We have isolated a novel 75-kDa gelatinase from a chicken macrophage cell line, HD11. Biochemical and immunological characterization of the purified enzyme demonstrated that it is distinct from the chicken 72-kDa gelatinase A (MMP-2). The enzyme is capable of specific gelatin binding and rapid gelatin cleavage. Incubation with an organomercurial compound (p-aminophenylmercuric acetate) induces proteolytic processing and activation of this enzyme, and the resultant gelatinolytic activity is sensitive to both zinc chelators and tissue inhibitors of metalloproteinases. A full-length cDNA for the enzyme has been cloned, and sequence analysis demonstrated that the enzyme possesses the characteristic multidomain structure of an MMP gelatinase including a cysteine switch prodomain, three fibronectin type II repeats, a catalytic zinc binding region, and a hemopexin-like domain. The 75-kDa gelatinase is produced by phorbol ester-treated chicken bone marrow cells, monocytes, and polymorphonuclear leukocytes, cell types that characteristically produce the 92-kDa mammalian gelatinase B (MMP-9). The absence of a 90–110-kDa gelatinase in these cell types indicates that the 75-kDa gelatinase is likely the avian counterpart of gelatinase B. However, the protein is only 59% identical to human gelatinase B, whereas all previously cloned chicken MMP homologues are 75–90% identical to their human counterparts. In addition, the new 75-kDa chicken gelatinase lacks the type V collagen domain that is found in all mammalian gelatinase Bs. Furthermore, the secreted enzyme appears structurally distinct from known gelatinase Bs and the activated enzyme can cleave fibronectin, which is not a substrate for mammalian gelatinase B. Thus the results of this study indicate that a second MMP gelatinase exists in chickens, and although it is MMP-9/gelatinase B-like in its overall domain structure and expression pattern, it appears to be biochemically divergent from mammalian gelatinase B.

The matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteases whose principal substrates are resident proteins of basement membranes and the extracellular matrix (ECM) (1–5). Members of the MMP family have been implicated in physiological and pathological processes that involve proteolytic cleavage or remodeling of the ECM (6–8). Regulation of MMP function involves controlling expression, activation, and activity of the MMPs. Expression of MMPs is differentially regulated by a variety of cytokines in a cell-type-specific manner (2, 3, 5), which represents one level of MMP regulation. Furthermore, most MMPs are secreted as inactive proenzymes, containing a regulatory sequence of amino acids in their amino-terminal domain (9–11). Therefore, these enzymes must undergo proteolytic removal of the propeptide in order to function as active proteases (12–21). Once activated in vivo, MMPs are susceptible to inhibition by the tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have been described (5, 22). These proteins are highly proficient inhibitors of the MMPs and constitute the third level of control over MMP function (3).

At least 23 distinct MMPs have been cloned thus far and can be grouped into at least four subclasses based on substrate specificity and domain structure (1–5, 23). One subclass is composed of the interstitial collagenases, which are able to cleave fibrillar collagens. Members of the stromelysin subclass can digest laminin, fibronectin, and proteoglycans. The membrane-type MMPs compose a subclass whose members have a carboxyl-terminal transmembrane domain and are anchored to the extracellular surface of the plasma membrane (5). MMP-2 and MMP-9 (gelatinase A and B) make up the gelatinase subclass and are the largest members of the family at 72 and 92 kDa, respectively. These two enzymes can hydrolyze denatured collagens (gelatins) with very high efficiency. The remaining members of the MMP family do not fit into the traditional subclasses since either their substrate specificity is novel, as is the case for MMP-20 (enamelysin), or they contain novel domains, such as the carboxyl-terminal interleukin-1 receptor-like sequence of MMP-23 (5).

The members of the MMP family share structural and catalytic properties. The matrix metallopeptinase family includes enzymes that function in a wide variety of physiological processes, including tissue remodeling, angiogenesis, and cell migration. The MMPs are typically classified into five subclasses based on their substrate specificity and domain structure (1–5, 23). One subclass is composed of the interstitial collagenases, which are able to cleave fibrillar collagens. Members of the stromelysin subclass can digest laminin, fibronectin, and proteoglycans. The membrane-type MMPs compose a subclass whose members have a carboxyl-terminal transmembrane domain and are anchored to the extracellular surface of the plasma membrane (5). MMP-2 and MMP-9 (gelatinase A and B) make up the gelatinase subclass and are the largest members of the family at 72 and 92 kDa, respectively. These two enzymes can hydrolyze denatured collagens (gelatins) with very high efficiency. The remaining members of the MMP family do not fit into the traditional subclasses since either their substrate specificity is novel, as is the case for MMP-20 (enamelysin), or they contain novel domains, such as the carboxyl-terminal interleukin-1 receptor-like sequence of MMP-23 (5).
lytic properties and appear to have undergone diversification through exon duplication and shuffling (1–3, 5). There are two structural domains that are found in all MMPs and function to stabilize the catalytic zinc ion in the active site. The catalytic zinc-binding site contains three histidines that secure the zinc ion in the active site (1, 3, 4). The propeptide region contains an unpaired cysteine whose sulfhydryl group occupies the fourth coordination of the catalytic zinc and maintains the latent state of pro-MMPs (9–11). Most MMPs also contain a hemopexin or vitronectin-like domain near their carboxyl termini that plays a role in substrate specificity, inhibitor binding (24–30), and integrin association (31, 32). The gelatinases contain three fibronectin type II repeats that contribute to their high affinity binding to gelatin (33–35). Gelatinase B also contains a domain that is homologous to the o2 chain of type V collagen (36). The role of this domain has yet to be determined.

