Human Procathepsin B Interacts with the Annexin II Tetramer on the Surface of Tumor Cells*

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To study potential roles of plasma membrane-associated extracellular cathepsin B in tumor cell invasion and metastasis, we used the yeast two-hybrid system to screen for proteins that interact with human procathepsin B. The annexin II light chain (p11), one of the two subunits of the annexin II tetramer, was one of the proteins identified. We have confirmed that recombinant human procathepsin B interacts with p11 as well as with the annexin II tetramer in vitro. Furthermore, procathepsin B could interact with the annexin II tetramer in vivo as demonstrated by coimmunoprecipitation. Cathepsin B and the annexin II tetramer were shown by immunofluorescent staining to colocalize on the surface of human breast carcinoma and glioma cells. Taken together, our results indicate that the annexin II tetramer can serve as a binding protein for procathepsin B on the surface of tumor cells, an interaction that may facilitate tumor invasion and metastasis.

During the processes of tumor cell invasion and metastasis, limited degradation of the extracellular matrix facilitates local invasion, angiogenesis, intravasation, and extravasation. This limited degradation is mediated by multiple proteases acting in an enzymatic cascade (1). The lysosomal cysteine protease cathepsin B is one of the multiple proteases and has been linked to tumor progression through observations that its activity, secretion, and membrane association are increased in malignant tumors, particularly at their invasive edges (2–6). Cathepsin B can degrade at neutral and acidic pHs the extracellular matrix proteins laminin, fibronectin, and collagen IV (7, 8). Digestion of fibronectin by cathepsin B results in the exposure of the CS-1 sequence, which is within the alternatively spliced type III connecting segment (IIICS) of fibronectin and is recognized by the integrin receptor, αvβ3 (9). In addition, cathepsin B can activate other proteolytic enzymes, such as urokinase-type plasminogen activator, which acts downstream in the proteolytic cascade, and collagenase I, which is capable of digesting fibrillar collagen in the extracellular matrices (10, 11). Therefore, cathepsin B may play important roles in extracellular proteolysis, and its degradation products may have an impact on subsequent cellular signaling pathways.

Cathepsin B is synthesized as a preproenzyme and is activated in prelysosomal acidic vesicles (late endosomes) before its delivery to lysosomes (12). In normal epithelial cells, the lysosomes containing mature cathepsin B are perinuclear (13). In tumor cells, cathepsin B is also localized in perinuclear lysosomes as well as in other yet unidentified vesicular compartments at the cell periphery. Redistribution of cathepsin B has been observed in human breast, colon, and esophageal carcinomas and gliomas (3, 14–17). Altered trafficking of cathepsin B is associated with increased secretion of mature enzyme or its precursor from tumor cells (18–20) and with cathepsin B being present on the external face of tumor cell plasma membranes (13, 15, 20). The mechanisms for trafficking of cathepsin B to the membrane and its association with the membrane are still unknown. Although lysosomal enzymes are trafficked to the lysosome primarily through mannose phosphate receptor pathways (22), alternative pathways include one that recognizes a sequence in the propeptides (23, 24), i.e. comparable with the pathway used for targeting of yeast vacuolar proteases (25, 26).

An understanding of the molecular events responsible for secretion and membrane localization of cathepsin B in tumors may lead to identification of new targets for therapeutic intervention. Therefore, we applied the yeast two-hybrid system to search for proteins that interact with procathepsin B. One of the clones encodes the annexin II light chain (p11), a member of the Sl100 family. There is little evidence of a function for p11 without its annexin II heavy chain (p36) partner, although a recent report suggests that p11 binds to the C-terminal region of the high molecular weight cytosolic phospholipase A2 and is able to inhibit its activity (27). The association of the light chain (p11) with the heavy chain (annexin II or p36) appears to mediate the interaction of the annexin II tetramer with the plasma membrane (28). In addition, p36 as well as the annexin II tetramer is thought to function as a cell surface receptor for several proteins (29). In this paper, we report that procathepsin B interacted with the annexin II tetramer both in vitro and in vivo. The interaction between procathepsin B and the annexin II tetramer on the tumor cell surface may play an important role in extracellular proteolysis as well as in tumor cell invasion and metastasis.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s minimal essential medium, bovine serum albumin, isopropyl-β-D-thiogalactopyranoside, imidazole, and 1-butanol were from Sigma; fetal bovine serum and Versene were from Life Technologies, Inc.; the BT20 human breast carcinoma line and the U87 human glioma line from ATCC (Manassas, VA). Ni-NTA1 resin was prepared as described.

