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Development and Characterization of a New Polyclonal Antibody Specifically against Tissue Inhibitor of Metalloproteinases 4 in Human Breast Cancer

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Tissue inhibitors of metalloproteinases (TIMPs) may regulate extracellular matrix turnover and cellular functions by modulating matrix metalloproteinase (MMP) activity and cell proliferation and apoptosis. To investigate the locations and functions of TIMP-4 in human breast cancer, a highly specific polyclonal anti-TIMP-4 peptide antibody (pAb-T4-S61) was developed. The potency and specificity of the purified IgG were characterized by an enzyme-linked immunosorbent assay, immunoblot, and immunohistochemistry. The optimal IgG concentration range was 0.1–10 μg/ml. pAb-T4-S61 did not cross-react with TIMP-1 and TIMP-2 and should not react with TIMP-3 according to the sequence analysis. Parental MDA-MB-435 breast cancer cells were TIMP-4 negative and a TIMP-4 transfected clone, TIMP-4-435-12, produced TIMP-4. Membrane type-1 MMP was detected although TIMP-2 was not found in these cells. Interestingly, the TIMP-4 protein was detected by immunohistochemical staining in infiltrating breast carcinoma cells in tumor tissues. Thus, pAb-T4-S61 is a useful tool to investigate expression patterns and functions of TIMP-4 in cancers.

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Abbreviations used: Ab, antibody; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; MMP, matrix metalloproteinase; MMP-2, matrix metalloproteinase 2, gelatinase A; MT1-MMP, membrane type 1 matrix metalloproteinase; pAb-T4-S61, a polyclonal antibody against a TIMP-4 peptide starting from residue Ser^{61}; TIMP, tissue inhibitor of metalloproteinases.

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Key Words: **tissue inhibitors of metalloproteinases (TIMPs); new TIMP-4-specific antibody; matrix metalloproteinases (MMPs); extracellular matrix (ECM); human breast cancer; proliferation and apoptosis; enzyme regulation; enzyme-linked immunosorbent assay (ELISA); enhanced chemiluminescence (ECL); immunohistochemistry.**

Extracellular matrix (ECM) turnover is regulated by the balance of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Under normal physiological conditions a delicate balance of MMPs and TIMPs is maintained, however, during a number of pathological situations the balance is disrupted leading to tumor metastasis and connective tissue diseases such as arthritis (1, 2). Thus, the balance between TIMP levels and activated MMP levels is important in the regulation of ECM metabolism.

Although important as MMP inhibitors, individual members of the TIMP family may have other physiological roles that are equally important. Particular interest has focused on the formation of pro-MMP/TIMP complexes that are involved in the activation of latent MMPs. A specific well-documented example is the activation of pro-MMP-2 (pro-gelatinase A) on the cell surface through a ternary MT1-MMP/TIMP-2/MMP-2 complex (3–5). Other non-inhibitory MMP/TIMP complexes have been demonstrated, including MMP-9/TIMP-1 (6) and MMP-2/TIMP-4 (7). TIMP family members may also play an important role in angiogenesis and apoptosis (8). The regulation of TIMP expression is unique for each member. Both TIMP-1 and TIMP-3 are induced by cytokines and hormones but TIMP-2 expression is mainly constitutive (9, 10). Additionally, TIMP-3 expression is subject to cell cycle regulation

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(11). TIMP-4 expression is tissue-specific with the highest expression levels in heart (12).

By overexpressing TIMP-4 in human breast cancer cells, tumor growth and metastasis may be inhibited (13). Although this inhibition is presumably due to anti-angiogenic activity, the complete biochemical mechanism and specific breast cancer tissue localization are unknown. To further investigate the function of TIMP-4 in human breast cancer, a polyclonal antibody (pAb) specifically against TIMP-4 was produced and characterized. This may lead to a more complete understanding of TIMP functions and new treatments specifically against the invasive spread of breast cancer.

MATERIALS AND METHODS

Cell culture. MDA-MB-435 human breast cancer cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD, and were cultured as described previously (13). The full-length sequence of human TIMP-4 was subcloned into the pCI-neo mammalian Expression Vector (Promega) and expressed as described previously (13). Recombinant TIMP-4 protein was also expressed in SF9 insect cells that were infected with TIMP-4 transfected baculovirus as described previously (14).

