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Characterization of Senescence- and Apoptosis-Dependent Forms of Terminin as Derived From a Precursor Found in Replicating and Nonreplicating Cells

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Abstract Previously we have reported the production of a monoclonal antibody (Mab 1.2) which recognizes a cytoplasmic protein, terminin, in three different molecular weights: 90 (Tp90), 60 (Tp60), and 30 kDa (Tp30) forms. Further characterization shows that Tp90 is found in young growing and nongrowing quiescent fibroblasts, while Tp60 is found in permanently growth-arrested senescent fibroblasts and Tp30 in cells committed to undergo programmed cell death (apoptosis). In tissue, Tp90 is found in embryonic brain; later, in neonatal brain after terminal differentiation is completed, only Tp60 is found. Tp30 is found in crude liver fractions extracted *without* the protective action of protease inhibitors. In all these circumstances, Tp90 is mostly seen in the detergent-soluble fraction, while Tp60 and Tp30 are detergent-insoluble. We now report that in cultured fibroblasts, as well as in tissues such as brain and liver, Tp60 and Tp30 are derived from the Tp90 polypeptide, indicated by the fact that only the Tp90 species is identified by both immunoblotting and immunoprecipitation assays, when the cell or tissue extracts are prepared *in the presence of protease inhibitors.* Further evidence shows that immunoprecipitation of in vitro translation products from brain, liver, and cultured fibroblasts also present a single band of Tp90 polypeptide. Pulse-chase experiments show that during apoptosis, Tp90 is processed to Tp60, and eventually to Tp30. However, when the total protein extracts are fractionated, only Tp90 is found in the detergent-soluble fraction, with diminishing quantities during the time course of apoptosis, and Tp30, in contrast, is found as the only protein species in the insoluble fraction, with increasing quantity during the same time course. Newly processed Tp60 is not found in either of the fractions, reflecting its loss during the fractionation procedure. Limited one-dimensional peptide mapping of Tp90 yields three different bands at 30, 28, and 25 kDa, but only the one at 30 kDa is recognized by Mab 1.2. These results lead us to suggest that terminin protein is synthesized in the Tp90 form, and cleaved to lower molecular weight forms depends upon different physiologic conditions, with Tp60 processed in the terminally differentiated or senescent state and rapidly to Tp30 in apoptosis. Our findings further suggest that Tp90's processing to either Tp60 or Tp30 produces insoluble protein forms. Furthermore, the presence of Tp90 in nonapoptotic (either replicating or nonreplicating) cells may reflect the absence of necessary proteolytic action required for the execution of apoptosis. Future experiments will allow us to determine the nature of this proteolytic action, as well as whether this action is due to the autocatalytic action of Tp90 or by other endogenous proteases, and then to determine the significance of this biochemical action in cells. © 1996 Wiley-Liss, Inc.

Key words: immunoprecipitation, in vitro translation, V-8 digestion, peptide mapping

In general, any given cell can be categorized into one of four major physiological states: a) the *rapidly growing* state, where cell division is a continual event; b) the *quiescent* state, when cell division is temporarily stopped but can be reinitiated in permissive conditions; c) the long-lived *nonreplicating* state, when growth-arrest is permanent and further cell division is no longer possible; and finally d) the *self-destructive* state, when cells are programmed to commit suicide and experience the active pathway of programmed cell death. Each of these four states seems to be controlled by precisely regulated

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molecular programs, which have been the subject of intense research recently. Previously we identified a 57 kDa protein, statin, whose presence in nuclei can be used as a marker to separate nonreplicating cells from their replicating counterparts. Statin presence does not differ qualitatively between temporarily and permanently growth-arrested cells, however, so as to make an unequivocal distinction between the two cell types. How then may one distinguish quiescent young human fibroblasts from their senescent sister cultures, other than by morphological differences in size, volume, and similar parameters?

We and others have been involved in the last decade in the search to understand why senescent human fibroblasts are permanently blocked from further replication; significant breakthroughs have begun to reveal an elaborate mechanism ensuring that senescent fibroblasts can maintain their long-lived, nondividing phenotype. For example, in senescent fibroblasts c- *fos* expression is found to be permanently repressed [Seshadri and Campisi, 1990], and RB protein is kept in the permanently unphosphorylated state [Stein et al., 1990]. Further characterization shows that senescent fibroblasts do not express *cdc*2 or cyclins A or B [Stein et al., 1991] and accumulate inactive *cdk*2/cyclin D1 and *cdk*2/cyclin E complexes [Dulic et al., 1993], which may be related to the presence of another protein, *Sdi1,* a 21 kDa protein identical to at least three other proteins which have all been characterized as inhibiting *cdk*2's kinase action [Noda et al., 1994; Serrano et al., 1993; Xiong et al., 1993; Harper et al., 1993; El-Deiry et al., 1993].

Along this line of attempting to study the unique biochemical features of senescent fibroblasts, we have used the immunosuppression technique to produce an unique monoclonal antibody (Mab 1.2) which exhibits positive cytoplasmic granular staining in senescent fibroblasts, but not in their young replicating or quiescent counterparts [Wang and Tomaszewski, 1991]. Biochemically, this antibody recognizes a soluble protein of 90 kDa (Tp90) in young cells, but an insoluble protein of 60 kDa (Tp60) in senescent fibroblasts; serendipitously we found that the antibody can also recognize a protein of 30 kDa (Tp30) in cells that are committed to programmed cell death [Hébert et al., 1994]. In tissue, antibody staining is found in terminally differentiated neurons [Schipper et al., 1994], as well as in prostatic epithelial cells committed to die due to castration [Mitmaker et al., 1993]; this positive histochemical reaction corresponds to the presence of Tp60 in the insoluble fraction of neonatal and adult brain extracts [Yang and Wang, 1993]. Since immunohistochemical assays usually use an insoluble cell scaffold as the reaction specimen, the positive staining activity may reflect the presence of either Tp60 or Tp30 in the samples. Nevertheless, this suggestion does not add to the clarification of whether the different forms of terminin, Tp90, Tp60, and Tp30, are three entirely unrelated proteins encoded by three different messages while sharing an identical antigenic epitope to monoclonal antibody 1.2, or whether the smaller forms, Tp60 and Tp30, may be products of Tp90 by posttranslational processing.

