SPARC/Osteonectin Induces Matrix Metalloproteinase 2 Activation in Human Breast Cancer Cell Lines

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ABSTRACT

Activation of the matrix metalloproteinase 2 (MMP-2) has been shown to play a major role in the proteolysis of extracellular matrix (ECM) associated with tumor invasion. Although the precise mechanism of this activation remains elusive, levels of the membrane type 1-MMP (MT1-MMP) at the cell surface and of the tissue inhibitor of MMP-2 (TIMP-2) appear to be two important determinants. Induction of MMP-2 activation in cells cultivated on collagen type I gels indicates that the ECM is important in the regulation of this process. In this study, we show that SPARC (osteonectin) in a small ECM-associated matricellular glycoprotein, can induce MMP-2 activation in two invasive breast cancer cell lines (MDA-MB-231 and BT549) but not in a noninvasive counterpart (MCF-7), which lacks MT1-MMP. Using a set of peptides from different regions of SPARC, we found that peptide 1.1 (corresponding to the NH2-terminal region of the protein) contained the activity that induced MMP-2 activation. Despite the requirement for MT1-MMP, seen in MCF-7 cells transfected with MT1-MMP, the activation of MMP-2 by SPARC peptide 1.1 was not associated with increased steady-state levels of MT1-MMP mRNA or protein in either MT1-MMP-transfected MCF-7 cells or constitutively expressing MDA-MB-231 and BT549 cells. We did, however, detect decreased levels of TIMP-2 protein in the media of cells incubated with peptide 1.1 or recombinant SPARC; thus, the induction of MMP-2 activation by SPARC might be due in part to a diminution of TIMP-2 protein. We conclude that SPARC, and specifically its NH2-terminal domain, regulates the activation of MMP-2 at the cell surface and is therefore likely to contribute to the proteolytic pathways associated with tumor invasion.

INTRODUCTION

MMPs are a family of proteases that degrade specific ECM components. Among them, MMP-2 (M, 72,000 type IV collagenase/gelatinase A) cleaves collagen type IV and has therefore been implicated in basement membrane degradation associated with tumor progression (1). More recently, it has been shown to digest collagen type I and could thus also be implicated in the invasion of interstitial connective tissue (2, 3).

MMP-2 is secreted as an inactive proenzyme, and its activation requires the proteolytic removal of the NH2-terminal profragment (4–7). The activation of MMP-2 is a membrane-associated mechanism that involves the expression at the cell surface of another MMP (MT1-MMP; Refs. 8 and 9). Unlike most other MMPs, MT1-MMP and the three other members of the MT-MMP subgroup contain a transmembrane domain (reviewed in Ref. 9). Earlier studies have described MT1-MMP as two major bands of Mr 63,000 and Mr 60,000, which would correspond to the pro and the active form of MT1-MMP, respectively (10–13). A proteolytically processed third form of Mr 43,000 has been correlated with MMP-2 activation induced by Con A and phorbol myristic acid in HT1080 fibrosarcoma cells (12). MMP-2 is unique in its binding of TIMP-2 before activation (7, 14–16). Moreover, Strongin et al. (17) have identified a complex of TIMP-2, MMP-2, and MT1-MMP at the plasma membrane of HT1080 cells treated with 12-O-tetradecanoylphorbol-13-acetate. Although the exact mechanism of MMP-2 activation has not been elucidated, the participation of these three proteins in the process has been established (7, 16–19).

A number of studies in vitro and in vivo support a significant role for MMP-2 and MT1-MMP in tumor invasion. In situ hybridization has shown that mesenchymal cells (and particularly peritumoral fibroblasts) are the major source of both proteases in most cancers (reviewed in Ref. 20). It has been suggested, however, that the expression of MMPs by tumor cells themselves might be representative of a late stage of tumor progression or of a migratory/invasive phenotype (21, 22). The contribution of invasive tumor cells to the expression and/or activation of MMP-2 and MT1-MMP has been clearly documented in vitro (1, 9, 23–25).

