Identification of a Precursor Protein to the Major Glycoproteins of Mouse Mammary Tumor Virus

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Mouse mammary tumor virus-producing cultures of mouse mammary tumor cells synthesize a viral-related polypeptide of molecular weight of 73,000 (gp 73) which is rapidly labeled during a short pulse but disappears during the chase concomitantly with the appearance of label in the virion glycoproteins gp 49 and gp 37.5/33.5. The addition of the protein synthesis-inhibitor cycloheximide to the chase medium has little effect on this conversion. Treatment of the proposed precursor with α-chymotrypsin leads to the formation of a polypeptide of molecular weight 49,000, similar to the major virion glycoprotein. A comparison of tryptic digest maps of the glycoproteins involved supports the hypothesis that both the viral glycoproteins gp 49 and gp 37.5/33.5 are derived from gp 73.

Mouse mammary tumor virus (MTV) is an oncornavirus containing an aggregate 70S RNA (6), a lipid-containing outer membrane, and nine or more structural polypeptides (4, 5, 9, 20, 21, 24, 26). At least two of these polypeptides are major glycoprotein species which appear to be located on the outer membrane of the virion (1, 4, 24, 26). Immunological precipitation techniques have been used to identify the cytoplasmically synthesized polypeptides of oncornaviruses, including MTV (4, 7, 8, 10, 19, 22). Previous reports have indicated that tumor cell cultures producing MTV contain a cytoplasmic precursor glycoprotein to one or both of the major viral glycoproteins (4). In this report we describe the characteristics of the precursor glycoprotein (molecular weight, 73,000) which gives rise to the major viral glycoproteins gp 49 and gp 37.5/33.5.

MATERIALS AND METHODS

Reagents. Reagents used are as follows: trypsin-TPCK (Worthington); α-chymotrypsin (Worthington); Pronase (B-grade; Calbiochem); [35S]methionine, (312 Ci/mmol [New England Nuclear]; 230 to 280 Ci/mmol [Amersham/Searle]); [14C]leucine, 324 mCi/mmol (New England Nuclear); [3H]dGTP, 9.3 mCi/mmol (New England Nuclear); poly rC:oligo dG12-18 (P. L. Biochemicals, Inc.).

Cell cultures. Tumor cells were prepared from spontaneous mammary gland tumors of the BALB/cfC3H/Crgl mouse strain as described (3, 14). Cells were seeded at 5×10^5 cells/cm² in 10-cm petri dishes (Falcon) in Dulbecco modified Eagle medium containing 15% fetal calf serum (GIBCO), 10 µg of bovine

insulin (Sigma) per ml, 5 µg of hydrocortisone (Sigma) per ml, 100 units of penicillin per ml, and 100 µg of streptomycin sulfate per ml.

Labeling of cultures. Cell cultures containing approximately 2×10^7 cells per 10-cm petri dish were pulsed with [35S]methionine or [14C]leucine in Hanks balanced salt solution. The isotopic concentration and duration of the pulse varied with the experiment and are described in the text.

Isolation of MTV. Labeled and unlabeled MTV was isolated from the tissue culture fluid of tumor cell cultures as described (5).

DNA polymerase assay. The DNA polymerase activity in tissue culture fluids was measured as described (3) using rC:dG12-18 and [3H]dGTP as template-primer and substrate.

Preparation of cell extracts. Cytoplasmic extracts were prepared as previously described (4). Cell cultures were washed several times with cold phosphate-buffered saline, scraped from the culture dishes, washed again, and homogenized in buffer (0.01 M Tris-hydrochloride [pH 7.5]–0.025 M KCl–0.005 M MgCl₂) in the presence of 0.5% (vol/vol) Triton X-100 and 0.5% (wt/vol) deoxycholate. The homogenate was clarified by centrifugations at 100 × g for 5 min and at 150,000 × g for 35 min. The resulting supernatant was used in the immunoprecipitation procedure.

Protein estimations. The method of Lowry et al. (13) was used with bovine serum albumin as a standard.