Antibody production. A peptide corresponding to a unique TIMP-4 sequence (Ser^{61}-Ala-Asp-Pro-Ala-Asp-Thr-Glu-Lys-Met-Leu-Arg-Tyr-Glu^{74}-NH_2) (residue numbering starts from Met^1 of the proTIMP-4 protein that contains a signal peptide) was synthesized by Dr. Umesh Goli at the Biochemical Analysis, Synthesis, and Sequencing Service Laboratory in the Department of Chemistry at Florida State University. The peptide was purified by Sephadex-G15 size exclusion chromatography, and the purity was verified by reverse phase high-performance liquid chromatography. The molecular mass of the peptide is 1.63 kDa, its isoelectric point is 4.2, and its molar extinction coefficient at 280 nm is 1280 $M^{-1}cm^{-1}$ based on calculations previously described (15, 16). The purified TIMP-4 peptide (61–74) was coupled to keyhole limpet hemocyanin (Sigma Chemical Co.) with 2% glutaraldehyde in 0.2 M phosphate buffer, pH 7.0. One milligram of the conjugated peptide was emulsified in Freund's complete adjuvant (Sigma Chemical Co.) and injected into each of three New Zealand white rabbits. Subsequently, 1 mg of the conjugated peptide was emulsified in Freund's incomplete adjuvant (Sigma Chemical Co.) and used to boost the rabbits at 6-week intervals. The rabbits were bled 1 week after each boost. Rabbit sera were taken before and after the immunization. Polyclonal antibodies against MT1-MMP were prepared as previously described (17). Human TIMP-1 and TIMP-2 proteins and monoclonal antibodies against them were kindly provided by Drs. L. Jack Windsor of the Indiana University, IN, and Rafael Fridman of the Wayne State University, MI, respectively.

Ab purification and characterization. The immunoglobulin G (IgG) molecules of the antipeptide antiserum were purified by two-step affinity chromatography. Firstly, IgG molecules in antiserum were purified by a protein A affinity column kit (Pierce Chem. Co.) according to the manufacturer's directions. Secondly, peptide antigen was coupled to Affi-gel 10 gel (Bio-Rad) to form an antigen affinity column. Then, the protein A purified IgG was further purified by the peptide antigen affinity chromatography. The final purified IgG molecules were stored at −80°C in 0.1 M glycine, 60 mM Tris, and 0.02% sodium azide, pH 7.4. Preimmune IgG molecules were purified from preimmune serum by a protein A affinity column for negative control experiments. An enzyme-linked immunosorbent assay (ELISA) was used to assess the potency and specificity of the rabbit polyclonal Ab as described previously (17, 18).

Immunoblot analysis and immunohistochemical staining. Immunoblot analysis was performed as described previously (17) with the following exceptions: electrophoresis was performed with 12% polyacrylamide gels, the secondary Ab was conjugated to horseradish peroxidase (Chemicon), and enhanced chemiluminescence (ECL) was performed (Amersham-Pharmacia). Immunohistochemical staining was performed as described previously (19). In brief, the fixed and paraffin embedded breast cancer tissue sections were deparaffinized. The sections were incubated with the two-step affinity purified pAb-T4-S61 (0.5 μg/ml IgG) as the primary antibody and the biotin-conjugated anti-rabbit IgG (DAKO) as the secondary antibody. The colorimetric detection was performed using a standard indirect streptavidin-biotin immunoreaction method by DAKO's Universal LSAB kit according to the manufacturer's instructions.

RESULTS

Specificity of the Antibody

The peptide antigen sequence Ser^{61}-Ala-Asp-Pro-Ala-Asp-Thr-Glu-Lys-Met-Leu-Arg-Tyr-Glu^{74}-NH_2 shares less than 45% homology with any other peptides. This peptide sequence was utilized in a search-and-sequence alignment analysis performed against all the most current protein data banks by using basic local alignment search tool (BLAST) programs (National Center for Biotechnology Information (Bethesda, MD), http://www.ncbi.nlm.nih.gov), which are used to perform database searches and rigorous statistical analyses for evaluating the significance of matches. The only sequences identified were the corresponding TIMP-4 peptides from different species. Therefore, this unique sequence is a specific antigen for generating a peptide Ab against human TIMP-4 and TIMP-4 of other species. However, it will not cross-react with the other three TIMPs, TIMP-1, -2, and -3.