1 The abbreviations used are: Ni-NTA, nickel nitrilotriacetic acid; CB, cathepsin B; CBpropeptide, cathepsin B propeptide; p11, annexin II light chain; p36, annexin II or annexin II heavy chain; t-PA, tissue-type plasminogen activator; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; x-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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obtained from Qiagen (Chatsworth, CA); the enhanced chemiluminescence Western blotting detection system was from Amersham Pharmacia Biotech; micro-BCA assay kits, horseradish peroxidase-labeled goat anti-rabbit IgG, and horseradish peroxidase-labeled goat anti-mouse IgG were from Pierce. Formaldehyde was bought from Polysciences (Warrington, PA); proteins for anti-fibrillar matrix antibodies (Eugene, OR); fluorescein-conjugated affinity-purified donkey antibody to mouse IgG, and normal donkey serum were from Jackson ImmunoResearch (West Grove, PA); tosylated Dynabeads M-280 were from Dynal (Naarden, CV); anti-p11 and anti-p36 monoclonal antibodies were from Transduction Laboratories (Lexington, KY); anti-p11 polyclonal antibodies were from Biodiagnostic International (Kennebunk, Maine); anti-procathepsin B monoclonal antibody (DC-1) was from Oncogene Science (Cambridge, MA); pGEX-2T vectors and glutathione-Sepharose beads were from Amersham Pharmacia Biotech. The mature form of human cathepsin B was obtained from Athens Research (Athens, GA). Protein concentrations in this paper were determined by the micro-BCA assay (Pierce).

**Construction of Plasmids for Yeast Two-hybrid Screening—**Plasmid pEG202 (30), containing the sequences for the LexA DNA binding domain (amino acids 1–202) as well as yeast HIS3 gene, was used to express the fusion proteins. Two cathepsin B cDNA fragments (186 bp, corresponding to the propeptide, and 825 bp, corresponding to the propeptide plus the full-length single chain protein) were amplified by polymerase chain reaction using a human cathepsin B cDNA plasmid as a template. The propeptide and full-length cathepsin B cDNA fragments were cloned into the pEG202 vector in the correct orientation and with the correct reading frame to express the LexA-cathepsin B fusion proteins. To obtain the cathepsin B cDNA fragments, two primer pairs were synthesized according to the cathepsin B cDNA sequence. The primer pair for the 186-bp propeptide cDNA fragment was composed of the following sequences: 5′ primer, 5′-CGGATCCGAGGAAACAAGGAGG-GCCC-3′ (corresponding to +52 to +63 of the coding region; containing the underlined BamHI site and CC dinucleotides for in-frame cloning); 3′ primer, 5′-GGAGGCTCTCGAGTTACTTCAGGTCCTCGGT-3′ (corresponding to +521 to +537 of the coding region and containing the underlined XhoI site). The identity and orientation of the constructs were confirmed by DNA sequencing. The plasmid containing the 186-bp cathepsin B cDNA fragment corresponding to the propeptide was designated as pEG202-CBpropep.

**Two-hybrid System Screening of Human cDNA Library—**Two-hybrid screening was conducted as described (31). RYF231 (32) yeast cells containing the reporter gene LEU2 and reporter plasmid pJK103lacZ were sequentially transformed with pEG202-CBpropep and DNA from the HeLa cDNA library cloned into the TRP1 vector, pEG2–5, in parallel. Transformants were plated in the dropout medium (Glusura ‘his’ ‘trp’ ) at a density of approximately 200,000 colonies/24-cm×24-cm plate. 2×105 transformants were collected and diluted to the concentration in which the plating efficiency would be on the order of 106 colony-forming units/100 μl. Library transformants containing cDNAs that encode proteins which interact with the bait exhibit galactosidase-dependent growth on media lacking leucine (Leu−) and galactoside-dependent β-galactosidase activity (lacZ+). 100 μl of the transformant dilution (106 colony-forming units/100 μl) was plated on the selection plates (Gal/Raf ura−his−trp−leu−), and 58 colonies grew. The 58 Leu− isolated were transferred to a glucose master plate and then replica-plate to four new plates to test for lacZ expression and galactoside dependence. These plates included two Leu− plates and two X-gal plates, one Leu− plate, and one X-gal plate, which contained galactoside to induce cDNA expression (plus rifampicin to enhance growth), whereas the other Leu− plate and the other X-gal plate contained glucose to repress cDNA expression. 24 out of the 58 exhibited galactoside-dependent activation of the interactors (Leu−, LacZ+). The pEG4–5 cDNA plasmid DNA samples were first isolated from 24 yeast clones and amplified through PCR. The PCR products were then digested with BamHI and then ligated into pEG202-CBpropep. The ligated DNA was used to transform Escherichia coli strain DH5α. A total of 186 bp of the complete length of the cDNA inserts was obtained by dideoxy sequencing using a 24-mer oligonucleotide primer (5′-CCAGCCTCCTTGTGAGTGGGATG-3′), which is derived from the coding sequence for the B42 activation domain. The cDNA sequences were then subjected to homologous sequence search through BLAST programs from nonredundant GenBank + EMBL + DDBJ + PDB sequences or GenBank EST division.