Activation of MMP-2 can be induced in vitro by several agents including Con A, 12-O-tetradecanoylphorbol-13-acetate, transforming growth factor β1, and tumor necrosis factor α (7, 16). Although these studies show a tightly controlled regulation of this process, molecules responsible for MMP-2 activation in vivo have not been identified. We have shown that MMP-2 activation can be induced in invasive human breast cancer cells cultivated in or on gels of native collagen type I, a configuration thought to mimic the invasion of interstitial tissue by tumor cells (24, 26). A similar activation has been documented in human fibroblasts by us (24, 26) and others (27–29) and, more recently, in endothelial cells (30). In general, the regulation of invasive behavior by ECM components or ECM-associated molecules has been documented in many models and appears to be a mechanism important for tumor invasion.

The matricellular proteins are a specific class of ECM-associated components that regulate interactions between cells and the ECM (31). Included within this group is SPARC (also known as osteonectin or BM-40; reviewed in Ref. 32). SPARC is associated with morphogenesis and tissue remodeling and has been shown to regulate several cellular functions contributing to invasive behavior, such as cell spreading and adhesion. It has also been shown to modulate the expression of several MMPs in fibroblasts and in human monocytes (33, 34). Recent studies have also described an association between SPARC expression and tumor progression in several cancers including those of the colon, breast, lung, kidney, adrenal cortex, brain, and ovary (35–39). Moreover, Ledda et al. (40) have demonstrated that...
human melanoma cells transfected with SPARC antisense RNA exhibited decreased tumorigenic and metastatic behavior.

In the present study, we asked whether SPARC might participate in collagen type I-induced MMP-2 activation. We now report that SPARC alone was sufficient to induce MMP-2 activation in invasive cancer cells in an MT1-MMP-dependent manner.

MATERIALS AND METHODS

Cell Culture. The six human mammary cancer cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD). Their invasive properties and differentiation states have been described previously (41). Briefly, four cell lines (MDA-MB-435, MDA-MB-231, BT549, and Hs578T) display high invasive capacities in vitro and have lost some epithelial features, such as expression of E-cadherin. In contrast, two cell lines (MCF-7 and T47D) have retained many epithelial features and do not display highly invasive properties. The M1T-MMP-transfected cell lines (nos. 2-6 and 8-5) were isolated after transfection of MCF-7 cells with a human MT1-MMP cDNA in the cytomegalovirus-promoter driven, neomycin-resistance vector pCNCXho. The M1T-MMP-transfected clones partially activate MMP-2 to the M=62,000 intermediate, which is activated further to the M=59,000 mature form in the presence of ConA. All cells were cultivated in IMEM supplemented with 10% FBS. Cultures were maintained at 37°C and confirmed as Mycoplasma-free with the Genprobe kit (Gen-Probe, San Diego, CA). All chemicals and culture media were purchased from Sigma Chemical Co. (St. Louis, MO) or Life Technologies, Inc. (Gaithersburg, MD).

Anti-Integrin Antibody Blocking Experiments. Blocking experiments were performed in 24-well plates using anti-α2 integrin antibody clones P16E (Chemicon, Temecula, CA) and 6F1 (kindly provided by Dr. B. Collier). The Mount Sinai Medical Center, New York) and anti-β1 blocking antibody clones P5D2 (Chemicon) and Mab13 (kindly provided by Dr. S. Akiyama, National Institute of Dental Research, NIH, Bethesda, MD). Cells (5×10⁵) were preincubated with the antibodies (used at concentrations ranging from 1 to 10 µg/ml for P5D2 and P16E and up to 50 µg/ml for Mab13; PD52 and P16E were also tried in combination) for 30 min at room temperature in IMEM containing 5% FBS. The cells were then either plated on plastic in 10% FBS-IMEM for 24 h or placed in a collagen type I solution (250 µl of the solution/well at 2 mg/ml collagen: Vitrogen, Collagen Corporation, Palo Alto, CA). After polymerization of the collagen, the gels were covered for 24 h in 10% FBS-IMEM containing the same concentration of antibody as in the gels. Cultures were then washed twice in SFM (IMEM supplemented with 1% HEPES buffer, 1% glucose, 1% nonessential amino acids, and 0.4% insulin-transferrin-selenium mixture) and incubated for 72 h in a mixture containing SFM (75%) and SFM conditioned for 72 h by MMP-2-transfected MCF-7 cells (25%). The same concentration of antibody was maintained in the SFM/MMP-2 medium, and the activation of MMP-2 was analyzed as described below.

rSPARC and SPARC Peptides. rSPARC was produced in Escherichia coli and was purified as described previously (42). The synthetic peptides used in this study are a series of 20- or 21-mers spanning different regions of the bovine (peptide 1.1: QEALPDETEVVEETVAEVAEV) or mouse (peptide 4.2: TCDLDNDKYIALEE-) SPARC sequences and rSPARC or peptides as that within the gel. After 72 h, the conditioned media were collected for zymography analysis, and the cells were harvested for Western blotting.