The purified antibody against the TIMP-4 peptide (pAb-T4-S61) was characterized by ELISA (Fig. 1). ELISA plates were coated with 50 μl of TIMP-4 peptide (61–74) in concentrations ranging from 0 to 256 nM (Fig. 1A) or recombinant TIMP-4 protein in concentrations ranging from 0 to 300 nM (Fig. 1B). This Ab is very potent. The sensitivity of 5 μg/ml IgG is approximately 5 picomole TIMP-4 protein with an absorbance value of 1 at 405 nm (Fig. 1B). The optimum dilution range of the 1 mg/ml IgG stock was 1:100 to 1:800. The Ab is also very specific. Crude TIMP-4 protein was used as a competitor (Fig. 1C). The crude TIMP-4 protein was pre-incubated with pAb-T4-S61 before addition to the peptide antigen coated ELISA plate to test the specificity of the Ab.

Immunoblot analysis was performed with the Ab directed against TIMP-4 (Fig. 2A). The conditioned media of the parental MDA-MB-435 and TIMP-4 transfected clone were examined. Specific Abs against TIMP-1 and TIMP-2 were used as controls (Figs. 2B and 2C). In addition, the glycosylated form (30 kDa) of

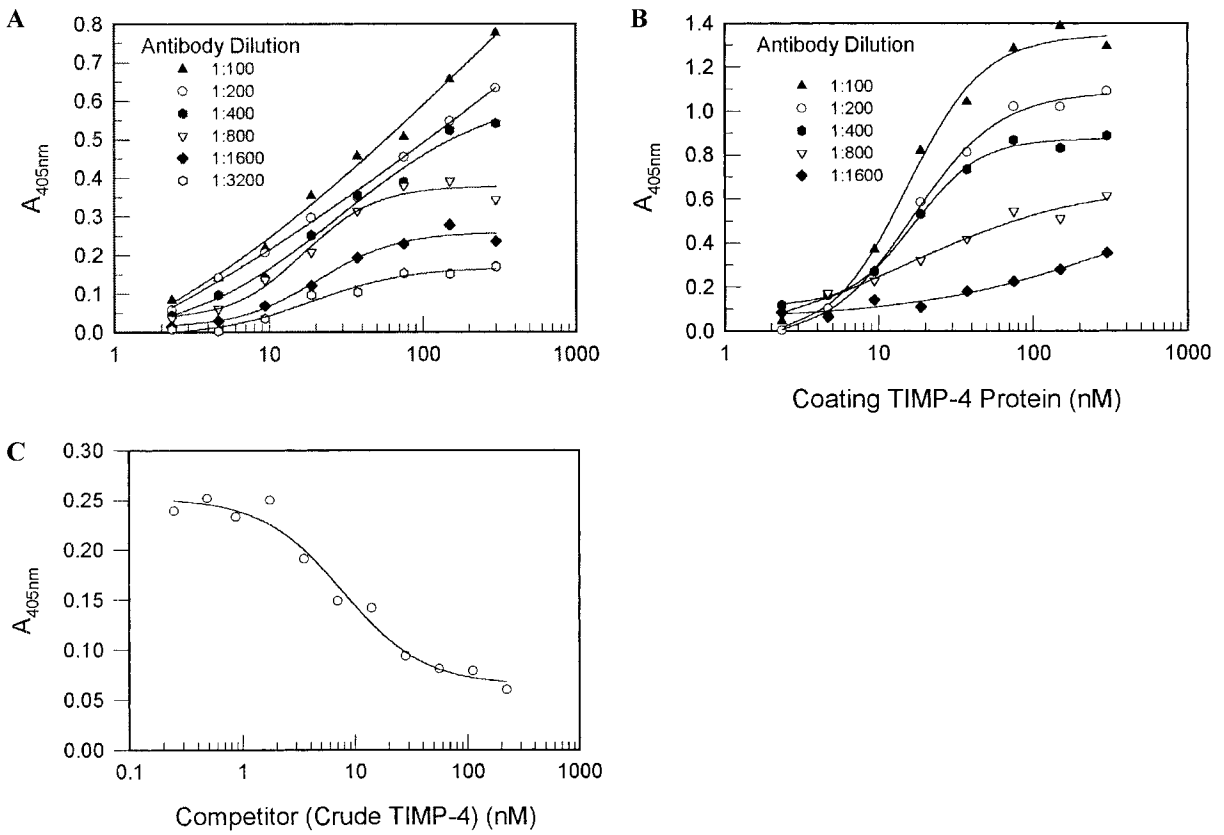

FIG. 1. ELISA characterization of antipeptide antibody pAb-T4-S61. ELISA plates were coated with various concentrations of TIMP-4 peptide (61–74) (A) and TIMP-4 protein (B). The antibody dilutions are indicated on each diagram. (C) Competition ELISA was performed using the synthetic peptide as a coating antigen and preincubating pAb-T4-S61 with selected concentrations of crude TIMP-4 protein as the competitor. Then, the TIMP-4 protein pretreated Ab was added to the wells. The stock pAb-T4-S61 used was the two-step affinity purified IgG at 1 mg/ml. A_{405nm} is the absorbance at 405 nm.