While studying activation of the 72-kDa MMP-2 in cultured avian cells, a 75–80-kDa gelatinase was detected by zymography in the conditioned medium and cell lysates of a chicken macrophage cell line, HD11. The zymographic activity appeared weak and diffuse yet distinct from the 72-kDa gelatinase. Expression of this 75-kDa gelatinase also was observed in cultures of primary chicken embryo bone marrow cells treated with PMA or LPS. Isolation and characterization of this gelatinase demonstrated that catalytically it is a member of the MMP family. Cloning and sequencing of the cDNA of the 75-kDa gelatinase confirmed that structurally this enzyme is an MMP gelatinase. The enzyme does appear to be MMP-9-like in its overall structure and cellular expression pattern; however, it possesses a number of biochemical and amino acid sequence features that are distinct from all known mammalian gelatinase B enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pathogen-free fertilized eggs and adult chicken blood were purchased from SPAFAS (Preston, CT). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). All other cell culture reagents and Benchmark pretested protein molecular weight standards were purchased from Life Technologies, Inc., SDS, acrylamide, bisacrylamide, and low molecular weight protein standards were purchased from Bio-Rad. Protease-free bovine serum albumin, Histopaque-1077, Histopaque-1119, methylcellulose (25 cps), and Echerichia coli 0111:B4 lipopolysaccharide (LPS) were purchased from Sigma. Gelatin-Sephrose, red agaroose, the ECL detection reagents, and the FPLC system were purchased from Amersham Pharmacia Biotech. Immobilon polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem. Oligonucleotide primers were synthesized by either Life Technologies, Inc., or Operon Technologies (Alameda, CA). Chicken type II collagen, human fibronectin, and murine laminin were purchased from Chemicon. Murine type IV collagen was obtained from Becton Dickinson. The quail fibrosarcoma cell line, QT6, and the human sarcoma cell line, HT 1080, were purchased from the ATCC (Manassas, VA). Freshly isolated human neutrophils were a kind gift from the laboratory of Dr. S. Simon (State University of New York, Stony Brook). The chicken macrophage cell line, HD11 (37), was a kind gift from Dr. M. Hayman (State University of New York, Stony Brook). Recombinant human MMP-9 (gelatinase B) was a kind gift from Dr. R. Friedman (Wayne State University, Detroit, MI) (38). Activated carboxyl-terminally truncated MMP-3 (AC-MMP-3) (39) was a kind gift from Dr. H. Nagase (Imperial College School of Medicine, London, UK).

**Isolation of Chicken Embryo Fibroblasts and Bone Marrow Cells**—Primary cultures of chicken embryo fibroblasts (CEF) and Rous sarcoma virus (RSV)-transformed CEF (RSVCEF) were prepared and cultured as described (40). Primary chicken embryo fibroblasts and chicken embryo bone marrow cells were isolated from femurs dissected out of 18–19-day-old embryos (37, 41). The epiphyses of the femur were cut off; cells were flushed out of the bone with a 3-cm syringe fitted with a 21-gauge needle that was filled with DMEM containing 10% heat-inactivated FBS, 2% heat-inactivated chicken serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin. HD11 cells and chicken embryo bone marrow cells were cultured under similar conditions using the above growth medium supplemented with 2% heat-inactivated chicken serum. In order to generate serum-free conditioned medium, the cultures were washed with DMEM and incubated for 24 h in the growth medium without serum. Cell lysates were prepared as follows. Monolayers were washed twice with PBS and lysed at 4 °C with constant shaking in the tissue culture dishes with 10 mM Tris, pH 8.0, 0.5% Triton X-100 supplemented with 10 mM EDTA and 40 μg/ml aprotinin. The cells were removed with a cell scraper, and the entire lysate was recovered from the culture dish and centrifuged at 4 °C for 10 min at 11,000 × g. The supernatant was harvested, and the pellet was discarded. The concentration of total protein in the extracts was determined using the BCA Protein Assay Kit (Pierce) as described by the manufacturer.

**Gelatin Zymography**—Gelatin substrate zymography was performed as described previously (40). Briefly, samples were diluted in SDS sample buffer in the absence of reducing agents and electrophoresed on 8% SDS-polyacrylamide gels co-polymerized with 0.1% gelatin. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100, incubated 16 h at 4 °C in calcium assay buffer (CAB), 50 mM Tris, 200 mM NaCl, 10 mM CaCl₂, and 0.05% Brij-35, pH 7.5. After incubation, gels were stained with Coomassie Brilliant Blue R-250 (0.2% in 50% methanol, 10% glacial acetic acid) for 2 h and destained in 10% glacial acetic acid, 20% methanol.

**Enzyme and Inhibitor Purification**—The 75-kDa chicken gelatinase was purified from serum-free conditioned medium (SFCM) harvested from HD11 cultures treated with PMA (100 ng/ml) using gelatin-Sephrose chromatography as described previously (40). SFCM (300 ml) was applied at 15–20 mL/h to a 7.5-mL gelatin-Sepharose column that had been equilibrated with CAB. The column was washed with 10 column volumes of CAB, and the 75-kDa gelatinase was eluted with CAB containing 10% Me₂SO. Purity and protein concentration were determined by analyzing samples on silver stained SDS-polyacrylamide gels under reducing conditions (42). To purify further the gelatinase, the enzyme-containing fractions of the Me₂SO eluate were concentrated and applied to an FPLC Superdex 200 HR16 gel filtration column (Amersham Pharmacia Biotech) equilibrated in CAB as described previously (43). The gelatinase fractionated into the 60–80-kDa range, and fractions containing the enzyme were analyzed as above. A partial amino acid sequence of 75-kDa gelatinase was determined as described previously (40).

Chicken gelatinase B was recombantly expressed and purified from the SFCM of transfected myeloma cells. Briefly, the coding region of chicken gelatinase A (44) was cloned into the HindIII/XbaI site of the pEE12 expression vector (45), transfected into the mouse myeloma cell line, NSO, and stable colonies were isolated as described (43, 45). Colonies were screened for expression of gelatinase by gelatin zymography. Positive NSO colonies were picked and expanded in culture. Recombinant gelatinase B was purified from SFCM harvested from the expanded NSO cells as described above for the 75-kDa gelatinase. Chicken TIMP-2 was purified using red agaroose chromatography and FPLC gel filtration as described previously (43).

**Activation of Purified Chicken Gelatinases and Enzyme Activity in Solution**—Gelatinases were activated with 2 mM APMA in CAB for the indicated times at 37 °C. Gelatinase activity in solution was measured using heat denatured 32H-acetylated type I collagen (gelatin) purified from rat tails (40). Activated enzymes were incubated with the labeled gelatin (1 μg/μl, 8000 cpm/μg) in CAB for 3 h followed by trichloroacetic acid precipitation and counting of the resulting supernatant as described previously (40).

