Identification of Some Components of Basal Lamina of Avian Ovarian Follicle

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ABSTRACT Experiments were conducted to identify components of the basal lamina of the ovarian follicle. Pure and intact basal lamina was isolated from preovulatory follicles of the chicken ovary. Some components of the basal lamina could be solubilized with guanidine-HCl (designated Fraction 1) and remaining components with β-mercaptomethanol containing guanidine-HCl (designated Fraction 2). With Western blot analysis, monoclonal and polyclonal antibodies raised against avian, mammalian, and human proteins recognized proteins in Fractions 1 and 2 of solubilized basal lamina. Thus, antibodies raised against extracellular matrix proteins, laminin, fibronectin, entactin or nidogen, tenascin, heparan sulfate proteoglycan, osteonectin, and Type IV collagen reacted positively with basal lamina proteins. Antibodies raised against the growth factors; epidermal growth factor; acidic and basic fibroblast growth factors; platelet-derived growth factor-AA; transforming growth factor-α; transforming growth factor-β1, -β2, -β3, and -β5; and insulin-like growth factor-I and -II cross-reacted with basal lamina proteins. Similarly, antibodies raised against insulin-like growth factor-binding proteins-2, -3, -4, -5, -6, and -7 cross-reacted with basal lamina proteins. In addition, antibodies generated against matrix metalloproteinases-1, -2, -3, -4, -8, -9, and -13 reacted positively with basal lamina proteins. Furthermore, antibodies produced against tissue inhibitors of matrix metalloproteinases-1, -2, -3, and -4 also reacted positively with basal lamina proteins. Moreover, interleukin-3, granulocyte macrophage-colony-stimulating factor, interferon-γ antibodies recognized proteins in basal lamina. These observations are consistent with the view that the basal lamina of avian ovarian follicle is a store or source of biologically active molecules, namely growth factors, growth factor-binding proteins, cytokines, matrix metalloproteinases, and their tissue inhibitors. The growth factors could exert major effects on ovarian cell behavior and function, and the enzymes could participate in tissue remodeling during follicular development.

(*Key words*: basal lamina, basement membrane, extracellular matrix proteins, ovarian follicle, chicken)

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INTRODUCTION

Basement membranes are specialized, extracellular matrix sheets that provide mechanical support and important signals for growth and differentiation to cells they are associated with (Yurchenco and Schittny, 1990). They also compartmentalize tissues, serve as a blood-tissue barrier, prevent transmigration of cells, and influence the exchange of macromolecules between tissue compartments. Basement membranes have been shown to have three layers; lamina lucida (rara) and lamina densa produced by epithelial cells and lamina fibroreticularis produced by connective tissue (Kefalides et al., 1979; Laurie and Leblond, 1985; Inoue and Leblond 1988). The lamina fibroreticularis is usually absent. The lamina lucida (rara) and lamina densa are collectively referred to as the basal lamina (Kefalides et al., 1979; Laurie and Leblond, 1985; Inoue and Leblond 1988). Basement membranes (or basal laminae) contain Type IV collagen and noncollagenous extracellular matrix proteins such as laminin, heparan sulfate proteoglycan (HSPG), entactin or nidogen (for reviews see Timpl, 1989; Yurchenco and Schittny, 1990). Type IV collagen, laminin, HSPG, and fibronectin have been immunolocalized to the lamina densa of basement membranes (Laurie et al., 1982, 1984). Basement membranes contain other components that are not classified as extracellular matrix proteins. For example, growth factors (Folkman et al., 1988; Vigny et al., 1988; Taub et al., 1990;

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Abbreviation Key: EGF = epidermal growth factor; EHS = Engelbreth-Holm-Swarm; FGF = fibroblast growth factor (a or b indicates acidic or basic); GM-CSF = granulocyte macrophage colony stimulating factor; HSPG = heparan sulfate proteoglycan; IGF = insulin-like growth factor; IGFBP = IGF binding protein; IL = interleukin; MMP = matrix metalloproteinase; PAI = plasminogen activator inhibitor; PGDF = platelet-derived growth factor; TGF = transforming growth factor; TIMP = tissue inhibitor of metalloproteinase; t-PA = tissue-type plasminogen activator; u-PA = urokinase-type plasminogen activator.

Vukicevic et al., 1992, Kleinmann et al., 1993; Jones et al., 1993a,b), matrix metalloproteinases (MMP) (Mackay et al., 1993), and their inhibitors (Leco et al., 1994) have been identified in basement membranes. In response to appropriate signals, these biologically active components of the basement membranes could become activated to regulate the functions of tissues locally. Basement membranes or basal laminae contain many more components of unknown identity or nature. The identity of several components of basal laminae are unknown partly due to the unavailability of pure preparations of this extracellular matrix sheet. Because of their potential physiological importance, it would be significant to identify the remaining components of basal lamina. The goal of the present study was to obtain a pure preparation of a complete and intact basal lamina and to begin to identify its components. Pure, complete, and intact basal lamina was obtained from the avian ovarian follicle due to its unique anatomical structure. In the mature avian ovarian follicle, the granulosal layer (membrana granulosa) consists of a single layer of granulosa cells located between the basal lamina and the perivitelline layer (Wyburn et al., 1965; Perry et al., 1978; Bakst, 1979; Callebaut et al., 1991), making possible the isolation of intact basal lamina with a hypotonic solution.

MATERIALS AND METHODS

Chemicals

(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Hepes), collagenase Type F, soybean trypsin inhibitor, BSA (Fraction V), penicillin G, streptomycin, fungizone, Tris, Triton X-100, leupeptin, EDTA-Na_2, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and Tween 20 were purchased from Sigma Chemical Company.[2] Medium 199 (M199) containing Hank's salts was purchased from Gibco- BRL.[3] Acrylamide, bis-acrylamide, SDS, glycine, β-mercaptoethanol, urea, bromophenol blue, ammonium persulfate, N,N,N',N'-tetra-methyl-ethylendiamine (TEMED), Coomassie blue R-250, Silver Stain Plus Kit, and nitrocellulose membrane were obtained from Bio-Rad Laboratories Inc.[4]

Monoclonal antibody, mouse anti-bovine osteonectin, was obtained from the Developmental Studies Hybridoma Bank.[5] Monoclonal antibodies, mouse anti-rat entactin/nidogen, mouse anti-rat HSPG, and mouse anti-human tenascin from the N-terminal region were purchased from Chemicon International, Inc.[6] Polyclonal antibodies, rabbit anti-mouse laminin, rabbit anti-chicken fibronectin, goat anti-human collagen Type IV, rabbit anti-human epidermal growth factor (EGF), rabbit anti-human basic fibroblast growth factor (bFGF), sheep anti-human TGF-α, rabbit anti-human insulin-like growth factor-I (IGF-I), rabbit anti-human IGF-II were obtained from Chemicon International, Inc.[6]

Rabbit anti-human MMP-1; rabbit anti-human MMP-2, -3, -8, -9, and -13; rabbit anti-human tissue inhibitor of metalloproteinase-1 (TIMP-1); rabbit anti-human TIMP-2, -3, -4; and goat anti-human tissue-type plasminogen activator (t-PA); goat anti-human urokinase-type plasminogen activator (u-PA); sheep anti-human Type 1 plasminogen activator inhibitor (PAI-1); rabbit anti-human interleukin-3 (IL-3); and rabbit anti-human interferon-γ were also obtained from Chemicon International, Inc.[6] Rabbit anti-bovine acidic FGF was obtained from Sigma Chemical Company.[2] Additional polyclonal antibodies, goat anti-human insulin-like growth factor binding protein-1 (IGFBP-1); goat anti-human IGFBP-2, -3, -4, -5, -6, and -7; and rabbit anti-human granulocyte macrophage colony-stimulating factor (GM-CSF) were obtained from Research Diagnostics, Inc.[7] Other polyclonal antibodies including the following: goat anti-human platelet-derived growth factor-AA (PDGF-AA), goat anti-human PDGF-BB, rabbit anti-chicken TGF-β1, and goat anti-human TGF-2, -3, and -5 (TGF-β2, -3, and -5) were purchased from R&D Systems.[8] Goat anti-rabbit IgG, rabbit anti-goat IgG, and rabbit anti-sheep IgG were obtained from Pierce Chemical Company.[9] Horse-radish peroxidase conjugated secondary antibodies, rabbit anti-rat IgG, rabbit anti-mouse IgG, and rabbit anti-chicken IgG were obtained from Chemicon International, Inc.[6]

Animals

Single Comb White Leghorn hens in their first year of reproductive activity were caged individually in a windowless, air-conditioned room with a cycle of 14 h light:10 h darkness. They had free access to a layer ration (2,870 kcal ME/kg BW, 16.28% protein, 3.51% calcium) and tap water. The time of egg lay of each bird in the colony was noted to the nearest 30 min (daily). The animals were killed by cervical dislocation approximately 10 to 12 h before the expected time of ovulation of the largest preovulatory follicle. The largest and second largest (F_1 and F_2) preovulatory follicles were removed and placed in ice-cold Hank's salt solution containing NaCl, 140 m*M*; KCl 5, m*M*; $MgCl_2$, 1.1 m*M*; $CaCl_2$, 2.5 m*M*; Hepes, 10 m*M*; and Glucose, 5.6 m*M*, pH 7.4. The thecal and granulosal layers were separated by the method described by Gilbert et al. (1977).