TIMP-1 protein was detected in the parental MDA-MB-435 cell medium (Fig. 2B), although neither TIMP-4 nor TIMP-2 was found in the same sample (Figs. 2A and 2C, respectively). Interestingly, the TIMP-4 transfected TIMP-4-435-12 cells no longer produced TIMP-1 protein when TIMP-4 was produced (Fig. 2A). pAb-T4-S61 did not cross-react with TIMP-1 and TIMP-2 proteins. Because this TIMP-4 peptide antigen sequence is only 14.3% identical to the corresponding region of TIMP-3 (15), pAb-T4-S61 should not recognize TIMP-3 protein. This experiment further confirms that pAb-T4-S61 is highly specific.

Detection of TIMP-4 Protein in an Infiltrating Breast Carcinoma Tissue

Immunohistochemical staining with the purified pAb-T4-S61 was performed in human breast tissue sections (Fig. 3). TIMP-4 was localized on the malignant breast epithelial cells. The specificity of the Ab was confirmed by preincubating the Ab with recombinant TIMP-4 (rTIMP-4) protein before immunostaining. Strong TIMP-4 staining was detected in the infiltrating breast cancer cells; however, no signal of TIMP-4 staining was detected in the same sample when the Ab was pre-absorbed by rTIMP-4. These results further confirm the antibody's TIMP-4 specificity and potency. Strong TIMP-4 staining was detected in the malignant infiltrating breast cancer cells compared with very weak signals of TIMP-4 staining on the residual normal ductal and lobular epithelial cells.

Expression of MT1-MMP and TIMP-4

The parental malignant human breast cancer cell line MDA-MB-435 expressed TIMP-1 protein, however, neither TIMP-2 nor TIMP-4 was expressed (Fig. 2). The TIMP-4 transfected clone expressed TIMP-4 protein as expected; however, it was surprising that the TIMP-1 expression was suppressed. The plasma-membrane fraction (detergent DT phase) of these breast cancer cells was analyzed for MT1-MMP using a specific Ab characterized previously (17). MT1-MMP at molecular mass of 63 kDa was detected in the samples