Gelatin Zymography Assays. Samples were denatured under nonreducing conditions and were resolved by SDS-PAGE on 10% gels containing 0.1% gelatin (w/v). The gels were washed for 1 h at room temperature in a 2% Triton X-100 solution (v/v), transferred to a buffer containing 10 mM CaCl₂, 50 mM Tris-HCl (pH 7.6), and incubated overnight at 37°C. The gels were stained for 30 min with 0.1% (w/v) Coomassie Blue G 250 in 45% methanol (v/v):10% acetic acid (v/v) and were destained in 10% acetic acid (v/v):20% methanol (v/v).

Northern Blot Analysis. For Northern blotting, experiments with the different SPARC peptides were scaled up into six-well plates (30×10⁴ to 50×10⁴ cells were used and placed in a volume of 1.5 ml) but were otherwise performed as described above. After 72 h, the conditioned media were collected for zymographic and/or for TIMP-2 analyses, and the cells were collected for Northern blotting. The cells were recovered from the gels by treatment with collagenase (type 1A, 1 mg/ml in PBS; Sigma). For analysis of SPARC mRNA expression by the breast cell lines, total RNAs were isolated from subconfluent cells cultivated under standard culture conditions. Total RNA was extracted with guanidium isothiocyanate and was purified on a CsCl cushion (44). Four µg of each RNA was analyzed by electrophoresis on 1% agarose gels containing 10% formaldehyde and was subsequently transferred onto nylon membranes (Hybond TM-N; Amersham, Aylesbury, United Kingdom). The membranes were hybridized with a cDNA probe for either MT1-MMP (provided by Dr. Motoharu Seiki, Kanazawa University, Kanazawa, Japan), TIMP-2 (provided by Dr. William G. Stetler-Stevenson, CNCI, NIH), or SPARC (provided by Dr. Larry Fisher, National Institute of Dental Research, NIH). 32P-labeled with a random-priming labeling kit (Boehringer Mannheim, Germany). The amounts of blotted RNA were normalized to GAPDH mRNA (Clontech, Palo Alto, CA). The hybridization signals were quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Western Blotting Analyses. Analyses of SPARC expression in the breast cancer cell lines were performed on 72 h-conditioned media (10 ml in a 10-cm Petri dish) obtained from cells cultured on plastic in SFM. Loading was normalized with respect to cell density, determined by cell counting after the 72-h incubation. For MT1-MMP analyses of cells stimulated with SPARC peptides (see above), the assays were performed in 24-well plates (15×10⁴ to 25×10⁴ cells in 300 µl). After 72 h, conditioned media were collected for zymographic analyses, and the cells were reserved for protein extraction. Total protein lysates were prepared in a HEPES buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA (pH 8.0)) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin; Merck, Darmstadt, Germany). Protein concentration was determined with the BCA kit (Pierce, Rockford, IL). Transfer was monitored with Ponceau red reversible staining. The filters were blocked in 5% milk in PBS for 4 h before exposure to the primary antibody overnight at 4°C (rabbit polyclonal anti-SPARC, G-protein Sepharose-primed IgG (1/500: provided by Dr. Larry Fisher) or mouse monoclonal anti-MT1-MMP antibody (10 µg/ml; clone 118-3B1 (8) provided by Dr. Motoharu Seiki, Institute of Medical Science, University of Chicago).
RESULTS

We had shown previously that, in contrast to noninvasive cell lines, invasive breast cancer cells activate exogenous MMP-2 when cultivated on collagen type I gels. These assays were performed after plating of the cells for 24 h with serum-supplemented IMEM before they were incubated with MMP-2-supplemented, SFM medium for 72 h (24). We found here that the 24 h preincubation with serum was necessary to obtain the collagen/serum-stimulated activation in these cells. Indeed, cells plated directly in collagen gels in SFM remained viable but did not activate MMP-2 (data not shown). These data indicate the participation of exogenous factor(s) for collagen-stimulated MMP-2 activation by these cells.