**Polyclonal Antibody Preparation**—Purified chicken 75-kDa gelatinase or gelatinase A was electrophoresed on SDS-polyacrylamide gels under non-denaturing conditions. The proteins were excised from the gel; the gel slices were homogenized in water by repeated serial passage through two 21-gauge needles, and the antigen-containing slurry was sent to Biodesign (Renebnumpark, ME) for subcutaneous injection into rabbits. After two successive booster injections, blood was drawn from the immunized animal and serum was isolated and tested for immuno-reactivity to the appropriate antigen by Western blotting.

**Affinity Purification of Monospecific Polyclonal Antibodies**—75-kDa gelatinase and gelatinase A were electrophoresed on 8% SDS-polyacrylamide gels under non-denaturing conditions and transferred to nitrocellulose membranes (46). The membrane was stained with 1.5% Pon
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cesu S, 5% trichloracetic acid, denatured with water, and the portions of the membrane containing either the 75-kDa gelatinase or the 72-kDa gelatinase A proteins were excised using a scalpel. The immobilized proteins were blocked for 1 h in PBS with 1% Tween 20 and then were incubated with agitation in the appropriate rabbit antiserum for 4 h at room temperature. The membranes were washed at 65 °C. The membranes were washed 10 min in PBS, 1% Tween 20 and again two times for 10 min in PBS. Affinity purified antibodies were eluted with 0.1 M glycine, pH 2.7. The antibody preparations were neutralized with 100 mM Tris, pH 9.0, and consists of the nucleotide sequence 5′-GGTTACCTT-NCTNAGCCGNCNCCAGGAGTN-3′ (GSP 1) where N is any nucleotide. The 3′ primer sequence is complementary to the nucleotide sequence 5′-GGCATCGTACTGGC10-NF CREB-3′ (GSP 2, Y = T or C; R = A or G; D = A, G, or T). The underlined bases were added to facilitate cloning and encode a Kpn1 site and a SacI site, respectively. This PCR generated a 1.1-kb fragment that was isolated and cloned into the EcoRV site in the multicloning region of pBluescript KS(-), the gene-specific primer is the complement of the sequence found near the 5′ end of the first fibronectin type II repeat domain in the 1.14-kb fragment (releasate) was collected.

RESULTS

The Detection of a New Gelatinolytic Enzyme Produced by a Chicken Macrophage Cell Line—A number of avian cell types were cultured in the presence or absence of the phorbol ester PMA. The SFCM were collected, and putative gelatinases were detected using gelatin zymography (Fig. 1). SFCM from parallel cultures of untreated and PMA-treated human fibrosarcoma cells (HT 1080) were used as a standard for thezymogen and active forms of MMP-2 (gelatinase A) and the zymogen form of MMP-9 (gelatinase B). RSVCEF and quail fibrosarcoma cells (QT6) produce detectable amounts of 72-kDa gelatinase A in the presence or absence of PMA (lanes 3, 4, 7, and 8). However, these avian cells do not produce any detectable gelatinase B-like enzymes under the culture conditions. The SFCM from the untreated chicken macrophage cell line (HD11) contains little gelatinase A compared with the other avian cells (lane 5 versus lanes 3, 4, 7, and 8). However, there is a broad zone of gelatinolytic activity between 70 and 80 kDa in the lane containing SFCM from the PMA-treated HD11 cells (lane 6). This 70–80-kDa zone of activity appears to be derived from a metalloproteinase as the zymographic activity is completely abolished by the presence of the zinc chelator 1,10-phenanthroline (lane 10).

Substrate Specificity of the 75-kDa Gelatinase—Native rat tail type I collagen was prepared as described (40). Native collagen stored in 0.1 N acetic acid was neutralized with 1 N NaOH when necessary, and 20 μg were incubated in CAB, pH 7.5, with 2 μg APMA in a total volume of 200 μl in the absence or presence of the indicated enzymes for 48 h at 25 °C. Heat-denatured collagen (gelatin) was prepared by heating native collagen (gelatin) at 100 °C for 10 or 60 min at 37 °C in 100 μl of CAB, pH 7.5, in the absence or presence of 75-kDa gelatinase, which had been preincubated with 2 μg APMA for 1 h at 37 °C. Fibronectin and laminin were incubated in 200 μl of CAB, pH 7.5, for 48 h at 37 °C in the absence or presence of enzyme and 2 μg APMA. Following incubation the reaction was stopped with EDTA (50 mM), reduced with β-mercaptoethanol, and electrophoresed in SDS-polyacrylamide gels (8 or 10%), which were then stained with Coomassie Blue.
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through, and the beads were washed. Proteins that were bound to gelatin were eluted with 10% (v/v) Me 2SO. These samples were subjected to analysis using gelatin zymography and silver staining of SDS-polyacrylamide gels (Fig. 2). The zymographically active material in the SFCM is able to bind to gelatin, as little to no lytic zones are apparent in the flow-through or the column wash. Most of the zymographic activity found in the original SFCM is recovered in the Me 2SO elute (E1–E5) indicating that the ability of this enzyme to bind gelatin is specific (Fig. 2A). This gelatin affinity chromatography significantly enriches the purity of the gelatinase as most of the proteins in the conditioned medium are present in the flow-through fraction, whereas only a few protein species are eluted (Fig. 2B). The eluted fractions exhibited a major protein band with a diffuse electrophoretic mobility ranging between 70 and 75 kDa and a less intense band with a mobility of approximately 60 kDa and corresponded to the zones of lysis detected by gelatin zymography (Fig. 2A). The fractions containing the HD11 gelatinase were concentrated and subjected to fractionation using gel filtration as described under “Experimental Procedures” in order to purify further the gelatinase.