In this report, we provide the first evidence to suggest that Tp60 and Tp30 are derived from Tp90; thus the two lower molecular weight forms seem to exist in a precursor and product relationship with the higher molecular weight form. This suggestion is based on the fact that both cultured fibroblasts and tissues (brain and liver) show only Tp90 presence in the enriched presence of protease inhibitors, while without these inhibitors Tp60 and Tp30 are recognized. Furthermore, Tp90 is the only immunoprecipitable species from in vitro translated products of cell and tissue extracts. Pulse-chase experiments with mouse 3T3 fibroblasts show that Tp90 is processed to Tp60, and eventually to Tp30 during apoptosis. However, further cell fractionation studies show that in apoptotic cells, Tp90 is detergent-soluble and is rapidly processed to detergent-insoluble Tp30. The half life of Tp60 in the detergent-insoluble fraction is short to almost undetectable. Peptide map analysis of gel-eluted Tp90 shows that it can be cleaved to the antigenic-positive Tp60, and later Tp30, with two additional cleavage products of 28 and 25 kDa which do not retain positive reaction to Mab 1.2. These results lead us to reason that during senescence and terminal differentiation, Tp90 is processed to Tp60; and during programmed cell death, it is processed to Tp30. It is this cleavage action to lower molecular weights which renders Tp60 and Tp30 insoluble. The enzymatic process regulating the change from Tp90 to either Tp60 or Tp30 may be part of the crucial steps orchestrating events leading to cellular senescence, terminal differentiation or even programmed cell death. The presence of Tp90

may serve as a reflection of the lack of essential apoptotic proteolytic action. At the very least, the presence of any of the three different forms of terminin may be used as a powerful marker to distinguish objectively cells in the physiological states described above.

MATERIALS AND METHODS

Cell Culture and Conditions for Induction of Programmed Cell Death

Mouse 3T3 fibroblasts were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics, including 50 U/ml penicillin and 50 μg/ml streptomycin. The monolayer cultures were maintained in humid air containing 5% CO_2 at 37°C. Confluent cultures were used for all experiments, with cell density of approximately 3×10^4 cells/cm^2 per 100 mm tissue culture plate. To activate programmed cell death in these cultures, the confluent cultures were left at the contact-inhibition state for 24–48 h, followed by washing with DMEM without fetal bovine serum and subsequent maintenance in this serum-free medium. Afterwards, at 0, 6, 12, 18, and 24 h, cultures were harvested and processed for detergent extraction for soluble and insoluble fractions and SDS-PAGE, as described below.

Animals

The brain and liver RNA and protein specimens used in this study were obtained from male Fischer 344 rats 3 months of age, obtained from the breeder colony of the National Institute on Aging of the National Institutes of Health (Bethesda, MD). They were maintained in animal facilities with a 12:12 h light-dark cycle, and were fed ad lib. with standard rat chow and water. Animals were sacrificed by decapitation; immediately afterwards, intact brain and liver tissues were removed and immersed directly either into precooled (4°C) homogenizing buffer for protein extraction, or into precooled solution D as described below.

Antibody

For all immunoprecipitation and immunoblotting assays described in this study, monoclonal antibody (Mab 1.2) was used as the specific probe to characterize the presence and different molecular weights of terminin. This monoclonal antibody was produced according to the procedure described in our previous publication [Wang and Tomaszewski, 1991]. Another monoclonal antibody (*pai*), characterized to possess no specific antigenic reaction, was used as the control probe in all assays described below.

Protein Extraction From Mouse Fibroblast Cultures and Brain and Liver Tissues

Monolayer cultures of mouse 3T3 fibroblasts cultured in serum-containing and serum-deprived states were harvested by scraping with a rubber policeman, after washing by phosphate buffered saline (pH 7.2) three times. Cell pellets were obtained by centrifugation at 1,000 rpm for 10 min. Detergent-soluble and -insoluble fractions were obtained by incubating resuspended cell pellets in extraction buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF); and aprotinin, leupeptin, and pepstatin at 2 μg/ml each; this extraction was carried out at 4°C for 10 min. Subsequently, the samples were centrifuged for 10 min at 7,000 rpm. The resulting supernatant was collected and used as the "detergent-soluble" fraction; the pellets were then used as the "detergent-insoluble" fraction, and were further extracted for protein samples with RIPA buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and the three protease inhibitors listed above. The extraction was performed at 4°C for 1 h, and followed by sonication for 10 min; after further centrifugation at 7,000 rpm for 15 min, the supernatant was then used as protein samples of "detergent-insoluble" fractions.

For protein extraction from brain and liver samples, tissue blocks were meshed and homogenized with 20 strokes of a glass tissue grinder on ice. The homogenization was done in 5 volumes of lysing medium containing 50 mM triethanolamine, pH 7.5, 250 mM sucrose, 25 mM KCl, 5 mM MgCl, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 5 μg/ml each of the following protease inhibitors: aprotinin, leupeptin, and pepstatin. After stroking, tissue homogenates were passed through three layers of cheesecloth to removed residual intact tissue chunks. The flow-throughs were further incubated in the same lysing medium but containing DNase (0.1 mg/ml), and incubated for 1 h at 4°C. After this incubation, the reaction mixture was sonicated for 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant obtained after this cen-

trifugation was then used for immunoblotting and immunoprecipitation assays.

Protein concentrations for all assays were determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA) using bovine plasma γ-globulin as the standard.

DNA Fragmentation

Assays for the activity of programmed cell death were performed according to the procedure of [Blin and Stafford, 1976], with minor modification as previously described [Hébert et al., 1994]. In brief, mouse 3T3 fibroblasts harvested at 0, 6, 12, 18, and 24 h after serum deprivation were then processed for DNA extraction by phenol and ethanol/sodium acetate precipitation, before precipitation with ethanol. The precipitated DNA samples were then electrophoretically separated on 1% agarose gel (ICN Biomedicals, Mississauga, Ontario) in the presence of 50 μg/ml *ethidium bromide, in buffer containing* 10 mM Tris-HCl, 1 mM EDTA, at pH 8.0, for 18 h at 20 V.

SDS-PAGE and Immunoblotting

Protein samples extracted according to the above procedure were boiled for 10 min and processed for electrophoretic separation according to molecular weight, as described [Laemmli, 1970]. For immunoblotting assays, the proteins separated on SDS-PAGE gels were transferred to nitrocellulose paper as described [Towbin et al., 1979]; the blots were then incubated with either antiterminin monoclonal antibody (Mab 1.2) or the control (*pai*) monoclonal antibody. Detection of the positive reaction was performed by subsequent incubation with rabbit anti-mouse IgG (Cappel Organon, Teknika, NY), using peroxidase-conjugated goat antirabbit IgG (Cappel) as the tertiary antibody. The final detection step was accomplished by incubation with 4-chloronaphthol (Sigma Chemicals) and H_2O_2 as the peroxidase substrate for reaction. Background nonspecific reaction was evaluated by comparison between Mab 1.2 and *pai* antibody in their final reaction profile and intensity.

RNA Isolation, In Vitro Translation and Immunoprecipitation

Total RNA was obtained according to the one-step isolation procedure described by [Chomczynski and Sacchi, 1987]. Monolayer cultures of mouse 3T3 fibroblasts were harvested by scraping, and cell pellets were collected by centrifugation as described above. The cell pellets were then suspended in solution D containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 1% β-mercaptoethanol. Brain and liver tissue samples in 1 g volume were resuspended in 10 ml of this solution D, followed by homogenization with 10–15 strokes in a glass grinder. Solution D buffer containing either the cell materials or tissue homogenates was further processed for total RNA isolation by sequential steps involving interspersed centrifugation and extraction by phenol-chloroform precipitation, isopropanol solubilization, and ethanol precipitation; the detailed procedure is included in the above reference. The isolated total RNA samples were then processed for poly(A)$^+$RNA purification following the procedure of Aviv and Leder [1972], by incubation with oligo (dT) cellulose beads (Boehringer Mannheim, Montreal, Québec), with modified steps described by [Sambrook et al., 1989].