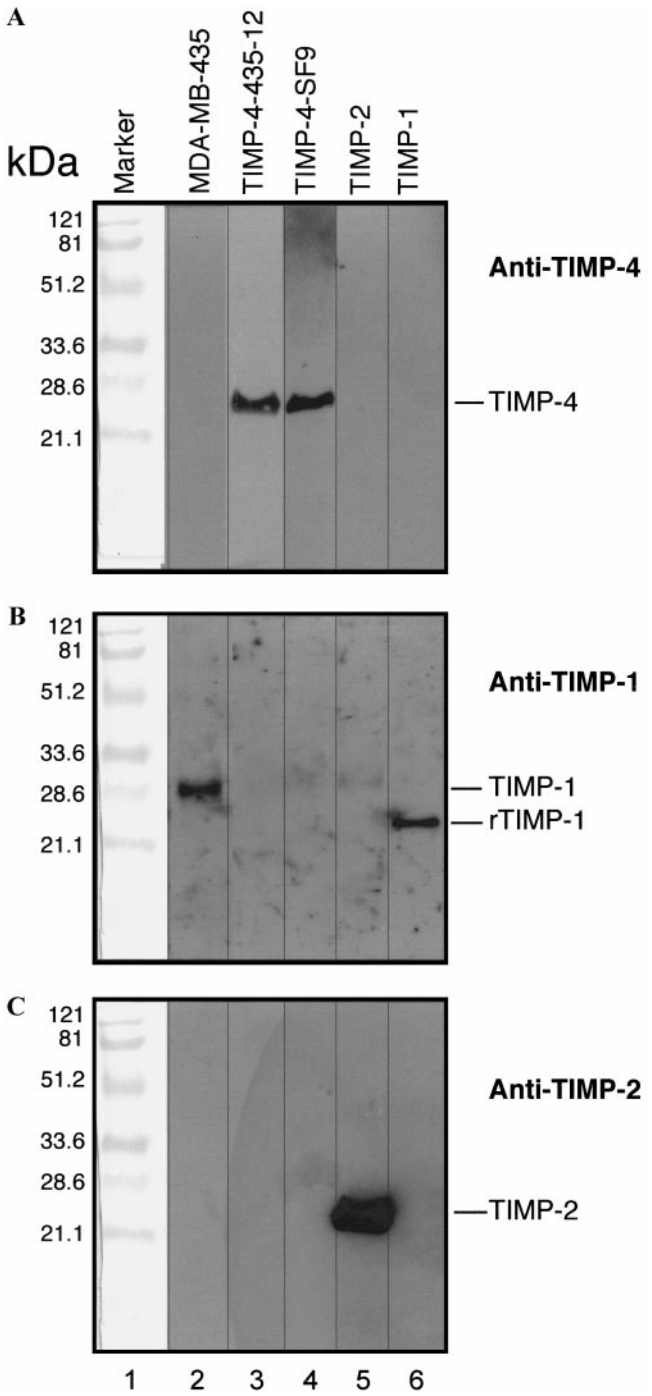

FIG. 2. Characterization of pAb-T4-S61 by immunoblot analysis. The conditioned media of MDA-MB-435 and the TIMP-4 transfected clone, TIMP-4-435-12, were subjected to 12% SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with pAb-T4-S61 (A), anti-TIMP-1 (B), and anti-TIMP-2 (C) and were developed using enhanced chemiluminescence (ECL). The insect cell line TIMP-4-SF9 was used as a positive TIMP-4 control and recombinant TIMP-1 and -2 were used as negative controls. Prestained low molecular weight markers were also electrophoresed and transferred, however, were not subject to ECL.

of both the parental cell line and the transfected clone (Fig. 4). TIMP-2, which has been shown to mediate the activation of pro-MMP-2 by MT1-MMP on the cell surface, was not detected in these cell lines.

DISCUSSION

We report here that a specific rabbit Ab against a unique human TIMP-4 peptide sequence has been produced, purified, and characterized. This peptide sequence corresponds to residues 61–74 of TIMP-4 (Ser^{61}-Ala-Asp-Pro-Ala-Asp-Thr-Glu-Lys-Met-Leu-Arg-Tyr-Glu^{74}-NH_2). pAb-T4-S61 is highly specific to TIMP-4 and did not cross-react with TIMP-1 or TIMP-2. Because this TIMP-4 peptide antigen sequence is only 14.3% identical to the corresponding region of TIMP-3 (15), pAb-T4-S61 should not recognize TIMP-3 protein. Although commercial polyclonal antibodies directed against TIMP-4 are available (Chemicon), they are not very specific and do not work for immunohistochemistry staining (data not shown). Additionally, pAb-T4-S61 proves useful in a number of experimental techniques, including ELISA, immunoblot, and immunohistochemistry.

A TIMP-4 transfected clone of the human breast cancer cell line (MDA-MB-435) was probed with pAb-T4-S61. Although the parental cell line did not express TIMP-4, the transfected clone produced a high level of TIMP-4 protein. Interestingly, the parental cell line expressed TIMP-1, however, the transfected clone suppressed TIMP-1 expression. The regulation and coordination of the expression of different TIMPs are not fully understood. The mechanism by which TIMP-4 suppresses TIMP-1 expression is not known. Future investigations may reveal the mechanisms of coordination, competition, and compensation related to the gene regulation and protein expression of different TIMPs in cells.

The TIMP-4 expression pattern in human breast cancer tissue is very intriguing. It seems that the TIMP-4 protein expression level is correlated with the degree of malignancy. Very low levels TIMP-4 protein was detected in the normal ductal and lobular breast epithelial cells, however, very high levels of TIMP-4 protein was found in the malignant infiltrating breast cancer carcinoma cells (Fig. 3). These results may be paradoxical to the traditional belief that TIMPs can suppress tumor invasion and metastasis. It is possible that cancer cells are expressing high levels of TIMP-4 to overcome the elevated MMP expression levels, thus, controlling the excessive ECM degradation that is harmful to cancer cell survival. In addition, TIMP-4 may be required for pro-MMP activation and cell interaction with ECM. Furthermore, TIMP-4 may have functions unrelated to its anti-metalloproteinase activity, such as preventing cancer cell apoptosis or promot-

