing Enzyme

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Translation of middle-component RNA of cowpea mosaic virus in vitro produced two polypeptides of 95 and 105 kilodaltons (95K and 105K, respectively) with overlapping amino acid sequences, which were specifically cleaved by a protease encoded by the bottom-component RNA. The proteolytic cleavage was studied by the addition of antibodies raised against various bottom-component RNA-encoded proteins to extracts prepared from bottom-component RNA-inoculated cowpea protoplasts. Since antiserum to the 32K polypeptide efficiently inhibited the proteolytic activity of such extracts, although antiserum to VPg or to the 170K polypeptide did not, evidence was obtained which indicates that the 32K polypeptide represents the protease involved. Fractionation of proteolytically active extract by glycerol gradient centrifugation demonstrated that 32K polypeptides do not exist as free proteins but are aggregated to the bottom-component RNA-encoded 170K, 84K, 60K, or 58K polypeptides. Maximal proteolytic activity was observed for 32K polypeptides associated with 170K polypeptides, suggesting that the activity was unstable and confined to newly synthesized molecules.

Among plant viruses, cowpea mosaic virus (CPMV), the type member of the comoviruses, has become one of the most extensively studied viruses. The genome of this virus consists of two separately encapsidated plus-stranded RNA molecules with molecular weights of 2.01×10^6 (bottom-component [B] RNA) and 1.22×10^6 (middle-component [M] RNA), respectively (18, 26, 27). Both RNAs possess a genome-linked protein, denoted as VPg, at their 5′-terminus and are polyadenylated (1–3, 22, 23). Expression of the viral RNAs does not involve the generation of subgenomic mRNAs but occurs by means of proteolytic cleavages of long, primary translation products. Thus, it has been shown that B-RNA is translated into a 200-kilodalton (200K) polyprotein which is first cleaved to give a 32K and a 170K polypeptide (8, 16). The 170K polypeptide is then further cleaved to give either 60K and 110K or 84K and 87K polypeptides (20). Subsequent cleavage of the 60K polypeptide provides VPg and a 58K polypeptide (7, 28). M-RNA is translated into two polypeptides of 95K and 105K with overlapping amino acid sequences (4, 16). These polypeptides are proteolytically cleaved at the same position to give polypeptides of 60K, 58K, and 48K (4, 16). The 60K polypeptide is derived from the COOH-terminal half of both these polypeptides and represents the precursor to both capsid proteins VP37 and VP23, whereas the 58K and 48K cleavage products are derived from the NH_2-terminal parts of the longer (105K) and smaller (95K) polypeptides, respectively. The function of the overlapping 58K and 48K polypeptides is unknown as yet, but they may be involved in the spreading of viral RNA throughout the leaf (19). The protease responsible for the cleavage of the M-RNA-encoded primary translation products is obviously coded for by the B-RNA, since both in vitro translation products obtained from this RNA (i.e., the 170K and 32K polypeptides [8, 16]) and extract from cowpea mesophyll protoplasts inoculated with purified B-RNA (4) exhibit this activity. Until now, however, it has not been possible to elucidate which B-RNA-encoded polypeptide actually represents the protease involved. This report presents evidence that the proteolytic activity resides in the 32K polypeptide.

MATERIALS AND METHODS

Virus and RNA. CPMV was propagated in cowpea plants (*Vigna unguiculata* L. "California Blackeye") as described previously (12, 25). Separate B- and M-components were obtained by three cycles of centrifugation in a linear 15 to 30% (wt/vol) zonal sucrose gradient (16 h, 23,000 rpm at 10°C; Beckman Ti 15 rotor) as previously described (6, 20). M-RNA was isolated as follows: purified M-components were disrupted by adding an equal volume of 4% (wt/vol) Sarkosyl NL97–2% (wt/vol) sodium tri-isopropylnaphthalene sulfonate–0.02 M Tris-hydrochloride (pH 7.4)–0.2 M NaCl–0.004 M EDTA and by heating for 5 min at 60°C. The RNA was purified by three extractions with phenol (saturated at pH 8) and precipitated with two volumes of ethanol at −20°C. The precipitate was dried and dissolved in water and then subjected to a linear 15 to 30% (wt/vol) sucrose gradient centrifugation (16 h, 22,500 rpm at 20°C; Beckman SW27 rotor). Fractions containing intact RNA were pooled and precipitated with two volumes of ethanol at −20°C. The RNA precipitate obtained was washed three times with absolute ethanol, dried, and dissolved in water at a concentration of 1 mg/ml.