Isolation of Basal Lamina

The granulosal layer was placed in a hypotonic solution containing Tris-HCl, 10 m*M* (pH 7.4); leupeptin, 0.5 mg/L; EDTA-Na_2, 1 m*M*; pepstatin, 0.7 mg/L; and phenylmethylsulfonyl fluoride (PMSF), 0.2 m*M*, in a petri dish. The granulosa cells, sandwiched between the basal lam-

[2]Sigma Chemical Co., St. Louis, MO 63178.
[3]Gibco-BRL, Gaithersburg, MD 20897.
[4]Bio-Rad Laboratories Inc., Hercules, CA 94547.
[5]Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA 52242.
[6]Chemicon International, Inc., Temecula CA 92590.
[7]Research Diagnostics, Inc., Flanders, NJ.
[8]R&D Systems, Minneapolis, MN 55413.
[9]Pierce Chemical Company, Rockford, IL 61105.

ina and perivitelline layer, were lysed, and the basal lamina and the perivitelline layer were separated. The duration of time required for the complete separation of the two layers (basal lamina and perivitelline layer) was dependent upon the hypotonicity of the solution. The separation was much faster (1 to 3 min) in the absence of Tris-HCl than in Tris-HCl-containing solution (4 to 8 min). The basal lamina of avian ovarian follicle was removed intact and complete.

One-Step Solubilization of Basal Lamina

Basal laminae were placed in a microfuge tube, and solubilization buffer containing 6 *M* guanidine-HCl; 50 m*M* Tris-HCl, pH 7.4; and 5 m*M* β-mercaptoethanol was added (100 μL per basal lamina per follicle). After shaking for 60 min at 4 C, the entire basal lamina was dissolved. Similar results were obtained when 8 *M* urea was substituted for guanidine-HCl. Exclusion of β-mercaptoethanol from the solubilization buffer led to incomplete solubilization of the basal lamina (fragments remained). A longer period of time was required for complete basal lamina solubilization when a less concentrated solution of guanidine-HCl or urea was used. The basal lamina could also be completely solubilized after overnight shaking at 4 C in β-mercaptoethanol containing 2 *M* guanidine-HCl or 2 *M* urea solution (referred to as total fraction). When examined under the light microscope, the guanidine-HCl-solubilized or urea-solubilized mixture was clear. The guanidine-HCl or urea-solubilized mixture was placed in a 3-kDa cutoff dialysis membrane and dialyzed against 150 m*M* NaCl and 50 m*M* Tris-HCl, pH 7.4, at 4 C overnight. The mixture became cloudy due to precipitation of proteins. Tissue isolation, solubilization, and dialysis were carried out under sterile conditions.

Two-Step Solubilization of Basal Lamina

Fraction 1. Basal laminae were placed in a microfuge tube, and solubilization buffer containing 6 *M* guanidine-HCl and 50 m*M* Tris-HCl, pH 7.4, was added (100 μL buffer per basal lamina). After shaking at 4 C overnight, intact pieces of basal lamina could be seen. Similar results were obtained when 8 *M* urea was substituted for guanidine-HCl. The mixture was centrifuged at 1,000 to 2,500 × *g* for 10 min. The supernatant, designated Fraction 1, was placed in a 3-kDa cutoff dialysis membrane and was dialyzed overnight at 4 C against 150 m*M* NaCl and 50 m*M* Tris-HCl, pH 7.4. After dialysis, Fraction 1 turned cloudy, because of the precipitation of some proteins. The dialyzed Fraction 1 was aliquoted and stored frozen in the same buffer.

Fraction 2. The basal lamina fragments collected by centrifugation (see preparation of Fraction 1) were solubilized by shaking for 60 min at 4 C with β-mercaptoethanol (5 m*M*) containing 6 *M* guanidine-HCl and 50 m*M* Tris-HCl, pH 7.4, and were designated Fraction 2. Similar results were obtained when 8 *M* urea was substituted for guanidine-HCl. The Fraction 2 solution was placed in a 3-kDa cutoff dialysis tube and was dialyzed overnight at 4 C against 150 m*M* NaCl and 50 m*M* Tris-HCl, pH 7.4. Aliquots of the dialysate were stored in the same buffer. The dialysate of Fraction 2 did not turn cloudy. The exclusion of β-mercaptoethanol from the buffer led to incomplete solubilization of the basal lamina (fragments remained).

Gel Electrophoresis

The SDS-PAGE was carried out with the Mini-PROTEAN II gel system.[4] The stacking gel contained 3% acrylamide. The separating (running) gels contained 12.5, 7.5, or 5% acrylamide. The samples were dissolved in 2× SDS gel-loading buffer (100 m*M* Tris, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; and 5% β-mercaptoethanol) and boiled for 5 min; 10 μL was applied to the sample wells. The electrophoresis was run at 200 V until the bromophenol blue dye front reached the bottom of the gel (about 35 min). Some gels were fixed and stained with Coomassie blue R-250 and destained with 5% glacial acetic acid overnight (destaining solution was changed several times). Other gels were fixed and stained with a Bio-Rad Silver Stain Plus Kit.[4] Another group of gels was fixed in 20% trichloroacetic acid for 30 min, rinsed overnight in deionized water, stained with GelCode Blue[9] for 60 min, and then rinsed in deionized water for 120 min. The gels were dried using the Novex DryEase Mini-Gel Drying System.[10]

Western Blot Analysis

Solubilized basal lamina proteins (Fraction 1 and 2) were resolved in gels containing 7.5 or 5% acrylamide by SDS-PAGE and were electrophoretically transferred (23 V at room temperature) to nitrocellulose membranes[4] using Trans-Blot SD Semi Dry electrophoretic transfer cell.[4] The nitrocellulose membranes were rinsed twice with PBS-Tween (0.9% NaCl, 0.14% $NaH_2PO_4 \cdot H_2O$, and 0.05% Tween 20 vol/vol) for 10 min with constant shaking at room temperature. The nitrocellulose membrane was blocked with PBS containing 5% BSA and 0.1% sodium azide for 2 h at room temperature with constant shaking. The membrane was then incubated with the appropriate primary antibody diluted in PBS containing 5% BSA and 0.1% sodium azide for 1.5 h at room temperature with constant shaking. The primary antibody dilutions were as follows: extracellular matrix proteins: anti-laminin, (1:5,000), anti-osteonectin (full strength), anti-fibronectin (1:45,000), anti-entactin/nidogen (1:1,500), anti-tenascin (1:1,000), anti-HSPG (1:1,500), and anti-collagen Type IV (1:1,000); growth factors: anti-EGF

[10]Novex, San Diego, CA 92121.