**Expression of His6-p11 Protein in Bacteria—**The bacterial expression plasmid pET30α-p11 (a generous gift from Dr. James H. Shellenberger (27)) containing the His tag and full-length human p11 cDNA sequences was transformed into bacteria BL21(DE3), a λ–mutant strain containing the T7 polymerase under the control of lacUV5 promoter. The addition of isopropyl-β-D-thiogalactopyranoside induced expression of His6-p11. The expressed p11 was purified using histidine binding resin Ni-NTA according to the manufacturer’s instruction.

**Expression of GST-Cathepsin B Propeptide (GST-CBpropeptide) Protein in Bacteria—**The CBpropeptide cDNA fragment was amplified by polymerase chain reaction and inserted into a bacterial expression vector pGEX-2T (Amersham Pharmacia Biotech). Expression of GST-CBpropeptide fusion was induced by adding isopropyl-β-D-thiogalactopyranoside. The expressed GST-CBpropeptide was purified through glutathione-Sepharose beads.

**Interaction Assays between Recombinant Procathepsin B and Recombinant His6-p11 in Vitro—**Assays were performed at 4°C. 5 μg of purified His6-p11 was loaded on 50 μl of the Ni-NTA resin, and the resin was washed with 1 ml of 300 mM NaCl, pH 6.0, to remove unbound His6-p11. Human procathepsin B was produced by a vaccinia virus expression system in HeLa cells and purified on an immunoadfinity column (34). 3 μg of procathepsin B and 3 μg of mature cathepsin B were added to the resin and incubated at 4°C with rotation for 12 h. The mixture was then washed 10 times with 200 μl of 300 mM NaCl, pH 6.0. His6-p11, and bound proteins were eluted with 200 μl of 300 mM imidazole in the NaCl solution. The wash and eluted fractions were collected and subjected to 15% SDS-PAGE and immunoblot analysis.

**Interaction Assays between Recombinant GST-CBpropeptide and Recombinant p11 in Vitro—**Assays were performed at 4°C. 2 μg of GST-CBpropeptide was loaded on 50 μl of glutathione-Sepharose beads and washed with PBS, pH 7.4, buffer to remove unbound fusion proteins. Purified wild-type and mutated p11 were described above. Recombinant containing the 825-bp cathepsin B cDNA fragment corresponding to the full-length protein was designated as pEG202-CBfull.

**Immunoprecipitation of Recombinant Procathepsin B and Recombinant Annexin II Tetramer—**Purified annexin II tetramer was obtained as described (35). Before immunoprecipitation, purified annexin II tetramer was mixed with purified procathepsin B in buffer A (20 μl Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 5 mM EDTA, and 1 μM diithreitol) and kept at 4°C for 10 min. About 1 μg of the isolated antibody was mixed with protein A-Sepharose and used in each experiment. 2 μg of the tetramer and/or 1 μg of procathepsin B were added to each experiment. After overnight incubation at 4°C with rotation, the Sepharose beads were washed five times with buffer A containing 0.1% Triton X-100. Sample loading buffer was then used to elute the bound fraction from the beads. The bound fraction was subjected to 15% SDS-PAGE and immunoblot analysis.

**Immunoblotting—**Following SDS-PAGE, the separated proteins were electrophoretically transferred onto a nitrocellulose membrane in 25 mM Tris, 2000 mM glycerol; 20% (v/v) methanol, pH 8.3. The membrane was blocked with 5% nonfat milk in 0.1% Tween 20, 20 μl Tris-HCl, pH 7.4 (T-TBS) overnight and then incubated with 1:4000 dilution of rabbit anti-human cathepsin B IgG (developed and characterized in our laboratory (36)) in T-TBS or a 1:5000 dilution of anti-p11 or anti-p36 monoclonal antibody in T-TBS for 2 h. The blots were then probed with 1:4000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG or horseradish peroxidase-labeled goat anti-mouse IgG in T-TBS and detected using an enhanced chemiluminescence Western blotting detection system. The blot was stripped at 65°C for 30 min.
with stripping buffer (2% w/v SDS, 62.5 mM Tris, pH 6.8, and 100 mM β-mercaptoethanol) before probing with a second primary antibody.

Cell Culture—The BT20 human breast carcinoma line and the U87 human glioma line were grown in minimal essential medium (MEM) containing 10% fetal bovine serum as recommended by the ATCC (Manassas, VA). All cell lines were screened on a routine basis with 4,6-diamidin-2-phenylindole-dihydrochloride and shown to be free of mycoplasma. All cells were treated in the serum-free minimal essential medium overnight and washed three times in cold PBS, pH 7.4, before harvesting for different experiments.

Versene Extraction—BT20 or U87 cells grown to ~80% confluence were first washed with cold PBS three times and then incubated with Versene at 37 °C for 10 min. The supernatant from the Versene incubation was collected by centrifugation and concentrated for further analysis.