Blocking Collagen-Type I/Serum-induced MMP-2 Activation with Anti-Integrin Antibodies. Because α2β1 is the main cellular receptor for collagen type I, we tried to block this collagen-induced activation with anti-α2β1 antibodies. We used two invasive breast cancer lines for this purpose, selected for their differential response in terms of MT1-MMP regulation: MDA-MB-231, which did not overexpress MT1-MMP mRNA level on collagen gels; and BT549, which showed the highest up-regulation of MT1-MMP mRNA by collagen, as we described previously (24). Because Seltzer et al. (27) had shown an inhibition by α2β1 antibodies of MMP-2 activation induced in fibroblasts cultivated within collagen type I gels, we cultivated our breast cells under the same conditions (the cells were also placed in the collagen gels to avoid the detachment of the cells) and used the same antibodies. Although these antibodies clearly inhibited the adhesion of both cell lines to collagen type I in adhesion assays, we were still unable to block the MMP-2 activational response of either breast cancer cell line (data not shown).

Taken together, these observations indicated that the interaction with collagen was mediated by a non-integrin binding factor present in serum. We therefore studied the potential involvement of SPARC in the collagen-induced MMP-2 activation because: (a) it is present in serum; (b) it binds collagen type I; and (c) it has been shown to regulate expression of other MMPs in fibroblasts and monocytes (33, 34).

Invasive Human Breast Cancer Cells Express SPARC. We first evaluated the endogenous expression of SPARC in several human breast cancer cell lines displaying various degrees of invasion and MMP-2 activation. We found that SPARC mRNA was prevalent in invasive BT549 and Hs578T cells, was produced at lower levels by invasive MDA-435 cells, and was undetectable in invasive MDA-MB-231 cells or in noninvasive T47D or MCF-7 cells (Fig. 1A). By Northern analysis, SPARC mRNA levels were not affected by the presence or absence of the collagen gel (data not shown). Similar results were seen with respect to SPARC protein, except that SPARC protein could not be detected in the conditioned medium of the MDA-435 cell line, which showed a low level of mRNA (Fig. 1B). Interestingly, the SPARC secreted by the BT549 cells migrated slightly faster than the M, 43,000 form secreted by the Hs578T cells. The BT549 and the MDA-MB-231 cells were used for further study, because BT549 cells produce high levels of SPARC and increase MT1-MMP mRNA in response to collagen/serum (24), whereas MDA-MB-231 cells, which also activate MMP-2, do not express SPARC or increase levels of MT1-MMP mRNA in response to collagen type I/serum (24).

SPARC Induces MMP-2 Activation. We next looked at the ability of these two cell lines to activate exogenous MMP-2 in the presence of different concentrations of rSPARC, in the absence of serum. As shown in Fig. 2A, rSPARC induced MMP-2 activation by MDA-MB-231 cultured in collagen gels or on plastic in a concentration-dependent manner. Cultivation of the MDA-MB-231 cells in collagen gels clearly potentiated the response to SPARC, because MMP-2 activation could be detected at the lowest concentration (25 μg/ml, 0.75 μM) in collagen gels, whereas the highest concentration (100 μg/ml, 3 μM) was necessary to induce MMP-2 activation on plastic. The potentiation with collagen was not seen in BT549 cells, and the effect of rSPARC on BT549 cells cultured on plastic appeared to be stronger than that seen for cells in collagen (Fig. 2B). In contrast to the results obtained with invasive breast cancer cell lines, we did not observe any MMP-2 activation in noninvasive MCF-7 cells treated with rSPARC, whether they were cultured on plastic or in collagen gels (data not shown).