(1:1,500), anti-aFGF (1:6,000), anti-bFGF (1:3,000), anti-PDGF-AA (1:3,000), anti-TGF-α (1:2,000), anti-TGF-β1 (1:1,500), anti-TGF-β2 (1:1,000), anti-TGF-β3 (1:1,500), anti-TGF-β5 (1:2,000), anti-IGF-I (1:1,000), and anti-IGF-II (1:3,000); growth factor binding proteins: anti-IGFBP-1 (1:400), anti-IGFBP-2 (1:1,000), anti-IGFBP-3 (1:1,000), anti-IGFBP-4 (1:2,000), anti-IGFBP-5 (1:1,000), anti-IGFBP-6 (1:1,000), and anti-IGFBP-7 (1:2,000); cytokines: anti-IL-3 (1:1,000), anti-interferon-γ, (1:2,000), and anti-GM-CSF (1:3,000); MMP: anti-MMP-1 (1:4,000), anti-MMP-2 (1:4,000), anti-MMP-3 (1:4,000), anti-MMP-8 (1:5,000), anti-MMP-9 (1:5,000), and anti-MMP-13 (1:5,000); TIMP: anti-TIMP-1 (1:4,000), anti-TIMP-2 (1:3,000), anti-TIMP-3 (1:5,000), and anti-TIMP-4 (1:5,000); plasminogen activators: anti-tPA (1:2,000), anti-uPA (1:2,000), and anti-PAI-1 (1:2,000). After incubation, the nitrocellulose membranes were washed three times with PBS-Tween for 10 min each at room temperature and then were incubated with the appropriate horse-radish peroxidase-conjugated secondary antibody diluted in PBS-Tween for 60 min at room temperature with shaking. Dilutions of the secondary antibodies were goat anti-rabbit IgG (1:100,000 to 600,000), rabbit anti-rat IgG (1:250,000), rabbit anti-mouse IgG (1:200,000 to 300,000), rabbit anti-goat IgG (1:250,000), rabbit anti-sheep IgG (1:250,000 to 500,000), and rabbit anti-chicken IgG (1:200,000). After incubation with secondary antibody, the nitrocellulose membranes were washed five times with PBS-Tween for 10 min each. The nitrocellulose membranes were incubated with SuperSignal Ultra substrate solution[9] in a 1:1 mixture of ultra stable peroxide and ultra luminol/enhancer solutions[9] for 5 min and then were placed in a plastic sheet protector and exposed to CL-XPosure Film[9] for 1 to 10 min (some samples were exposed for only a few seconds, whereas a few were exposed overnight). The molecular weights of purified proteins (intact molecules) against which the antibodies used in this study were raised are provided in the legend to each figure.

Zymography of Solubilized Basal Lamina Proteins

Samples of Fractions 1 and 2 of solubilized basal lamina and a protease standard, collagenase Type F, were mixed with zymogram sample buffer and were applied to ready gels impregnated with 10% gelatin or 12% β-casein (BioRad Laboratories[4]). Electrophoresis was at 100 V (constant) for 90 min in a Mini-PROTEAN II gel system.[4] The gels were placed in renaturation buffer[4] (100 ml/gel) and incubated for 30 min at room temperature with constant shaking. They were then placed in development buffer[4] (100 ml/gel) and allowed to incubate at 37 C for 16 h with gentle agitation. The gels were stained with 0.5% Coomasie Blue R250 in 40% methanol/10% acetic acid for 90 min at room temperature followed by destaining in 40% methanol/10% acetic acid for 45 min.

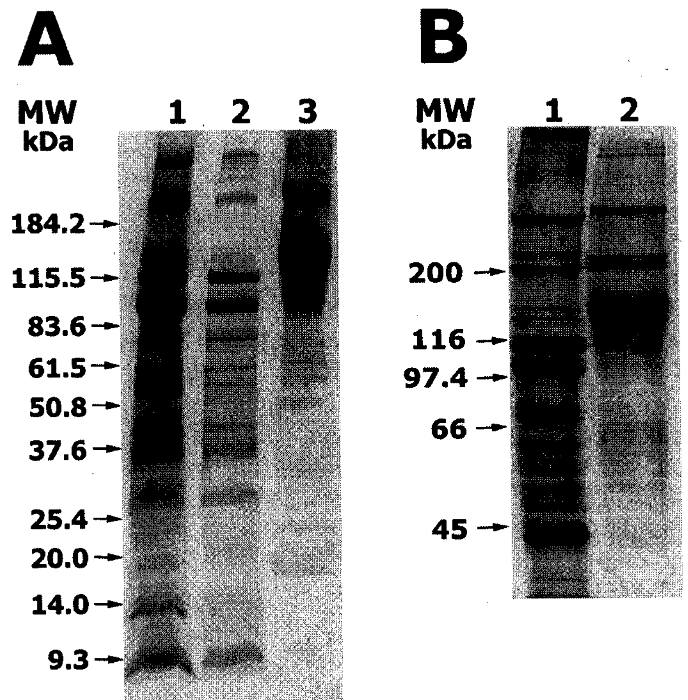

FIGURE 1. Basal lamina proteins were resolved by SDS-PAGE on (A) 4 to 20% acrylamide gradient gels or (B) 7.5% acrylamide gels and were stained with GelCode blue (Pierce Chemical Co., Rockford, IL 61105). In panel (A) Lane 1: 10 μg of proteins solubilized in one step; Lane 2: 5 μg of Fraction 1 proteins; Lane 3: 5 μg of Fraction 2 proteins. In panel (B) Lane 1: 5 μg of Fraction 1 proteins; Lane 2: 5 μg of Fraction 2. Molecular weights are denoted on the left border.

RESULTS

Gel Electrophoretic Separation of Basal Lamina Proteins

Basal lamina proteins obtained by one-step or two-step solubilization were resolved with SDS-PAGE on 4 to 20% or 7.5% acrylamide gels containing GelCode blue (Figure 1A,B). There were major differences between the number and apparent molecular weights of proteins in the two fractions. In Fraction 1, the molecular weight range of the proteins was 10 to <400 kDa (Figure 1A, Lane 2; Figure 1B, Lane 1). The molecular weight range of proteins in the Fraction 2 was 40 to <400 kDa; however, most of them had molecular masses between 40 and 130 kDa (Figure 1A, Lane 3; Figure 1B, Lane 2). Some proteins present in Fraction 2 with molecular weights between 130 and 200 kDa were not readily apparent in Fraction 1. Specifically, a 130-kDa protein in Fraction 2 was absent from Fraction 1 (Figure 1A,B).

Western Blot Analysis of Basal Lamina Proteins

With Western blot analysis, polyclonal antibodies raised against avian, mammalian, and human proteins cross-reacted with several protein bands of solubilized basal lamina in either Fraction 1 or 2. When commercially available, monoclonal antibodies were used.

Extracellular Matrix Proteins. Antibodies raised against several extracellular matrix proteins reacted positively with protein bands in Fraction 1 or 2. The antisera that cross-reacted with Fraction 1 proteins were anti-fibronectin (218- and 173-kDa bands), tenascin (360- and 225-kDa bands), and osteonectin (SPARC or BM-40; bands at 290, 205, and 105 kDa) (Figure 2). Those that reacted with Fraction 2 proteins include Type IV collagen antibody (bands at 370, 225, 212, and 200 kDa), anti-entactin/nidogen (360- and 200-kDa bands), and HSPG antibody (protein bands at 370, 212, and 200 kDa) (Figure 3). Antibody produced against laminin gave positive reactions with a 285-kDa protein in Fraction 1 and 285- and 172-kDa bands in Fraction 2 of solubilized basal lamina (Figure 3).