In vitro translation assays were carried out according to the procedure described by Krieg and Melton [1984]; we used the commercially available rabbit reticulocyte lysate (Promega, Madison, WI). The oligo (dT) affinity column-purified poly(A)$^+$ RNA samples were incubated in a reaction mixture containing Flexi Rabbit reticulocyte lysate, amino acids excluding methionine, 1 mM ^{35}S-methionine, 25 mM magnesium acetate, 2.5 M KCl, 100 mM DTT, 40 U/μl RNase ribonuclease inhibitor, and RNA substrate in nuclease-free water; the incubation was carried out at 30°C for 1 h. Control for translation efficiency was established by the RNA of Brome Mosaic Virus (BMV), included in the Promega in vitro translation kit.

Immunoprecipitation assays were performed with either the radioactive labelled in vitro translated products, or with protein samples prepared as described above; the detailed procedure was previously published [Ching and Wang, 1990]. In brief, protein specimens were incubated with the monoclonal antibody overnight in the above-described lysing medium, except that the concentration of the three protease inhibitors was increased to 10 μg/ml. The reaction was carried out at 4°C with constant agitation. Subsequently, the reaction mixtures were incubated with rabbit anti-mouse immunoglobulin G (ICN Biomedica, Montréal, Québec), followed by subsequent incubation with protein A-sepharose beads (Pharmacia, Montréal, Qué-

bec). The beads were then pelleted by centrifugation at 10,000 rpm for 10 min, followed by subsequent washing with either RIPA buffer (for cell extracts) or lysing buffer (for tissue extracts). The resulting reaction products were then processed by boiling the pelleted protein A sepharose beads in double-strength SDS-PAGE sample buffer, and processed for analysis by polyacrylamide gel electrophoresis. For in vitro translation samples, the immunoprecipitates were evaluated by autoradiography; for protein extracts, by Coomassie-blue staining.

Pulse-Chase Experiments and Densitometric Quantitation

Quiescent confluent cultures were rinsed three times with methionine-free MEM, and pulse-labelled with 800 μCi [^{35}S]-methionine (> 1,000 Ci/mmol) in 2 ml methionine-free medium at 37°C for 2 h, in the presence of 10% fetal calf serum previously dialyzed against methionine-free medium. The cultures were then washed twice and incubated in 10 ml of methionine-containing MEM, but without serum, for various lengths of time up to 24 h, when they were harvested for protein extraction, immunoprecipitation, and SDS-PAGE analysis. Densitometric scans were performed on autoradiographic X-ray film, with a program which includes measurement of background intensity in several areas of the films; averages of these measurements were subtracted to derive valid Tp90, Tp60, or Tp30 band intensities.

Limited Proteolysis

Protein extracts of mouse 3T3 fibroblasts prepared according to the above procedure were used for immunoprecipitation assays with terminin antibody (Mab 1.2). The immunoprecipitated products were then processed for SDS-PAGE analysis, and verified to contain a single precipitated band of Tp90. The gel pieces containing Tp90 were then processed for in-gel proteolysis, according to the well-described procedure of limited protease action [Cleveland et al., 1977]. The *Staphylococcus aureus* protease (Sigma, St. Louis, MO) of strain V8 type, with 538 units/mg, was prepared in 0.1 M Tris-HCl buffer at pH 6.8. The gel pieces containing the Tp90 protein were cut off from the main gel and re-equilibrated in a buffer containing 0.125 M Tris-HCl, pH 6.8, 1% SDS, and 0.01 M sodium EDTA (pH 7.0) for 20 min at room temperature, followed by meshing into small pieces; these were kept in separate groups to control for equal protein amounts, and then loaded into individual wells of a 17% SDS gel. To each well, the *S. aureus* V8 protease was added in doses of 0, 1, 3, or 5 ng. After the completion of electrophoresis, the resulting peptide profiles were visualized by silver staining following the procedure established by Wray et al. [1981], or processed for immunoblotting assays with monoclonal antibody 1.2 as described above.

RESULTS

Total withdrawal of serum supply from confluent cultures of mouse 3T3 fibroblasts is the simplest model of programmed cell death (apoptosis) established in our laboratory. We have found that fetal calf serum may contain survival factors; removing it activates the "death or suicide genes" in a synchronized fashion, and final cell demise is observed 24 h afterwards. While undergoing programmed cell death, these cells exhibit characteristic morphological and biochemical changes, including membrane blebbing, chromosomal condensation, and DNA fragmentation (Fig. 1). Since our identification of the 30 kDa form of terminin in cells undergoing programmed cell death, we have found that during the 24 h leading to the eventual death, Tp90 presence in the soluble fractions of mouse 3T3

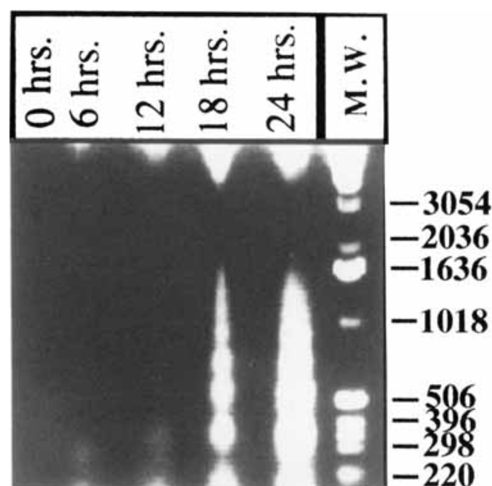


Fig. 1. Demonstration of programmed cell death by the fragmentation of DNA into oligonucleosomal ladders. DNA specimens obtained at 0, 6, 12, 18, and 24 h after serum deprivation were harvested, and 10 μg was loaded onto each lane. A very small degree of fragmentation was seen at 12 h; by 18 h, the fragmentation is clearly significant. Mass DNA fragmentation and final death are seen at 24 h after serum is withdrawn from the culture. Molecular weight markers in base pair unit are shown on the **extreme right lane** (M.W.).

fibroblasts diminishes in quantity after induction of programmed cell death by total deprivation of serum. In contrast, the presence of Tp30 in the insoluble fraction increases in quantity during the same period, with the maximal amount achieved at 12 h after serum deprivation (Fig. 2). When the same extraction procedures are applied to senescent human fibroblasts or postnatal rat brain tissues, only Tp60 is found in the insoluble fractions of both samples [Wang and Tomaszewski, 1991; Yang and Wang, 1993]. Similarly, in rat liver Tp30 is the only species found in the nuclear/cytoskeletal fraction without the presence of protease inhibitors; just as in cultured fibroblasts and tissue extracts of brain, Tp90 is not present in this fraction (data not shown).

