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Articles

Characteristics of a 95-kDa Matrix Metalloproteinase Produced by Mammary Carcinoma Cells†

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ABSTRACT: A M_r 95 000 matrix metalloproteinase (MMP) produced by rat mammary carcinoma cells has been isolated and characterized. The MMP was secreted in a proteolytically inactive form that was free from bound tissue inhibitor of metalloproteinases. The enzyme was highly glycosylated as evident from an apparent drop of M_r from 95 000 to 83 000 after treatment with *N*-glycanase. Rotary shadowing electron micrographs of purified proenzyme preparations revealed a uniform set of ellipsoidal molecules. Treatment of the proenzyme with 1% SDS resulted in generation of catalytic activity and exposed a cryptic unpaired Cys residue. The latent proenzyme may be activated in at least three additional ways: either spontaneously upon storage, by treatment with organomercurials, or by limited proteolysis by trypsin. Each mode of activation yielded a distinct pattern of cleavage of the enzyme. The activated enzyme cleaved gelatin (denatured type I collagen) and native type IV and V collagen at 30–37 °C. Noncollagenous proteins including α1-proteinase inhibitor, casein, and fibrinogen also were cleaved. The rat mammary carcinoma cell line that produces the M_r 95 000 MMP is composed of two distinct (epithelial- and myoepithelial-like) cell types. The enzyme is expressed constitutively by the epithelial cells. This suggests that expression of the M_r 95 000 MMP is regulated differently from that of interstitial collagenase, which is produced by the epithelial cells only in response to specific inductive factor(s) from the myoepithelial-like cells. Monoclonal antibodies raised against the purified latent M_r 95 000 form of the enzyme bind specifically to the M_r 95 000 MMP and have been used to localize the enzyme to the Golgi region and cytoplasmic granules of the epithelial cells.

Matrix metalloproteinases (MMPs)[1] are believed to have major roles in the degradation of connective tissues that occurs in normal physiological processes and in pathological conditions such as wound repair, rheumatoid arthritis, periodontal disease, and tumor invasion and metastasis (Harris et al., 1984). The MMPs constitute a family of structurally and functionally homologous metal-dependent proteinases (Whitham et al., 1986; Birkedal-Hansen et al., 1988; Muller et al., 1988; Collier et al., 1988). Common structural features include an NH_2-terminal propeptide sequence, a catalytic domain containing a zinc-binding motif, a hemopexin-like domain, and some highly conserved oligopeptide sequences. These structural similarities have prompted the use of the term "collagenase family of metalloproteinases" (Muller et al., 1988). Several investigators have described gelatin-cleaving proteinases in the M_r 90 000–95 000 range from neutrophils (Sopata & Dansewicz, 1974; Murphy et al., 1980; Hibbs et

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[1] Abbreviations: MMP, matrix metalloproteinase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; APMA, (4-aminophenyl)mercuric acetate; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; PBS, Dulbecco's phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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al., 1985) and macrophages (Vartio et al., 1982; Hibbs et al., 1987). Recently, Wilhelm et al. (1989) cloned and sequenced a M_r 92000 MMP (MMP-9),[2] which degrades type IV and V collagens, from SV40 transformed human lung fibroblasts. The biological function of the enzyme has not been established, but the enzymatic degradation of type IV collagen has been implicated in invasive and metastatic growth by facilitating the penetration by tumor cells of the basement membrane (Liotta et al., 1983). Moreover, metalloproteinases in the M_r 90000–95000 range, possibly MMP-9, have been identified although not extensively characterized in the culture medium of several cell lines of neoplastic origin (Yamagata et al., 1988; Ballin et al., 1988). We have shown recently that the highly invasive and metastatic rat mammary carcinoma cell line BC1 is capable of secreting high levels of interstitial collagenase (MMP-1) (O'Grady et al., 1981; Lyons et al., 1989a). In this paper, we demonstrate that BC1 cells also express high levels of a type IV and V collagen degrading the M_r 95000 MMP that appears to be regulated in a manner different from interstitial collagenase.

Materials and Methods

Materials. Materials were obtained from the following sources: culture media from Flow, McClean, VA, or Mediatech, Washington, DC; penicillin and amphotericin B from Squibb, Princeton, NJ; gentamycin from Solo Park Labs, Franklin Park, IL; Hepes and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) from Research Organics, Cleveland, OH; SDS–PAGE M_r markers, heparin–Sepharose, and Sepharose 4B from Pharmacia, Uppsala, Sweden; Labtek chamber slides from Miles Laboratories, Naperville, IL; dimethyl sulfoxide from Aldrich, Milwaukee, WI; 2-methoxy-2,4-diphenyl-3-(2*H*)-furanone from Fluka, Ronkonkoma, NY, or Calbiochem, San Diego, CA; Coomassie Brilliant Blue G250 from Kodak, Rochester, NY; electrophoresis reagents from Bio-Rad, Richmond, CA; [^{3}H]acetic anhydride from Amersham, Arlington Heights, IL; Co-Bind 96-well plates from Micromembranes Inc., Newark, NJ; Brij 35 from Technicon Corp., Tarrington, NY; *Clostridium histolyticum* collagenase and α1-proteinase inhibitor from Worthington, Freehold, NJ; *N*-glycanase from Genzyme, Boston, MA; immunological reagents from Southern Biotechnology, Birmingham, AL; all other biochemical reagents from Sigma, St. Louis, MO.

Cell Culture. The rat mammary carcinoma cell line, BC1, and its epithelial clonal derivative, BC1-E2 (Lyons et al., 1989b), were cultured under permanently serum-free conditions as described (Stevenson et al., 1985), except that bovine serum albumin was omitted from the medium. BC1-M3, a myoepithelial clonal derivative of BC1 (Lyons et al., 1989b), was supplemented with 20% medium conditioned by BC1-E2, which had been passed first through a gelatin–Sepharose column to remove the endogenous M_r 95000 MMP. Large-scale cultures of BC1-E2 were grown in Nunc 6000-cm^2 cell factories in basal medium (a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's media) supplemented with 10 mM Hepes, 1 μg/mL transferrin, 5 μg/mL insulin, and antibiotics. Large-scale cultures of BC1 and cocultures of BC1-E2 and BC1-M3 were grown in basal medium supplemented with 30 mM Pipes and 1 μM retinoic acid (Lyons et al., 1989a).