Growth Factors. Antibodies generated against several growth factors reacted primarily with proteins in Fraction 1 of solubilized basal lamina (Figures 4 to 6), although a few gave positive reactions with Fraction 2 proteins as well (Figure 4). Thus, aFGF antibody reacted with a 160-kDa Fraction 2 band, and bFGF antibody reacted with 115- and 100-kDa protein bands in Fraction 1 and a 208-kDa band in Fraction 2. Epidermal growth factor (EGF) antiserum cross-reacted strongly with a 225-kDa protein and weakly with a 360-kDa Fraction 1 band, whereas TGF-α antibody reacted strongly with Fraction 1 proteins at 360 kDa but weakly at 345 kDa (Figure 4). Insulin-like growth factor-I (IGF-I) antibody reacted with a 300-kDa band in Fraction 1, whereas IGF-II (IGF-II) antibody reacted with a 330-kDa band Fraction 1 protein (Figure 5). Platelet-derived growth factor-AA (PDGF-AA) antibody reacted with a 350-kDa Fraction 1 band (Figure 5). Antibody raised against the PDGF-BB isoform of PDGF did not react with any protein band in Fraction 1 or 2. Trans-

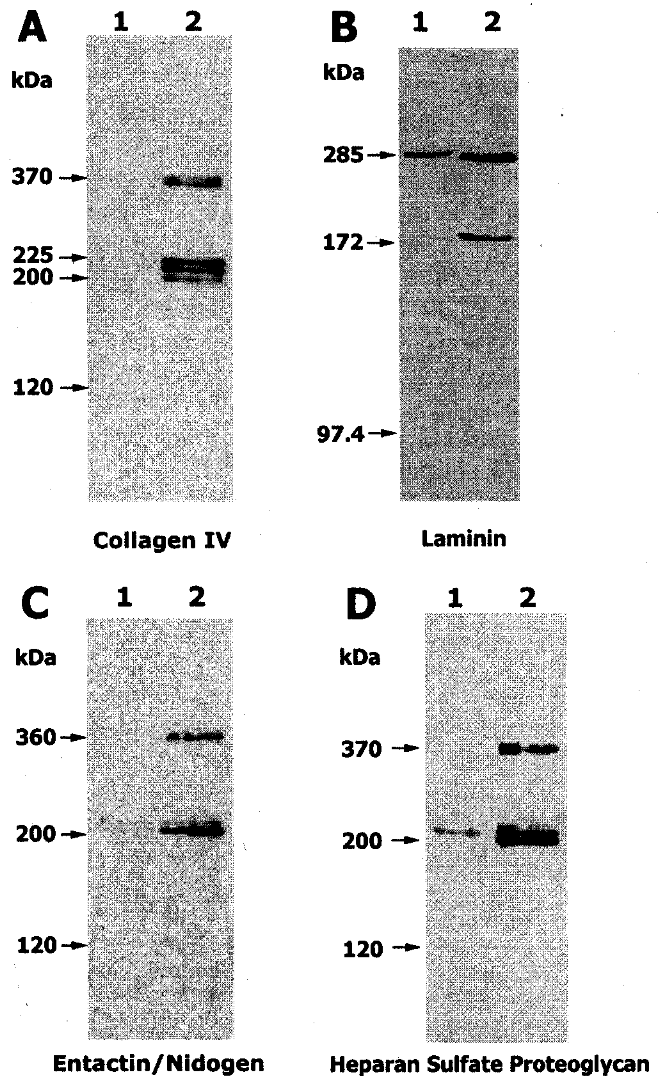

FIGURE 3. Western blot of basal lamina proteins using antibodies raised against extracellular matrix proteins (A) collagen Type IV, (B) laminin, (C) entactin/nidogen, and (D) heparan sulfate proteoglycan (HSPG). Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are collagen Type IV (170 and 185 kDa), laminin (220 and 400 kDa), entactin/nidogen (146 to 150 kDa), and HSPG (400 kDa).

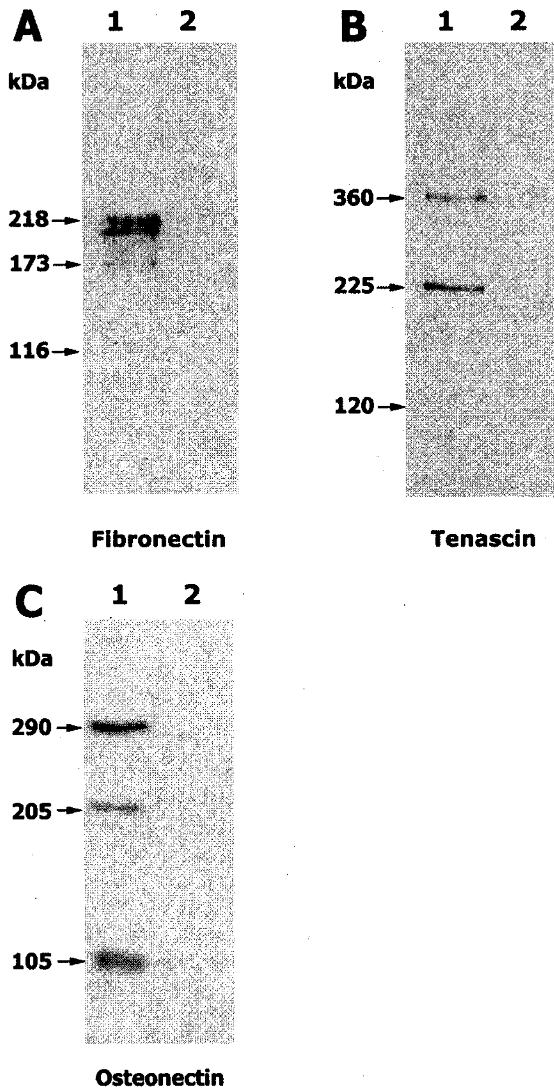

FIGURE 2. Western blot of basal lamina proteins using antibodies raised against extracellular matrix proteins (A) fibronectin, (B) tenascin, and (C) osteonectin. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are fibronectin (235 kDa), tenascin (333 kDa), and osteonectin (30 kDa).

forming growth factor-β, (TGF-β1, -2, -3, and -5) antibody reacted with bands at 280, 225, 205, and 112 kDa in Fraction 1 (Figure 6).

IGFBP. Antibodies generated against IGFBP cross-reacted mainly with proteins in Fraction 1 of solubilized basal lamina (Figure 7). The IGFBP-2 antibody reacted with a 350-kDa band in Fraction 1; IGFBP-3 antibody reacted with 310-, 228-, and 108-kDa bands in Fraction 1, and IGFBP-4 antibody reacted with 310-kDa bands in Fraction 1. The IGFBP-5 antibody reacted with a 310-kDa Fraction 1 band and a 310-kDa band in Fraction 2; IGFBP-6 antibody reacted with a 105-kDa Fraction 1 band. The IGFBP-7 antibody reacted strongly with a 380-kDa band and weakly with a 310-kDa band in Fraction 1 (Figure 7). The IGFBP-1 antibody (even at a dilution of 1:100) did not react with any Fraction 1 protein.

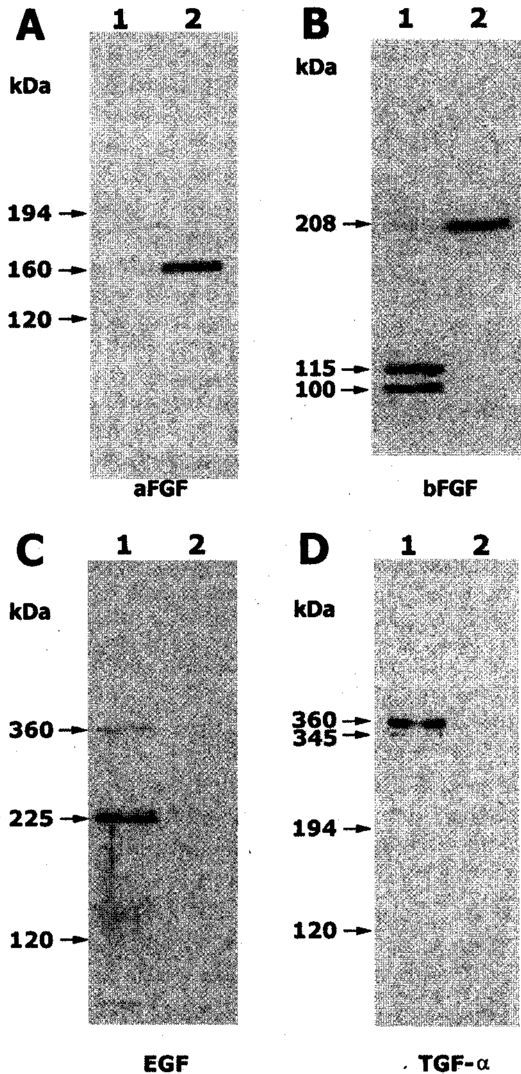

FIGURE 4. Western blot of basal lamina proteins using antibodies raised against growth factors (A) acidic fibroblast growth factor (FGF), (B) basic FGF, (C) epidermal growth factor (EGF), and (D) transforming growth factor (TGF)-α. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are acidic FGF (18 kDa), basic FGF (18 kDa), EGF (6 kDa), TGF-α (6 kDa).