Antiserum. Rabbit anti-MTV serum was prepared and absorbed, and the specificity was determined against purified, disrupted MTV and cytoplasmic extracts from both MTV-producing and murine leukemia virus-producing (Gross passage A) cultures as described (4).

Immunological precipitation. Cytoplasmic extracts were immunoprecipitated with an excess of anti-MTV serum (immunoprecipitation titrations

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were shown to plateau in a small excess of antibody [4, 22]) at 37 C for 30 min and overnight at 4 C. The resulting precipitates were collected by centrifugation at 13,000 × g for 30 min and washed three times with 500 µl of phosphate-buffered saline containing 0.5% Nonidet P-40.

Polyacrylamide gel electrophoresis. For disc gel electrophoresis, the dissociation and electrophoresis of samples on columns was based on the procedure of Maizel et al. (15, 16). Slab gel electrophoresis of samples on 10% slab gels (10 by 15 by 0.075 cm) was performed by a method adapted from the disc gel system. The immunoprecipitates or virus preparations were disrupted with 5 M urea, 0.1% sodium dodecyl sulfate (SDS), and 2% β-mercaptoethanol at 100 C for 2 min, and after cooling, 12.5-µl samples were applied directly into the gel slots. The gels were electrophoresed in a continuous SDS-neutral pH system at 25 V (10 mA) for 18 to 20 h. After electrophoresis, the gel slabs were fixed for 1 h in 10% trichloroacetic acid at 4 C and washed in 7% acetic acid. The slabs were either dried in vacuo or stained for 1 h with Coomassie blue and destained at 56 C for 4 h in 7% acetic acid (23) and dried in vacuo. The gels were autoradiographed using Kodak blue-sensitive single-coated X-ray film for 1 to 3 days.

Isolation of viral-related polypeptides. Labeled viral polypeptides of cytoplasmic and virion origin were separated by electrophoresis on 10% polyacrylamide disc gels. After electrophoresis the gels were sliced longitudinally (15) and dried in vacuo. The polypeptides were visualized by autoradiography, cut from the dried gels, and eluted by hydration with 0.1% SDS. After 24 h at 37 C with at least two changes of extractant, the polypeptide solutions were filtered and concentrated by one of two methods: (i) [^{35}S]methionine-labeled polypeptides in 1 to 2 ml of 0.1% SDS were dialyzed against distilled water, and the dialysate was lyophilized; (ii) [^{14}C]leucine-labeled polypeptides were precipitated by the addition of 0.2 volume of 50% trichloroacetic acid in the presence of 100 µg of bovine serum albumin as carrier. The precipitates were dissolved in 1 N NaOH and reprecipitated twice with 20% trichloroacetic acid. The precipitates were washed twice with a 70% ethanol-30% diethyl ether solution and dried by lyophilization.

Protein analysis by two-dimensional separation of tryptic digests. The extracted polypeptides were oxidized with performic acid, lyophilized, and digested with trypsin-TPCK in 0.05 M ammonium bicarbonate (pH 8.6) as described (2). The peptic digests were lyophilized and dissolved in 0.1 M acetic acid (insoluble material was removed by centrifuging for 1 min in a microfuge), and 5,000 to 10,000 counts/min of sample were applied near one corner of a cellulose-coated (MN300) thin-layer chromatography plate (20 by 20 by 0.1 cm; Brinkmann) for electrophoresis at pH 4.6 (2.5% acetic acid-2.5% pyridine) for 100 min at 500 V. The chromatography plates were dried and chromatographed in the second dimension in n-butanol-acetic acid-water-pyridine (150:30:120:120). The labeled peptides were localized by autoradiography for periods of 1 to 4 weeks.