Incubation of protoplasts, labeling of proteins, and subcellular fractionation. Cowpea mesophyll protoplasts were prepared, inoculated, and incubated as described previously (11, 20). When labeled proteins were required, protoplast suspensions (5 ml, 5×10^5 cells per ml) were supplied with portions (150 μCi) of [^{35}S]methionine (1,100 Ci/mmol; New England Nuclear Corp.) at 18 and 25 h after inoculation. Forty-four hours after inoculation, protoplasts were collected by centrifugation (2 min, 600 × *g*), suspended in 0.5 ml of

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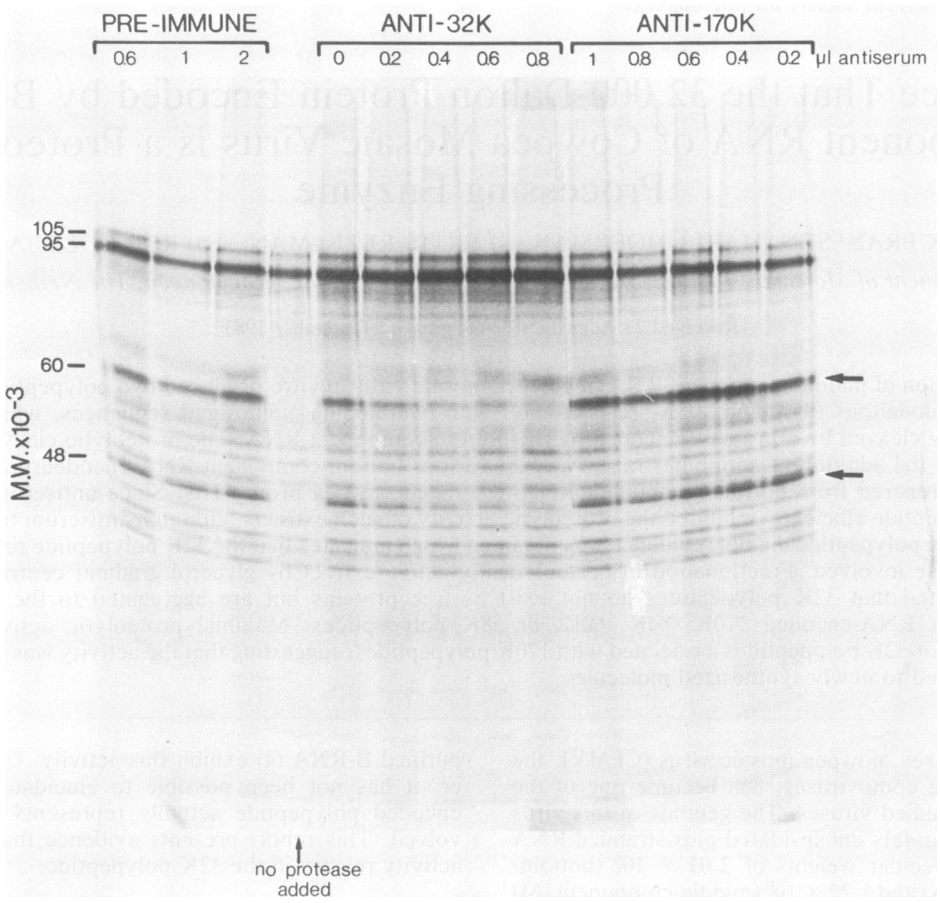

FIG. 1. Inhibition of the protease activity by antiserum raised against the 32K polypeptide. Increasing volumes of anti-32K, anti-170K, or preimmune sera were added to 2-μl portions of the S30 fraction from B-component-inoculated protoplasts, in a total volume of 20 μl of TKED buffer. After incubation for 1.5 h at 0°C, 2 μl of [^{35}S]methionine-labeled in vitro translation products of M-RNA was added, and incubation was continued for 1.5 h at 30°C. Proteolytic cleavage of the 105K and 95K polypeptides was determined by electrophoresis in a 12.5% polyacrylamide gel. The arrows indicate the 60K and 48K cleavage proteins from the 95K polypeptide (the input amounts of the 105K polypeptide were too low to allow detection of the 58K cleavage product). Molecular weights (M.W. $\times 10^{-3}$) are indicated to the left of the figure.

TKEDP buffer (50 mM Tris-acetate [pH 7.4], 10 mM potassium acetate, 1 mM EDTA, 10 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride) containing 10% (wt/vol) sucrose, and disrupted by homogenization for 2.5 min at 0°C in a small Thomas tissue homogenizer. The homogenate thus obtained was centrifuged for 30 min at 30,000 × *g* and 4°C to give the 30,000 × *g* supernatant (S30) fraction, which contained (most of) the virus-specific proteolytic activity.

SDS-polyacrylamide slab gel electrophoresis. Portions of radiolabeled proteins were mixed with one-third volume of a fourfold-concentrated sample buffer (40 mM Tris-hydrochloride [pH 8.0], 4 mM EDTA, 40% [vol/vol] glycerol, 8% [wt/vol] sodium dodecyl sulfate [SDS], 20% [vol/vol] β-mercaptoethanol, 0.004% [wt/vol] bromophenol blue) and heated for 3 min at 100°C. The samples were then electrophoresed at 150 V in an SDS-polyacrylamide gel containing 12.5% acrylamide (with 0.09% bisacrylamide) by using a stacking gel of 4% acrylamide and 0.10% bisacrylamide, as previously described (13, 20). After electrophoresis, gels were dried either with or without preceding staining and autoradiographed with Kodak Royal X-omat X-ray film.