Purification of the M_r 95000 MMP. Conditioned media from large-scale cultures of BC1-E2 or cocultures of BC1-E2 and BC1-M3 were made to 0.04% NaN_3 and pH 7.5 by the addition of 10% NaN_3 and 5 M NaOH and filtered through glass filter paper. Each batch of medium (4–6 L) was applied directly, at 50 mL/h, to a 2.6 × 6 cm column of heparin–Sepharose CL-6B equilibrated in 50 mM Tris-HCl, 75 mM NaCl, 1 mM $CaCl_2$, and 0.02% NaN_3, pH 7.5. Alternatively, the medium was concentrated 10-fold by ultrafiltration on an Amicon RA2000 device fitted with an S1Y10 spiral membrane and applied to the heparin–Sepharose column at 25 mL/h. The run-through from the heparin–Sepharose column was applied at 25 mL/h to a 1.6 × 6 cm column of gelatin–Sepharose 4B equilibrated in 50 mM Hepes–NaOH, 1 mM $CaCl_2$, and 0.02% NaN_3, pH 7.5, and washed with 1 column volume of that buffer. The column was then washed with 10 column volumes of 50 mM Hepes–NaOH, 5 mM $CaCl_2$, 200 mM NaCl, 1 μM ZnC12, and 0.02% NaN_3, pH 7.5, containing 1 M arginine hydrochloride. M_r 95000 MMP was eluted subsequently with 50 mM Hepes–NaOH, 5 mM $CaCl_2$, 200 mM NaCl, 1 μM $ZnCl_2$, and 0.02% NaN_3, pH 7.5, containing 7.5% v/v dimethyl sulfoxide. Concentrations of the purified enzyme were determined by assuming an extinction coefficient of $A_{280nm}^{1mg/mL;1cm} = 1.29$.

Electrophoresis. SDS–PAGE was performed according to Laemmli (1970). Prior to electrophoresis, samples were added to a sample buffer, which contained 16 mM EDTA, in order to ensure a rapid inactivation of proteolysis. Gels were fixed in 12% trichloroacetic acid, stained in 0.2% Coomassie Brilliant Blue G250 in 30% methanol and 7.5% acetic acid, and destained in water. Subsequently, some gels were destained totally in 50% methanol and 12% acetic acid and then silver stained according to Merril et al. (1980). Standard proteins for M_r determination were reduced with 5% 2-mercaptoethanol, and M_rs of samples were determined by fitting their mobilities to a linear regression plot of the mobilities of the standards run in a 5–15% gradient polyacrylamide gel. Standard proteins were myosin (M_r 200000), β-galactosidase (M_r 116000), phosphorylase B (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20000), and lysozyme (M_r 14000).

Zymography. For zymography, the method of Heussen and Dowdle (1980) was used, except that in some experiments the substrate was gelatin to which a fluorescent label had been introduced with 2-methoxy-2,4-diphenyl-3(2*H*)-furanone by the method of O'Grady et al. (1984). This modification has considerable advantages over the original method for both direct (Heussen & Dowdle, 1980) and reverse (Herron et al., 1986) zymography in that lysis can be monitored continuously by visual and photographic inspection under long-wave ultraviolet light and that the intrinsic capacity of proteins in the samples to bind Coomassie Brilliant Blue does not interfere with the pattern of lysis. Both methods produced the same proteolytic bands for any given sample. Zymography was performed either in 0.75-mm 10% polyacrylamide slab gels containing 2 mg/mL gelatin or a 1.5-mm 5–15% gradient gels containing 1 mg/mL gelatin. Standard proteins for M_r determination were reduced with 5% 2-mercaptoethanol. The gels were photographed under long-wave ultraviolet illumination. In some cases, the gel was washed subsequently with water at 22 °C for 20 min and then stained in 0.2% Coomassie Brilliant Blue R250 in 30% methanol and 7.5% acetic acid and destained in 30% methanol and 7.5% acetic acid. Samples for zymography were preincubated for 1 h at 22 °C with 1–2% SDS in electrophoretic sample buffer.

Proteolytic Assays. The ability of M_r 95000 MMP to cleave a variety of protein substrates was asssessed by incu-

[2] MMPs are referred to by the numerical nomenclature system proposed by Okada et al. (1986) and extended at the Matrix Metalloproteinase Conference held at San Destin, FL, in 1989.

bating the purified proteinase with each protein substrate in a buffer containing 0.5 mM APMA, 50 mM Tris-HCl, 5 mM $CaCl_2$, 200 mM NaCl, and 15 mM NaN_3, pH 7.5. Glycerol (final concentration 10% v/v) was included in collagen-containing samples to prevent fibril formation. Commercially prepared α_1-proteinase inhibitor was purified further by a single passage over Cibacron blue agarose to remove contaminating serum albumin. Cleavage rate estimates were obtained by determining the rates of disappearance of the substrates (for collagens α- and β-chain-sized components) by scanning the Coomassie Blue stained gels with a densitometer. Gelatinolytic activity was determined by a solid-phase assay, to be described in detail elsewhere (Lyons and Birkedal-Hansen, unpublished). Briefly, rat tail tendon collagen, radioactively labeled with [^{3}H]acetic anhydride (Birkedal-Hansen, 1987) (~8000 cpm/μg), was heat denatured (56 °C for 15 min) and covalently coupled to Co-Bind 96-well microtitration plates (0.5 μg per wall). Coupling was in 0.04 M sodium phosphate buffer, pH 7.4, for 16 h at room temperature. Unreacted sites were blocked by subsequent incubation with 1 M ethanolamine, pH 8.0 (1 h, 22 °C). Prior to use in assays, the plates were washed first with 1% SDS and then with assay buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, 15 mM NaN_3, pH 7.5) containing 0.05% Brij 35 to remove noncovalently bound gelatin and SDS. Samples were prepared in assay buffer containing 0.05% Brij 35 and 0.5 mM APMA and were preactivated by incubation at 37 °C for 1 h. Assays were performed routinely at 35 °C by incubating 100 μL of sample for 0–4 h and then removing the entire volume of fluid for liquid scintillation counting. The assay is sensitive to trypsin, *C. histolyticum* collagenase, and M_r 95000 MMP with detection limits of 5, 45, and 45 pg, respectively. One unit of activity was defined as the enzyme activity that released 1 μg of gelatin/min under these conditions.

Activation by APMA and Trypsin. A range of concentrations of APMA and trypsin were tested for ability to activate the purified M_r 95000 MMP (1 μg/mL; >98% latent). APMA was dissolved in 0.1 M NaOH, neutralized with 2 M Tris-HCl, pH 7.5, and added to the proenzyme from a 10X stock solution. Activation was for 1 h at 37 °C. Activation with trypsin (TPCK treated) was for 10 min at 37 °C and was stopped by the addition of 5 mg/mL soybean trypsin inhibitor. Activated samples were diluted and assayed for gelatinolytic activity (1 h at 35 °C). Control samples containing activating agents but no M_r 95000 MMP had no gelatinolytic activity.