RESULTS

Pulse-chase experiments. Previous studies have indicated that a viral-related glycoprotein of molecular weight 76,000 (now estimated at 73,000) is synthesized in the cytoplasm of MTV-infected cells and that it incorporates labeled amino acids prior to the appearance of the cell-associated major virion glycoproteins (4). The relationship between this possible precursor and the major virion glycoproteins was examined in cultures of mouse mammary tumor cells. The cell cultures were pulsed for 15 min with [^{35}S]methionine at 50 µCi/ml and chased with unlabeled culture medium for periods ranging from 30 min to 7 h. Immunoprecipitated cytoplasmic extracts were prepared and separated on 10% polyacrylamide slab gels as described above. Immediately after the pulse, [^{35}S]methionine was detected in the viral-related glycoprotein gp 73. After 30 min of chase, label began to appear in the major viral glycoproteins gp 49 and gp 37.5/33.5 (Fig. 1a). The amount of each polypeptide present at various times was quantitated by scanning the autoradiograph in a densitometer and calculating the area for each peak. From a plot of area versus duration of chase, the approximate half life of gp 73 was estimated at 50 to 60 min, a result which coincides with the maximum incorporation of label into gp 49 and gp 37.5/33.5 (Fig. 1b). These two cell-associated virion glycoproteins appeared at approximately equal rates. Labeled viral proteins were continuously lost from the cells in the form of extracellular MTV. The released virions for each chase period were concentrated on sucrose density gradients in the presence of unlabeled carrier MTV, and the radioactivity was measured by scintillation counting.

The presence of cycloheximide at 50 µg/ml in the cell culture medium during a chase period of 2 h appeared to have little effect on the appearance of gp 49 and gp 37.5/33.5 (Fig. 2). However, this concentration was sufficient to inhibit protein synthesis by greater than 95%, as determined by the incorporation of label into total trichloroacetic acid-insoluble material. Virion production was measured with the DNA polymerase assay, which showed that cyclohexamide also had little effect on virus production.

