Transcription factor early growth response 1 activity up-regulates expression of tissue inhibitor of metalloproteinases 1 in human synovial fibroblasts

ARTICLE *in* ARTHRITIS & RHEUMATOLOGY · FEBRUARY 2003

Impact Factor: 7.76 · DOI: 10.1002/art.10774 · Source: PubMed

ARTHRITIS & RHEUMATISM
Vol. 48, No. 2, February 2003, pp 348–359
DOI 10.1002/art.10774

Transcription Factor Early Growth Response 1 Activity Up-Regulates Expression of Tissue Inhibitor of Metalloproteinases 1 in Human Synovial Fibroblasts

Wilhelm K. Aicher,[1] Dorothea Alexander,[2] Christian Haas,[3] Stefan Kuchen,[4] Axel Pagenstecher,[3] Steffen Gay,[4] Hans-Hartmut Peter,[3] and Hermann Eibel[3]

Objective. **To investigate the regulatory potential of early growth response 1 (Egr-1) on tissue inhibitor of metalloproteinases 1 (TIMP-1) expression in synovial fibroblasts.**

Methods. **Egr-1 and TIMP-1 transcripts were detected by in situ hybridization in synovial tissue. Egr-1–regulated TIMP expression was studied in immortalized fibroblast lines using gel retardation assays, RNase protection analysis, reporter gene studies using the human TIMP-1 promoter, and by enzyme-linked immunosorbent assay.**

Results. **TIMP-1 and Egr-1 were coexpressed in synovial fibroblasts of inflamed joints, and Egr-1 activated the expression of TIMP-1. Egr-1 binding to a recognition sequence in the TIMP-1 promoter was demonstrated in gel retardation and reporter gene assays. Since the same DNA sequence was also recognized by the transcription factor Sp-1, our results suggest that the expression of TIMP-1 in synovial fibroblasts may be differentially regulated by Egr-1 and Sp-1. In addition, fibroblasts expressing Egr-1 at high levels were found to express increased levels of TIMP-2 and TIMP-3 messenger RNA.**

Conclusion. **The enhanced expression of Egr-1 may regulate the activity of matrix metalloproteinases in synovial fibroblasts by enhancing the expression of the TIMP-1, -2, and -3 genes.**

Destruction of the extracellular matrix defines one of the most critical steps when transformed cells spread from a primary tumor and begin to generate metastases. Joint destruction is also a key feature in rheumatoid arthritis (RA). Matrix degradation and erosion of the connective tissues, including cartilage, tendon, and bone, start at sites of attachment of synoviocytes to cartilage (1). The process is induced by synoviocytes aggressively invading the cartilage and subchondral bone (2) and is driven in particular by highly activated synovial fibroblasts (3–6) with a distinct phenotype characterized by elevated levels of adhesion molecules and expression of proinflammatory cytokines (7–12). At sites of cartilage erosion, the synovial fibroblasts are found to express high levels of matrix metalloproteinases (MMPs) such as MMP-1 (collagenase) and MMP-3 (stromelysin) (13–16). The activity of these enzymes is tightly regulated on a transcriptional as well as a posttranslational level by interactions with specific inhibitors of the enzymatic activity (15). An imbalance of MMPs and their specific inhibitors has been postulated as one of the pathologic mechanisms underlying RA (17–19). Although the regulation of MMP expression in fibroblasts is well studied (13,15–18), much less is known about the regulatory mechanisms acting on the expression of the tissue inhibitors of metalloproteinases (TIMPs).

Supported by Deutsche Forschungsgemeinschaft grants Ei235/3-1 and Ai16/10-1, the Kompetenznetz Rheuma (to Dr. Eibel), a Hench Foundation scholarship (to Dr. Haas), a Bode Foundation grant (to Dr. Aicher), and by institutional funding.

[1]Wilhelm K. Aicher, PhD: University Hospital Tubingen, Tubingen, Germany, and University Hospital Freiburg, Freiburg, Germany; [2]Dorothea Alexander, MS, University Hospital Tubingen, Tubingen, Germany; [3]Christian Haas, MD, Axel Pagenstecher, MD, Hans-Hartmut Peter, MD, Hermann Eibel, PhD: University Hospital Freiburg, Freiburg, Germany; [4]Stefan Kuchen, MD, Steffen Gay, MD: University Hospital Zurich, Zurich, Switzerland.

Dr. Aicher and Ms Alexander contributed equally to this work.

Address correspondence and reprint requests to Hermann Eibel, PhD, Clinical Research Unit for Rheumatology, University Hospital Freiburg, Breisacher Strasse 64, D-79106 Freiburg, Germany. E-mail: eibel@nz11.ukl.uni-freiburg.de.

Submitted for publication July 16, 2001; accepted in revised form October 23, 2002.

TIMP-1, a glycoprotein of 28 kd, strongly and stoichiometrically binds to both MMP-1 and MMP-3. Like MMP-1 and MMP-3, TIMP-1 is produced by synovial fibroblasts at sites of synovial attachment to cartilage (15,17,20). We previously studied the expression of the transcription factor early growth response 1 (Egr-1) by in situ hybridization using samples obtained from the synovium of RA patients, and constitutive Egr-1 overexpression was found only in RA synovial fibroblasts, and not in controls (21,22). Further analyses using cell lineage–specific markers demonstrated that Egr-1 is overexpressed only in fibroblast-like lining cells but not in other cell types present in the inflamed synovium of RA patients.