Identification of Free Thiols. The M_r 95000 MMP (90 μg/mL) in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 15 mM NaN_3 was mixed with $^1/_{20}$ volume of 0.4 mM DACM in acetone, essentially as described by Yamamoto et al. (1977), and incubated for 1 h at 22 °C. SDS (1% final concentration) or 1,10-phenanthroline (1 mM final concentration) was added to some samples. The reaction was stopped by adding 2-mercaptoethanol (5% final concentration) and boiling in SDS–PAGE sample buffer. The protein samples were resolved by electrophoresis, and incorporation of the fluorescent compound was visualized by long-wave ultraviolet illumination.

Immunological Studies. Monoclonal antibodies to the M_r 95000 MMP were produced as described previously (Birkedal-Hansen et al., 1988). Briefly, regional lymph node cells obtained from mice immunized with the purified M_r 95000 MMP according to a brief immunization schedule (five injections over 15 days) with relatively high doses of antigen (40–80 μg/injection per mouse) were fused with P3-X63Ag8.653 murine myeloma cells. Hybridomas that were positive in an enzyme-linked immunosorbent assay for immunoreactivity with the M_r 95000 MMP were cloned and recloned by limiting dilution. Isotyping was done by the enzyme-linked immunosorbent assay as described (Birkedal-Hansen et al., 1988) with isotype-specific second antibodies. Antibodies were purified from ascites obtained from pristane-primed mice by sequential precipitation with 2.5% caprylic acid and 45% saturated $(NH_4)_2SO_4$ (McKinney & Parkinson, 1987). Western blots prepared by electrophoretic transfer of crude culture medium protein to nitrocellulose were stained as described (Birkedal-Hansen et al., 1988) by using a two-layer technique with biotinylated monoclonal antibody as the first layer and horseradish peroxidase conjugated streptavidin as the second. Color development was with diaminobenzidine–H_2O_2–$NiCl_2$. For immunofluorescent staining, cells seeded in Lab-Tek chamber slides were fixed for 15 min in 4% formaldehyde in PBS and permeabilized by 1% Triton X-100 in Tris-buffered saline. Staining with the first antibody (50 μg/mL biotinylated Ig in PBS containing 1% bovine serum albumin) was for 90 min at 22 °C. Development with Texas Red conjugated streptavidin (1 μg/mL in PBS containing 1% bovine serum albumin) was for 1 h at 22 °C. The slides were mounted in PBS.

Electron Microscopy. Rotary shadowing of the purified M_r 95000 MMP was performed according to Tyler and Branton (1980).

Results

Expression of Metalloproteinases by Rat Mammary Carcinoma Cells (BC1). The rat mammary carcinoma cell line BC1 contains two clonally stable cellular phenotypes: a flattened, epithelial-like and a rounded, elongated, myoepithelial-like cell type (Lyons et al., 1989b). We have shown previously that expression of collagenase by these cells is dependent on a unique reciprocal and synergistic relationship: clonal growth of the myoepithelial cells is dependent on a soluble growth factor released by the epithelial cells, whereas expression of collagenase, which is a product of the epithelial cells, is dependent on a soluble factor released by the myoepithelial cells (Lyons et al., 1989b). In this study, we have analyzed the expression of the M_r 95000 MMP by the original two-component parental line and by clonal derivatives of its epithelial and myoepithelial components. When cultured in serum-free medium, BC1 cells expressed two major metal-dependent gelatin-cleaving activities at M_r 95000 and 65000, in addition to the M_r 59000 interstitial collagenase (Figure 1). The M_r 65000 activity (Figure 1A) was immunologically distinct from the M_r 95000 MMP and from interstitial collagenase since it was not recognized by mouse monoclonal antibodies GeBC1-1D4 and CoBC1-IID1 against these enzymes (Figure 1C). The M_r 95000 MMP was expressed by clonal progeny of the epithelial component (clone E2) of the parental BC1 line in the absence of the myoepithelial phenotype or any of its soluble secretory products (Figure 1A). In the two-component parental BC1 cultures, the M_r 95000 MMP was expressed only by a fraction (5–15%) of the epithelial cells and never by the myoepithelial cells (Figure 2). It was concluded that the source of the M_r 95000 MMP in cultures of BC1 was the epithelial phenotype and that its expression was constitutive and independent of the epithelial–myoepithelial interactions that regulate expression of collagenase.

Purification. The M_r 95000 MMP was purified from serum-free culture medium obtained from confluent cultures by successive passage over heparin–Sepharose and gelatin–Sepharose. Collagenase and several other contaminant proteins

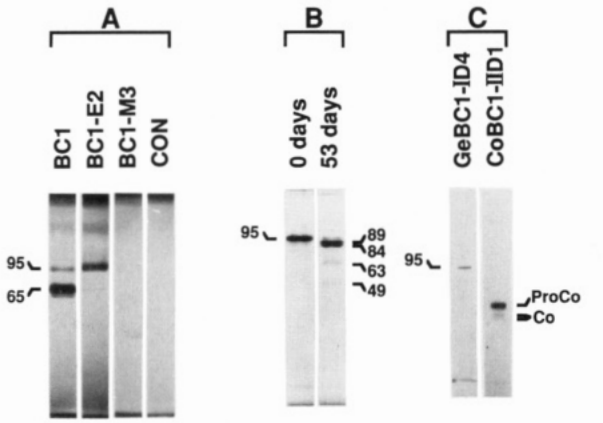

FIGURE 1: Expression of matrix metalloproteinases by different BC1 phenotypes. (A) Fluorescent zymogram of culture medium (3 day) conditioned by the parental cell line, BC1, an epithelial clone, BC1-E2, and a myoepithelial-like clone, BC1-M3. The control medium (CON) was that used for incubation of BC1-M3 cells (see Materials and Methods). The major proteolytic bands at M_r 95000 and 65000 are indicated. The 5–15% polyacrylamide gradient gel contained 1 mg/mL fluorescent gelatin. (B) The M_r 95000 MMP proenzyme (106 μg/mL) purified by heparin- and gelatin–Sepharose chromatography. Upon extended storage (4 °C; 53 days) the proenzyme underwent quantitative autolytic conversion to M_r 89000 and 84000 forms and minor M_r 63000 and 49000 forms. The SDS–PAGE in a 10% gel was stained with Coomassie Brilliant Blue G250. (C) Western blot of BC1 culture medium (3 day) stained with antibodies to the M_r 95000 MMP and interstitial collagenase. The culture medium was concentrated 20-fold by ultrafiltration in a Centricon 10 vessel, subjected to SDS–PAGE under nonreducing conditions in a 7.5% gel, and electroblotted onto nitrocellulose. Strips from the blot were stained for the M_r 95000 MMP with monoclonal antibody GeBC1-ID4 (5 μg/mL) and for interstitial collagenase with monoclonal antibody CoBC1-IID1 (1 μg/mL). The bands corresponding to the M_r 95000 MMP, procollagenase (ProCo), and activated forms of collagenase (Co) that form during concentration are indicated.