Tryptic digest maps of MTV proteins. The [^{35}S]methionine-labeled viral proteins were isolated from both cytoplasmic extracts and purified virions and subjected to trypsin digestion as described. A comparison of the tryptic digest maps shows that the main methionine-containing peptides in the digest of gp 73 were also
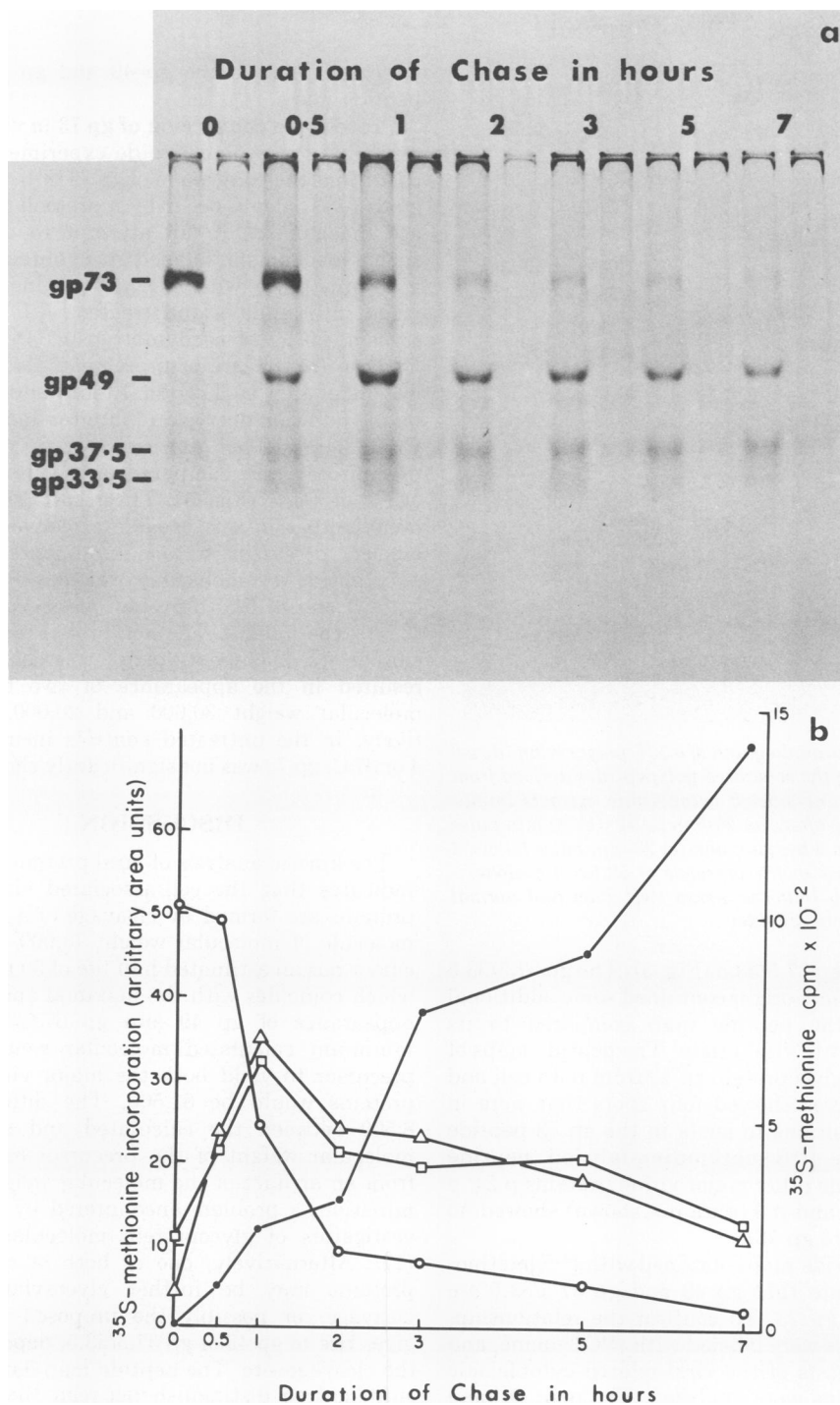
Fig. 1. (a) *Autoradiograph of a 10% polyacrylamide gel slab showing the separated polypeptides obtained after a 15-min [³⁵S]methionine pulse of cell cultures followed by unlabeled chase varying from 0.5 to 7 h. Cytoplasmic extracts were prepared from cells obtained from two 10-cm petri dishes, divided in two, and immunoprecipitated with anti-MTV serum (left side) or normal rabbit serum (right side). (b) The autoradiograph (a) was scanned densitometrically, and the area under each peak was determined by the weight of paper in each peak. The weights for gp 73 (○), gp 49 (△), and gp 37.5/33.5 (□) are plotted against time. The label in extracellular virus (●) is also shown as total counts/minute per culture.*
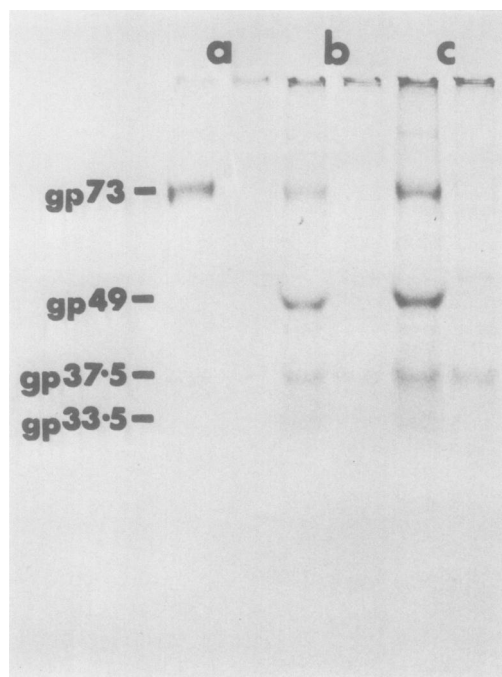
FIG. 2. *Autoradiograph of a 10% polyacrylamide gel slab showing the separated polypeptides derived from [³⁵S]methionine-labeled cytoplasmic extracts immunoprecipitate after: (a) 20-min pulse; (b) 20-min pulse followed by a 2-h chase; and (c) 20-min pulse followed by a 2-h chase in the presence of 50 µg of cycloheximide per ml. Immune serum (left side) and normal rabbit serum (right side).*