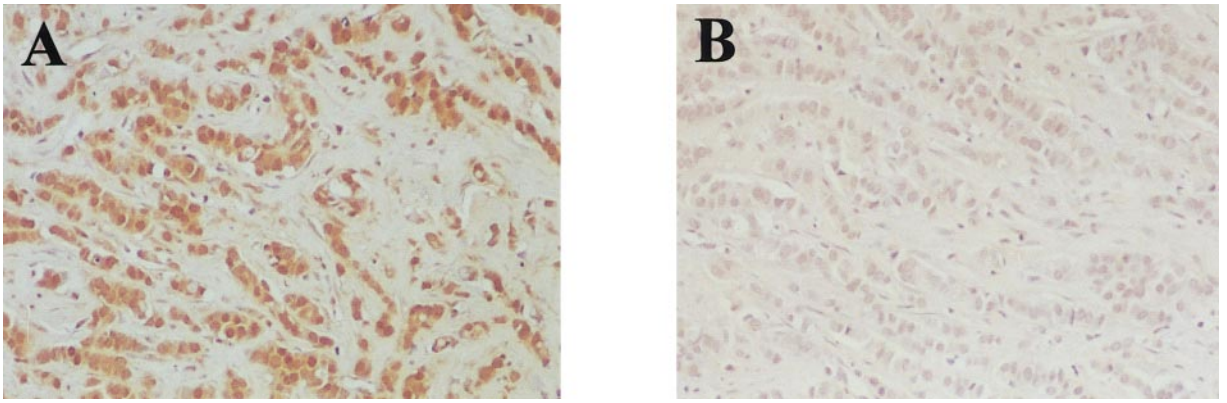

FIG. 3. Immunohistochemical staining of TIMP-4 protein in an infiltrating breast carcinoma tissue. (A) Strong TIMP-4 protein staining was detected in the infiltrating breast cancer cells with the two-step purified pAb-T4-S61 (0.5 μg/ml IgG). (B) No signal of TIMP-4 protein was detected in the same samples when the primary antibody was preincubated with rTIMP-4 (0.3 mg/ml), showing that the antibody is highly specific to TIMP-4 protein.

ing cancer cell proliferation (15). Thus, the roles of TIMPs and MMPs in cancer are very intricate and not well-understood. Further investigations are required to understand why high levels of TIMP-4 protein were found in malignant breast cancer tissues and not normal breast tissues.

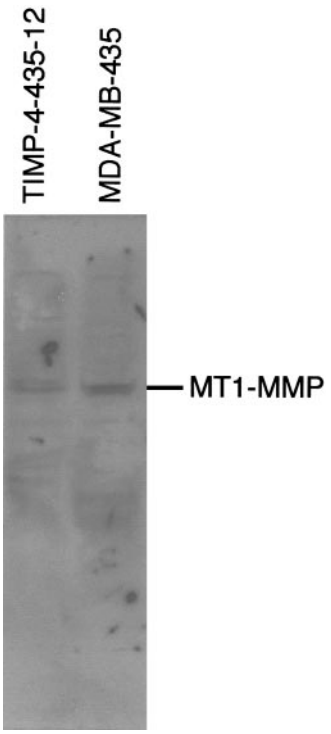

FIG. 4. Detection of MT1-MMP in the cell lysate. The breast cancer cell lysates of the parental MDA-MB-435 and the TIMP-4 transfected clone, TIMP-4-435-12, were analyzed by immunoblotting (as described in the legend to Fig. 2) with an antibody (pAb-MT1-160) against MT1-MMP that was previously characterized (17). A MT1-MMP Ab-positive band at approximately 63 kDa was detected in both cell lines.

The MT1-MMP/TIMP-2/MMP-2 ternary complex has been well documented (3–5). The N-terminal inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP while the C-terminal of TIMP-2 binds to the hemopexin domain of pro-MMP-2. This concentrates the pro-MMP-2 near the cell surface and enables an active MT1-MMP free of TIMP-2 to process the latent MMP-2 to the active form. TIMP-4 is most similar to TIMP-2 according to the primary structure analysis (15). It has also been shown to bind C-terminally to the hemopexin domain of MMP-2 leaving the N-terminal domain of TIMP-4 free (7). An efficient membrane anchored ternary activation complex with TIMP-4 may also exist and TIMP-4 may facilitate pro-MMP2 activation in the absence of TIMP-2. The specific pAb-T4-S61 will be useful to further characterize the involvement of TIMP-4 in human breast cancer and many other normal and pathological processes.

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