Antisera and immunoprecipitation. Antiserum against the electrophoretically separated virus-coded 170K polypeptide was prepared by directly immunizing a New Zealand white rabbit with polyacrylamide containing the denatured antigen, in principle as described by Tijan et al. (24) and Schiff and Grandgenett (21) but with some modifications as described elsewhere (P. Zabel and F. van Straaten, manuscript in preparation). Antiserum against the electrophoretically separated 32K polypeptide was prepared by immunizing rabbits with 32K polypeptide eluted with buffer (12.5 mM Tris-hydrochloride [pH 6.8], 0.1 mM EDTA) from gel slices of a nonfixed polyacrylamide gel. Antiserum against the genome-linked protein VPg was prepared as described previously (28). Specificity of the various antisera raised against CPMV-encoded proteins was tested by immunoprecipitation of proteins from the S30 fraction of radiolabeled B-component-inoculated protoplasts in a buffer (TKE-TDS) containing 50 mM Tris-acetate (pH 7.4), 10 mM potassium acetate, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. Immunoprecipitation was performed in TKE-TDS buffer as described previously (4), except as stated otherwise. None of the antiserum preparations used showed reactivity against host proteins.

Glycerol gradient centrifugation. Typically, 80 μl of the S30 fraction obtained from B-component-inoculated proto-

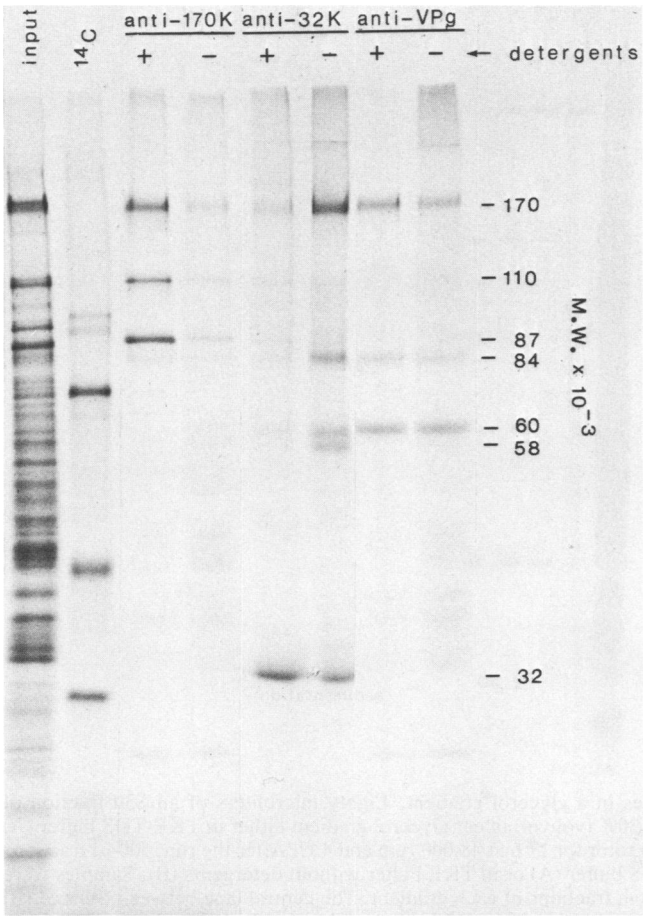

FIG. 2. Autoradiogram of a polyacrylamide gel with immunoprecipitated CPMV polypeptides. Portions of the S30 fraction (2 μl) from [^{35}S]methionine-labeled B-component-inoculated protoplasts were incubated with 2 μl of anti-32K serum, 5 μl of anti-170K serum, or 5 μl of anti-VPg serum, in a final volume of 20 μl of TKE buffer (without detergents, −) or TKE-TDS buffer (with detergents, +). The two lanes on the left contain the [^{35}S]methionine-labeled polypeptides from B-RNA-inoculated protoplasts without immunoprecipitation (input) and ^{14}C-methylated protein markers (^{14}C) (myosin [M.W., 210,000]; phosphorylase *b* [M.W., 100,000 and 92,500]; bovine serum albumin [M.W., 68,000]; ovalbumin [M.W., 46,000]; and carbonic anhydrase [M.W., 30,000]), respectively. The numbers to the right of the figure refer to the molecular weights of the B-RNA-encoded polypeptides.

plasts was layered on linear 15 to 30% (vol/vol) glycerol gradients in TKED buffer (i.e., TKEDP buffer minus phenylmethylsulfonyl fluoride). Centrifugation was carried out in a Beckman SW41 or SW50 rotor under conditions described in the figure legends. After the run, gradients were fractionated in 500-μl portions which were assayed for both protease activity and occurrence of viral proteins.

In vitro translation of M-RNA. CPMV M-RNA was translated in an mRNA-dependent rabbit reticulocyte lysate (a generous gift of R. J. Jackson, Department of Biochemistry, University of Cambridge, Cambridge, England) under conditions as previously described (8, 16, 17). [^{35}S]methionine (10 μCi per 10 μl of reaction mixture) was used as the radioactive amino acid, and incubation was for 1 h at 30°C.

Viral protease assay. For detection of the viral protease, 2 to 20 μl of samples to be analyzed was mixed with 2 μl of [^{35}S]methionine-labeled in vitro translation products from CPMV M-RNA (as obtained after 1 h of translation) in a total volume of 22 μl of TKED buffer and incubated at 30°C for 1.5 h. To determine proteolytic activity, 5-μl portions of each sample were electrophoresed in a 12.5% polyacrylamide gel.