The purified gelatinase was incubated with the organomercurial APMA for varying times and analyzed both by silver staining of a reducing SDS-PAGE gel and by gelatin zymography (Fig. 3, A and B). Prior to treatment with APMA, the purified enzyme preparation displays a major 75-kDa band and a minor 60-kDa band under reducing conditions (Fig. 3A, time 0), in contrast to the diffuse staining observed under non-reducing conditions (Fig. 2B, E1–E5). The 75-kDa band diminishes with increasing time of incubation with APMA until it disappears between 3 and 8 h of treatment, whereas the 60-kDa band also disappears slowly as the time of incubation increases. A faint 55-kDa band is visible between 1 and 24 h of treatment. A protein band with a molecular mass of 50-50 kDa appears after 1 h of incubation, becomes the dominant protein band by 8 h of treatment, and persists throughout the remainder of the time course. The zymographic analysis, carried out under non-reducing conditions (Fig. 3B), reveals a pattern of lytic zones that closely corresponds to the silver-stained proteins. However, there is a pronounced enhancement of zymographic activity associated with the proteins in the APMA-treated samples. This enhancement is most apparent when the silver-stained 75-kDa protein at time 0 (Fig. 3A) and its corresponding weak zymographic activity (Fig. 3B) are compared with that of the less intensely stained 50-kDa protein and its very intense zymographic activity at 8 and 24 h after APMA treatment.

In order to determine if conversion of the purified 75-kDa gelatinase to the 60-, 55-, and 50-kDa forms represents actual zymogen activation, the enzyme samples were assayed in solution for their ability to hydrolyze [3H]gelatin before and after treatment with APMA (Fig. 3C). The untreated, purified gelatinase is not able to degrade gelatin, consistent with it being a zymogen. However, after incubation with APMA, the gelatinolytic activity increases substantially. Gelatin degrading activity is readily detectable after 1 h of incubation and reaches its maximum between 3 and 8 h. The enzyme preparation continues to be active even after 24 h of incubation with APMA. The appearance of gelatinolytic activity in solution is coincident with the appearance of both the 50-kDa silver-stained protein and the zone of lysis with an apparent mobility of 50 kDa. These results indicate that the isolated HD11 gelatinase exists mainly as a 75-kDa zymogen with a small amount of an undefined 60-kDa form and that APMA treatment yields transient intermediates of 60 and 55 kDa, and a relatively stable 50-kDa highly active product. The specific activity of the active enzyme was calculated to be 5500–6000 mCi of gelatin hydrolyzed per mg of gelatinase, which was comparable to that of chicken gelatinase A assayed in parallel (data not shown). The apparent molecular weights of the zymogen and active forms of the HD11 gelatinase are distinct from those of chicken gelatinase A and mammalian gelatinase B, so in order to distinguish the HD11 gelatinase the enzyme will be referred to as the 75-kDa gelatinase.

The 75-kDa Gelatinase Is Immunologically Distinct from Gelatinase A—To confirm that 75-kDa gelatinase is not a variant of gelatinase A, specific antibodies were raised to both 75-kDa gelatinase and gelatinase A. Purified 75-kDa gelatinase and gelatinase A were analyzed by both protein staining (Fig. 4A) and Western blotting (Fig. 4B and C) before and after APMA treatment. The polyclonal antibody raised against purified 75-kDa gelatinase is capable of recognizing both the zymogen and
converted forms of the enzyme (Fig. 4B, lanes 1 and 2) in a pattern that correspond closely to the proteins stained with Coomassie Blue (Fig. 4A, lanes 1 and 2). The antibody raised against gelatinase A is able to detect the zymogen and APMA-converted products of gelatinase A (Fig. 4C, lanes 3 and 4). However, neither purified zymogen nor APMA-treated gelatinase A at 10-fold excess is detected by the polyclonal antibody directed against 75-kDa gelatinase (Fig. 4B, lanes 3 and 4). The antibody directed against gelatinase A also is unable to detect either purified or APMA-treated 75-kDa gelatinase also at 10-fold excess (Fig. 4C, lanes 1 and 2). This reciprocal lack of immunoreactivity indicates that the 75-kDa gelatinase is not a variant of gelatinase A.

**Inhibition of 75-kDa Gelatinase by Chicken TIMP-2**—To characterize further this enzyme as a true MMP, sensitivity to TIMP was examined. Purified chicken TIMP-2 (chTIMP-2) was examined for its ability to inhibit the enzymatic activity of both 75-kDa gelatinase and gelatinase A (Fig. 5). In this assay, the IC$_{50}$ for chTIMP-2 was determined to be 14 nM for 5 nM gelatinase A (solid boxes) and 20 nM for 5 nM 75-kDa gelatinase (solid circles). Although the IC$_{50}$ for each enzyme was similar, greater than a 50-fold excess of chTIMP-2 was needed to inhibit the 75-kDa gelatinase activity, whereas only a 5–10-fold excess of inhibitor was sufficient to inhibit completely the gelatinolytic activity of gelatinase A. The 75-kDa gelatinase was inhibited by human TIMP-1 as well (data not shown). Thus, 75-kDa gelatinase is inhibited by members of the TIMP family but is not as sensitive to TIMP-2 as gelatinase A.

**The Multiple Forms of Secreted 75-kDa Gelatinase Are Resolved as a Single Protein upon Reduction**—The secreted 75-kDa gelatinase that accumulates in the SFCM of PMA-treated HD11 cultures exhibits a broad, diffuse zone (60–75 kDa) of lytic activity on gelatin substrate gels. However, gelatin zymographic analysis of cell lysates prepared from the same cultures yields a single band with an apparent mobility of 75 kDa (Fig. 6A). Immunostaining of the secreted 75-kDa gelatinase also yields 2–3 diffuse protein bands, whereas immunostaining of the corresponding cell lysate yields a single 75-kDa band (Fig. 6B). Under reducing conditions, the apparent heterogeneity of the secreted form and the discrepancy between secreted and cell-associated 75-kDa gelatinase disappear. The diffuse bands in the SFCM collapse into a single 75-kDa immunoreactive band that co-migrates with the 75-kDa gelatinase immunoreactivity in cell lysates (Fig. 6C). The purified secreted enzyme also displays a diffuse staining pattern under non-reducing conditions (Fig. 2B, E1–E5), which is resolved into a major 75-kDa protein band after disulfide bond reduction (Fig. 3A, time 0). These data indicate that the broad zone of lysis detected by gelatin zymography in samples of SFCM from cultures of HD11 cells is generated by multiple forms of the same immunoreactive protein. The multiple forms would appear to be the result of some heterogeneity in intrachain disulfide bonds.
Both PMA and Endotoxin (LPS) Can Induce 75-kDa Gelatinase Production in HD11 Cells and in Primary Cultures of Chicken Embryo Bone Marrow Cells—The 75-kDa gelatinase originally was detected (Fig. 1) in cultures of an established, virally transformed, bone marrow cell line, HD11, that exhibits some macrophage-like properties (37). It was important to demonstrate that 75-kDa gelatinase can be produced by primary cell cultures and be induced by a physiological stimulus, in order to affirm that 75-kDa gelatinase expression is not exclusive to a highly selected cell line. Chicken embryo bone