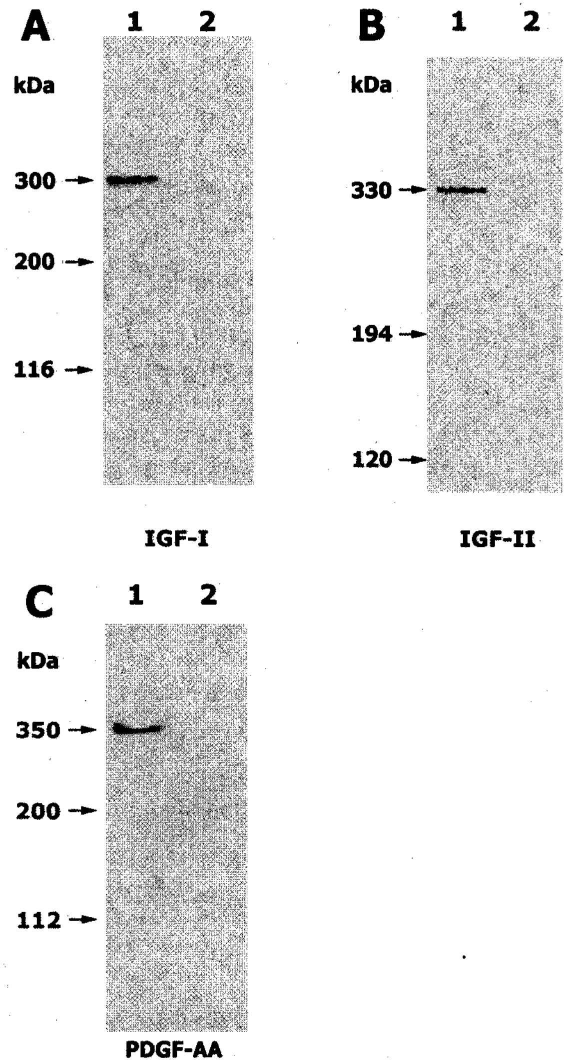

FIGURE 5. Western blot of basal lamina proteins using antibodies raised against growth factors (A) insulin-like growth factor (IGF-I), (B) IGF-II, and (C) platelet-derived growth factor (PDGF-AA). Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are IGF-I (7.5 kDa), IGF-II (7.5 kDa), and PDGF-AA (29 kDa).

Cytokines and Hematopoietic Factors. Antibodies produced against some hematopoietic factors and cytokines gave positive reactions with proteins in Fraction 1 and 2 of solubilized basal lamina (Figure 8). Anti-interferon-γ (IFN-γ) serum reacted with 310- and 210-kDa bands in Fractions 1 and 2, respectively. Anti-interleukin-3 (IL-3) serum reacted with a 350-kDa Fraction 1 band. Antibody generated against granulocyte macrophage-colony stimulating factor (GM-CSF) also reacted with a 350-kDa Fraction 1 band (Figure 8).

MMP. Antisera raised against MMP reacted positively with proteins in Fractions 1 and 2 (Figure 9). The MMP-1 antibody reacted with 328- and 112-kDa Fraction 1 bands and a 320- and 228-kDa Fraction 2 band. The MMP-2 antiserum gave positive reaction with a 112-kDa Fraction 1 band. Anti-MMP-3 serum reacted with 350-, 112-, 92-, and 85-kDa Fraction 1 bands. The MMP-8 antiserum

FIGURE 6. Western blot of basal lamina proteins using antibodies raised against growth factors (A) transforming growth factor (TGF)-β1, (B) TGF-β2, (C) TGF-β3, and (D) TGF-β5. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are TGF-β1 (25 kDa), TGF-β2 (25 kDa), TGF-β3 (25 kDa), and TGF-β5 (25 kDa).

recognized 285-, 105-, and 100-kDa Fraction 1 bands and 190-, 180-, and 120-kDa Fraction 2 bands. The MMP-9 antiserum reacted with a 290- and 280-kDa Fraction 1 band and 290- and 215-kDa bands in Fraction 2. Anti-MMP-13 serum reacted with 310-, 210-, and 105-kDa Fraction 1 bands and a 205-kDa band in Fraction 2 (Figure 9).

TIMP. Antisera produced against TIMP reacted positively with Fraction 1 and 2 proteins (Figure 9). The TIMP-1 antibody reacted with 328- and 112-kDa Fraction 1 bands and a 228-kDa Fraction 2 band. The TIMP-2 antiserum gave positive reactions with 328-, 112-, and 105-kDa Fraction 1 bands and a 228-kDa band in Fraction 2. The TIMP-3 antibody reacted with a 310 kDa Fraction 1 band. The TIMP-4 antiserum reacted positively with a 290-kDa Fraction 1 band and 290-, 215-, and 145-kDa bands in Fraction 2 (Figure 10).

Plasminogen Activators and Their Inhibitors. Antibodies raised against t-PA, u-PA, and Type 1 PAI-1 cross-

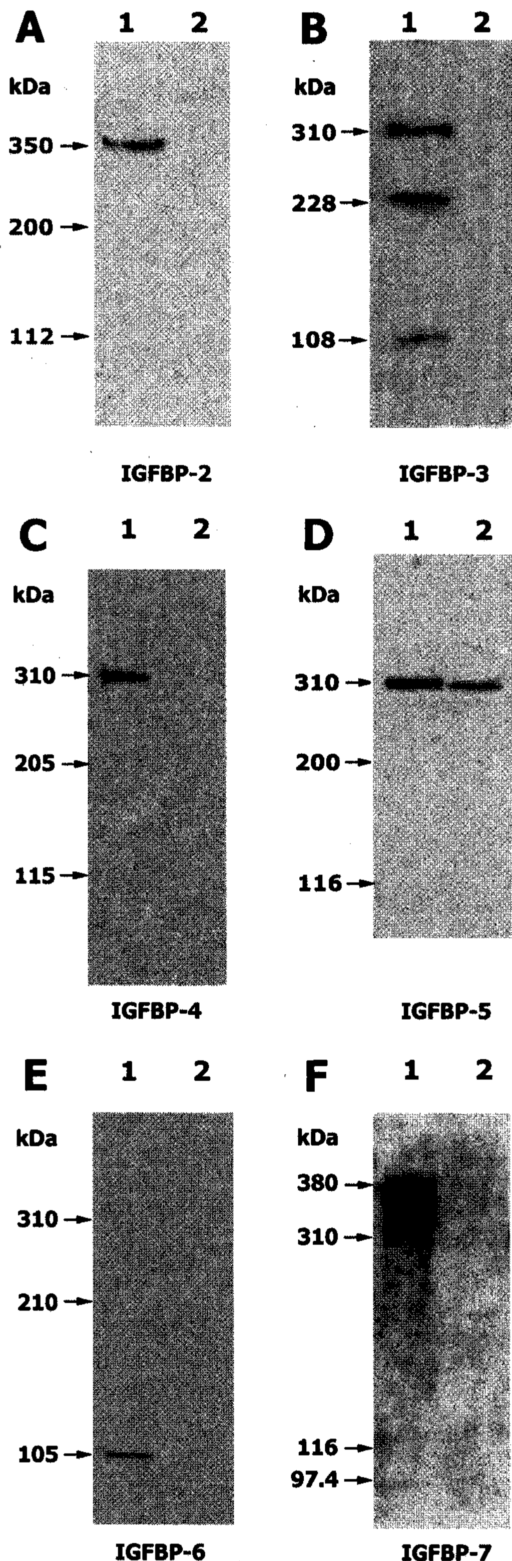

FIGURE 7. Western blot of basal lamina proteins using antibodies raised against insulin-like growth factor-binding proteins (A) IGFBP-2, (B) IGFBP-3, (C) IGFBP-4, (D) IGFBP-5, (E) IGFBP-6, and (F) IGFBP-7. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are IGFBP-2 (31 kDa), IGFBP-3 (46 to 53 kDa), IGFBP-4 (24 kDa), IGFBP-5 (31 kDa), IGFBP-6 (24 to 26 kDa), and IGFBP-7 (31 kDa).