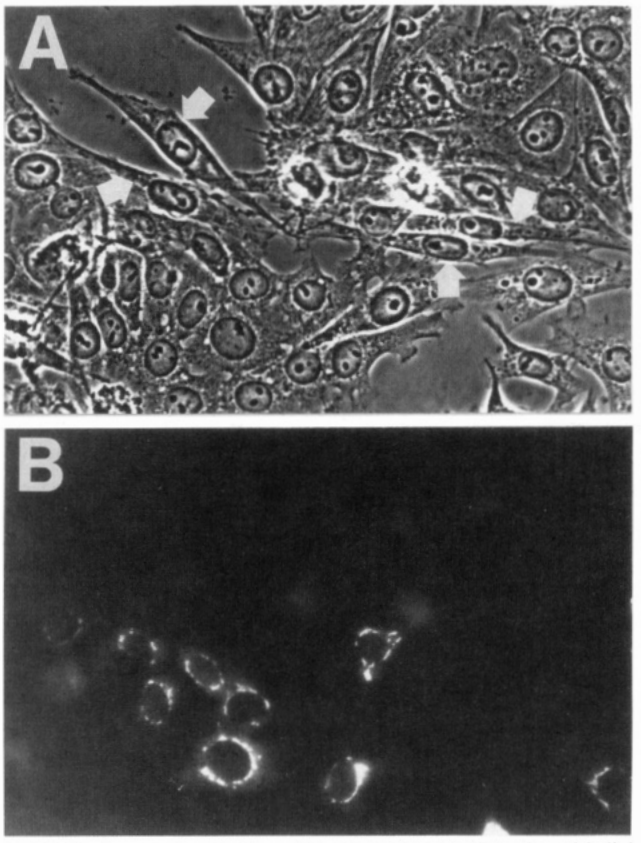

FIGURE 2: Immunolocalization of the M_r 95000 MMP to the epithelial cells of BC1 cultures. Fixed and permeabilized BC1 cells were first reacted with biotinylated antibody (GeBC1-VIIC2) to the M_r 95000 MMP and then with Texas Red conjugated streptavidin. Frame A shows a phase-contrast image of the field. The two epithelial cell colonies (lower left and upper right) are separated by several elongated myoepithelioid cells (arrows). All of the immunofluorescence positive cells (frame B) are epithelial.

were removed first by a single passage over heparin–Sepharose (Lyons et al., 1989a). The M_r 95000 MMP bound to the gelatin–Sepharose column and was eluted with 7.5% dimethyl sulfoxide. The purity at this stage was greater than 99% by SDS–PAGE (Figure 1B). No evidence of a copurifying tissue inhibitor of metalloproteinase-like molecules similar to that observed in human U937 cells (Wilhelm et al., 1989) was observed. The yield was typically about 100 μg/L of culture medium, and the specific activity of the fully activated, purified enzyme was 1430 units/mg as determined by a solid-phase, gelatin-based assay. The proteolytic activity of the activated enzyme was inhibited by the metal-chelating agents EDTA, 1,10-phenanthroline, α,α'-bipyridyl, and 8-hydroxyquinoline, indicating that the enzyme was a metalloproteinase. Rotary shadowing electron micrographs of the purified enzyme revealed a uniform set of ellipsoidal globular molecules without any notable features and measuring approximately 10 × 12 nm (data not shown).

Activation by APMA and Trypsin. The gelatinolytic activity of the M_r 95000 MMP remained latent in the culture medium and during purification, but the activity could be unmasked by exposure to SDS, to APMA, and to trypsin. Slowly progressive "spontaneous" activation occurred during storage both at 22 °C and at 4 °C. The predominant products formed were M_r 89000 and 84000 components, in addition to minor conversion products at M_r 63000 and 49000 (Figure 1B). Exposure to either APMA or trypsin resulted in generation of catalytic activity, but the activation process was slow, required comparatively high concentrations of reagents, and lead to the generation of a number of different activation products. The optimal concentration of trypsin for activation was 200 μg/mL, with both lower and higher concentrations yielding lower activities of the M_r 95000 MMP. A concentration of 1 mM APMA activated the enzyme to an extent similar to that produced by 200 μg/mL trypsin, while lower concentrations yielded lower degrees of activation.

Exposure of the M_r 95000 MMP to APMA resulted in the formation of a major M_r 63000 protein (Figure 3A) that was active in gelatin zymograms. The emergence of the M_r 63000 species coincided with the rise in gelatinolytic activity (Figure 3A,C), suggesting that this form of the enzyme was responsible for gelatin cleavage in the solid-phase assay. The M_r 84000 and 89000 species seen after "spontaneous" activation either were not formed or were converted rapidly to the M_r 63000 species. After longer incubation, an additional M_r 49000 gelatin-cleaving band was observed, as well as inactive autolytic products of M_r 35000 and 29000. Activation of the BC1 M_r 95000 MMP with trypsin resulted in formation of a major M_r 87000 product, which upon further incubation yielded several additional bands in the M_r 44000–65000 range (Figure 3B).

Presence of Glycosides and Free Sulfhydryls. The enzyme contained N-linked glycosides, particularly in the part of the molecule that is removed during activation. *N*-Glycanase treatment of the M_r 95000 latent MMP resulted in a substantial M_r reduction of 12000 (to M_r 83000), whereas similar treatment of the M_r 63000 fully activated enzyme reduced the M_r by less than 2000 (to M_r 61500) (Figure 4). This finding is consistent with recent amino acid sequence information that shows three potential glycosylation sites in the