**Fig. 4.** 75-kDa gelatinase is immunologically distinct from gelatinase A. Purified 75-kDa gelatinase (75 k Gel) or chicken gelatinase A (Gel A) was analyzed following incubation in the absence (−) or presence (+) of APMA for 6 or 1 h, respectively. For each condition, 1 µg of enzyme was assayed by Coomassie Blue staining of a reducing 8% SDS-PAGE (A). Five ng of 75-kDa gelatinase and 50 ng of chicken gelatinase A were blotted and probed with a polyclonal antibody directed against 75-kDa gelatinase (B). Five ng of chicken gelatinase A and 50 ng of 75-kDa gelatinase were blotted and probed with a polyclonal antibody specific for gelatinase A (C).

**Fig. 5.** Inhibition of the gelatinolytic activity of chicken 75-kDa gelatinase and gelatinase A by chTIMP-2. 75-kDa gelatinase (●) and gelatinase A (■) were activated with 2 mM APMA for 6 or 1 h, respectively. Five nM of each enzyme was incubated with the indicated concentrations of chTIMP-2 for 1 h, followed by the addition of 10 µg of 3H-gelatin. Samples were incubated at 37 °C for 3 h, and insoluble gelatin was precipitated using trichloroacetic acid, and acid-soluble fragments in the supernatant were quantitated by a scintillation counter. Complete inhibition of gelatinolytic activity was defined as acid-soluble cpm released in the absence of enzyme. The acid soluble cpm released in the absence of chTIMP-2 was set to 100%.

**Fig. 6.** The multiple forms of secreted 75-kDa gelatinase are resolved by a reducing agent (2-mercaptoethanol, β-ME). HD11 cells (1 × 10^7 cells/ml) were cultured in serum-free medium in the presence of 100 ng/ml PMA for 20 h. Conditioned medium (CM) was collected and a cell lysate (CL) prepared. Samples were analyzed by gelatin zymography (A, 2 µl) and by Western blotting of an 8% SDS-polyacrylamide gel electrophoresed in the (−) absence (B, 5 µl) or (+) presence (C, 5 µl) of the reducing agent 100 mM β-mercaptoethanol.
Characterization of a 75-kDa Gelatinase B-like Enzyme

The zymogen form of 75-kDa gelatinase was induced by PMA and endotoxin (LPS) in primary cultures derived from chicken embryo bone marrow. HD11 cells, primary bone marrow cells, and CEF were cultured for 20 h in serum-free medium alone or with the addition of either 100 ng/ml PMA or 10 μg/ml endotoxin (LPS). At the end of the incubation, cell lysates were prepared. Samples of cell lysates (5–60 μg) were analyzed on non-reducing SDS-polyacrylamide gels, which were blotted and probed with an antibody directed against 75-kDa gelatinase.

Marrow cells were isolated from 19-day embryonic femurs and were cultured as primary cells in the presence or absence of PMA or endotoxin (LPS). After 20 h in culture, cell lysates were prepared and analyzed by Western blotting for the presence of 75-kDa gelatinase. Cultures of HD11 and chicken embryo fibroblasts were examined in parallel as positive and negative controls, respectively. LPS can induce the production of 75-kDa gelatinase in HD11 cultures (Fig. 7, lane 3), although not to the same extent as PMA (lane 2). Treatment of primary chicken embryo bone marrow cells with either PMA or LPS leads to production of 75-kDa gelatinase (lanes 5 and 6), and PMA also is a more effective inducer of the enzyme than LPS. A similar pattern of 75-kDa gelatinase induction is observed when the conditioned media of these cultures are analyzed (data not shown). These results demonstrate that 75-kDa gelatinase is produced by primary cells from the bone marrow and can be induced by a natural inflammatory agent, LPS. The results also confirm that normal fibroblasts do not express 75-kDa gelatinase.

Amino-terminal Protein Sequencing and Cloning of 75-kDa Gelatinase—The zymogen form of 75-kDa gelatinase was isolated and subjected to Edman degradation and amino acid sequence analysis that yielded a 20-amino acid sequence, LL-SAPSDE_LAENYLLRFGY. This sequence shows limited homology (50%) to amino acid residues 35–64 of human gelatinase B, (36). Although these 20 amino acid residues are distinct from residues found in human gelatinase B, the length of this sequence was inadequate to elucidate whether or not the 75-kDa gelatinase is an avian homologue of gelatinase B. In order to obtain sufficient amino acid sequence information to execute the comparison of 75-kDa gelatinase with gelatinase B, the full-length cDNA of the 75-kDa gelatinase was generated as described under “Experimental Procedures.” The deduced amino acid sequence for the full-length protein (Fig. 8) predicts a molecule with a mass of 76.6 kDa. Based on the position of the 20-amino acid residues obtained by sequencing of the purified gelatinase, the predicted molecular mass for the secreted protein is 73.2 kDa, which closely corresponds to the 75-kDa mass indicated by reducing SDS-PAGE analysis (Figs. 3A and 6C). The close correspondence between the deduced molecular mass of secreted 75-kDa gelatinase and the apparent molecular weight observed in SDS-PAGE indicates that the enzyme secreted by PMA-treated HD11 cells is not heavily glycosylated. The amino acid sequence of the secreted 75-kDa gelatinase shares 59% identity with human gelatinase B (36), its closest mammalian relative. A direct comparison between the complete amino acid sequence of 75-kDa gelatinase and human gelatinase B (Fig. 8) demonstrates that these proteins contain some domains (catalytic region and fibronectin repeats) that share high amino acid identity, yet they possess a number of domains and subunits (propeptide domain, hinge region, and hemopexin domain) that are divergent in the two gelatinases (see “Discussion”).