Immunoprecipitation Using Antibody-coated Dynabeads—Tyrosyl-activated Dynabeads M-2000 were incubated at 37 °C on a rotator with rabbit anti-human cathepsin B IgG or anti-p36 monoclonal antibody at a concentration of 3 μg of antibody/107 Dynabeads (~20 μg/ml) in borate buffer (100 mM H3BO3, pH 9.5) for 24 h. For this and all subsequent steps, beads were collected by brief centrifugation and use of a magnet (magnetic particle concentrator, Dynal, Inc.). About 200 μg of protein from the Versene-extraction of BT20 cells was precloned with rabbit IgG or mouse IgG and protein A-Sepharose beads and incubated with rabbit anti-human cathepsin B IgG or anti-p36 monoclonal antibody cross-linked to Dynabeads at 4 °C overnight. The beads were collected and washed three times with buffer A (without reducing reagents) containing 0.1% Triton X-100. Sample loading buffer (without reducing reagents) was then used to elute the bound fraction from the beads. The eluted fraction was subjected to 15% SDS-PAGE and immuno blot analysis.

Immunocytochemical Staining and Confocal Microscopy—Surface stainings were performed using a modification (37) of the general immunocytochemical methodologies described by Willingham (38). Non-permeabilized cells (BT20 or U87) grown on glass coverslips were fixed with 4% formaldehyde at 4 °C for 10 min. After being washed with buffer containing 136.59 mM NaCl, 2.68 mM KCl, 8.06 mM Na2HPO4, 1.47 mM KH2PO4, 1.0 mM CaCl2, and 1.0 mM MgCl2, pH 7.4 (PBS-Ca2+), cells were blocked with 2 mg/ml bovine serum albumin in PBS-Ca2+. To stain only for surface antigens, the cells were not permeabilized with detergents. All subsequent antibody incubations and washes were performed at 4 °C. Cells were incubated with different combinations of primary antibodies (rabbit anti-human cathepsin B IgG (1:500 dilution) plus mouse anti-p11 IgG1 (1:125 dilution), rabbit anti-human cathepsin B IgG plus mouse anti-p36 IgG1 (1:125 dilution), or rabbit anti-p36 IgG (1:125 dilution) plus mouse anti-p11 IgG1) for 2 h. After six rapid washes with PBS-Ca2+, cells were incubated with fluorescein-conjugated affinity-purified donkey anti-rabbit and Texas red-conjugated affinity-purified donkey anti-mouse IgG (20 μg/ml) plus mouse anti-p11 IgG1 (1:125 dilution), rabbit anti-human cathepsin B IgG (1:500 dilution) and 4′,6-diamidin-2-phenylindole-dihydrochloride and shown to be free of mycoplasma. All cells were treated in the serum-free minimal essential medium overnight and washed three times in cold PBS, pH 7.4, before harvesting for different experiments.

RESULTS

Procathepsin B Interacts with p11 in the Yeast Two-hybrid Interaction Trap—To look for proteins that interact with cathepsin B, we screened a HeLa cell cDNA library using a yeast two-hybrid system (39). We used the LexA version of the two-hybrid system in which bait proteins are expressed as fusions to LexA, cDNA-encoded proteins are fused to an activation domain, and interactions between the bait and the cDNA-encoded protein are detected by activation of two reporter genes, Leu2 and LacZ, with LexA binding sites (30). We constructed two bait vectors for expressing LexA fusions to different fragments of cathepsin B. One vector, pEG202-CBpro, encodes LexA fused to the propeptide fragment of procathepsin B. The second vector, pEG202-CBfull, encodes LexA fused to full-length procathepsin B. Both pEG202-CBpro and pEG202-CBfull vectors were tested for background interaction with the reporter genes, and no background activation of the reporters was found. We also expressed LexA fused to the mature form of cathepsin B but found that it activated transcription of the reporters on its own (data not shown). Since the propeptide of lysosomal and yeast vacuolar enzymes may serve as a signal peptide for their trafficking (23–26), we initially used the pEG202-CBpro vector to screen the HeLa cell cDNA library as described (see “Experimental Procedures” and Ref. 31). From 106 library transformants, we identified 24 clones that encoded proteins that interacted with CBpropeptide. 13 of these also interacted with procathepsin B full-length protein but not with 10 unrelated baits (data not shown). Of 13 candidates isolated, 4 of them coded for annexin II light chain (p11). The remaining 9 represented 6 unique cDNAs that will be described elsewhere.