We have further established that maintaining the 90 kDa size of Tp90 is dependent upon the protective action of protease inhibitors. When a total protein extract is prepared in the absence of protease inhibitors, the major protein species recognized by monoclonal antibody 1.2 on Western blots is Tp30; this is true with total protein extracts from cultured mouse 3T3 fibroblasts (Fig. 3A), rat brain (Fig. 3B), and rat liver (Fig. 3C). A single Tp90 band is observed in lane c of Figure 3A–C in the protein samples extracted in the presence of all four protease inhibitors. However, when protein extracts are prepared without them, a single band (Tp30) is recognized in the mouse 3T3 fibroblast sample (Fig. 3A, lane e); a weak Tp90 band with strong Tp60 and Tp30 bands is observed in the rat liver sample (Fig. 3B, lane e); but in rat brain samples, Tp90, Tp60, and Tp30 are all seen, along with two bands positioned between Tp90 and Tp60 (Fig. 3C, lane e). None of these immunoreactive bands is seen when the control monoclonal antibody (*pai*) replaces terminin monoclonal antibody in the primary antibody incubation steps during the immunoblotting assays (lanes b and d of Fig. 3A–C).

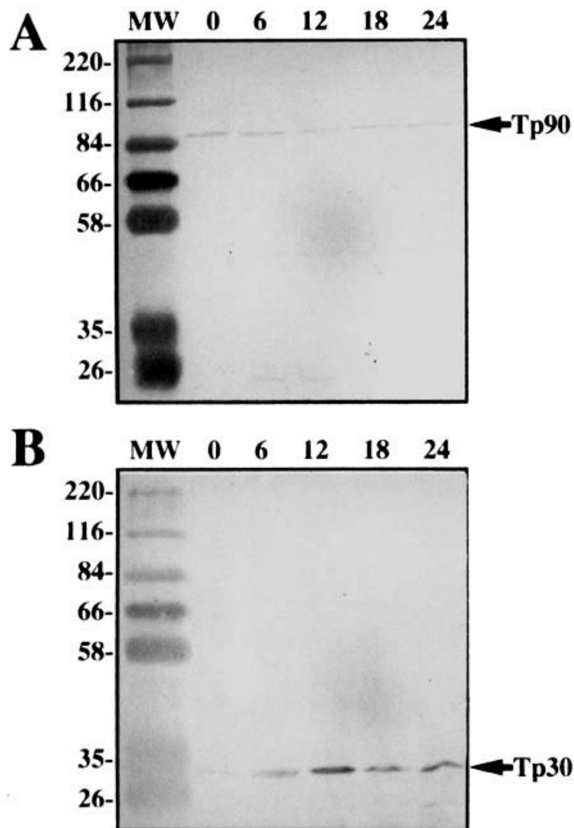
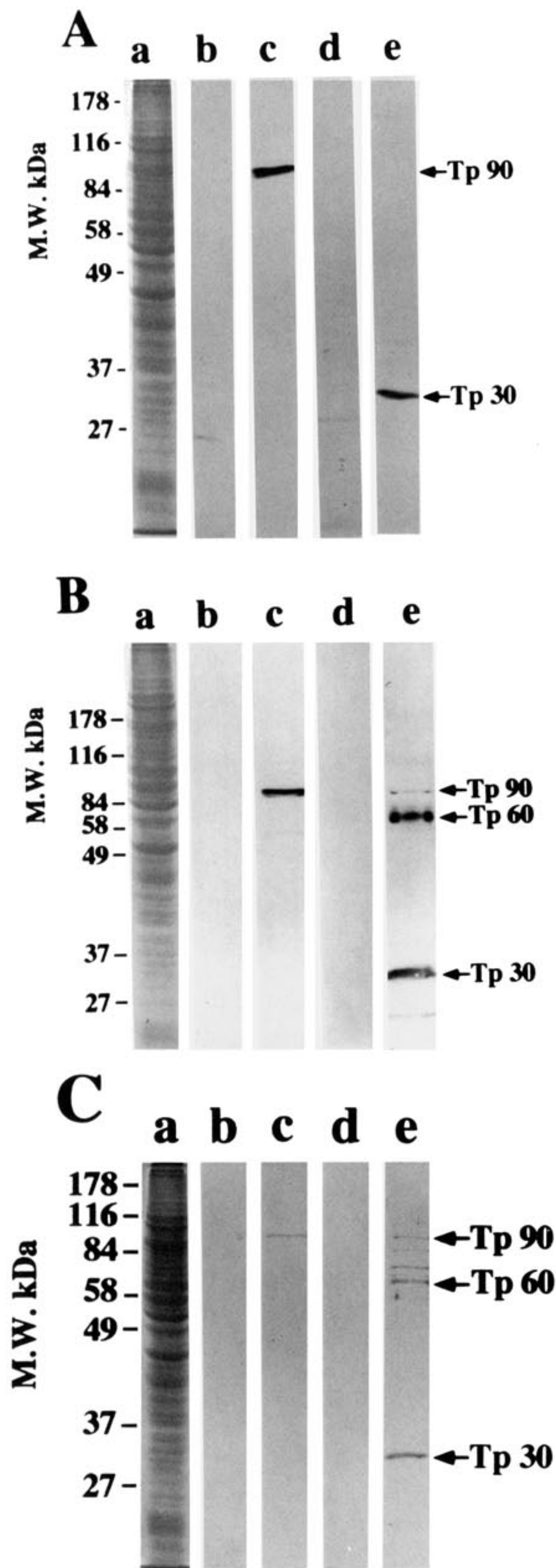


Fig. 2. Quiescent mouse 3T3 fibroblasts processed for extraction of soluble (**A**) and insoluble (**B**) fractions at 0, 6, 12, 18, and 24 h after serum deprivation, followed by immunoblotting with monoclonal antibody 1.2. Note that the presence of Tp90 in the soluble fraction decreases with time, while Tp30 increases to the maximal level at 12 h.