SPARC Peptide 1.1 Induces MMP-2 Activation. To define the sequence(s) in SPARC responsible for this activity, we monitored MMP-2 activation by MDA-MB-231 and BT549 cells exposed to several synthetic peptides from the different domains of SPARC. We used peptide 1.1 (NH2-terminal domain I), peptide 3.2 (domain III), and peptide 4.2 (COOH-terminal domain IV). As shown in Fig. 3,
SPARC-INDUCED MMP-2 ACTIVATION

rSPARC (µg/ml)/plastic  
0 25 50 100  
72kDa 62kDa 59kDa

rSPARC (µg/ml)/collagen
0 25 50 100 cont

Fig. 2. MMP-2 activation induced by rSPARC in MDA-MB-231 and BT549 cells. Zymographic analysis of 72-h conditioned media obtained from MDA-MB-231 (A) and BT549 (B) cells, cultured on plastic or in collagen type I gels, with increasing concentrations of rSPARC is shown. MMP-2 (M, 72,000) proenzyme and MMP-2 activated forms (M, 62,000 and M, 59,000) are shown. Control lane (cont) is conditioned medium from a cell-free collagen gel. Experiments were performed three times; one representative experiment is shown.

Peptide 1.1 strongly potentiated MMP-2 activation by both MDA-MB-231 and BT549 cells on plastic or in collagen type I gels. In contrast, we observed a weak response to peptides 4.2 and 3.2, which was most evident in BT549 cells. As with rSPARC, none of the three peptides induced MMP-2 activation in the noninvasive MCF-7 cell line.

Peptide 1.1-induced MMP-2 Activation Does Not Alter MT1-MMP mRNA Level. Because MT1-MMP is considered a major MMP-2 activator, we investigated the effect of SPARC on MT1-MMP expression. Using Northern blotting, we examined the steady-state levels of MT1-MMP in MDA-MB-231 and BT549 cells incubated with peptide 1.1 in collagen gels or on plastic. The cultures showed MMP-2 activation responses to collagen and peptide 1.1, as described above (Fig. 4A). As shown in Fig. 4B and reported previously (24), MT1-MMP mRNA was expressed by both cell lines. We also observed increased levels of MT1-MMP mRNA in BT549 cells cultivated in collagen gels (Fig. 4, B and C). However, exposure to peptide 1.1 did not increase the basal level of MT1-MMP in either cell line, whether it was cultured in collagen type I gels or on plastic.

Peptide 1.1-induced MMP-2 Activation Does Not Alter the MT1-MMP Protein Level. Because we could not detect significant changes in MT1-MMP mRNA by peptide 1.1 in either of the two cell lines, we looked for evidence of posttranslational modifications of MT1-MMP by Western blotting. Three major bands of M, 63,000, M, 60,000, and M, 43,000 have been described for MT1-MMP, which are thought to represent the proenzyme, mature form, and a form associated with MMP-2 activation, respectively (12). We were unable to detect any clear difference on Western blots in MDA-MB-231 cells treated with peptide 1.1 relative to cells treated with peptides that did not induce MMP-2 activation on plastic or in collagen gels. The M, 43,000 band was detectable in all samples, although it was weak in the untreated cells, especially those on plastic (data not shown). In BT549 cells (Fig. 5), a clear increase of the M, 60,000 and the M, 43,000 forms was observed in the cells cultivated in the collagen gels relative to the cells cultured on plastic, in agreement with results obtained from Northern blotting. However, as we observed with MDA-MB-
MMP-2 activation and MT1-MMP expression in MCF-7 cells transfected with MT1-MMP. As seen in Fig. 6, MT1-MMP-transfected clones already partially activated MMP-2 under basal conditions on plastic (no MMP-2 activation or MT1-MMP protein was detected in the vector control 3-1 cells). As observed for MDA-MB-231 and BT549 cells, activation was enhanced somewhat in cells treated with peptide 1.1, a result supporting the involvement of MT1-MMP. Again, no qualitative or quantitative changes of MT1-MMP expression that could correlate with MMP-2 activation were obvious on Western blots, in agreement with our data for MDA-MB-231 and BT549 cells. In contrast, we confirmed that ConA induces an overexpression of the \( M_r \) 63,000 and \( M_r \) 60,000 forms of MT1-MMP as published previously (11, 13) and observed the \( M_r \) 43,000 form associated with the induction of MMP-2 activation in all of the cell lines including the MT1-MMP transfectants (data not shown), as described previously in HT1080 cells (12).