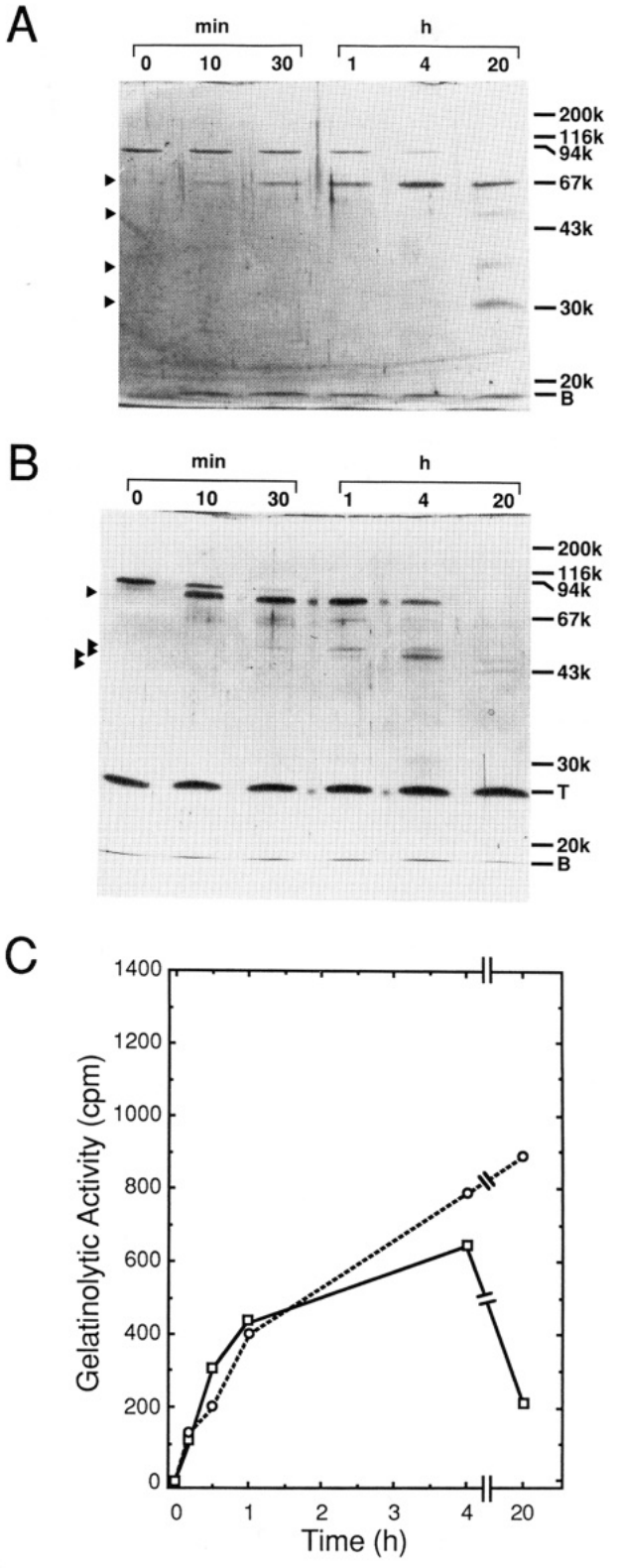

FIGURE 3: M_r conversion of the M_r 95000 MMP following activation. Samples of the purified M_r 95000 MMP (10 μg/mL) were exposed to APMA (0.5 mM) or trypsin (10 μg/mL) for 20 h at 37 °C. During the course of incubation, aliquots were removed and assayed for gelatinolytic activity (1 h at 35 °C) and prepared for SDS–PAGE. Samples for SDS–PAGE (0.2 μg of the M_r 95000 MMP loaded per lane) were electrophoresed under reducing conditions in a 10% polyacrylamide gel, which was stained subsequently with silver nitrate. (A) The SDS–PAGE gel of the APMA-activated M_r 95000 MMP shown was incubated for the time indicated above the lane. Arrowheads indicate the major breakdown products. (B) The SDS–PAGE gel of the trypsin-activated M_r 95000 MMP is shown. (C) The gelatinolytic activity over the course of incubation is shown for APMA activation (dashed line) and for trypsin activation (solid line).

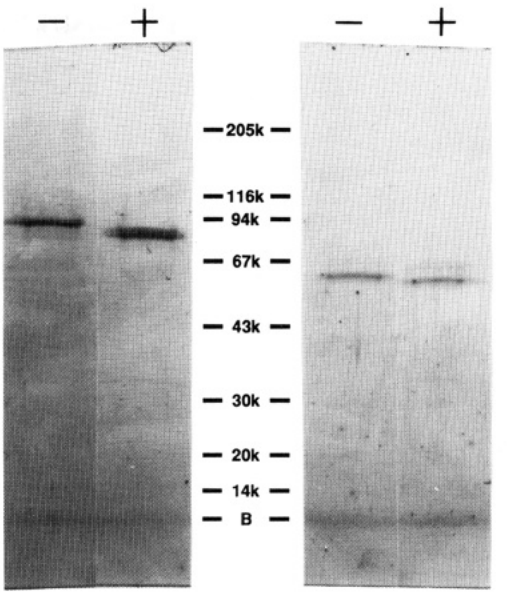

FIGURE 4: *N*-Glycanase treatment of precursor and activated forms of the M_r 95000 MMP. The precursor (left) and 0.5 mM APMA-activated (right) forms of the M_r 95000 MMP were incubated in parallel with or without *N*-glycanase (10 units/mL) according to the manufacturer's instructions. Samples (1 μg per lane) were analyzed by SDS–PAGE under reducing conditions in a 5–15% gradient gel and stained with Coomassie Brilliant Blue G250.

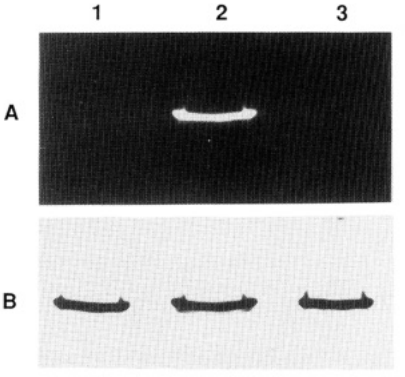

FIGURE 5: Detection of free sulfhydryl with fluorescent maleimide (DACM). Samples of the M_r 95000 MMP proenzyme were incubated with the sulfhydryl-reactive reagent DACM in assay buffer in either the absence (lane 1) or the presence of 1% SDS (lane 2) or 1 mM 1,10-phenanthroline (lane 3) and analyzed by SDS–PAGE. Immediately following electrophoresis, the gel was photographed under long-wave ultraviolet light to locate DACM-labeled protein bands (A). The gel was then stained with Coomassie Brilliant Blue G250 (B) and rephotographed to locate all protein bands.

amino-terminal region of a homologous human M_r 92000 MMP (Wilhelm et al., 1989).