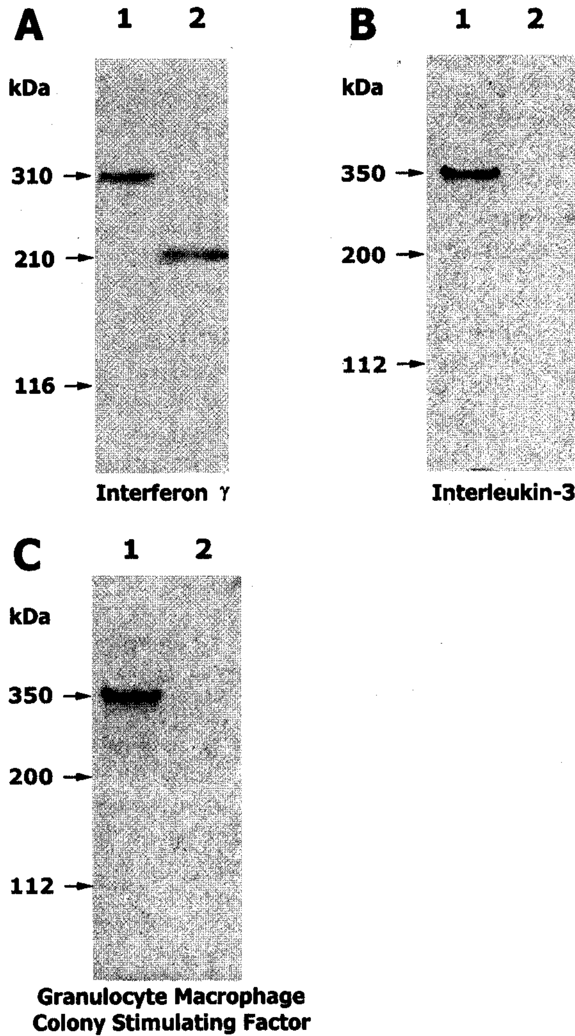

FIGURE 8. Western blot of basal lamina proteins using antibodies raised against cytokines (A) Interferon-γ, (B) Interleukin-3, and (C) granulocyte macrophage colony-stimulating factor (GM-CSF). Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are Interferon-γ (40 to 70 kDa), interleukin-3 (70 kDa), and GM-CSF (14 kDa).

reacted mainly with proteins in Fraction 2 of solubilized basal lamina (Figure 11). Antibody produced against t-PA reacted with 305- and 200-kDa Fraction 2 bands. Anti-uPA serum gave positive reactions with a 305- and 300-kDa Fraction 1 band and 425-, 305-, and 200-kDa bands in Fraction 2. Antibody generated against PAI-1 gave a positive reaction with a 103-kDa Fraction 1 band and 200- and 140-kDa bands in Fraction 2 (Figure 11).

It should be noted that antibodies raised against unrelated proteins reacted with solubilized basal lamina protein bands of identical or similar molecular mass. For example, antibodies of some MMP and TIMP reacted positively with identical bands (compare Figure 9 with Figure 10). Similarly, some MMP and laminin were colocalized to the same band (compare Figure 2 with Figure 9). In addition, t-PA or u-PA and PAI-1 antisera cross-reacted with certain protein bands of similar molecular weight (see Figure 11). Furthermore, the antibodies of some IGFBP and IGF reacted positively with protein bands with similar molecular weight (compare Figure 5 with Figure 7). Moreover, antisera produced against growth factors such as FGF, EGF, and TGF-β cross-reacted

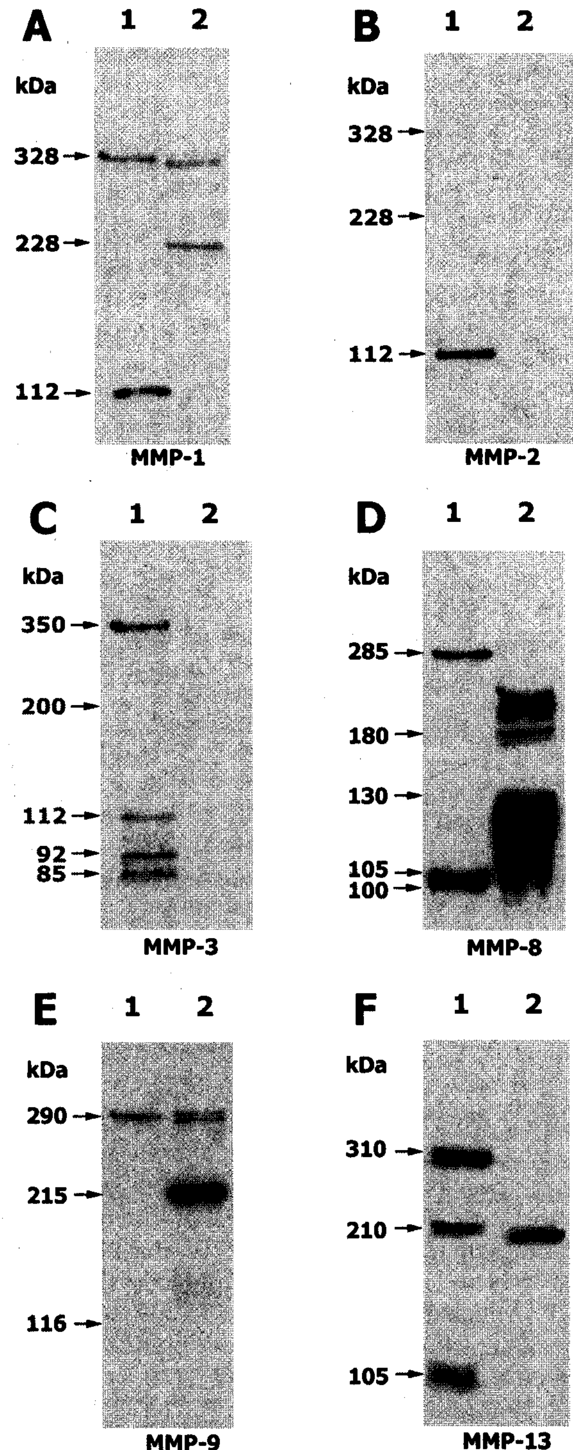

FIGURE 9. Western blot of basal lamina proteins using antibodies raised against matrix metalloproteinases (A) MMP-1, (B) MMP-2, (C) MMP-3, (D) MMP-8, (E) MMP-9, and (F) MMP-13. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are MMP-1 (53 and 55 kDa), MMP-2 (62 to 66 kDa), MMP-3 (57 to 59 kDa), MMP-8 (58 and 64 kDa), MMP-9 (88 and 92 kDa), and MMP-13 (48 and 60 kDa).

with bands that antisera, raised against extracellular matrix proteins, had recognized (compare Figure 3 with Figures 4 and 6). It seemed that in the basal lamina, some of the proteins exist as complexes that were not dissociated under the present experimental conditions.

Zymography. By using 12% β-casein and 10% gelatin as substrates, zymography was performed to determine if basal lamina proteins possess proteolytic activity. A 55-kDa protein of Fraction 1 of solubilized basal lamina gave a clear band in the zymogram (Figure 12), suggesting that it possesses proteolytic activity. The 55-kDa Fraction 1 protein gave positive reactions with casein and gelatin substrates; however, the reaction was stronger with gelatin substrate. Proteins in Fraction 2 did not give any positive reaction with either casein- or gelatin-containing gels (Figure 12).