Recombinant Procathepsin B Interacts with Recombinant His6-p11 in Vitro—To confirm the direct interaction between procathepsin B and annexin II light chain (p11), we performed an in vitro assay (Fig. 1). The bacterial expression plasmid pET30a-p11 (27) containing the His tag and full-length human p11 cDNA sequences was used to produce recombinant His6-p11 in Escherichia coli strain BL21(DE3). We used a vaccinia virus system to generate the properly modified and folded proenzyme (34). Using Ni-NTA resin to which purified His6-p11 had been bound, we tested whether immunopurified procathepsin B and mature human cathepsin B could interact with p11. After incubation of procathepsin B and mature cathepsin B with His6-p11 bound to the Ni-NTA resin, the resin was washed with NaCl buffer. Wash fractions were collected and analyzed for protein content (Fig. 1, lanes 1–10). After proteins could no longer be eluted with the NaCl buffer, we eluted the bound proteins with NaCl buffer containing imidazole. The wash and eluted fractions were then analyzed by 15% SDS-PAGE and immunoblotting (Fig. 1, lanes 1 to 10 and Elution). Procathepsin B and p11 were eluted with imidazole buffer (Fig. 1, Elution). Mature cathepsin B was not eluted, indicating that mature cathepsin B did not interact with His6-p11 under these conditions. Mature cathepsin B was detected in the samples from earlier washes (lanes 1 and 2). Since procathepsin B was not present in the 10th wash (Fig. 1, lane 10), the simultaneous elution of procathepsin B and His6-p11 (Fig. 1, Elution) indicated that procathepsin B was bound to p11. Thus, the interaction of cathepsin B with His6-p11 in vitro appears to be specific for the cathepsin B proenzyme.

Recombinant p11 Interacts with GST-CBpropeptide, Which Is Bound to Glutathione-Sepharose Beads—To further confirm that the propeptide fragment of procathepsin B is the fragment responsible for the interaction with p11, recombinant GST-CBpropeptide fusion proteins were prepared and tested for...
their ability to interact with p11. In addition, we tested the interaction between GST-CBpropeptide and a p11 C-terminal deletion mutant protein. Waisman and co-workers (40) recently demonstrated that p11 binds to plasminogen and participates in the stimulation of t-PA-dependent activation of plasminogen by the annexin II tetramer. A deletion mutant of the p11 subunit, missing the last two C-terminal lysine residues, retains only 15% of the ability of the wild-type p11 subunit to bind plasminogen (40). Purified recombinant GST and GST-CBpropeptide are shown in Fig. 2 (panel A). The recombinant GST protein is ~26 kDa, and the propeptide of cathepsin B is ~6 kDa, so the GST-CBpropeptide protein has an estimated molecular size of ~32,000 (Fig. 2, panel A). We tested for interaction between p11 or its deletion mutant protein and GST-CBpropeptide or GST. The GST-CBpropeptide or GST had been previously bound to glutathione-Sepharose beads. After incubation and subsequent washes, protein components remaining on the beads were eluted with glutathione buffer. The eluted fractions were analyzed by SDS-PAGE and immunoblotting (Fig. 2, panel B). Both p11 and its deletion mutant interacted with GST-CBpropeptide (lanes 3 and 6). No interaction was detected between p11 or the p11 deletion mutant and GST (lanes 2 and 5). These data indicate that both p11 and its deletion mutant can interact with the propeptide fragment of procathepsin B in vitro. The p11 mutant could lessen the interaction between p11 and plasminogen, but it did not affect the interaction between procathepsin B and p11. Thus, p11 appeared to interact with procathepsin B through a different domain than that used by plasminogen.

**Procathepsin B Interacts with the Annexin II Tetramer in Vitro**—Two p11 monomers serve as the regulatory subunits in the annexin II tetramer in vivo (see “Discussion”). The formation of the tetramer results in the association of the tetramer with the plasma membrane (28). To test if procathepsin B also interacts with the annexin II tetramer, we immunoprecipitated a mixture of recombinant annexin II tetramer and procathepsin B with either anti-p11 or anti-p36 monoclonal antibodies. The mixture of annexin II tetramer and procathepsin B is shown in Fig. 3 (panel A). Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting (Fig. 3, panel B). As illustrated in panel B, neither anti-p11 or anti-p36 antibodies could immunoprecipitate procathepsin B from the solution containing only procathepsin B (lanes 1 and 3). After procathepsin B was mixed with the annexin II tetramer, it could then be co-precipitated with p11 and p36 by either anti-p11 or anti-p36 monoclonal antibodies (lanes 2 and 4). Our results indicate that recombinant procathepsin B could interact with the recombinant annexin II tetramer in vitro. Interaction between procathepsin B and the annexin II tetramer may be of functional significance in tumor progression and metastasis as both cathepsin B (13, 15, 20) and the annexin II tetramer (42) have been found on the surface of tumor cells.