The Tp90 found in the protein extract of mouse 3T3 fibroblasts, shown in Figure 4A, lane c, can be immunoprecipitated as a single band electrophoretically at the 90 kDa position (Fig. 4A, lane e); the similar presence of a single band at 90 kDa can also be observed in the resulting product of immunoprecipitation assays with rat brain and liver extracts (Fig. 4B,C, lanes e). As shown in Figure 3, only Tp90 is recognized as a major positive band in all three protein samples, except for a very faint band of Tp60 in lane c of Figure 4A and C. In addition to the heavy immunoglobulin band seen in both control and Mab 1.2 reaction lanes, Tp90 is the major immunoprecipitated band present in the precipitates from the terminin antibody reaction assays, but not in the control samples. In addition, a weak band is seen immediately below the immunoglobulin position in the terminin antibody precipitates. Upon re-evaluation of the identity of this 90 kDa species, the immunoprecipitated Tp90 from all three protein samples retains reactivity with Mab 1.2, thus confirming the suggestion that this immunoprecipitated 90 kDa protein is the same Tp90 seen in the total protein extracts of cultured fibroblasts, as well as rat brain and liver (Fig. 4A–C, lane g). Again, the positive single band seen at 90 kDa is present only in the terminin antibody immunoblotting reaction, and not in the control antibody reaction, thus con-

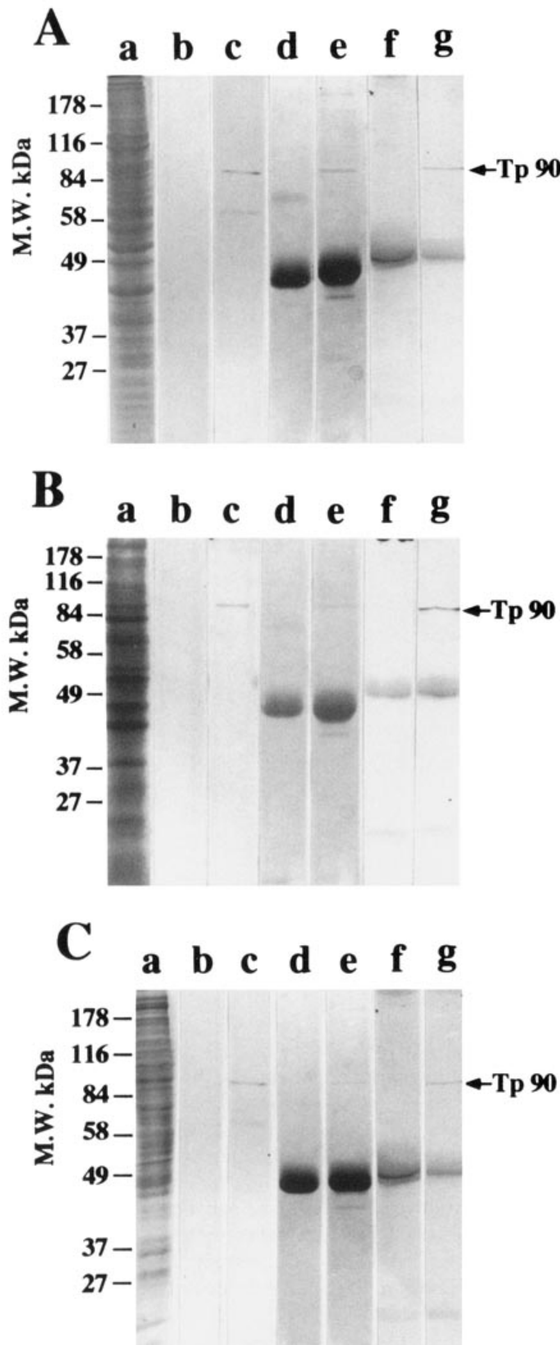


firming the identity of this protein as Tp90. The band present below the immunoglobulin band in lane e does not react with the terminin antibody in the reblotting assays, indicating that it may be a nonspecific or co-immunoprecipitated reaction during the prior immunoprecipitation assays. The presence of Tp90 as a single band in lanes c, e, and g of Figure 4A–C suggests that in the presence of protease inhibitors, this terminin subspecies is the major band, and is immunoprecipitable by the Mab 1.2 antibody.

To determine whether the Tp90 seen in protein extracts from cultured mouse 3T3 fibroblasts, rat brain, and liver tissue extracts is indeed synthesized as one single polypeptide of the same or higher molecular weight, we used the in vitro translation procedure coupled with immunoprecipitation assays. As shown in Figure 5A (lane a), the message-derived synthesis of mouse 3T3 fibroblasts produces a large number of cellular protein products at various molecular weights. When this reaction product is subjected to immunoprecipitation assay with monoclonal antibody 1.2, a single prominent band of 90 kDa is seen as the only ^{35}S-methionine-labelled protein in the reaction product (Fig. 5A, lane d); efficiency of the in vitro translation assay is controlled for by using the commercial kit for viral messages (Fig. 5A, lane b). Control for possible nonspecific reactions is established with another monoclonal antibody bearing no particular antigenic reaction; the absence of an identifiable band in the immunoprecipitation reaction with this control antibody is seen in lane c of Figure 5A. The two additional bands at slightly above 58 kDa and 45 kDa seen in Figure 5A, lane d, are due, respectively, to

Fig. 3. Illustration that terminin antibody (Mab 1.2) recognizes Tp90 as the positive band when protein extracts are prepared in the presence of protease inhibitors, but Tp30 when the same protease inhibitors are omitted. Protein extracts of confluent cultured mouse 3T3 fibroblasts (**A**), rat liver (**B**), and rat brain (**C**) were prepared according to the procedure described in Materials and Methods, except that in *lanes b and c,* protease inhibitors, including PMSF, aprotinin, leupeptin, and pepstatin, were included but in *lanes d and e,* these inhibitors were not included during the extraction steps. *Lane a* in all three panels represents the protein profiles detected by Coomassie-blue staining; since the patterns of total extract proteins did not differ significantly between conditions with and without protease inhibitors, only one lane is shown here as a representation. Results of the control antibody reaction are shown in lanes b and d in all three panels.

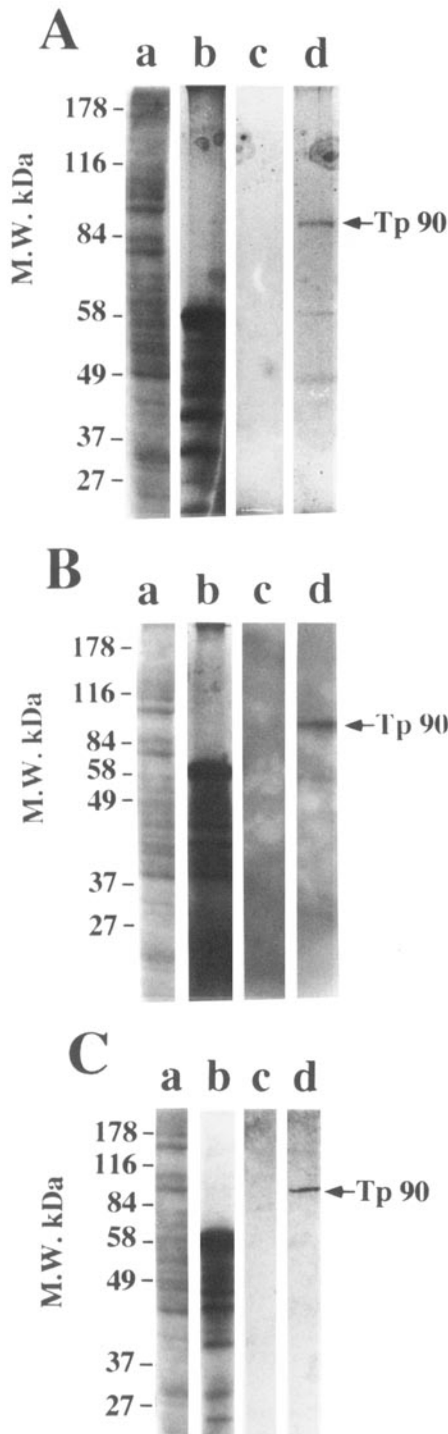
incomplete protection of Tp90 from proteolysis and a nonspecific reaction of the rabbit anti-mouse IgG. Similar results showing Tp90 as the single translatable and immunoprecipitated band are also observed with message-derived protein synthesis performed with rat brain and liver samples (Fig. 5B,C). All these results suggest that terminin is synthesized as Tp90 in confluent cultures of mouse 3T3 fibroblasts as well as in liver and brain of 3-month-old rat.