rSPARC and Peptide 1.1 Induce a Decrease in TIMP-2. Because we could not associate any apparent modifications in MT1-MMP that were associated with the SPARC/collagen-induced MMP-2 activation, we examined the effect of rSPARC and SPARC peptides on the levels of TIMP-2 in the conditioned media of the cells on plastic and in collagen gels. Fig. 7A shows a concentration-dependent diminution of TIMP-2 levels in the samples that received rSPARC, both on plastic or in collagen, concomitantly with increased MMP-2 activation (as shown in Fig. 2). Similarly, a decreased level of TIMP-2 was found in the samples incubated with peptide 1.1 and to a slight extent with peptide 3.2, which induces MMP-2 activation minimally (Fig. 7B). The decreased levels of TIMP-2 protein were not correlated with decreases in TIMP-2 mRNA (data not shown).

Both rSPARC and peptide 1.1 caused a diminution of cellular metabolism (as seen with the Wst-1 reagent: data not shown), particularly at high concentrations in collagen gels (100 \( \mu \)g/ml, 3 \( \mu \)M rSPARC and 2 \( \mu \)M peptides). This combination probably increases the local concentration of SPARC around the cells and could thus account for the fact that BT549 cells, already producing endogenous SPARC, activated MMP-2 less in collagen gels than on plastic. Additionally, it could explain why...
TIMP-2 levels remain low at 3 μM SPARC in BT549 cells with no the presence of increasing concentrations of rSPARC (Ai and of 1 mM SPARC peptides Tremble et al. (33), who reported the induction of MMP-1, MMP-9, and collagen-induced activation of MMP-2 by two invasive breast cancer cell lines, MDA-MB-231 and BT549. Our results showed that SPARC was expressed by all but one of those with invasive characteristics, a result further implicating SPARC in the invasive phenotype. Ledda et al. (38) have also noted that SPARC expression was correlated with the invasive potential of human melanoma cell lines and have furthermore shown that suppression of SPARC by transfection of melanoma cells with an antisense SPARC expression vector resulted in decreased expression of MMP-2 and diminished invasive capacities in vitro with marked inhibition of the tumorigenicity of these cells in vivo (40). The involvement of SPARC in tumor invasion is consistent with its participation in morphogenesis and angiogenesis and in the regulation of cell shape, adhesion, and proliferation (32, 47–51). In apparent contrast to these results, Mok et al. (52) reported that SPARC was expressed at a higher level in normal human ovarian surface epithelial cells relative to the corresponding carcinoma cells and that transfection of SPARC into a tumorigenic ovary cell line inhibited its tumorigenic potential. Such a discrepancy could, however, be cell type dependent, because the responses induced by SPARC are different from one cell type to another. For instance, a growth-inhibitory effect of SPARC was observed in endothelial cells but not in melanoma cells (40, 50).

Our results have also indicated an enhancement of SPARC-induced MMP-2 activation by MDA-MB-231 cells cultured in collagen gels in comparison with culture on plastic. This effect could be masked in the BT549 cells because they produce high levels of endogenous SPARC which, added to the experimental concentrations of SPARC, might interfere with the overall cellular metabolism. The high endogenous expression of SPARC by BT549 cells may also explain why they begin to activate MMP-2 under control conditions (i.e., cultivated in SFM on plastic), whereas MDA-MB-231 cells, which do not produce SPARC, do not activate MMP-2 under such conditions. The observations with the MDA-MB-231 cells are, however, in agreement with several studies indicating an enhancement of effects induced by SPARC in the presence of different types of collagen. For example, Tremble et al. (33) have reported that the induction by SPARC of several MMPs in fibroblasts was augmented when the cells were grown on different collagen types. Accordingly, SPARC has been shown to bind to several collagens including collagen type I (48, 53, 54). Because SPARC is present in serum, it should also be considered in the interpretation of our previous results that showed MMP-2 activation in invasive breast cancer cells lines cultivated on collagen gels after preincubation with serum (24, 26, 55). Collectively, these data indicate that the interaction between SPARC and collagen type I contributes to the MMP-2 activation process. Association of SPARC with collagen type I could increase the concentration of SPARC around cells but could also modify interactions between the cells and collagen type I (56).