RESULTS

Inhibition of the protease activity by anti-32K serum. Since translation products obtained from B-RNA after 1 h of in vitro translation were proteolytically active (4, 16), either the 32K polypeptide or the 170K polypeptide must be responsible for this activity. If the proteolytic activity resides in the 170K polypeptide, then it might be expected that one of the known, final cleavage products of the 170K polypeptide represents the mature protease. To discriminate between these possibilities, antisera raised against the 32K and 170K polypeptides were prepared and tested for their ability to inhibit the proteolytic activity. For this purpose, increasing amounts of anti-32K, anti-170K, or preimmune sera were added to 2-μl portions of the S30 fraction from B-component-inoculated protoplasts in a total volume of 20 μl of TKED buffer and incubated for 1.5 h at 0°C to allow binding of the immunoglobulins to the viral proteins. Possible inhibition of the proteolytic activity in these mixtures was then determined by addition of 2 μl of [^{35}S]methionine-labeled (95K and 105K) in vitro translation products from M-RNA and by analysis for the lack of appearance of the 60K, 58K, and 48K cleavage products. A significant decrease in proteolytic activity was obtained with anti-32K serum, even at lower amounts (Fig. 1). On the other hand, preimmune serum, tapped from the anti-32K rabbit before immunization, and anti-170K serum did not show any inhibitory effect (Fig. 1). These results suggest that the proteolytic activity resides in the 32K polypeptide and not in the 170K polypeptide. Some complications which may be important should be regarded, however. First, although the titer of the anti-170K serum used was reasonable under normal immunological conditions (i.e., in TKE-TDS buffer), reacting with both the 170K polypeptide and the 110K and 87K polypeptides derived from this polypeptide, this antiserum showed a reduced reactivity under conditions where its possible inhibitory effect on the proteolytic cleavage was tested (in TKE buffer, i.e., in the absence of any detergent [Fig. 2]). Moreover, in the absence of detergents, the anti-32K serum apparently bound to complexes of 32K polypeptides with the viral 58K, 60K, 84K, and 170K polypeptides (Fig. 2). Therefore, the possibility that one of these four other B-RNA-encoded polypeptides represented the protease, the activity of which was inhibited by sterical hindrance of immunoglobulin G (IgG) molecules bound to associated 32K polypeptides, had to be considered. The following observations, however, provide evidence against this idea. First, since the 87K and 110K polypeptides were not detectable in complexes with the 32K polypeptide (Fig. 2), the conclusion can be drawn that at least these polypeptides were not responsible for the proteolytic cleavage. Second, antiserum raised against VPg did not interfere with the proteolytic cleavage (data not shown). This antiserum was capable of binding the VPg-containing 170K, 60K, and, to a lesser extent, 84K polypeptides, both in the presence and absence of detergents (Fig. 2). The results obtained with all three antisera are therefore consistent and indicate that neither the 170K polypeptide nor any of its cleavage products represent the protease, but instead, point out that the 32K polypeptide bears the activity in question.