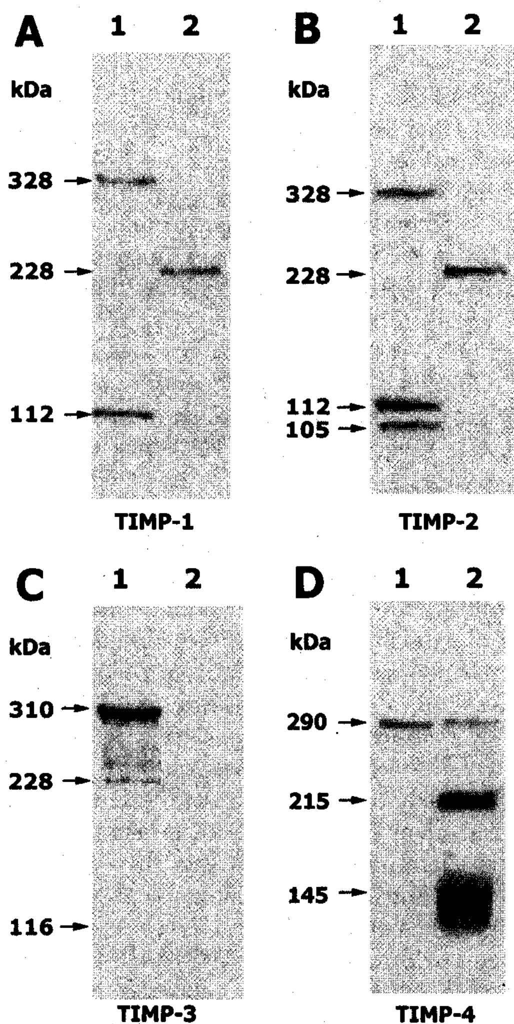

FIGURE 10. Western blot of basal lamina proteins using antibodies raised against tissue inhibitors of metalloproteinases (A) TIMP-1, (B) TIMP-2, (C) TIMP-3, and (D) TIMP-4. Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are TIMP-1 (30 kDa), TIMP-2 (21 kDa), TIMP-3 (24 and 30 kDa), and TIMP-4 (23 and 29 kDa).

DISCUSSION

The present results demonstrate that basal lamina isolated from avian ovarian follicle contains the major components of basement membranes—the extracellular matrix proteins, as well as several accessory molecules such as growth factors and their binding proteins, cytokines, MMP, and their tissue inhibitors. Basal laminae or basement membranes have different proportions of collagen as well as noncollagenous extracellular matrix proteins such as, laminin and HSPG (Timpl, 1989; Yurchenco and Schittny, 1990). Type IV collagen is unique to basement membranes and is a major structural component (Timpl, 1989; Yurchenco and Schittny, 1990). Type IV collagen has been immunolocalized to the basal lamina of rat (Bagavandoss et al., 1983; Bortolussi et al., 1989) and to porcine and bovine (Bortolussi et al., 1989) ovarian follicles. The present results indicate that Type IV collagen is a component of the basal lamina of the avian ovarian follicle. Fibronectin has been immunolocalized to the basal lamina of rat (Bagavandoss et al., 1983) and bovine (Bortolussi et al., 1989; Yoshimura et al., 1991) ovarian follicles. The present results confirm earlier studies in which fibronectin was immunolocalized to the basal lamina of the chicken ovarian follicle (Yoshimura et al., 1985; Callebaut et al., 1988). Laminin has been immunolocalized to the basal lamina of rat (Bagavandoss et al., 1983; Bortolussi et al., 1989) and murine (Wordinger et al., 1983), human (Christiane et al., 1988), and porcine, and bovine (Bortolussi et al., 1989) ovarian follicles. The present data are consistent with the notion that laminin is a component of basal lamina of avian ovarian follicles.

The source and regulation of production of the components of the basal lamina of the avian ovarian follicle is largely unknown. Recent in vitro studies showed that granulosa cells synthesized and deposited large quantities of fibronectin (Novero and Asem, 1993; Asem and Novero, 1994), a component of the basal lamina. Thus, granulosa cells may contribute to the formation of basal lamina.

The results obtained from the present study confirm and extend previous reports that basal laminae or basement membranes immobilize and store several bioactive molecules such as growth factors, cytokines, and enzymes. It was shown that EGF, TGF-β, bFGF, IGF-I, and PDGF are components of basement membranes (Folkman et al., 1988; Vigny et al., 1988; Taub et al., 1990; Vukicevic et al., 1992, Kleinmann et al., 1993). The present results indicate that in addition to the growth factors shown to be components of basement membranes several other growth factors, TGF-α, aFGF, and IGF-II, are localized to basal lamina of the avian ovarian follicle. Earlier studies showed that extracellular matrix contains IGFBP-2 (Hakeda et al., 1996) and IGFBP-5 (Jones et al., 1993a, Hakeda et al., 1996). In the present study, in addition to anti-IGFBP-2, -5 sera, antibodies raised against IGFBP-3, -4, -6, and -7 cross-reacted with basal lamina proteins, suggesting that these IGFBP may be integral components of some basement membranes. In the present study, the

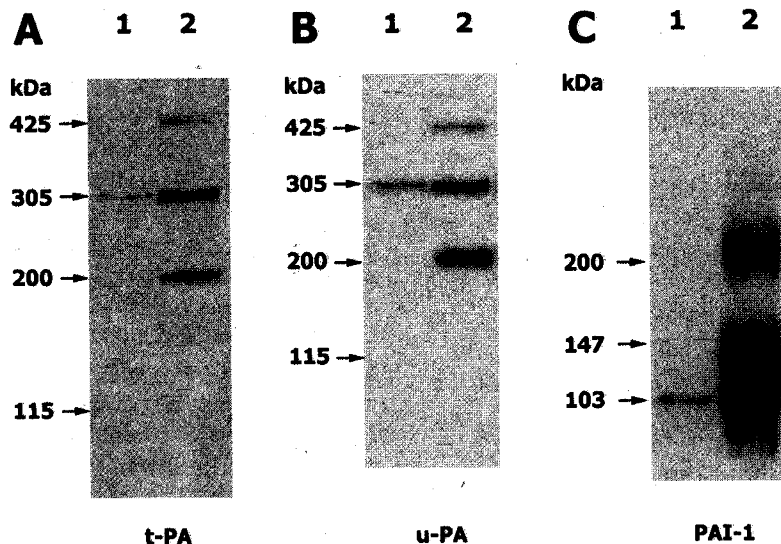

FIGURE 11. Western blot of basal lamina proteins using antibodies raised against (A) tissue plasminigen activator (t-PA), (B) urokinase plasminogen activator (u-PA), or (C) Type 1 plasminogen activator inhibitor (PAI-1). Lane 1: Fraction 1 proteins; Lane 2: Fraction 2 proteins. Molecular weights are indicated on the left border. The molecular weights of purified intact molecules used to raise antibodies are t-PA (70 kDa), u-PA (52 kDa), PAI-1 (43 and 52 kDa).

molecular weights of protein bands that reacted positively with the IGFBP antisera are larger than the molecular weights reported for IGFBP. Thus, the bands that gave positive reactions with IGFBP antisera in the present study may actually be nondissociated, large protein complexes containing IGFBP. The molecular weights of IGFBP, especially those in serum and other body fluids, are between 24 to 44 kDa (see for example Jones and Clemmons, 1995). However, using Western ligand and immunoblotting techniques, proteins of larger molecular weights (≥ 120 kDa) have been identified as IGFBP in serum and other body fluids of different species (see for example Bicsak et al., 1990; Prosser and McLaren, 1992; Gerard and Monget, 1988). Therefore, it was suggested that the large molecular weight proteins identified as IGFBP may actually be complexes formed by 24 to 44 kDa IGFBP with other proteins that could not be dissociated with SDS in the Western ligand blotting technique (Bicsak et al., 1990).

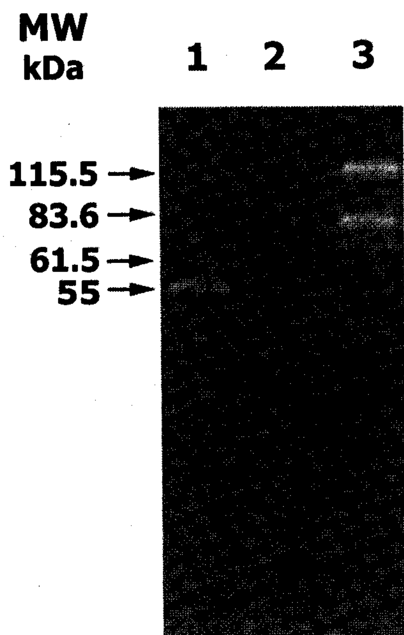

FIGURE 12. Zymography of basal lamina proteins, Fraction 1 and Fraction 2 was performed on gelatin-containing (10%) ready gels. Lane 1: authentic collagenase F standard; Lane 2: Fraction 1; and Lane 3: Fraction 2. Molecular weights of proteins are denoted on the left border.