Several studies in vivo support the relevance of SPARC/collagen type I-induced MMP-2 activation to tumor invasion. Such a mechanism could indeed occur in vivo when invasive cells interface with the interstitial connective tissue. Because our in situ hybridization data from breast cancer specimens had shown increased levels of both collagen type I and MT1-MMP mRNAs in fibroblasts located around invasive and preinvasive clusters but not around benign or normal breast tissue, we suggested a functional relationship of both molecules in tumor progression (24). Moreover, SPARC/collagen type I interactions appear to be relevant in vivo, because collagen type I-deficient Mov-13 mice do not retain SPARC in the ECM (56). Several recent reports have also described an association between SPARC expression and the progression of several cancers including those of the breast, lung, colon, kidney, ovary, adrenal cortex, and brain (35–39).

Although the biological functions of different forms and fragments of

**DISCUSSION**

In this study, we have investigated the potential role of SPARC in collagen-induced activation of MMP-2 by two invasive breast cancer cell lines, MDA-MB-231 and BT549. Our results showed that rSPARC can induce MMP-2 activation in these two invasive cell lines but not in the noninvasive MCF-7 line. These results implicate SPARC in the proteolysis of ECM associated with tumor invasion. Regulation of MMP expression by SPARC has also been shown by Tremble et al. (33), who reported the induction of MMP-1, MMP-9, and MMP-3 in rabbit synovial fibroblasts cultured with SPARC.

**Fig. 7.** Effect of SPARC peptides on TIMP-2 protein. TIMP-2 ELISA on conditioned media of MDA-MB-231 cells and BT549 cells cultured on plastic or in collagen gels in the presence of increasing concentrations of rSPARC (A) and of 1 mM SPARC peptides 1.1, 3.2, or 4.2 (B). Experiments were performed three times; one representative experiment is shown.

**TIMP-2 levels remain low at 3 μM SPARC in BT549 cells with no activation, especially in collagen gels. However, at the lower concentrations of either SPARC or peptide 1.1 that have been used in other studies (43), the drop in cellular metabolism was minimal and not sufficient to account for the decrease in TIMP-2 protein that we observed.**

**SPARC-INDUCED MMP-2 ACTIVATION**

Similarly, Shankavaram et al. (34) found that SPARC induced MMP-9 and MMP-1 in human monocytes. In a screen of six breast cancer cell lines, we found that SPARC was expressed by all but one of those with invasive characteristics, a result further implicating SPARC in the invasive phenotype.
SPARC are not fully understood, it is known that specific domains of the molecule trigger different cell responses (57). For example, in agreement with other studies, the COOH-terminal region of SPARC has been implicated in selective binding to several collagen types (54, 58–60). A peptide from domain II has been shown to act in synergism with a COOH-terminal peptide to inhibit endothelial cell proliferation (50), and these two domains also appear to regulate focal adhesion disassembly in these cells (61). Our results showed that peptide 1.1 from the NH2-terminal region (acidic domain I) can potentiate MMP-2 activation in two invasive breast cell lines and indicate that the NH2-terminal domain of SPARC contributes to the activation of MMP-2. In agreement with these results, the same peptide has been shown to trigger other cellular responses, such as antispeading effects on fibroblasts and endothelial cells (47). Studying the effects of several peptides on the synthesis of MMP-1 by rabbit synovial fibroblasts, Tremblé et al. (33) found that peptide 3.2 (in domain III, now part of the extracellular, Ca2+-binding domain; Ref. 62) stimulated the greatest increase, whereas peptide 1.1 produced only a 1.1-fold induction on plastic. Shankavaram et al. (34) also found that peptide 3.2 was the major peptide inducing MMP-1 and MMP-9 production in human monocytes. Although these experiments were performed under conditions different from ours and with different cell types, the data indicate specific regulation of several MMPs by different regions of SPARC.