present in gp 37.5/33.5 (Fig. 3). The gp 37.5/33.5 of cytoplasmic origin contained some additional spots in the peptide map compared to its counterpart of viral origin. The peptide maps of the major glycoprotein gp 49 from both cell and virion sources showed four spots that were in similar positions to spots in the gp 73 peptide map. The [³⁵S]methionine-labeled peptide maps for the other major virion proteins p 24, p 17, p 13.5, and p 8 (data not shown) showed no similarity to gp 73.

The peptide maps obtained with [³⁵S]methionine indicate that gp 49 and gp 37.5/33.5 are related to gp 73. To confirm the relationship, cell cultures were labeled with [¹⁴C]leucine, and tryptic digests of the viral-related cytoplasmic glycoproteins were analyzed. The peptide map for gp 49 showed 13 out of 16 spots in common with gp 73, indicating a significant sequence homology. The majority of the remaining spots present in the gp 73 peptide map could be found in the peptide map of gp 37.5/33.5 (Fig. 4), which showed a total of 31 spots in common with gp 73. However, many of these spots

appeared in both the gp 49 and gp 37.5/33.5 peptide maps.

Proteolytic conversion of gp 73 in vitro. The results of the cycloheximide experiments indicated that the conversion of gp 73 into gp 49 and gp 37.5/33.5 could occur by a proteolytic cleavage mechanism. In an attempt to duplicate such a mechanism, labeled gp 73 obtained from a 30-min pulse with [³⁵S]methionine was divided into aliquots and treated with 50 µg of trypsin, 50 µg of α-chymotrypsin, or 50 µg of Pronase (a mixture of proteases). Each digest was incubated at 37 C for 10 min and stopped by the addition of trypsin inhibitor and 0.5 mM Cu^{2+} ions. The extracts were immunoprecipitated and compared on a gel slab with MTV proteins (Fig. 5). The results show that α-chymotrypsin was capable of converting the labeled precursor to an immunoprecipitable polypeptide of molecular weight 49,000, but little if any 37.5/33.5 protein was recovered in these experiments. The action of Pronase was similar to α-chymotrypsin, whereas trypsin resulted in the appearance of two bands at molecular weight 30,000 and 20,000, respectively. In the untreated controls incubated at 4 or 37 C, gp 73 was not significantly changed.

DISCUSSION

The kinetic analysis of viral protein synthesis indicates that the cell-associated viral glycoproteins are formed by cleavage of a precursor molecule of molecular weight 73,000. The precursor has an estimated half life of 50 to 60 min, which coincides with the maximal and parallel appearance of gp 49 and gp 37.5/33.5. The minimum calculated molecular weight for a precursor to yield both the major viral glycoproteins would be 81,500. The difference of 8,500 between the calculated and estimated molecular weight of the precursor may result from an artifact of the molecular weight determination, a problem encountered by other investigators of glycoprotein molecular weights (17). Alternatively, one or both of the glycoproteins may be further glycosylated after cleavage, or possibly the proposed precursor gives rise to gp 49 or gp 37.5/33.5, depending on the cleavage site. The peptide map data are not sufficient to distinguish between these mechanisms, although the latter would seem an unlikely possibility.