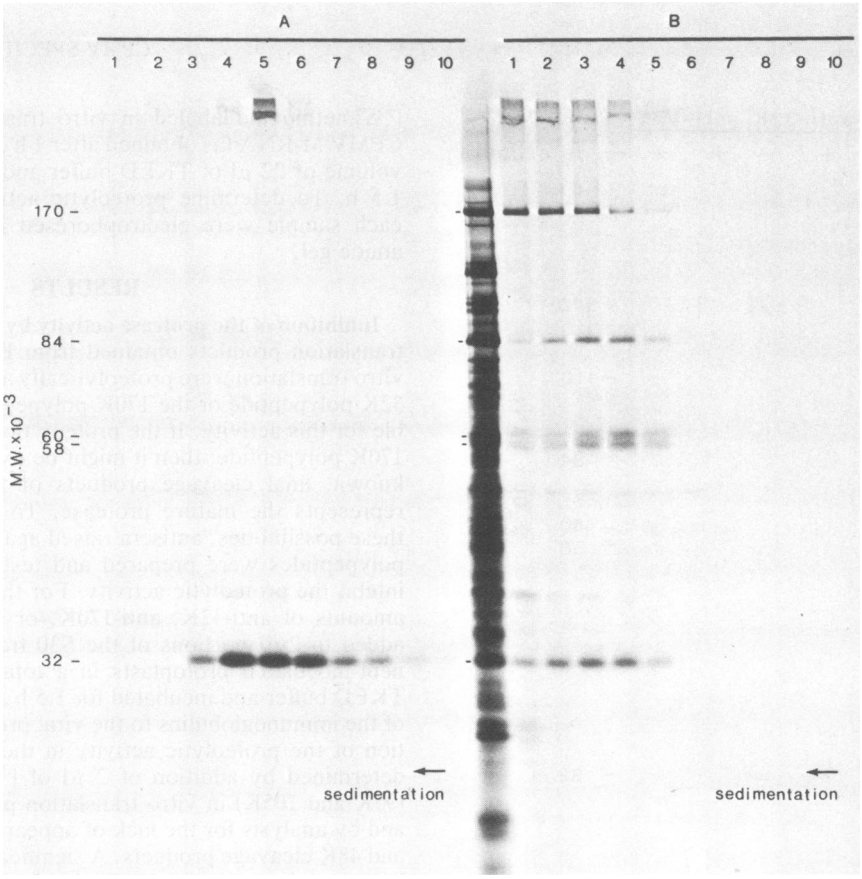

FIG. 3. Sedimentation of [^{35}S]methionine-labeled CPMV polypeptides in a glycerol gradient. Eighty microliters of an S30 fraction of radiolabeled B-component-inoculated protoplasts was layered on a 10 to 30% (vol/vol) linear glycerol gradient either in TKE-TDS buffer (A) or in TKE buffer (B). Centrifugation was carried out in a Beckman SW50 rotor for 17 h at 48,000 rpm and 4°C. After the run, 500-μl fractions were collected and immunoprecipitated with anti-32K serum in TKE-TDS buffer (A) or in TKE buffer without detergents (B). Samples were analyzed in a 12.5% polyacrylamide gel. Lanes 1 correspond to the bottom fractions of each gradient. The central lane between (A) and (B) contains unfractionated [^{35}S]methionine-labeled proteins from B-component-inoculated protoplasts. Numbers to the left of the figure refer to the molecular weights of some of the B-RNA-encoded proteins.

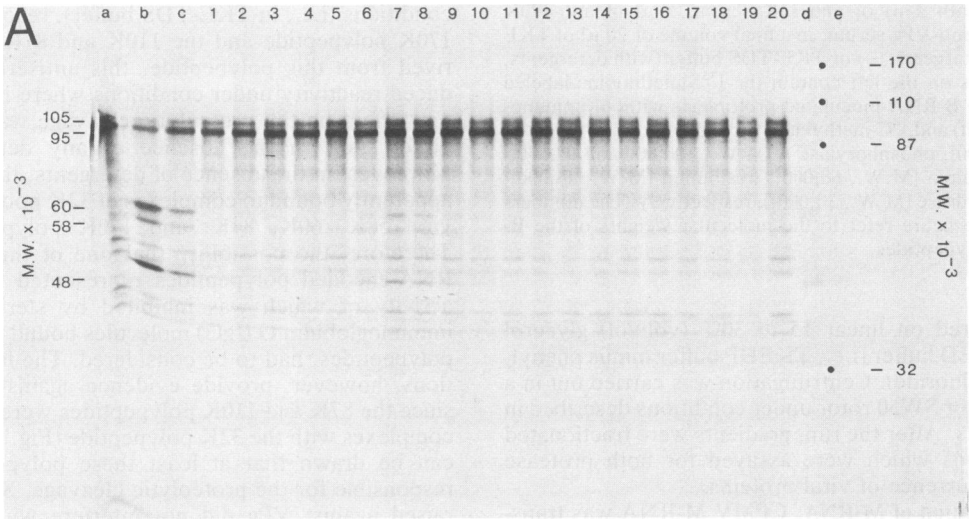

FIG. 4. Glycerol gradient fractionation and protease activity of B-RNA-encoded proteins. Eighty microliters of an S30 fraction of [^{35}S]methionine-labeled, B-component-inoculated protoplasts was layered on a 15 to 30% (vol/vol) linear glycerol gradient (in TKED buffer) and centrifuged for 42 h in a Beckman SW41 rotor at 38,000 rpm and 4°C. After centrifugation, the gradient was fractionated into 20 portions of 500 μl which were tested for protease activity (A) and the presence of viral polypeptides (B and C). (A) From each fraction, 20-μl portions were taken and tested for protease activity on M-RNA-encoded polypeptides, as described in the text. Proteolytic cleavage was analyzed in a 12.5% polyacrylamide gel (lanes 1 to 20). Lane 1 corresponds to the bottom fraction of the gradient. Lane a contains 2 μl of [^{35}S]methionine-labeled in vitro translation products of M-RNA not further treated. Lane b contains the same polypeptides which were incubated for 1.5 h at 30°C with 2 μl of S30 fraction from B-component-inoculated protoplasts. Lane c is as lane b, but the S30 fraction was preincubated on ice for 42 h after addition of glycerol to a final concentration of 20% (vol/vol). Lanes d and e contain [^{35}S]methionine-labeled proteins of B-component-inoculated (lane d) and noninoculated (lane e) protoplasts. Numbers indicated to the left of the gel refer to the molecular weights