We have further examined the inter-relationship between Tp90 and Tp60 or Tp30 by pulse-chase experiments with mouse 3T3 fibroblasts undergoing apoptosis by serum deprivation. Cells were prelabelled with ^{35}S-methionine for 2 h; then both fetal calf serum and radioactive labels were withdrawn from the culture, and at designated times cell cultures were processed for protein extraction, immunoprecipitation, and SDS-electrophoresis analysis. As shown in Figure 6a, Tp90 was the only immunoprecipitable radioactive labelled protein at the initial 0-h time point; the proportion of this protein decreases with time, so that by 12 h after serum removal we can detect Tp60, and by 18 h Tp30 as well. Finally, after 24 h of serum deprivation, the Tp30 and Tp60 bands are of equal intensity, while Tp90 is significantly diminished in quantity. The changes in intensity for the three species are illustrated by changes in area of the densitometric tracing, reflected graphically in Figure 6b. Further immunoprecipitation experiments were performed with similar pulse-chase cell samples, but separated into detergent-soluble (Fig. 6c) and detergent-insoluble (Fig. 6d) fractions. As shown in Figure 6c, Tp90 again is the only protein species seen in the detergent-soluble fraction, with diminishing pulse-chased quantity in the later hours of the time course. In addition, a very weak band of 60 kDa is seen at early time points, possibly reflecting an uncontrollable minute degree of protease action during in vitro processing of the immunoprecipitated products. Results of immunoprecipitation

Fig. 4. Immunoprecipitation and immunoblotting assays showing that Tp90 can be precipitated by the terminin monoclonal antibody. Protein extracts were obtained from confluent cultures of mouse 3T3 fibroblasts (**A**), rat liver (**B**), and rat brain (**C**), according to the procedure described in Materials and Methods. All extraction procedures were performed in the presence of protease inhibitors, including PMSF, aprotinin, leupeptin, and pepstatin. The protein profiles detected by Coomassie-blue staining are presented in *lane* a of all three panels. *Lanes* b *and* c of all three panels are the immunoblotting results of the same three extracted protein samples when subjected to reaction with either a control antibody (lane b) or the terminin monoclonal antibody (lane c). *Lanes* d *and* e of all three panels show the Coomassie-blue staining pattern of immunoprecipitation assays when the same three protein extracts are reacted with either control antibody (lane d) or terminin antibody (Mab 1.2, lane e). The identity of the 90 kDa band seen in lane e of all three panels was further verified when similar electrophoretically separated protein samples were transferred to nitrocellulose blots and further incubated with either control pai antibody (*lane f*) or terminin antibody (Mab 1.2, *lane g*).

of the detergent-insoluble fraction, on the other hand, provide the picture that Tp30 is the only protein species present, with increasing quantity at the later time points (Fig. 6d). In particular, at 18 to 24 h time points, there seems to be further degradation of Tp30 to one lower molecular weight band. No trace of Tp60 is seen in the immunoprecipitation products of the detergent-insoluble fraction at any time, nor in the 6 to 24 h time points of the detergent-soluble fraction. This result shows that Tp60's association with the detergent-insoluble fraction as seen by immunohistochemical experiment may be a weak biochemical association, which is lost from this fraction during cell fractionation. Together, these findings demonstrate again that Tp90 is the precursor and the apoptosis-associated Tp30 is its product, and confirm the result shown in Figure 2.



Further investigation into whether Tp90 is the precursor of Tp60 or Tp30 was pursued by eluting Tp90 from the SDS-PAGE gel of mouse 3T3 fibroblasts, and subjecting it to in-gel digestion with V-8 protease at concentrations ranging from 1 to 5 ng/μl. As shown in Figure 7A, lanes a and b, at 0 or 1 ng V-8 digestion, Tp90 remains a major band, migrates at the same molecular weight position when it is re-run on the SDS-PAGE gel, and retains its antigenic activity with Mab 1.2 (Fig. 7B, lanes a and b). At 3 ng, the gel-purified Tp90 is now digested into two separate bands at 90 and 60 kDa (Fig. 7A, lane c), both of which can be identified by Mab 1.2 (Fig. 7B, lane c). When the concentration of the V-8 protease is increased to 5 ng, Tp90 generates three distinct bands migrating at 30, 28, and 25 kDa (Fig. 7A, lane d), and immunoblotting results of these reaction products show that only the 30 kDa species retains antigenic activity with Mab 1.2; neither the 28 nor the 25 kDa protein band seen earlier in Figure 7A, lane d, can be recognized by the immunoblotting assay, indicating that neither of these two fragments possesses the specific antigenic epitope to monoclonal antibody Mab 1.2.

Fig. 5. Tp90 is the only subspecies of the terminin multiforms immunoprecipitable from in vitro translation products. Message-derived protein synthesis was performed with poly(A)$^+$ RNA isolated from confluent cultures of mouse 3T3 fibroblasts (**A**), rat liver (**B**), and rat brain (**C**). Detailed procedures regarding the in vitro translation and immunoprecipitation procedures are included in Materials and Methods. The translated protein profiles of all three samples with the rabbit reticulocyte lysate are shown in *lane a* of all three panels. Control for translation efficiency was performed with the message of Brome Mosaic Virus (BMV) (available in the commercially supplied translation kit), and shown in *lane b* of all three panels. When the same in vitro translated cell or tissue protein samples were reacted with either control monoclonal (pai) antibody (*lane c*) or terminin (Mab 1.2) antibody (*lane d*) for immunoprecipitation assays, a single radioactive-labelled prominent band of Tp90 is seen in the reaction products of all three samples.