**Cathepsin B, p11, and p36 Are Bound to the Surface of Tumor Cells in a Ca**$_{2+}$-Dependent Manner—We determined if procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. Both the tetramer and the p36 heavy chain have been reported to be present on the surface of several types of cells (42–46). We chose BT20 cells, a human breast carcinoma cell line, and U87 cells, a human glioma cell line, to perform our study because cathepsin B had previously been observed to be present on the surface of these cells (37). First, we confirmed that cathepsin B and the annexin II tetramer were present on the surface of these tumor cell lines. We then used Versene (0.5 mM EDTA in PBS; Life Technologies, Inc.) to wash the tumor cells. Because annexin II tetramers are Ca$_{2+}$-dependent phospholipid-binding proteins (28), the tetramers and their associated proteins should be stripped from the cell surface by Versene. We collected Versene-wash fractions as described under “Experimental Procedures” and analyzed them by SDS-PAGE and immunoblotting (Fig. 4, lane W). p11, p36, and several species of cathepsin B (procathepsin B (46 kDa), single-chain form of cathepsin B (31 kDa), and heavy chain of double-chain form of cathepsin B (26 kDa)) were all detected in Versene-wash fractions from both cell lines. The additional bands around 46 kDa may be variant glycosylated forms of procathepsin B on the plasma membrane as glycosylation variants of procathepsin B have been observed in human colon carcinomas (19, 21, 47). Neither cathepsin B nor the annexin II complex were detected when the cells were washed with PBS that did not contain EDTA (data not shown). The cells treated with Versene were shown to be viable under these experimental conditions. We also checked overnight serum-free medium from both BT20 and U87 cells for possible release of cathepsin B and annexin II subunits from the two cell lines (Fig. 4, lane M). Procathepsin B could be detected in overnight media from both cell lines; p11 or p36 were not detected. The
large quantity of procathepsin B secreted from both the BT20 and U87 cells was consistent with previous observations that tumor cells secret high levels of procathepsin B (19, 21). In addition, procathepsin B and mature cathepsin B also appeared to be bound in a Ca\(^{2+}\)-dependent manner to the surface of both tumor cell lines.

**Procathepsin B, p11, and p36 Are Co-immunoprecipitated from the Versene-wash Fraction of Intact Tumor Cells**—To determine whether procathepsin B, mature cathepsin B, p11, and p36 interact with one another on the surface of tumor cells, we immunoprecipitated the Versene-wash fractions from BT20 cells using rabbit anti-human cathepsin B IgG (Fig. 5A). Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting. Procathepsin B, p11, and p36 could be detected with anti-procathepsin B (DC-1) (upper panel), anti-p11 (lower panel), and anti-p36 monoclonal antibodies (middle panel), respectively, indicating that procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. We also used anti-p36 monoclonal antibody to immunoprecipitate the Versene-wash fractions (Fig. 5B). Procathepsin B and p11 could be detected in the immunoprecipitate with rabbit anti-human cathepsin B IgG (upper panel) and anti-p11 monoclonal antibody (lower panel), respectively. Mature cathepsin B was not detected in the immunoprecipitate. Our data suggest that the annexin II tetramer interacts with procathepsin B on the tumor cell surface but not with mature cathepsin B. This is consistent with the results of the yeast two-hybrid and in vitro experiments in which p11 interacted with procathepsin B but not with mature cathepsin B (Fig. 1). Nonetheless, mature cathepsin B does associate with the tumor cell membrane in a Ca\(^{2+}\)-dependent manner (see Fig. 4, lane W).

**Immunofluorescent Staining Demonstrates that Cathepsin B and the Annexin II Tetramer Co-localize on the Surface of Human Tumor Cells**—To further examine the location of these proteins on the cell surface, we used confocal immunomicroscopy to determine the relationship between cathepsin B and the annexin II tetramer. First, we studied the localization of p11 and cathepsin B on the surface of BT20 cells. Nonpermeabilized BT20 cells grown on glass were stained as described under “Experimental Procedures.” To ensure that the staining observed was on the cell surface, all procedures were performed at 4 °C, and detergents were not used. Panel A in Fig. 6 depicts the immunostaining for cathepsin B (green), and panel B depicts the immunostaining for p11 (red). The regions staining yellow indicate that cathepsin B and p11 were co-localized on the surface of the BT20 cells (Fig. 6, panel C). We also determined whether the two chains of the annexin II tetramer, p11 and p36, were colocalized on the surface of BT20 cells by double-staining using an anti-p11 monoclonal antibody and an anti-p36 polyclonal antibody. The green staining in panel E depicts for p36, the red staining in panel F depicts for p11, and the yellow indicates the colocalization of these two proteins (panel G). In addition, we performed a series of double immunofluorescent stainings for p11 and p36, p11 and cathepsin B, and p36 and cathepsin B on U87 glioma cells. The confocal images indicated that cathepsin B, p11, and p36 all co-localized with one another on the surface of U87 cells (data not shown). Taken together, these results demonstrate that cathepsin B co-localizes with the annexin II tetramer on the surface of two human tumor cell lines, one of epithelial origin and one of mesenchymal origin.