The Substrate Specificity of 75-kDa Gelatinase—Native and heat-denatured collagens and the resident ECM glycoproteins, fibronectin and laminin, were tested as substrates for 75-kDa gelatinase (Fig. 9). Native type I collagen is completely resistant to digestion by 75-kDa gelatinase over a 48-h incubation at 25°C (Fig. 9A, lane 4). Native type II collagen appears to have limited susceptibility to degradation by both gelatinase B and 75-kDa gelatinase (lanes 7 and 8); however, neither enzyme generates the characteristic ¼- and ¼-length fragments that occur with an interstitial collagenase or gelatinase A (lane 6). Instead, 90–115-kDa fragments of type II collagen are generated by both gelatinase B and 75-kDa gelatinase (lanes 7 and 8) implicating these enzymes in limited digestion of native type II collagen rather than a specific cleavage. The 75-kDa gelatinase appears to be incapable of cleaving or fragmenting type IV collagen under conditions that maintain the native type IV collagen structure (lane 12). Gelatinase A (lane 10) and gelatinase B (lane 11) also are unable to process native type IV collagen after 48 h of incubation at 25°C. In contrast, heat-denatured collagens (gelatins) are easily degraded by the 75-kDa gelatinase (Fig. 9B), as the activated enzyme completely hydrolyzes type I, II, and IV gelatin in 10–60 min.

Surprisingly, fibronectin appears to be a substrate for the 75-kDa gelatinase (Fig. 9C, lane 4). Active 75-kDa gelatinase cleavage of fibronectin yields products of 110, 85, and 40-kDa, whereas MMP-3 (stromelysin-1), one of the few MMPs reported to cleave fibronectin (1, 2, 4), completely degrades the substrate (lane 5). Neither gelatinase A nor gelatinase B are able to cleave fibronectin under identical conditions (lanes 2 and 3). The 75-kDa gelatinase is not capable of cleaving the ECM protein, laminin (lane 7), whereas MMP-3 cleaves laminin into two fragments of 130 and 150 kDa (lane 8). The inability of 75-kDa gelatinase to cleave laminin indicates that the observed fibronectin cleavage is specific and not the result of trace contamination by a stromelysin or any other enzyme capable of cleaving both ECM glycoproteins. Thus, 75-kDa gelatinase prefers to cleave denatured collagens rather than native collagens, a defining characteristic of the known gelatinases. The ability of 75-kDa gelatinase to extensively cleave fibronectin, however, appears to be a novel activity for this gelatinase.

Chicken Leukocytes Produce 75-kDa Gelatinase—It was unclear from the amino acid sequence comparison data (Fig. 8) whether the 75-kDa gelatinase is the true chicken homologue of gelatinase B. However, the induction of the 75-kDa gelatinase by PMA treatment of bone marrow cultures (Fig. 7) does suggest a gelatinase B-like expression pattern. In order to determine whether distinct chicken cells produce any 90–110-kDa gelatinase B-like enzyme, chicken monocytes and the avian equivalent of PMNs or neutrophils (i.e., heterophils) were
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Fig. 8. Comparison of the deduced amino acid sequence of chicken 75-kDa gelatinase with human gelatinase B. The deduced amino acid sequence of 75-kDa gelatinase was aligned to the published sequence of human gelatinase B (36). Amino acid residues are denoted by their one-letter IUPAC code. The numbering of amino acids starts at the first methionine (M) of each protein. The 1st line in each set of three is the deduced amino acid sequence for 75-kDa gelatinase (75k Gel), and the 2nd line is the amino acid sequence of gelatinase B (Gel B). The 2nd line in each group of three lines represents the consensus sequence. Dashes (−) indicate gaps in the sequence alignment. The 20-amino acid residues obtained from sequencing of the 75-kDa gelatinase are highlighted. The cysteine switch regions are boxed; the catalytic zinc binding sequences are within dashed lines; the fibronectin type II repeats are in bold, and the hemopexin domain is underlined.

Freshly isolated and analyzed for the production of gelatinases by gelatin zymography (Fig. 10A). These cell types, along with bone marrow cells, are the known sources of gelatinase B in mammals (36, 49). Chicken monocytes and bone marrow cells were cultured in the absence or presence of 100 ng/ml PMA. Both untreated and PMA-treated chicken monocytes and bone marrow cells produce a 72-kDa gelatinolytic enzyme (lanes 3–6), which co-migrates with thezymogen form of chicken gelatinase A (lane 11) and is immunoreactive with the antibody directed against chicken gelatinase A (data not shown). PMA-treatment of both monocytes and bone marrow cultures induced secretion of a gelatinase that displayed a broad, diffuse zone of zymographic activity, which migrates slower than gelatinase A (lanes 4 and 6). This broad zone of activity co-migrates with 75-kDa gelatinase present in the SFCM of PMA-treated HD11 cells (lane 2). Western blot analysis of the SFCM from both PMA-treated monocytes and PMA-treated bone marrow cells using the antibody directed against purified 75-kDa gelatinase confirmed that 75-kDa gelatinase has characteristics, such as induction by PMA and LPS, and enhanced expression of gelatinase A (lanes 3). As expected, purified 75-kDa gelatinase is active in gelatin zymographs (Fig. 1) and susceptible to inhibition by a zinc chelator (Fig. 1) and TIMP-2 (Fig. 5). Therefore, the biochemical characteristics of 75-kDa gelatinase clearly indicate that the enzyme is a member of the gelatinase subclass of the MMP family. Nucleotide sequence analysis of this enzyme confirmed that 75-kDa gelatinase has the conserved domain structure of an MMP gelatinase as it contains a cysteine switch, a catalytic zinc binding domain, a hemopexin-like region, and three type II fibronectin repeats (Fig. 8). Comparative analysis of the deduced amino acid sequence (Fig. 8) and the immunoreactivity (Fig. 4) established that 75-kDa gelatinase is distinct from the known chicken MMP gelatinase, gelatinase A (MMP-2) (40, 44).