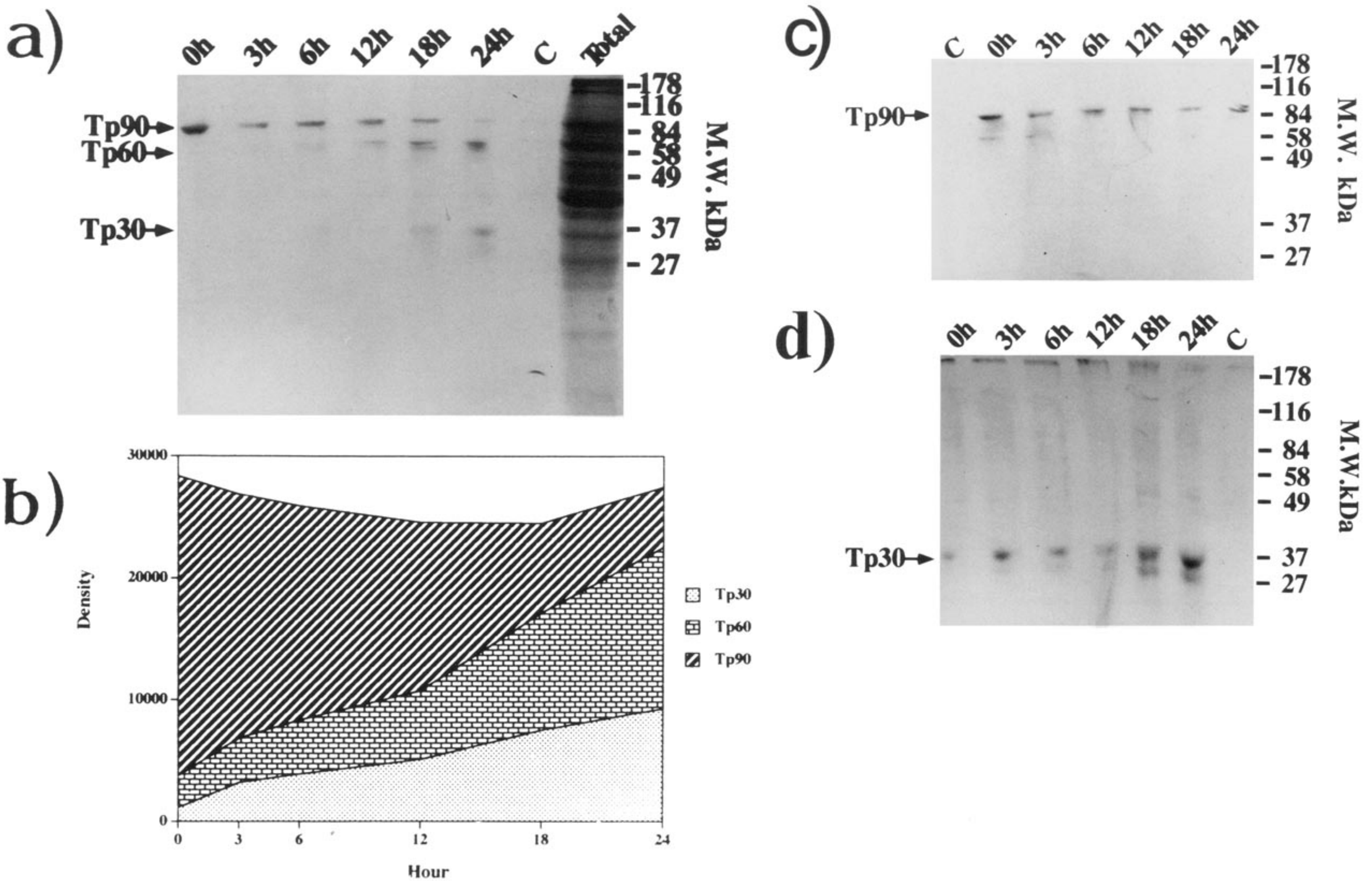


Figure 6

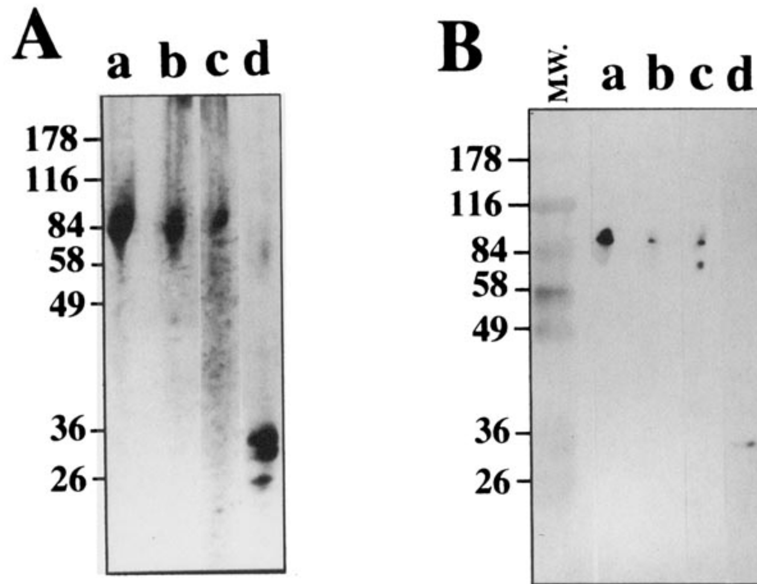


Fig. 7. Limited proteolysis of Tp90. A single Tp90 polypeptide was obtained from immunoprecipitation assays of confluent cultures of mouse 3T3 fibroblasts with the terminin monoclonal antibody. Protein extracts were prepared in the presence of protease inhibitors, followed by reaction with monoclonal antibody (Mab 1.2) to terminin as described in Materials and Methods. After SDS-PAGE and evaluation by Coomassie-blue staining (as shown inFig. 4A, lane e) the Tp90 was cut off from several similar gels and processed for V8 protease digestion, as described in Materials and Methods. **A:** A silver-staining gel, showing the digestion of gel-purified proteins into lower molecular weights. *Lane a* is the digested products of 0 ng V8 protease digestion, showing Tp90 as the major band, while *lane b* is the digested products of 1 ng V8 action, showing both Tp90 and another band immediately below it. *Lane c* shows the digested product of 3 ng V8 protease, showing the presence of a Tp90 band and a minor band at 60 kDa, and *lane d* shows the digested products of 5 ng V8 protease, showing three protein bands at 30, 28, and 25 kDa. **B:** The immunoblotting pattern of the same samples, when the proteins were transferred to a nitrocellulose blot and reacted with terminin monoclonal antibody.