MMPs contain a highly conserved oligopeptide sequence in the propeptide region (Pro-Arg-Cys-Gly-Val-Pro-Asp) that surrounds an unpaired Cys residue involved in the maintenance of catalytic latency (Muller et al., 1988; Sanchez-Lopez et al., 1988; Springman et al., 1990). This Cys residue is also present in the putative human homologue of the rate M_r 95000 MMP (Wilhelm et al., 1989). We have investigated the existence of such unpaired and presumably highly reactive thiol groups by use of the thiol-reactive fluorescent compound DACM (Figure 5). No DACM-reactive sites were exhibited when the purified, latent M_r 95000 MMP was dissolved in normal assay buffer. Similarly, treatment of the enzyme with the metal-chelating agent 1,10-phenanthroline was not sufficient to expose the DACM-reactive site. However, treatment of the latent M_r 95000 MMP with SDS under mild conditions

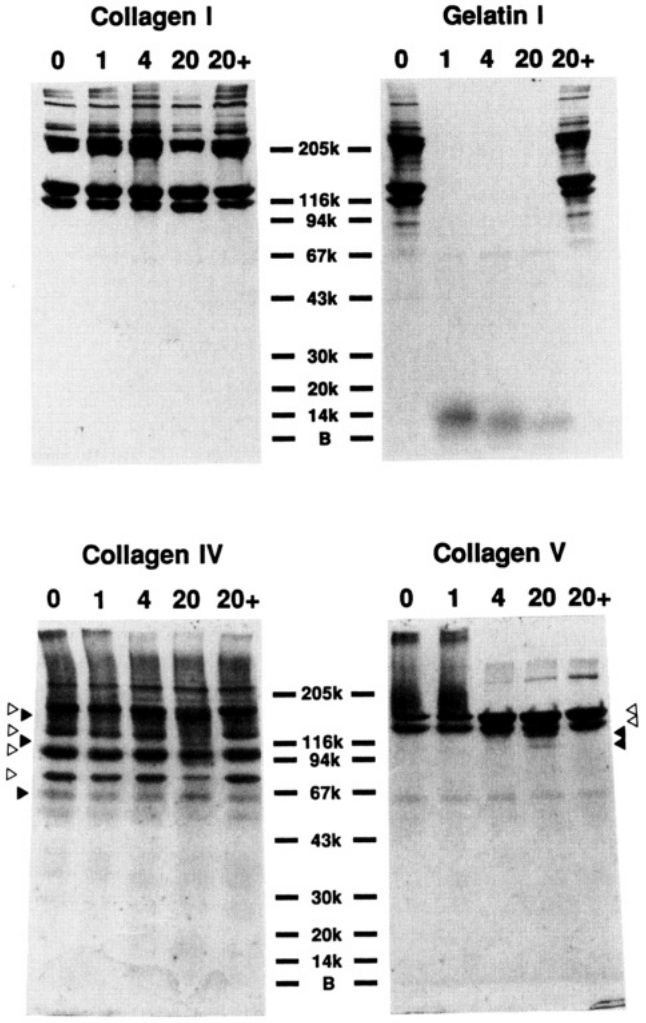

FIGURE 6: Cleavage of type I, IV, and V collagens by the M_r 95 000 MMP at 30 °C. M_r 95 000 MMP (7.8 μg/mL final concentration), activated with 0.5 mM APMA for 1 h at 37 °C, was incubated at 30 °C with collagen types I, IV, or V or gelatin derived from type I collagen (1 mg/mL) for 0, 1, 4, and 20 h, as indicated above the lanes. EDTA (25 mM) was included in 20-h incubations marked 20+. Samples were examined by SDS–PAGE under reducing conditions in 5–15% gradient gels, which were stained subsequently with Coomassie Brilliant Blue G250. Arrowheads indicate the positions of substrate (open) or cleavage products (closed). No proteolysis was observed in incubations of substrate without added enzyme.

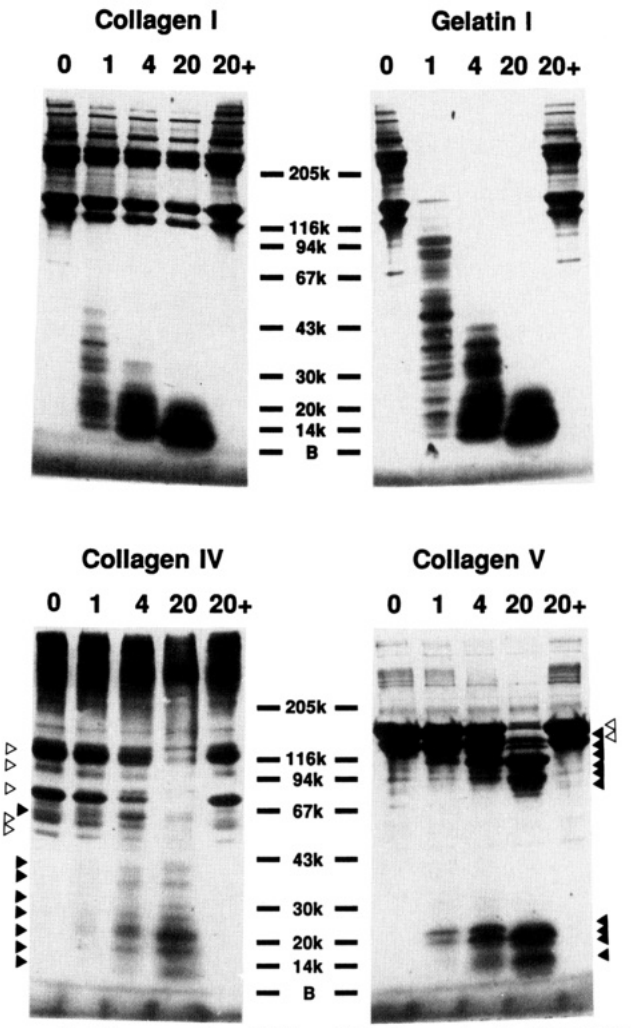

FIGURE 7: Cleavage of type I, IV, and V collagens by the M_r 95 000 MMP at 37 °C. The M_r 95 000 MMP (0.78 μg/mL final concentration), activated with 0.5 mM APMA for 1 h at 37 °C, was incubated at 37 °C with collagen types I, IV, or V or gelatin derived from type I collagen (1 mg/mL) and analyzed as in Figure 6.

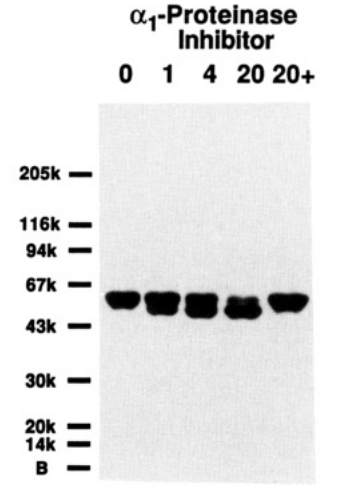

FIGURE 8: Cleavage of α_1-proteinase inhibitor by the M_r 95 000 MMP. The M_r 95 000 MMP (7.8 μg/mL final concentration), activated with 0.5 mM APMA for 1 h at 37 °C, was incubated at 37 °C with α_1-proteinase inhibitor (2 mg/mL) and analyzed as in Figure 6. The inhibitor is almost quantitatively converted to a M_r 53 000 form.