DISCUSSION

From a chicken macrophage cell line, we have detected, isolated, cloned and characterized a member of the MMP family, which we have named 75-kDa gelatinase. The avian enzyme appears to be gelatinase B (MMP-9)-like in some characteristics but distinct and unique in other properties. The 75-kDa gelatinase is active in gelatin zymographs (Fig. 1) and binds specifically to gelatin (Fig. 2). The zymogen can be converted and activated by treatment with organomercurials (Fig. 3) and once activated preferentially cleaves a variety of denatured collagens with a much higher efficiency than native collagens (Fig. 9). The activity of 75-kDa gelatinase also is susceptible to inhibition by a zinc chelator (Fig. 1) and TIMP-2 (Fig. 5). Therefore, the biochemical characteristics of 75-kDa gelatinase clearly indicate that the enzyme is a member of the gelatinase subclass of the MMP family.
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are distinct from mammalian gelatinase B. The apparent electrophoretic mobility of the zymogen form of the chicken enzyme (75 kDa) is quite different from that of gelatinase B (92 kDa). The appearance of multiple forms of secreted 75-kDa gelatinase, which collapse into a single protein upon reduction (Fig. 6), has not been observed or reported for gelatinase B. APMA treatment of 75-kDa gelatinase generates 60-, 55-, and 50-kDa conversion products (Fig. 3), whereas similar exposure of gelatinase B to APMA yields 86-, 82-, and 68-kDa conversion products (17). The close correspondence between the deduced molecular mass of the chicken gelatinase (73.2 kDa) and the apparent electrophoretic mobility of the secreted enzyme indicates that 75-kDa gelatinase contains little or no post-translational modification, whereas gelatinase B is a highly glycosylated protein (36). Phorbol ester-treated human fibroblasts are unable to produce any detectable 75-kDa gelatinase when cultured with or without PMA (Fig. 7) or when transformed by Rous sarcoma virus (Fig. 1). Interestingly, 75-kDa gelatinase is capable of cleaving fibronecin into discrete 110-, 85-, and 40-kDa fragments, whereas gelatinase B does not appear to be able to process or cleave fibronecin even in a limited manner (Fig. 9C). Furthermore, the deduced amino acid sequence of 75-kDa gelatinase demonstrates that the chicken enzyme lacks the type V collagen domain that all known mammalian gelatinase Bs contain (1).

The 75-kDa gelatinase is only 59% identical to human gelatinase B in its overall sequence. This is in sharp contrast to other MMP family members that exhibit much higher levels of amino acid conservation between chicken and human species (Table I). In the case of gelatinase A, the chicken enzyme is 84% identical to human gelatinase A (44). The chicken MT3-MMP is 89% identical to its human counterpart (52), and recently it was determined that chicken MMP-13 shares 76% identity with human MMP-13 (53). More detailed examination of the amino acid sequence identity within the individual structural domains shared by both 75-kDa gelatinase and gelatinase B (Table I, column 4) reveals that the inter-domain amino acid conservation of 75-kDa gelatinase and gelatinase B is much lower than would be expected if these gelatinases were true homologues (Table I, columns 1–3). The propeptide regions of the known chicken homologues of human MMPs are 90–92% identical, whereas 75-kDa gelatinase and gelatinase B share only 49% identity (47:96 residues) in this region. The identity of the catalytic regions shared by human MMPs and their chicken homologues ranges from 83 to 95%, whereas the catalytic region of 75-kDa gelatinase is 71% identical to that of gelatinase B (116:164 residues). This 71% identity between the catalytic domains, although not as high an identity as with other true homologues, argues that the 75-kDa gelatinase is gelatinase B-like in its MMP enzymatic characteristics. The 75-kDa gelatinase possesses a domain that encodes three fibronecin type II repeats and is moderately homologous to that of gelatinase B (72%, 129:180 residues). This is in contrast to the 89% identity

FIG. 9. The substrate specificity of 75-kDa gelatinase. Native collagens (A, 20 μg) types I (lanes 1–4), II (lanes 5–8), or IV (lanes 9–12) were incubated in 200 μl of CAB at 25 °C for 48 h with 2 mM APMA in the absence (−) or presence of 2 μg of either chicken gelatinase A (GelA), human gelatinase A (GelA), or 75-kDa gelatinase (75k Gel) as indicated. Following the incubation, EDTA was added to 50 mM to the samples, which were then boiled in SDS sample buffer containing 100 mM 2-mercaptoethanol for 5 min. The samples were electrophoresed in 10% SDS-polyacrylamide gels, which were subsequently stained with Coomassie Blue. Denatured collagens (B, 20 μg) types I (lanes 1–3), II (lanes 4–6), and IV (lanes 7–9) were incubated in 100 μl CAB at 37 °C for 10 or 60 min in the absence (−) or presence (+) of 200 ng of 75-kDa gelatinase, which was preactivated with 2 mM APMA for 30 min at 37 °C. At the end of the incubation, EDTA was added, and the samples were analyzed as in A. Fibronectin (20 μg, C, lanes 1–5) were incubated in 200 μl of CAB at 37 °C for 48 h in the absence or presence of 2 μg of either activated human ΔC-MMP-3 (Str) (39) or gelatinase A, gelatinase B, or 75-kDa gelatinase and 2 mM APMA. EDTA was added to terminate the reactions and analyzed as above. Following electrophoresis, the gel was stained with Coomassie Blue. 20 μg of laminin was incubated for at 37 °C for 48 h with 200 ng of either activated human ΔC-MMP-3 (Str) or 75-kDa gelatinase and 2 mM APMA (C, lanes 6 and 7). The reactions were terminated with EDTA. The samples were boiled, reduced, and electrophoresed in an 8% SDS-PAGE that was subsequently stained with Coomassie Blue. The electrophoretic positions of the 68-kDa active gelatinase B, 62-kDa active gelatinase A and 50-kDa active 75-kDa gelatinase are indicated by an arrow, a dash, and an arrowhead, respectively. Active ΔC-MMP-3 cannot be resolved by 8% SDS-PAGE.
that is shared by the same region of human
and chicken gelatinase A. The hemopexin domain of 75-kDa
gelatinase is only 52% identical (99:192 residues) to the he-
napexin domain found in human gelatinase B. In contrast, the
hemopexin-like regions of homologous human and chicken
MMPs are 68–87% identical.