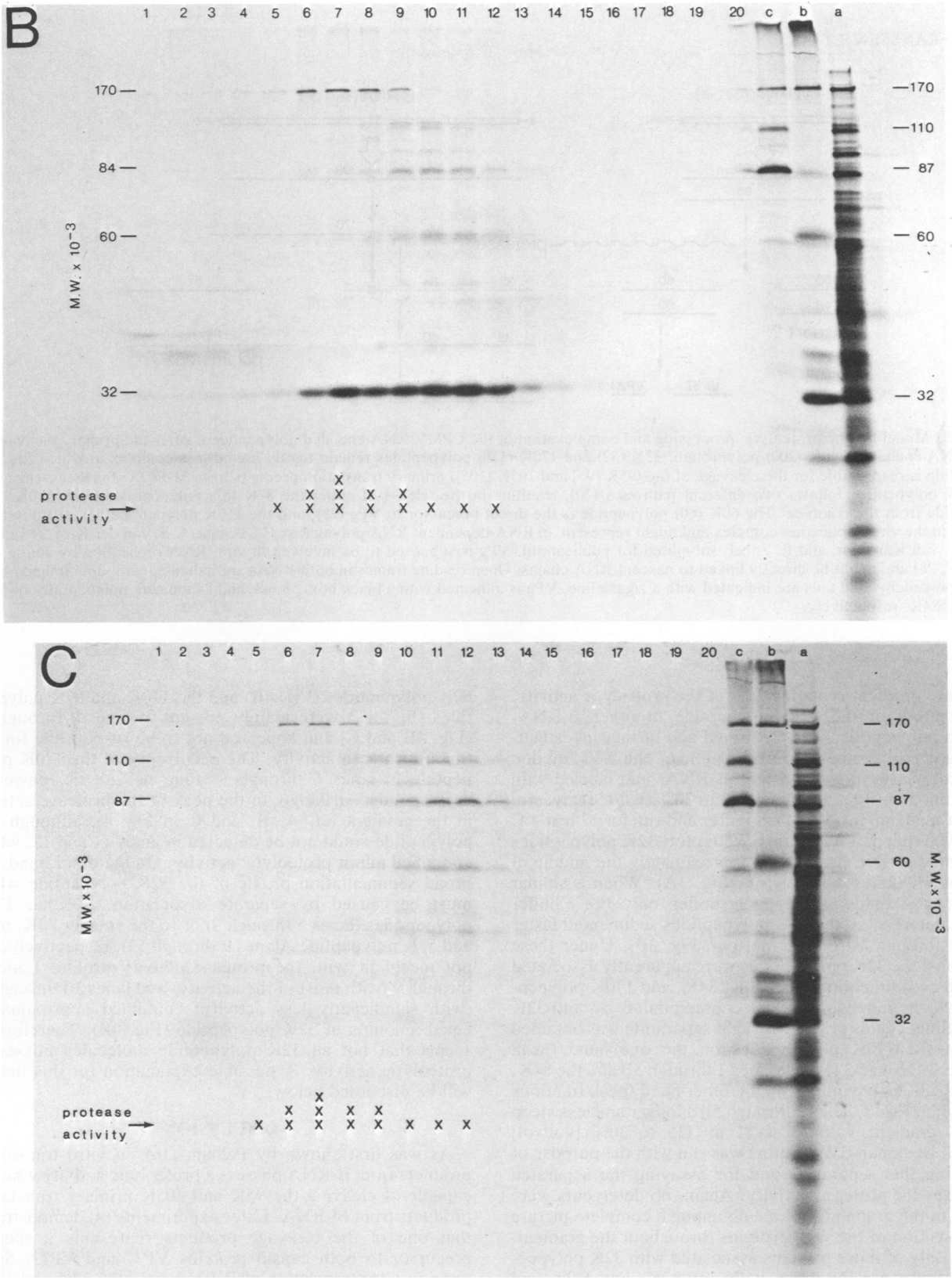

FIG. 4—*Continued*

of the in vitro translation products of M-RNA and the polypeptides generated by the proteolytic activity. Numbers to the right of the gel refer to the molecular weights of some of the B-RNA-encoded polypeptides. (B and C) Equal amounts (150 μl) of the glycerol gradient fractions were incubated either with a mixture of anti-32K and anti-VPg sera (B) or with anti-170K serum (C) in TKE-TDS buffer, and the immunoprecipitates were analyzed in 12.5% polyacrylamide gels. Each gel includes lanes containing the unfractionated [^{35}S]methionine-labeled S30 fraction of B-component-infected protoplasts (input, lane a) and immunoprecipitates of unfractionated S30 fraction obtained with anti-VPg and anti-32K sera (lane b) or with anti-170K serum (lane c) in TKE-TDS buffer. Numbers indicated at both sides of the gels refer to the molecular weights of the viral polypeptides. The number of crosses below the gel indicates the amount of proteolytic activity in the fractions, as deduced from (A).