Fig. 6. Pulse-chase analysis of the precursor and product relationship between Tp90, Tp60, and Tp30. Confluent cultures of mouse 3T3 fibroblasts were radioactively labelled with ^{35}S-methionine and then deprived of serum to activate apoptosis, as described in Materials and Methods. At designated time points, cells were processed for protein extraction and immunoprecipitation. **a** shows the protein profile of immunoprecipitate products of total protein pool subjected to SDS-PAGE; the positions of Tp90, Tp60, and Tp30 are indicated by arrows on the left. Time points designated as 0, 3, 6, 12, 18, and 24 h are number of hours after serum deprivation; *lane C* is an immunoprecipitation profile using a control antibody with no specific immunoreactivity. The total labelled protein profile is shown in the **extreme right lane,** with electophoretic mobility positions marked in the right margin. **b** shows the densitometric intensity measurements of the same Tp90, Tp60, and Tp30 immunoprecipitates seen in a; quantitative evaluation is represented graphically at different times with the density measurements shown in relative value in increments at 10^4 ratio. Further fractionation of total protein extracts of the pulse-chase samples was performed with 0.5% Triton buffer, as described in Materials and Methods, and the immunoprecipitation products of the detergent-soluble (**c**) and the detergent-insoluble (**d**) fractions are shown. Comparison of a, c, and d protein profiles shows that Tp90 is the precursor form in the soluble fraction, and chased to the insoluble Tp30 form during apoptosis, and Tp60 is lost during the cell fractionation procedure.

DISCUSSION

The cytoplasmic presence of terminin in the 60 kDa form has been used as a biochemical marker for senescent fibroblasts [Wang and Tomaszweski, 1991] and terminally differentiated neurons [Yang and Wang, 1993]. Furthermore, the presence of terminin in the 30 kDa form has been identified as a marker for defining the stage of commitment to programmed cell death [Hébert et al., 1994]. However, a third form of terminin exists, which migrates at the 90 kDa position and is found mostly in the soluble fraction of young fibroblasts or in embryonic brain tissues. It seems that all three forms of terminin, Tp90, Tp60, and Tp30, bear the same antigenic epitope to the monoclonal antibody (Mab 1.2), and their presence is dependent on the different physiological states of individual cells. The question then arises as to whether all three proteins are synthesized from three different messages but share the same antigenic epitope, or are synthesized from the same message but posttranslationally modified to different forms.

In this report, we describe preliminary evidence suggesting that a single polypeptide of Tp90 is synthesized, with the potential to be cleaved to Tp60 and Tp30 by step-wise proteolytic action.

Clearly demonstrated here is the sensitivity of Tp90 to protection from proteolysis. During in vitro manipulation, while protein extracts are processed, the presence of protease inhibitors is crucial; without their protective action, terminin may be rapidly processed and fail to reflect the true physiological state of the producing cells. In our earlier studies we found that in liver terminin is present as Tp30, and is associated with the endoplasmic reticulum fraction (data not shown). Since our other findings define the presence of Tp30 as a marker for commitment to programmed cell death, does this mean that the 3-month-old rat liver is experiencing massive apoptosis? Certainly not, since our recent results with more refined processing in obtaining liver extracts, particularly in preventing proteolysis by including three different kinds of protease inhibitors, display a positive reaction on immunoblots as Tp90. This explains that our earlier finding of Tp30 presence in liver is an experimental artifact, due to insufficient protection by protease inhibitors during the protein extraction procedure.

How does Tp90 become proteolytically processed to Tp60 or Tp30? We see two possibilities: 1) that there may exist an associated protease present in minute quantity, beyond detectability of any co-immunoprecipitated protein species on routine SDS-PAGE gels by either Coomassie-blue or silver-staining techniques, which acts (unless inhibited) to cleave Tp90 during apoptosis; and 2) that either Tp90 or one of its proteolytic products, Tp60 or Tp30, is itself a protease, and the autocatalytic or associated protease's proteolytic processing activates terminin's putative action. Answers to this question can only be obtained through future experiments on cloning the terminin gene, or characterizing the possible common moiety for protease action by sequencing analysis and/or biochemical characterization with purified native Tp90, Tp60, or Tp30.

Regulation of proteolysis for key proteins is well recognized as a crucial biochemical action, monitoring or arbitrating the success of many important physiological events. Failed or abnormal proteolysis may contribute to the etiology of Alzheimer's disease [Caputo and Salama, 1989; Kang et al., 1987], amyloid angiopathy [Van Broeckhoven et al., 1990; Levy et al., 1990], Gerstmann-Straussler syndrome [Prusiner, 1989], Icelandic hereditary cerebral haemorrhage [Cohen et al., 1983], and Bence-Jone proteins of myeloma [Linke et al., 1973; Shirahama et al., 1973]. Recently *ced*-3, a *C. elegans* cell-death gene, has been found to be homologous to the mammalian protein interleukin-1β converting enzyme (ICE) [Yuan et al., 1993], a cysteine protease that cleaves polypeptides at aspartate residues [Thornberry et al., 1992]. However, for this protease action to occur, ICE itself must undergo self-proteolysis to two subunits [Cerretti et al., 1992]. The fact that Tp30 is present only in cells experiencing the programmed cell death mechanism and is demonstrated in this report to be the proteolytic product of Tp90, suggests that the proteolytic process regulating the multiple forms of terminin may be similar to that observed with the ICE proteins. We are in the process of examining whether, after being cleaved from its precursor protein, Tp90, Tp30 itself becomes a protease, and if so, whether its action is also aspartate-residue dependent.

The presence of Tp90 in nonapoptotic cells may imply that the necessary proteolytic action for apoptosis is inhibited or sequestered. We do not know whether this inhibitory or sequestering action is actually contributed by the Tp90 protein itself, with the processing of it to Tp30 negating its potency. Future experiments on functional studies with either purified Tp90 or purified Tp30 will allow us to decipher the answer to this question. Furthermore, the pulse-chase experiments shown in Figure 6 presenting Tp60 as an intermediate between Tp90 and Tp30 do not mean that the senescent state is an intermediate stop before cells' entrance to the apoptotic state. Our reason is that apoptotic cells in general also show the up-regulation of c-*fos* and RB-phosphorylation, which are clearly repressed in senescent cells [Pandey and Wang, 1995]. Therefore, these results suggest that Tp60 is really a biochemical intermediate for producing Tp30 rather than a reflection of a senescent fibroblast state, before apoptosis commences. Moreover, using Tp60 as a marker for cellular senescence can only be meaningful if it is the final product, and no further processing to Tp30 occurs.

So far we have performed all our experiments with isolated protein in in vitro conditions. How in the intracellular environment Tp90 is cleaved to Tp60 upon the establishment of either the senescent or terminally differentiated state, or

how it is further cleaved to Tp30 in cells that are committing suicide, can only be studied once the cDNA clone or purified native protein is available for functional studies via transfection or microinjection. Nevertheless, our results show that the solubility of terminin proteins is changed by proteolysis: i.e., Tp90, after cleaving to Tp60 or Tp30, loses its solubility. Future investigations with cell fractionation studies will indicate the intracellular location of the proteolytic processing of terminin, and whether this location is actually the functional site for the various forms of terminin polypeptides. In all, what is clear is that the lower molecular weight forms of terminin, Tp60 and Tp30, are derived from Tp90 via a sensitive proteolytic process, which is dependent upon two different unique physiological states: the permanent growth-arrest state for the 60 kDa polypeptide, and programmed cell death for the 30 kDa subspecies.

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