The percent identity values revealed by the above inter-
domain comparison of human gelatinase B and chicken 75-kDa
gelatinase are not only quite different from true homologues
but appear more similar to percent identity values obtained
when the domains of non-homologous MMP gelatinases are
compared (Table I, column 5). Chicken gelatinase A is clearly
not the avian homologue of human gelatinase B. However, the
inter-domain and overall sequence identity values between
these two different gelatinases are more comparable to the
values attained for 75-kDa gelatinase versus gelatinase B (Ta-
ble I, compare column 5 with column 4). If 75-kDa gelatinase
were the true homologue of human gelatinase B, one would expect
inter-domain identities of 75–95% as in the compar-
isons of human gelatinase B versus rabbit gelatinase B (Table
I, column 6) or human gelatinase A versus chicken gelatinase A
(Table I, column 3). Instead, inter-domain identities of 49%
(propeptide) and 52% (hemopexin) and overall identities of only
59% (Table I, column 4) offer arguments that 75-kDa gelatin-
ase may not simply be the avian homologue of mammalian

FIG. 10. 75-kDa gelatinase is pro-
duced by chicken leukocytes. The in-
dicated cell types were cultured in the
absence or presence of PMA (100 ng/ml).
The resulting SFCM (CM) or releasates
(R) were collected as indicated along with
cell lysates (CL) from PMA-treated HD11
cells. The samples were analyzed by gel-
atin zymography (A) and Western blot-
ing (B) under non-reducing conditions for
the presence of 75-kDa gelatinase. Sample
volumes for A and B are 1 μl/lane
(lanes 1, 2, 9, and 10), 20 μl/lane (lanes 3
and 4), and 60 μl/lane (lanes 5–8). Lane
11 (A) is 0.5 ng of purified chicken gela-
tinase A. The relative zymographic posi-
tions of the zymogen and active forms of
gelatinase A (Gel A), gelatinase B (Gel B),
and 75-kDa gelatinase (75k Gel) are indi-
cated on the right in A. Avian cells do not
produce a gelatinase that co-migrates
with human gelatinase B.

### Table I

| Domain boundaries were based on those defined by Massova et al. (58). |
|---|
| 1. Human MT3-MMP versus chicken MT3-MMP | 2. Human MMP-13 versus chicken MMP-13 | 3. Human gelatinase A versus chicken gelatinase A | 4. Human gelatinase B versus 75-kDa gelatinase | 5. Human gelatinase B versus chicken gelatinase A | 6. Human gelatinase B versus rabbit gelatinase B |
| Overall identity | 89 | 76 | 84 | 59 | 46 | 83 |
| Propeptide | 92 | NA<sup>a,b</sup> | 90 | 49 | 41 | 78 |
| Catalytic region<sup>c</sup> | 90 | 83 | 95 | 71 | 60 | 94 |
| Fibronectin repeats | NA | NA | 89 | 72 | 64 | 88 |
| Hemopexin | 87 | 68 | 75 | 52 | 33 | 80 |

<sup>a</sup> NA, not applicable.
<sup>b</sup> The complete propeptide sequence for chMMP-13 is not available.
<sup>c</sup> For the gelatinases, the identity of the catalytic domain was calculated exclusive of the fibronectin repeats.
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gelatinase B.

Additional evidence for the distinct nature of this new gelatinase is that 75-kDa gelatinase possesses a large hinge region that does not appear to be closely related to that of gelatinase B. The chicken enzyme is only 25% identical to gelatinase B in this region and has a stretch of highly acidic residues in its hinge that is not found in any gelatinase B (Fig. 8). Furthermore, 75-kDa gelatinase lacks the proline-rich, type V collagen homology region that appears to be a signature domain of mammalian gelatinase B. Nevertheless, even with all of these structural, enzymatic, and sequence differences between chicken 75-kDa gelatinase and mammalian gelatinase B, 75-kDa gelatinase does possess some of the characteristics of the MMP-9 sub-group. 75-kDa gelatinase contains the characteristic long hinge region (56 amino acids) and contains a third cysteine in the hemopexin region. 75-kDa gelatinase also is highly responsive to phorbol ester treatment, is expressed in monocytes and bone marrow cells, and most significantly is the only detectable gelatinase in PMNs. Thus, 75-kDa gelatinase, at this stage of its characterization, likely belongs to the MMP-9 sub-group.

A small number of non-mammalian gelatinases have been cloned from newt (54), frog (Xenopus laevis, GenBankTM accession number AF072455), and fish (Oryzias latipes, GenBankTM accession number AB033755) and have been defined as gelatinase B-MMP-9-like. These enzymes, however, also display some homology (50–60%) to chicken 75-kDa gelatinase. Unfortunately, the detailed biochemical and enzymatic characteristics of these non-mammalian MMP-9s have not yet been determined. Nevertheless, there is a distinct structural feature that these non-mammalian gelatinases and 75-kDa gelatinase share; although all of these proteins exhibit approximately 50% identity with human gelatinase B, none contain the type V collagen homology domain. The biological function of the type V collagen domain has not been identified yet. Deletion of the domain does not affect the enzymatic activity of gelatinase B (33). It may be that the type V collagen domain has a unique function in mammals and is not essential for normal development and physiology in other vertebrates including chickens, newts, frogs, and fish. It is possible, therefore, that MMP-9-like enzymes may consist of two classes of functionally related enzymes: the 75-kDa gelatinase class that lacks the type V collagen domain, and the gelatinase B class that contains the type V collagen region. All the MMP-9-like, non-mammalian enzymes may be related more closely to the chicken 75-kDa gelatinase described in this study rather than to the known mammalian gelatinase B.

To understand better the potential role of 75-kDa gelatinase, we have analyzed expression of this enzyme in several cell and tissue types. Determining the expression pattern of 75-kDa gelatinase class that lacks the type V collagen domain, and the gelatinase B class that contains the type V collagen region. All the MMP-9-like, non-mammalian enzymes may be related more closely to the chicken 75-kDa gelatinase described in this study rather than to the known mammalian gelatinase B.

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