Because MMP-2 activation might require high expression of MT1-MMP (9), we studied the regulation of MT1-MMP by peptide 1.1. No relationship could be established between the levels of expression of MT1-MMP mRNA or protein and SPARC-induced MMP-2 activation. Collagen alone did not appear sufficient to induce MMP-2 activation. Moreover, peptide 1.1, despite its stimulation of MMP-2 activation, did not result in any modifications of MT1-MMP mRNA or protein. Although not definitively proven in the MDA-MB-231 or BT-549 cells, our results obtained with the MCF-7 cells transfected with MT1-MMP nevertheless strongly indicated the need for MT1-MMP expression in the MMP-2 activation process. In contrast, and in agreement with published data, we showed that ConA treatment induced the Mr 60,000 and Mr 43,000 forms of MT1-MMP concomitantly with MMP-2 activation in all cell lines tested. Although we cannot exclude the possibility that SPARC induces posttranslational modifications of MT1-MMP that we could not detect by Western blotting, the regulation of MMP-2 activation by SPARC appears to involve additional mechanisms. These could include: (a) a reorganization of the MMP-2 complex at the cell surface that would affect the activity of MT1-MMP, and/or (b) direct interactions between SPARC and the members of this complex. Lee et al. (28) reported that MMP-2 activation induced in fibroblasts cultivated in collagen lattices did not involve any apparent regulation of MT1-MMP mRNA or protein. These authors have instead found that MMP-2 activation in normal fibroblasts occurs intracellularly, after increased production of MMP-2 rather than of MT1-MMP. This mechanism seems unlikely in our system, because these human breast cancer cell lines do not produce MMP-2 endogenously (26).

Interestingly, we saw that diminution of TIMP-2 protein levels correlated with the activation of MMP-2 stimulated by SPARC or SPARC peptide. Our results indicate that the control of TIMP-2 levels occurs principally at the protein level, because no obvious modulation of TIMP-2 mRNA expression was detected. Other authors have reported that there were no changes in TIMP-2 mRNA levels correlated with the activation of MMP-2 in HT1080 or CCL-137 cells stimulated with different agents (including ConA or growth factors; Ref. 12) or in fibroblasts cultivated in collagen lattices (28). One possibility is that SPARC or peptide 1.1 increases the binding of TIMP-2 to the cell surface and thus diminishes the levels of soluble TIMP-2. Allenberg and Silverman (63) have previously documented reduced levels of TIMP-2 in the conditioned media of kidney mesangial cells after cytochalasin D-induced MMP-2 activation and have suggested that this diminution was due to the appearance of MT1-MMP and activated MMP-2 at the cell surface, each of which can bind TIMP-2. However, in our model, we did not observe qualitative or quantitative modifications of MT1-MMP that could mediate TIMP-2 binding at the cell surface (17, 19). It is also possible that a decrease in soluble TIMP-2 would cause a diminution of TIMP-2 at the cell surface and would allow MMP-2 activation, but this remains to be proven. Indeed, Strongin et al. (17) have shown that stoichiometric amounts of TIMP-2 are necessary for MMP-2 activation but that increased amounts of TIMP-2 inhibit activation of the proenzyme. Presently, we do not know whether diminution of soluble TIMP-2 is a cause or an effect of the observed MMP-2 activation. However, our observations with SPARC are consistent with the idea that an imbalance between MMP-2/MTI-MMP and TIMP-2 at the cell surface could facilitate the activation of MMP-2. In studies with neutralizing antibodies against TIMP-2, collagen/serum-induced MMP-2 activation in breast cancer cells was blocked by the removal of TIMP-2 and could be rescued by exogenous TIMP-2. Clearly, soluble and/or cell-associated TIMP-2 is a regulatory factor for collagen-stimulated and/or SPARC-stimulated MMP-2 activation. The mechanism(s) of TIMP-2 diminution triggered by SPARC and/or collagen are presently under study.

In conclusion, our data indicate that SPARC, through domain I, induced MMP-2 activation in invasive breast cancer cell lines. SPARC is thus implicated in the proteolysis of basement membrane and ECM associated with tumor invasion. Because the presence of collagen type I appeared to potentiate this response to SPARC in MDA-MB-231 cells, SPARC and collagen type I might act in concert. The activation of MMP-2 by SPARC was not associated with increased steady-state levels of MT1-MMP mRNA or with posttranslational modification of MT1-MMP, despite the apparent requirement of MT1-MMP. In contrast, SPARC/peptide 1.1 mediated a decrease in levels of soluble TIMP-2 protein, a change that could facilitate MMP-2 activation. These data add another dimension to the cell surface activation of MMP-2 that involves the matricellular protein SPARC.

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