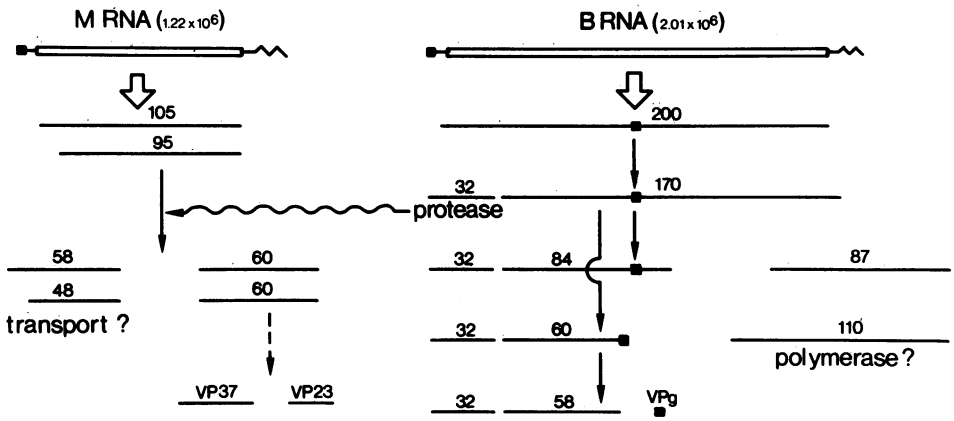

FIG. 5. Model for the proteolytic processing and complexation of the CPMV RNA-encoded polypeptides. After the primary cleavage of the B-RNA-encoded, 200K (200) polyprotein, 32K (32) and 170K (170) polypeptides remain tightly bound in a complex in which the 32K polypeptide is responsible for the cleavage of the (95K [95] and 105K [105]) primary translation products from M-RNA. Further cleavage of the 170K polypeptide follows two different pathways (20), resulting in the release of either the 87K (87) polypeptide or the 110K (110) polypeptide from the complex. The 60K (60) polypeptide is the direct precursor to VPg (28), and the 110K polypeptide has recently been detected in the viral replication complex and might represent an RNA-dependent RNA polymerase (L. Dorssers, S. van der Krol, J. van der Meer, A. van Kammen, and P. Zabel, submitted for publication). VPg is supposed to be involved in viral RNA replication by acting as a primer (7, 28) and might be directly linked to nascent RNA chains. Open reading frames in both RNAs are indicated with double-lined bars, and the polyadenylated tails are indicated with a zigzag line. VPg is indicated with a black box. 58, 48, and 84 indicate polypeptides of 58K, 48K, and 84K, respectively.

Glycerol gradient centrifugation of the proteolytic activity. Complexation of the 32K polypeptide to other B-RNA-encoded polypeptides was confirmed and further investigated by glycerol gradient centrifugation of the S30 fraction from protoplasts inoculated with B-RNAs and labeled with [^{35}S]methionine. In a 5-ml linear 10 to 30% (vol/vol) glycerol gradient made up in TKE-TDS buffer and run for 17 h at 4°C and 48,000 rpm in a Beckman SW50 rotor, 32K polypeptides sedimented as free proteins to approximately the middle of the gradient (lanes 4 through 6, Fig. 3A). When a similar gradient was made up in TKE buffer only (i.e., buffer without detergents), the 32K polypeptides sedimented faster (lanes 1 through 5 from the bottom, Fig. 3B). Under these conditions, the 32K polypeptides were apparently associated with the cosedimenting 58K, 60K, 84K, and 170K polypeptides, which, indeed, were all coprecipitated by anti-32K serum. Since in this gradient a slight separation was obtained between the 170K polypeptides on the one hand (peak fractions 1 through 3 [Fig. 3, lanes 1 through 3]) and the 84K, 60K and 58K polypeptides on the other hand (peak fractions 2 through 5 [Fig. 3, lanes 2 through 5]) a longer and less steep glycerol gradient volume of 11 ml (15 to 30% [vol/vol] glycerol, Beckman SW41 rotor) was run with the purpose of optimizing this separation and for assaying the separated complexes for protease activity. Again, no detergents were present in the gradient, but for obtaining a complete picture of the position of the viral proteins throughout the gradient, and not only of those proteins associated with 32K polypeptides, immunoprecipitations with anti-32K, anti-VPg, and anti-170K sera were carried out in buffer containing detergents (TKE-TDS). In this gradient, the protease activity sedimented into lanes 5 through 12 (Fig. 4A) from the bottom, with the main activity confined to lanes 6 through 9 (Fig. 4A). Screening of the gradient fractions with a mixture of anti-VPg and anti-32K sera (Fig. 4B) or with anti-170K serum (Fig. 4C) indicated that the protease-containing fractions all contained 32K polypeptide (Fig. 4B). The 84K and 60K polypeptides (Fig. 4B) and the 110K and 87K polypeptides (Fig. 4C) were mainly present in lanes 9 through 12 (Fig. 4B and C) and appeared not to be responsible for the (main) protease activity. The occurrence of the 170K polypeptide in lanes 6 through 9 (Fig. 4B and C), however, corresponded strikingly to the peak of the protease activity in the gradient (cf. A, B, and C in Fig. 4), although this polypeptide could not be detected in lanes 11 and 12, which contained minor proteolytic activity. On the other hand, the broad sedimentation profile of the 32K polypeptide which must be caused by separate association to either 170K polypeptides (lanes 5 through 9) or to the smaller 84K, 60K, and 58K polypeptides (lanes 9 through 13), respectively, did not nicely fit with the protease activity profile. Lanes 6 through 9 (with most of the activity) and lanes 10 through 12 (with significantly less activity) contained approximately equal amounts of 32K polypeptide (Fig. 4B). Therefore, it seems that not all 32K polypeptide molecules possessed proteolytic activity. A possible explanation for this finding will be discussed below.