**DISCUSSION**

An important property of metastatic cells is their ability to degrade and move through extracellular matrices. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis, and migration of tumor cells through the proteolytically modified region (48). There is evidence that invasive and metastatic tumors synthesize higher levels of various classes of degradative enzymes, including matrix metalloproteases, aspartyl, serine, and cysteine proteases, than do surrounding normal tissues or benign lesions (49). Local proteolysis during tumor invasion is facilitated by proteases bound to the tumor cell surface as well as proteases secreted from tumor cells and tumor-associated host cells (50). Proteases other than the cysteine protease cathepsin B studied herein have been found on the tumor cell surface; these include urokinase-type plasminogen activator bound to its receptor (uPAR) (51), tissue-type plasminogen activator (t-PA) and plasminogen also bound to cell surface receptors (46), and MT-MMPs, transmembrane matrix metalloproteases (52). Precursor forms of membrane-associated proteases can be activated by soluble proteases, and secreted precursors can be activated by membrane-associated proteases (53). Therefore, we speculate that the binding proteins responsible for the localization of procathepsin B to discrete regions on the external surface of tumor cells and their activation at those sites may delineate the role(s) of this enzyme in tumor invasion and metastasis.
In the present study, we have identified p11 (one of the two subunits of the annexin II tetramer) as a putative binding protein for procathepsin B using a yeast two-hybrid system. We confirmed that recombinant procathepsin B interacted with recombinant p11 and the annexin II tetramer in vitro and in vivo, including on the surface of tumor cell lines (BT20 and U87) of epithelial and mesenchymal origin. Furthermore, the binding of procathepsin B to the annexin II tetramer may result in activation of procathepsin B. Thus, our results provide a link between membrane association and activation of cathepsin B.

Annexin II (p36) is a Ca^{2+}-dependent phospholipid-binding protein (54), and p11 belongs to the S100 family of proteins (55), with two subunits of p11 and two subunits of p36 forming the annexin II heterotetramer (35). Although p11 and p36 lack signal peptides, membrane-bound p36 has been found on the extracellular surface of a diversity of cell types including keratinocytes (43), endothelial (46), glioma, and smooth muscle cells (44), as has the annexin II tetramer on endothelial (45) and epithelial cells (42). Extracellular p36 may be important in several biological processes, such as fibrinolysis, cell adhesion, ligand-mediated cell signaling, and viral infection (41). A wealth of evidence has suggested that p11 modulates the activity of the p36 subunit (56–59) and that binding of p36 to phospholipid is p11- and Ca^{2+}-dependent (28). The annexin II tetramer (p36 and p11) on the surface of human umbilical vein endothelial cells has been shown to stimulate t-PA-dependent plasminogen activation (40). Thus, it is of interest that the annexin II tetramer has been reported to be up-regulated on the surface of tumor cells (42) and that t-PA has been shown to activate procathepsin B in vitro (60). Whether t-PA can activate procathepsin B on the surface of tumor cells requires further investigation. Our data do show that mature cathepsin B was present on the cell surface (Fig. 4), although mature cathepsin B did not directly interact with the annexin II tetramer. We cannot rule out the possibility that other membrane proteins are involved in regulating the interaction between mature cathepsin B and the annexin II tetramer.

The annexin II tetramer on the cell surface also plays roles in cell and matrix interaction. p36 had previously been isolated as a collagen-binding protein from plasma membrane fractions of mammary tumors (61). p36 has also been found to serve as a cell surface receptor for tenascin-C (62). Tenascin-C is an extracellular protein and shows a restricted expression pattern during development (63). Although tenascin-C is missing from most adult tissues, it reappears at places where active tissue regeneration and cell migration occurs, namely in a large range of tumors (64, 65), in wound healing (66–69), and in regenerating nerves (70, 71). Perhaps, the interaction between the annexin II tetramer and matrix proteins may facilitate selec-
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...ative degradation of extracellular matrices during tumor invasion... 

Althought we know that the annexin II tetramer interacts with proteases and matrix proteins on the cell surface, how these interactions affect the extracellular matrix degradation remains unkown. The annexin II tetramer also interacts with membrane proteins such as integrins (72) and caveolin (73). Recently, Wei et al. reported that urokinase-type plasminogen activator receptor complexes with caveolin and β1 integrin on the cell surface (74). Such a common locus for these proteins is of potential functional importance, as cathepsin B has been shown to activate soluble and receptor-bound prourokinase (75). In this regard, in ovarian cancer cells, inhibition of cell surface cathepsin B prevents activation of prourokinase and, thereby, their invasion through Matrigel (75). Taken together, it is tempting to assume that the annexin II tetramer along with other proteins (such as urokinase-type plasminogen activator receptor, caveolins, and integrins) may serve as an orga... 