The use of cycloheximide, which inhibits protein synthesis but not the formation of the major viral glycoproteins from gp 73, indicates proteolytic cleavage as the processing mechanism. A more direct approach is the use of
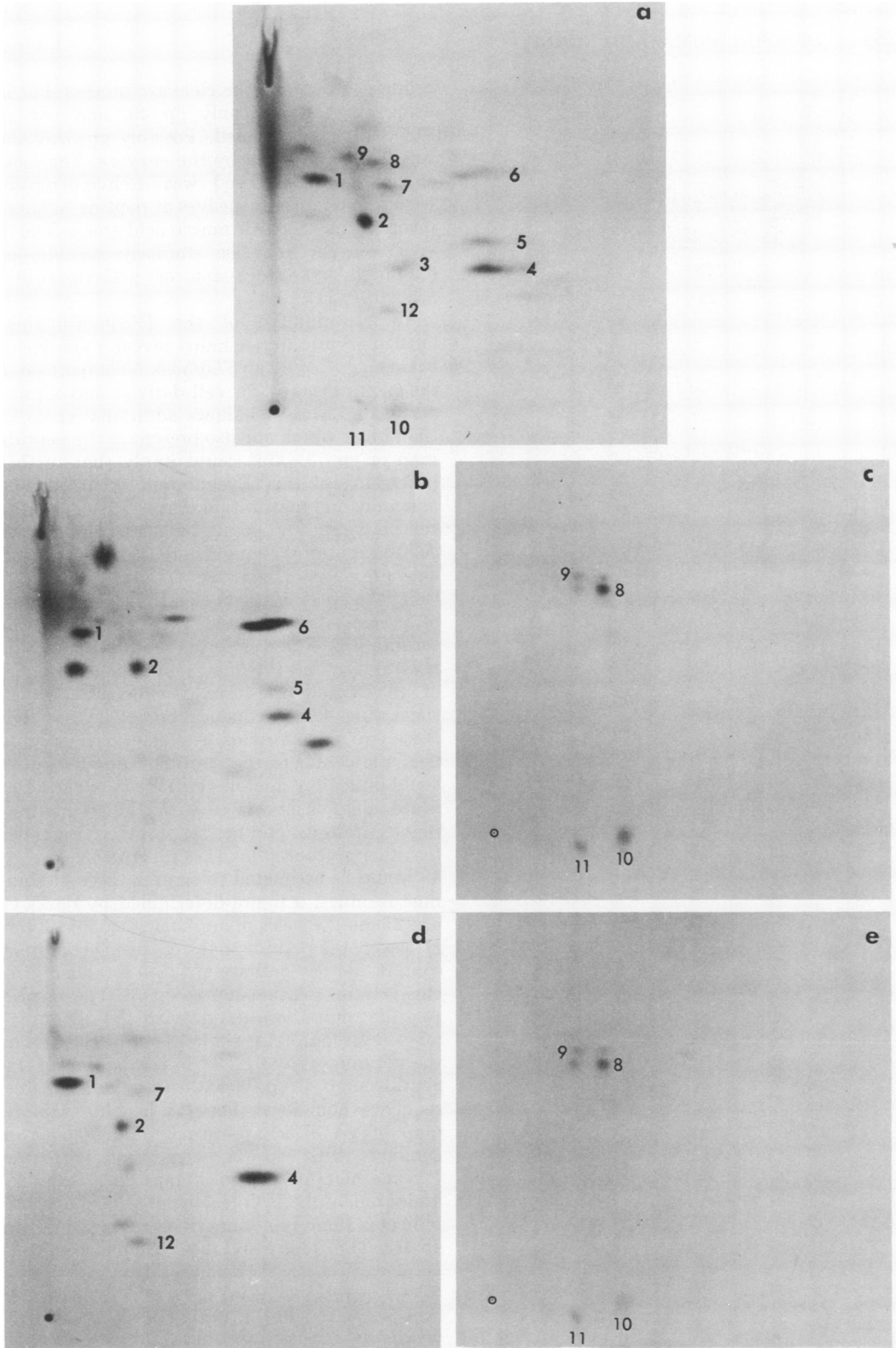
FIG. 3. *Autoradiograph of tryptic digest peptide maps of [³⁵S]methionine-labeled viral proteins. The maps were prepared on cellulose thin-layer chromatography plates by electrophoresis in one direction (left to right) and chromatography in the second direction (bottom to top). (a) gp 73; (b) cytoplasmically derived gp 37.5/33.5; (c) cytoplasmically derived gp 49; (d) virion-derived gp 37.5/33.5; (e) virion-derived gp 49. Spots are numbered for easy comparison.*
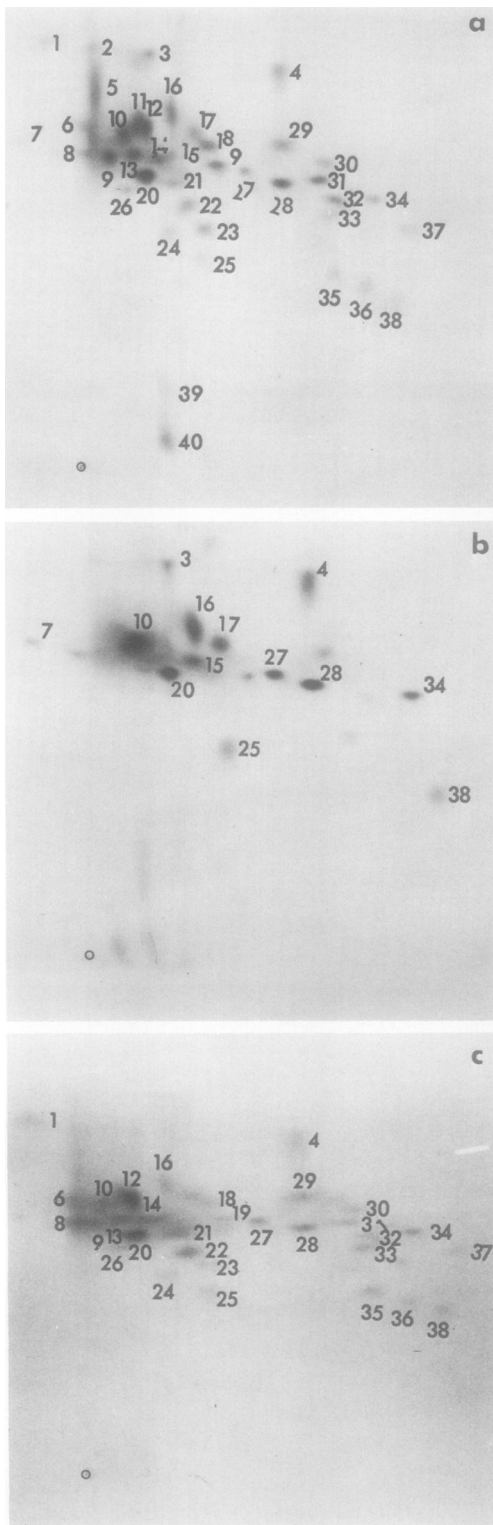
proteases to mimic the cleavage process, but in using this method only gp 49, and not gp 37.5/33.5, was detected. Possibly gp 37.5/33.5 was further digested by the enzyme. The most efficient protease used was α-chymotrypsin, which preferentially cleaves at peptide linkages involving an aromatic amino acid group. The in vivo cleavage may be similarly specific, as found in the processing of poliovirus and echovirus (11).