Some IGFBP may exert direct effects on cells in an IGF-independent manner (Jones and Clemmons, 1995). Amino acid analysis revealed that IGFBP-1 and IGFBP-2 have the RGD (Arg-Gly-Asp) sequence, the recognition site for binding of some extracellular matrix proteins to integrins (Baxter and Martin, 1989; Jones et al., 1993b; Rechler, 1993). The IGFBP-1 has been shown to influence CHO cell migration via $\alpha_5\beta_1$ integrin, independently of IGF-I (Jones et al., 1993b). The IGFBP contained in the extracellular matrix are more resistant to proteolytic degradation (Jones et al., 1993a). The affinity of IGFBP for IGF is reduced by interactions with extracellular matrix or cell surface proteins such that associations of IGFBP with extracellular matrix or cell surface can lead to an increase in the local level of IGF with an enhanced IGF effect (Jones and Clemmons, 1995). It was shown that IGFBP-5 enhanced the growth stimulatory actions of IGF-I on fibroblasts (Jones et al., 1993a).

Matrix metalloproteinases (MMP) such as collagenase Type 1 (MMP-1; 52 to 57 kDa), gelatinase A (MMP-2; 72 kDa), gelatinase B or collagenase Type IV (MMP-9; 92 kDa), and stromelysin (MMP-3; 57–60 kDa) are zinc-dependent (metal-dependent) enzymes (Woessner, 1991). They exist as inactive zymogens that must be activated before attaining catalytic activity (Woessner, 1991). Antisera raised against several MMP recognized ovarian basal lamina proteins in the present study in support of the

view that some MMP are integral components of basal laminae or basement membranes. This contention is supported by the fact that MMP-2 and MMP-9 activities were demonstrated in basement membrane reconstituted from Engelbreth-Holm-Swarm (EHS) tumor proteins (Matrigel) (Mackay et al., 1993). In the present study, in addition to anti-MMP-2 and -9 sera, antibodies raised against MMP-1, MMP-3, MMP-8, and MMP-13 cross-reacted with basal lamina proteins suggesting, that these MMP are integral components of some basal laminae or basement membranes. Tissue inhibitors of metalloproteinases (TIMP) are well known for their action as inhibitors of MMP in the degradation of extracellular matrix (Woessner, 1991). In general, the degradation of extracellular matrix proteins is determined by the balance of enzymes (MMP) and their inhibitors (TIMP). The TIMP-3 is primarily associated with the extracellular matrix (Leco et al., 1994). The present data suggest that TIMP-1, -2, -3, and -4 are components of basal lamina of avian ovarian follicle.

The current results also support the contention that t-PA and u-PA and Type 1 PAI-1 are components of basal laminae. It was shown that basement membrane extracted from murine EHS tumor contains active t-PA and u-PA (McGuire and Seeds, 1989). Similarly, extracellular matrix produced by endothelial cells contain active t-PA and u-PA (Vlodavsky et al., 1991b; 1993). In addition, active PAI-1 has been identified in endothelial cell extracellular matrix (Mimuro et al., 1987).

In the avian ovary, in response to appropriate signals, the equilibrium between the MMP, other proteases, and their inhibitors could be changed such that they could become activated to regulate the local and specific degradation of basal lamina in the rapidly enlarging follicle. In addition, the proteases and their inhibitors may play important roles in remodeling processes associated with follicular development and ovulation. Moreover, they may participate in the sequential degradation of proteoglycans and other binding proteins, resulting in the release and activation of sequestered growth factors. Depending on the tissue, the activated growth factors may modulate the proliferation or differentiation of cells in processes such as tumor development, wound healing, and inflammation. The results of the zymography experiments (using gelatin and casein as substrates) show that at least one basal lamina protein is a protease. Other proteases might have been inactive under the present experimental conditions. Indeed, the molecular weight (55 kDa) of the protein band that gave the positive results in the zymogram was different from the molecular weight of protein bands that cross-reacted with antibodies raised against many MMP and other proteases. Because proteins in Fraction 2 gave positive reactions with MMP antibodies, it would be expected that they would give positive zymograms as well. It was surprising that no Fraction 2 protein showed proteolytic activity in the zymography experiment. The reason for the lack of proteolytic activity in Fraction 2 is unknown. Again, the possibility remains that the proteases in Fraction 2 were not activated (remained in their latent forms) under the present experimental conditions. The present results demonstrate the putative localization of some hematopoietic factors or cytokines (GM-CSF, IL-3, interferon-γ) to basal lamina of the avian ovarian follicle. It has been reported that GM-CSF and IL-3 are bound to heparan sulfate in stroma of murine bone marrow (Roberts et al., 1988); therefore, it is possible that these hematopoietic factors or cytokines exist in the basal lamina bound to HSPG.

Some of the antibodies cross-reacted with protein bands of larger molecular weights than expected. The reasons for these observations are unknown; however, it has been reported that several active components of basement membranes exist as complexes formed with other macromolecular constituents such as HSPG. In basement membranes growth factors such as the FGF and EGF form complexes with proteoglycans and other components (Vlodavsky et al., 1987; Folkman et al., 1988; Vigny et al., 1988; Vlodavsky et al., 1991a,b). The TGF-β has been shown to form complexes with extracellular matrix proteoglycans (Ruoslahti and Yamaguchi, 1991) and cell-surface proteoglycans (Massague, 1990). The proteoglycans may regulate the physiological activities of the growth factors sequestered in basement membranes (Rouslahti and Yamaguchi, 1991). Insulin-like growth factors-I and -II may be associated with their binding proteins (IGFBP) that are bound to high molecular weight proteins in the basal lamina. It was shown that IGFBP-5 binds to Types III and IV collagen, laminin, and fibronectin (Jones et al., 1993a). Finally, it was demonstrated that MMP-2 and MMP-9 were obtained with laminin purified from basement membrane produced by murine Engelbreth-Holm-Swarm (EHS) tumor (Mackay et al., 1993).

The present results indicate that ovarian basal lamina contain osteonectin. It confirms earlier reports that osteonectin (BM-40 or SPARC) is an integral component of basement membranes. Osteonectin is a component of EHS tumor basement membrane (Dziadek et al., 1986; Mann et al., 1987). It has also been demonstrated in the extracellular matrices of bone, cartilage, and soft connective tissues (Holland et al., 1987; Wewer et al., 1988; Sage et al., 1989). Osteonectin binds to proteoglycan collagen IV, laminin, and fibronectin (Mayer et al., 1991).

A positive reaction obtained with an antibody does not prove that a particular protein exists in the avian ovarian basal lamina. The positive reaction may be the result of a mere recognition of an epitope in a related or an unrelated protein. For example, entactin from EHS tumor contains five consecutive EGF-like repeats (Durkin et al., 1988). Also, laminin purified from EHS tumor contains repeats with considerable homology to EGF (Sasaki et al., 1987; Sasaki and Yamada, 1987). In addition, EGF-like repeats have been demonstrated in HSPG purified from EHS tumor (Noonan et al., 1988). Purification and characterization (sequencing) of the components of Fractions 1 and 2 in future studies will reveal the identity of proteins in the basal lamina. The lack of positive reaction by an antibody may not be interpreted as the absence of a particular protein from the basal lamina. It may reflect lack of cross-reactivity between species.

It is noteworthy that solubilized basal lamina regulated the shape and function of chicken granulosa cells in vitro; Fraction 1 caused granulosa cells to assume rounded shape and stimulated progesterone production by them (Asem et al., 2000a,b). These findings indicate that the basal lamina of hen ovarian follicle is biologically active. The growth factor-like domains (epitopes) of components of the basal lamina that reacted positively to antibodies raised against growth factors may exert biological and physiological effects on cells adjacent to this extracellular matrix, as postulated by Engel (1989).

In summary, intact and complete basal lamina has been isolated from hen ovarian follicle. With Western blot analysis, antisera generated against proteins known to be components of basement membranes recognized basal lamina proteins. In addition, the antibody raised against some proteins that are not known to be components of basement membranes reacted positively with solubilized basal lamina of the avian ovarian follicle. Thus, the basal lamina of chicken ovarian follicle appears to contain, in addition to extracellular matrix proteins, several growth factors, growth factor binding proteins, MMP, TIMP and cytokines. These bioactive molecules can regulate ovarian cell proliferation and differentiation as well as tissue remodeling during follicular development.

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