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REFERENCES

1. Migone, P., Robbins, E., and Riklin, D. B. (1986) Cell 47, 487–498
2. Bergman, I., and Sussman, I. (1989) Intracellular Protein Catalysis (Suzuki, K. and Bond, J., eds) pp. 281–294, Plenum Publishing Corp., New York
3. Campo, E., Munoz, J., Miquel, R., Palacin, A., Sloane, B. F., and Emmert-Buck, M. (1994) Am. J. Pathol. 145, 301–309
4. Rempel, S. A., Rosenblum, M. L., Mikkelsen, T., Yan, P. S. Ellis, K. D., Rempel, S. A., Rosenblum, M. L. (1995) J. Biol. Chem. 270, 12335–12340
5. Lah, T. T., Buck, M. R., Honn, K. V., Crissman, J. D., Rao, N. C., Liotta, L. A., and Stetler-Stevenson, W. G. (1994) Am. J. Pathol. 145, 1291–1299
6. Frosch, B. A., Berquin, I., Emmert-Buck, M. R., Moin, K., and Sloane, B. F. (1999) Acta Pathol. Microbiol. Scand. 107, 28–37
7. Lah, T. T., Buck, M. R., Honn, K. V., Crissman, J. D., Rao, N. C., Liotta, L. A., and Sloane, B. F. (1989) Clin. Exp. Metastasis 7, 461–468
8. Ugarova, T. P., Ljubimov, A. V., Deng, L., and Plow, E. F. (1996) Biochemistry 35, 10913–10921
9. Kobayasahi, H., Schmitt, M., Goretzki, L., Chucholowska, N., Calvete, J., Kramer, M., Gunzler, W. A., Janicke, F., and Graefl, H. (1991) J. Biol. Chem. 266, 5417–5425
10. Eckehut, Y., Vaes, G. (1977) Biochem. J. 166, 21–31
11. Nishimura, Y., Kawahata, T., and Kata, K. (1988) Arch. Biochem. Biophys. 261, 84–71
12. Sloane, B. F., Moin, K., Samesi, M., and Sloane, B. F. (1999) Acta Pathol. Microbiol. Scand. 107, 28–37
13. Mikkelsen, T., Yan, P. S., Hjort, J., and Sloane, B. F. (1995) Pathol. Oncol. Res., 1, 43–52
14. Lah, T. T., Buck, M. R., Honn, K. V., Crissman, J. D., Rao, N. C., Liotta, L. A., and Sloane, B. F. (1989) Clin. Exp. Metastasis 7, 461–468
15. Sloane, B. F., Moin, K., Samesi, M., Tait, L. R., Rzehik, J., and Ziegler, G. (1994) J. Cell Sci. 107, 373–384
16. Ugarova, T. P., Ljubimov, A. V., Deng, L., and Plow, E. F. (1996) Biochemistry 35, 10913–10921
17. Kobayasahi, H., Schmitt, M., Goretzki, L., Chucholowska, N., Calvete, J., Kramer, M., Gunzler, W. A., Janicke, F., and Graefl, H. (1991) J. Biol. Chem. 266, 5417–5425
18. Eckehut, Y., Vaes, G. (1977) Biochem. J. 166, 21–31
19. Nishimura, Y., Kawahata, T., and Kata, K. (1988) Arch. Biochem. Biophys. 261, 84–71
20. Sloane, B. F., Moin, K., Samesi, M., Tait, L. R., Rzehik, J., and Ziegler, G. (1994) J. Cell Sci. 107, 373–384
21. Mikkelsen, T., Yan, P. S., Hjort, J., and Sloane, B. F. (1995) Pathol. Oncol. Res. 1, 43–52
22. Lah, T. T., Buck, M. R., Honn, K. V., Crissman, J. D., Rao, N. C., Liotta, L. A., and Sloane, B. F. (1989) Clin. Exp. Metastasis 7, 461–468
23. Ugarova, T. P., Ljubimov, A. V., Deng, L., and Plow, E. F. (1996) Biochemistry 35, 10913–10921
24. McIntrye, G. F., and Erickson, A. H. (1991) J. Biol. Chem. 266, 15438–15445
25. Johnson, L. M., Bankaitis, V. A., and Emr, S. D. (1987) Cell 48, 875–885
26. Valls, L. A., Hunter, G. P., Rothman, J. H., and Stevens, T. H. (1987) Cell 48, 887–897
27. Nakamura, M., and Fingleton, B. (1991) J. Biol. Chem. 266, 14335–14340
28. Sato, O., and Ohtsuki, K. (1991) J. Biol. Chem. 266, 9779–9783
29. Dethlefsen, L., and Kamen, R. D. (1995) J. Biol. Chem. 270, 26693–26696
Human Procathepsin B Interacts with the Annexin II Tetramer on the Surface of Tumor Cells

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