An examination of the [³⁵S]methionine-labeled tryptic digest maps reveal a similarity between gp 73 and gp 37.5/33.5. A comparison of the peptide maps for cell-derived and virion-derived gp 37.5/33.5 shows additional spots in the former which are also present in the peptide map of gp 73. This could imply that further processing of this glycoprotein occurs during assembly and maturation of the virus. Also, the cell-derived form could be contaminated by cellular material giving rise to additional spots. The presence of label at a similar molecular weight to gp 37.5/33.5 in electrophoresis profiles of precipitates obtained with normal rabbit serum (Fig. 1 and 2) adds credibility to this possibility. The [³⁵S]methionine peptide maps of gp 73 and gp 49 also show similarities, suggesting some sequence homology between these two glycoproteins. A cluster of four spots near the center of the peptide maps for gp 49 could indicate a microheterogeneity of carbohydrate moieties associated with a single methionine-containing peptide, a property described for other glycoproteins (12, 18). In this case gp 49 would be estimated to contain three methionine residues, a figure determined by Parks et al. (21) on the basis of amino acid composition. However, the peptide maps of this glycoprotein contain too few definite spots to establish conclusively its relationship to gp 73. The tryptic peptide maps obtained with [¹⁴C]leucine-labeled glycoproteins show 13 out of 16 spots in the peptide map of gp 49 in common with the peptide map of gp 73, indicating considerable sequence homology. The [¹⁴C]leucine peptide map of gp 37.5/33.5 reveals many spots in common with gp 73, consistent with the [³⁵S]methionine peptide map data. An examination of the [¹⁴C]leucine peptide maps also indicates some relationship between gp 49 and gp 37.5/33.5. This could result from a cross-con-