(22 °C, no reduction), which results in activation of the latent enzyme without M_r change (Figure 1A; Birkedal-Hansen, 1987), exposed a cryptic DACM-reactive site, presumably a free Cys thiol group (Figure 5).

Substrate Specificity. We have examined the activity of the M_r 95 000 MMP on a variety of native and denatured protein substrates in the 30–37 °C temperature range and found that gelatin (type I) is cleaved more rapidly than any other substrate (Figures 6–8). The rates of disappearance of α- and β-chain-sized components permitted us to estimate the order of magnitude of turnover numbers under the conditions specified in Figures 6 and 7. Type I gelatin was cleaved at a rate of greater than 40 mol mol^{-1} h^{-1} at 30 °C and greater than 400 mol mol^{-1} h^{-1} at 37 °C. Native triple-helical type I collagen was quite resistant at 30 °C. Although some limited cleavage took place at 37 °C, most of the type I collagen remained resistant at that temperature also. Type IV and V collagens were cleaved at 37 °C at similar rates in the 20 mol mol^{-1} h^{-1} range (Figure 7). At 30 °C the rate of cleavage was at least 10-fold lower with type IV collagen (<2 mol mol^{-1} h^{-1}) and at least 50-fold lower with type V collagen as a substrate (<0.4 mol mol^{-1} h^{-1}) (figure 6). The fragments of type V collagen, like the α1(V) and α2(V) chains of the intact molecule, occurred in pairs, suggesting that cleavage across all three chains at particular sites of the triple helix may have

occurred. Some noncollagenous proteins also were cleaved by the M_r 95000 MMP. α_1-Proteinase inhibitor (M_r 57000) was cleaved at one or more distal sites in a manner reminiscent of that of macrophage elastase (Banda et al., 1988), resulting in a truncated M_r 53000 product (Figure 8). Fibrinogen and casein also were cleaved by the M_r 95000 MMP, whereas bovine and human serum albumins and ovalbumin were not (not shown).

Discussion

Our studies have shown that rat mammary carcinoma cells (BC1) elaborate a M_r 95000 MMP that cleaves type IV and V collagens. The expression of this MMP is regulated differently from that of the interstitial collagenase also produced by BC1 cells. Both enzymes are produced by the epithelial component of the cell line (Figures 1 and 2; Lyons et al., 1989b); however, expression of the M_r 95000 MMP is constitutive, whereas that of collagenase is dependent upon a factor produced by the myoepithelial-like cell component of the parental line.

The M_r 95000 MMP shares several characteristics with other members of the collagenase family of metalloproteinases. These include its ability to be activated by APMA and SDS, inhibition by metal chelators, secretion as a latent enzyme containing an unpaired Cys residue, and autolysis following activation. It is likely that the enzyme is a rat homologue of a recently described human M_r 92000 MMP. The primary structure of the human M_r 92000 metalloproteinase contains elements common to most of this enzyme family, such as the putative Zn-binding site, the Pro-Arg-Cys-Gly-Val-Pro-Asp propeptide sequence including the unapired Cys residue, and a hemopexin-like domain (Wilhelm et al., 1989). The presence of an unpaired Cys residue as part of a highly conserved oligopeptide sequence in this family of enzymes prompted the search for a free Cys in the BC1 M_r 95000 MMP also. The lack of reactivity of the enzyme with DACM until exposed to SDS, a property shared with human fibroblast procollagenase,[3] suggested that the reactive group is shielded in some fashion in the absence of detergent. Recent evidence suggests that this Cys residue may constitute a fourth ligand on the reactive-site Zn^{2+} ion (Springman et al., 1990; Windsor et al., 1990). It is likely, but still remains to be proven, that SDS dissociates this Cys residue from coordination with the metal ion and thereby makes it available for reaction with DACM.

An important difference between the epithelial BC1 M_r 95000 MMP and the gelatin-cleaving proteinases in the same M_r range isolated from pig neutrophils (Murphy et al., 1989) and human fibroblasts (Wilhelm et al., 1989) lies in the events leading to and following activation. The neutrophil enzyme was activated slowly and incompletely by trypsin, whereas the BC1 enzyme was activated fully and somewhat more rapidly by trypsin, albeit at a much higher concentration than is required to activate the collagenase produced by the same cells (Lyons et al., 1989a). Conversely, APMA, which at low concentrations activated pig neutrophil gelatinase within 5 min (Murphy et al., 1989), required more than 4 h to fully activate the epithelial M_r 95000 MMP. The patterns of autolysis that followed activation also were distinct. The BC1 M_r 95000 MMP generated a fairly stable M_r 63000 active form, as well as a novel, proteolytically active M_r 49000 form, with little accumulation of intermediate forms in the M_r 80000–90000 range characteristic of neutrophil gelatinase. However, trypsin-induced activation resulted in relatively rapid formation of a partially active M_r 87000 species that was subject to extensive further proteolysis in a manner different from that seen after APMA activation (compare parts A and B of Figure 3).

The substrate specificity of the BC1 M_r 95000 MMP, including its ability to cleave type IV and V collagens, suggests that it may play a role in the destruction of the extracellular matrix, perhaps acting in concert with the collagenase produced by the same cells. However, the biological relevance of the ability of the M_r 95000 MMP to cleave type IV and V collagens in solution is not clear. It is known that type V collagen is a fibril-forming collagen, and it is the fibrillar form that is most likely to persist in vivo (Adachi & Hayashi, 1985; 1986). The proteolytic susceptibility of its soluble form may or may not have bearing on the susceptibility of fibrillar type V collagen, in analogy with the soluble and fibrillar forms of type III collagen (Birkedal-Hansen et al., 1985). Type IV collagen, however, is not a fibrillar collagen, and recent studies have shown that neutrophil gelatinase, which, like the BC1 M_r 95000 MMP, cleaves type IV collagen in solution, also has the ability to solubilize type IV collagen from intact basement membranes (Vissers & Winterbourn, 1988). Although our rate estimates are based on a limited range of conditions, their orders of magnitude to permit us to draw the conclusion that the rates of cleavage of type IV and V collagens by the M_r 95000 MMP (20 mol mol^{-1}) are much lower than the rate of cleavage of type I collagen by interstitial collagenase (1600 mol mol^{-1} h) (Birkedal-Hansen et al., 1985). This observation raises the question of whether type IV and V collagens actually serve as substrates for the M_r 95000 MMP in vivo.

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References

Adachi, E., & Hayashi, T. (1985) *Collagen Relat. Res. 5*, 225–232.

Adachi, E., & Hayashi, T. (1986) *Connect. Tissue Res. 14*, 257–266.