DISCUSSION

As was first shown by Pelham (16), in vitro translation products from B-RNA possess a proteolytic activity which is capable of cleaving the 95K and 105K primary translation products from M-RNA. Later experiments (4) demonstrated that one of the cleavage products represents a specific precursor to both capsid proteins VP37 and VP23. Since upon in vitro translation of B-RNA only the 32K and 170K polypeptides are produced (8, 16), it can be deduced that either the 32K or the 170K polypeptide possesses the proteolytic activity involved. The protease inhibition studies presented in this report provide evidence that the 32K polypeptide represents the protease in question. The proteolytic cleavage of the M-RNA-encoded 95K and 105K polypeptides by extract from B-component-inoculated protoplasts is efficiently inhibited by antiserum raised against the

32K polypeptide, but not by anti-170K, anti-VPg, or preimmune sera (Fig. 1). Further experiments, in which the reactivity of these antisera was analyzed under conditions where this inhibition was tested, demonstrated, however, that care should be taken from directly drawing conclusions from such experiments. Anti-32K immunoglobulins appeared to bind to 32K polypeptides associated in complexes with 58K, 60K, 84K, and 170K polypeptides. The binding of heterologous viral polypeptides, apparently present in protein aggregates, has also been reported for poliovirus with antiserum directed against the polioviral protease (10). Additional experiments, in which proteolytic extract from B-component-inoculated cells was fractionated in glycerol gradients, demonstrated that the protease activity can only be correlated with 32K polypeptides or uncleaved 170K polypeptides. Therefore, two possibilities still remain: the protease activity resides either in the 170K precursor polypeptide or in the 32K polypeptide. For several reasons the first possibility seems to be unlikely. (i) If the 170K polypeptide is proteolytically active, then one might expect that, in analogy with, for instance, the animal picornaviruses (9, 10, 14, 15), one of the cleavage products from this precursor represents the mature protease. There is no evidence for such a mature protease, however. The experiments shown in Fig. 2 and 4 indicate that none of the 110K, 87K, 84K, or 60K polypeptides possess significant proteolytic activity. (ii) Anti-VPg immunoglobulins which are capable of reacting with the 170K (and 60K) polypeptides under nondenaturing conditions (Fig. 2) do not interfere with the proteolytic cleavage. Therefore, it is reasonable to propose that the 32K polypeptide represents the protease. Indeed, antiserum raised against this polypeptide efficiently inhibits the proteolytic activity (Fig. 1). Although other viral polypeptides were associated with the 32K polypeptides which might have been blocked in their function by the binding of IgG molecules to the 32K polypeptide, it should be noted that the antibody-antigen complexes were not removed from the protease reaction mixture. The possibility that the 32K polypeptide is an activator of a cellular enzyme can readily be excluded by the observation that the proteolytic activity also resides in the in vitro product from B-RNA (16). A puzzling observation is that not all 32K polypeptides appear to be active molecules. Only 32K polypeptides associated with 170K polypeptides seem to be involved in the proteolytic cleavage of the M-RNA-encoded primary translation products (cf. Fig. 4A and B). A possible explanation for this phenomenon is that the 32K polypeptides connected to 170K polypeptides represent newly synthesized molecules which are freshly cleaved from the 200K primary translation product. The 32K polypeptides associated with the 84K, 60K, or 58K polypeptides may represent significantly older molecules, which may have lost much of their activity. Indeed, in vitro cleavage of the 200K primary translation product into the 32K and 170K polypeptides occurs as soon as the 200K polypeptide chain has been completed, whereas further cleavage of the 170K polypeptide into the 110K, 87K, 84K, and 60K polypeptides takes, at least in vitro, a significantly longer period of time, with the first cleavage products only visible after 6 to 8 h (H. Franssen, unpublished data).

The information available suggests the following pathway of the B-RNA-encoded polypeptides (Fig. 5): soon after its synthesis, the 200K primary translation product is cleaved into the 32K and 170K polypeptides, which remain associated after cleavage. The 32K polypeptide is the protease involved in the cleavage of the primary translation products of M-RNA, and its activity decreases over the course of time. Further cleavage of the 170K polypeptide occurs at a slower rate, giving rise to complexes consisting of the 32K polypeptide, with decreased activity, and 84K, 60K, or 58K polypeptides. The 110K and 87K polypeptides, which are cleaved from the COOH-terminal part of the 170K polypeptide (5), do not remain in the complexes but are released (Fig. 2). It should be mentioned, however, that the viral protein complexes described here have been found in the S30 fractions, whereas considerable amounts of the 58K and 60K polypeptides have been found in the membrane fraction (i.e., 30,000 × *g* pellet fraction) of infected cells (7, 28). Therefore, to obtain a complete picture of the viral protein complexes in infected cells, additional studies on this membrane fraction are necessary. The 32K polypeptide is probably not the only protease involved in the proteolytic processing of the viral polyproteins. According to the mapping of the coding sequences of VP37 and VP23 on the M-RNA sequence (27), the cleavage site of the 32K polypeptide should be the dipeptide sequence glutamine-methionine. The cleavage site used to release VP37 and VP23 from their 60K precursor (4), however, is a glutamine-glycine sequence (27). Therefore, at least a second (plant or viral?) protease should be involved in the generation of the M-RNA-encoded polypeptides. The cleavage sites used for the processing of the B-RNA-encoded polyprotein remain to be elucidated, and information about the nature of the protease charged with the cleavage of this protein is also not available.

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