FIG. 4. *Autoradiographs of tryptic digest peptide maps prepared with [¹⁴C]leucine-labeled proteins obtained from cytoplasmic extracts. The maps were prepared as described in the legend to Fig. 3. (a) gp 73, (b) gp 49, and (c) gp 37.5/33.5.*
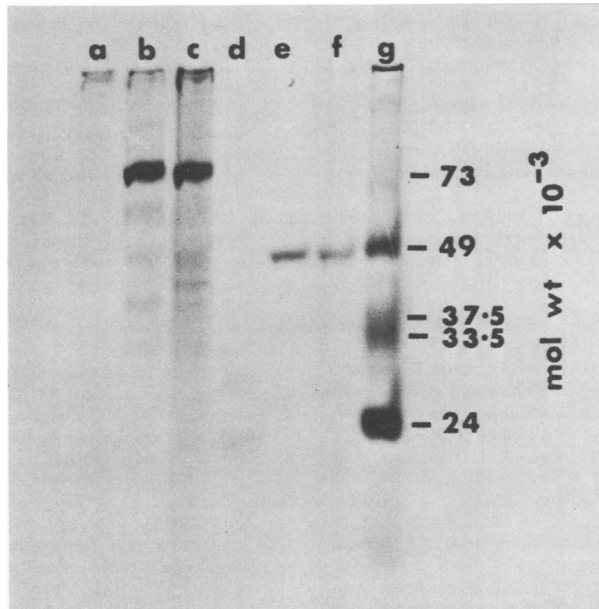
FIG. 5. *Autoradiograph of a 10% polyacrylamide gel slab showing electrophoretically separated polypeptides of a 30-min [³⁵S]methionine pulse-labeled cytoplasmic extract after various treatments. Aliquots were incubated for 10 min at: (b) 4 C; (c) 37 C; (d) 37 C with 50 µg of trypsin; (e) with 50 µg of α-chymotrypsin and (f) 50 µg of self-digested Pronase. The digestions were stopped as described and immunoprecipitated with anti-MTV serum. (a) Same as (b) but treated with normal rabbit serum. (g) Contains [³⁵S]methionine-labeled MTV polypeptides to serve as molecular weight standards.*

tamination of the glycoproteins or may indicate some sequence homology between gp 49 and gp 37.5/33.5. In experiments where isolates have been re-electrophoresed, no cross-contamination has been detected, although a partially degraded gp 49 could co-migrate in the gp 37.5/33.5 region and remain undetected. A more detailed examination of the components should resolve this ambiguity.

The mechanism whereby a precursor molecule is synthesized and subsequently cleaved has been recently demonstrated in other oncornavirus systems. A precursor and its intermediates have been characterized in the formation of the internal proteins of avian myeloblastosis virus (7, 25). A putative cell-associated precursor to a major glycoprotein of the avian sarcoma viruses, which requires additional carbohydrate moieties to mature, has been described (10). A number of precursors have also been implicated in the synthesis of a mouse leukemia virus (19). This study supports the notion that a precursor to the major virion glycoproteins of MTV is present in mouse mammary tumor cells, and yields further evidence for the generality of this type of maturation process.

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