Ballin, M., Gomez, D. E., Sinha, C. C., & Thorgeirsson, U. P. (1988) *Biochem. Biophys. Res. Commun. 154*, 832–838.

Banda, M. J., Rice, A. G., Griffin, G. L., & Senior, R. M. (1988) *J. Biol. Chem. 263*, 4481–4484.

Birkedal-Hansen, B., Moore, W. G. I., Taylor, R. E., Bhown, A. S., & Birkedal-Hansen, H. (1988) *Biochemistry 27*, 6751–6758.

Birkedal-Hansen, H. (1987) *Methods Enzymol. 144*, 140–171.

Birkedal-Hansen, H., Taylor, R. E., Bhown, A. S., Katz, J., Lin, H.-Y., & Wells, B. R. (1985) *J. Biol. Chem. 260*, 16411–16417.

Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., & Goldberg, G. I. (1988) *J. Biol. Chem. 263*, 6579–6587.

Harris, E. D., Welgus, H. G., & Krane, S. M. (1984) *Collagen Relat. Res. 4*, 493–512.

Herron, G. S., Banda, M. J., Clark, M. J., Gavrilovic, J., & Werb, Z. (1986) *J. Biol. Chem. 261*, 2814–2818.

Heussen, C., & Dowdle, E. B. (1980) *Anal. Biochem. 102*, 196–202.

Hibbs, M. S., Hasty, K. A., Seyer, J. M., Kang, A. H., & Mainardi, C. M. (1985) *J. Biol. Chem. 260*, 2493–2500.

[3] J. G. Lyons and H. Birkedal-Hansen, unpublished observations.

Hibbs, M. S., Hoidal, J. R., & Kang, A. H. (1987) *J. Clin. Invest. 80*, 1644–1650.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Liotta, L. A., Rao, C. N., & Barsky, S. H. (1983) *Lab. Invest. 49*, 636–649.
Lyons, J. G., Nethery, A., O'Grady, R. L., & Harrop, P. J. (1989a) *Matrix 9*, 7–16.
Lyons, J. G., Siew, K., & O'Grady, R. L. (1989b) *Int. J. Cancer 43*, 119–125.
McKinney, M. M., & Parkinson, A. (1987) *J. Immunol. Methods 96*, 271–278.
Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1980) *Science 211*, 1437–1438.
Muller, D., Quantin, B., Gesnel, M.-C., Millom-Collard, R., Abecassis, J., & Breathnach, R. (1988) *Biochem. J. 253*, 187–192.
Murphy, G., Bretz, U., Baggiolini, M., & Reynolds, J. J. (1980) *Biochem. J. 192*, 517–525.
Murphy, G., Ward, R., Hembry, R. Reynolds, J. J., Kuhn, K., & Tryggvason, K. (1989) *Biochem. J. 258*, 463–472.
O'Grady, R. L., Upfold, L. I., & Stephens, R. W. (1981) *Int. J. Cancer 28*, 509–515.
O'Grady, R. L., Nethery, A., & Hunter, N. (1984) *Anal. Biochem. 140*, 490–494.
Okada, Y., Nagase, H., & Harris, E. D., Jr. (1986) *J. Biol. Chem. 261*, 14245–14255.
Sanchez-Lopez, R., Nicholson, R., Gesnel, M.-C., Matrisian, L. M., & Breathnach, R. (1988) *J. Biol. Chem. 263*, 11892–11899.
Sopata, I., & Dansewicz, A. M. (1974) *Biochim. Biophys. Acta 370*, 510–523.
Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., & Van Wart, H. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 364–368.
Stevenson, G. A., Lyons, J. G., Cameron, D. A., & O'Grady, R. L. (1985) *Biosci. Rep. 5*, 1071–1077.
Tyler, J. M., & Branton, D. (1980) *J. Ultrastruct. Res. 71*, 95–102.
Vartio, T., Hovi, T., & Vaheri, A. (1982) *J. Biol. Chem. 257*, 8862–8866.
Vissers, M. C. M., & Winterbourn, C. C. (1988) *Collagen Relat. Res. 8*, 113–122.
Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P., & Docherty, A. J. P. (1986) *Biochem. J. 240*, 913–916.
Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., & Goldberg, G. I. (1989) *J. Biol. Chem. 264*, 17213–17221.
Windsor, L. J., Birkedal-Hansen, H., Birkedal-Hansen, B., & Engler, J. (1990) in *Matrix, Collagen and Related Research, Special Issue* (Birkedal-Hansen, H., Werb, Z., Welgus, H. G., & Van Wart, H., Eds.) Gustav Fischer Verlag, Stuttgart, Germany (in press).
Yamagata, S., Ito, Y., Tanaka, R., & Shimizu, S. (1988) *Biochem. Biophys. Res. Commun. 151*, 158–162.
Yamamoto, K., Sekine, T., & Kanaoka, Y. (1977) *Anal. Biochem. 79*, 83–94.

Kinetic Evidence for Two Nucleotide Binding Sites on the CaATPase of Sarcoplasmic Reticulum†

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ABSTRACT: The CaATPase of sarcoplasmic reticulum was reacted with [γ-^{32}P]ATP to form the covalent phosphoenzyme intermediate. Noncompetitive inhibition by reactive red-120 and chelation of calcium allowed us to monitor single-turnover kinetics of the phosphoenzyme reacting with water or added ADP at 0 °C. When ADP was added and the amount of product, [γ-^{32}P]ATP, formed was measured, we found that added cold ATP did not interfere with the phosphoenzyme reacting with ADP. We conclude that ATP cannot bind where ADP binds, the phosphorylated active site. This implies that when ATP at high concentrations causes an acceleration of phosphoenzyme hydrolysis, it must do so by binding to an allosteric site. Considering the monoexponential nature of product formation we observed, simple one-nucleotide-site models cannot account for the above result.

The ATPase of sarcoplasmic reticulum (SR)[1] membranes is an ionmotive pump which functions in myocytes to transport calcium from the cytosol to the membrane lumen (de Meis, 1981; Inesi 1985; Kirtley et al., 1990). In common with other membrane ATPases, it shows complex dependence of the velocity of ATP hydrolysis on substrate concentration (Inesi et al., 1967; Yamamoto & Tonomura, 1967), so that nonlinear Lineweaver–Burk and Eadie–Hofstee plots are obtained (non-Michaelis–Menten behavior). Among related enzymes with this property are the proton pumps of mitochondria (Schuster et al., 1975) and chloroplasts (Bowman, 1983; Koland & Hammes, 1986), the plasma membrane cation ATPases of the sodium–potassium (Kanazawa et al., 1978;

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EP, phosphorylated enzyme intermediate; FITC, fluorescein isothiocyanate (isomer I).

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