Egr-1 is a member of the Egr transcription factor family, of which all genes are transcriptionally induced by various activating stimuli in the absence of de novo protein synthesis. Egr expression contributes to the initial steps of cell activation, leading to growth, differentiation, and changes in downstream gene expression (23–25). For B cells, we could show that overexpression of Egr-1 accelerates development of pro-B and immature B cells (26). A similar function of Egr-1 was also described for developing thymocytes (27) and, likewise, Egr-1 may play an important role in the differentiation from resident synovial fibroblasts to an activated and invasive cell type. Egr-1 has a DNA binding domain with 3 Cys_2–His_2 zinc fingers (28) recognizing a GC-rich consensus sequence (GC/AGC/TGGGC/A/TG). It may act either as a transactivator (26,29,30) or as a transcriptional repressor (31).

In fibroblasts grown in the absence of external stimuli such as interleukin-1, tumor necrosis factor α, or other cytokines, the Egr-1 protein is barely detectable by immunoblotting using whole cell lysates (22), but Egr-1 expression is induced rapidly upon a wide variety of activating signals (32,33). However, in the joints of RA patients, Egr-1 is expressed at high levels in synovial fibroblasts, suggesting an important role in maintaining the activated phenotype of synoviocytes (21). Egr-1 target genes in synovial fibroblasts remain to be identified. Since the human TIMP-1 promoter sequence contains 2 potential Egr-1 binding sites, we speculated that the transcription factor activity of Egr-1 might regulate TIMP-1 expression. Here we show that the expression of TIMP-1 correlates strongly with expression patterns of Egr-1 in situ and that Egr-1 activates TIMPs 1, 2, and 3, but not TIMP-4, expression in immortalized human synovial fibroblast cell lines.

MATERIALS AND METHODS

In situ hybridization. During joint replacement surgery, synovial tissue samples were obtained from 5 patients who fulfilled the revised American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (34). The study was approved by the local ethics committees. After fixation for 6 hours in 4% formalin, the tissues were embedded in paraffin. Serial sections (5 μm) were obtained and stored at room temperature until use. Riboprobes of Egr-1 (246 bp) and TIMP-1 (339 bp) were prepared by polymerase chain reaction (PCR) amplification of specific fragments using the following primers: Egr-1 forward primer 5′-ATTGTGAGGGACATGCTCAC, reverse primer 5′-ACAAAAATCGCCGCCTACTC; TIMP-1 forward primer 5′-ATTCCGACCTCGTCATCAG, reverse primer 5′-ATTCCTCACAGCCAACAGTG.

The fragments were subsequently cloned using the PCR-Script Amp cloning kit (Stratagene, Heidelberg, Germany) and checked for sequence identity and orientation by automated sequencing (Microsynth, Balgach, Switzerland) and GenBank analysis. Digoxigenin (DIG)–labeled sense and antisense riboprobes were generated by T3- and T7-dependent in vitro transcription. In situ hybridization was performed as previously described (35). Briefly, after deparaffinization in graded ethanol baths, the slides were pretreated with 0.2*M* HCl for 8 minutes, washed with 0.1*M* triethanolamine–HCl buffer, and incubated with prehybridization solution for 1 hour. The sections were hybridized with DIG-labeled riboprobes (either sense or antisense) at 50°C overnight. Free and nonspecifically bound probes were removed by incubation with RNase A (20 μg/ml at 37°C for 1 hour) and washing steps were performed at 50°C at the following stringencies: 50% formamide/2× saline–sodium citrate (SSC) (5 minutes), 1× SSC + 1% sodium dodecyl sulfate (SDS) (15 minutes), 0.25× SSC + 1% SDS (15 minutes), and 0.1× SSC + 1% SDS (15 minutes). Immunologic detection was performed with anti-DIG alkaline phosphatase–conjugated $F(ab)_2$ (Roche Diagnostics, Mannheim, Germany) and nitroblue tetrazolium/BCIP substrate.

Synovial fibroblast lines. Immortalized synovial fibroblasts were generated from naive synovial fibroblasts K4 and heat-shock element (HSE) by transfection with a plasmid encoding the SV40 large T antigen (T-ag) (33). Cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Karlsruhe, Germany) containing penicillin/streptomycin (100 units/ml) supplemented with 10% fetal calf serum (FCS) at 37°C and 7.5% CO_2. SV40–T-ag expression was controlled by immunohistochemistry using monoclonal antibody 1605 (33).

Plasmids and in vitro mutagenesis. The 2.3-kb *Eco* RI/*Hind* III fragment from the pAlter-Egr-1 (31), containing the coding region of the murine Egr-1 gene, was subcloned into the plasmids pCR-Neo and pEF-neo. The pCR-Neo vector contains the cytomegalovirus (CMV) early promoter, which was replaced by the elongation factor 2 promoter in the pEF-neo vector. Both expression vectors yield high Egr-1 expression levels in fibroblasts, and both plasmids carry the neomycin resistance gene for selection in G418-containing medium.

The human TIMP-1 promoter–reporter plasmid was generated from p1338–chloramphenicol acetyltransferase

(CAT; a generous gift of Professor H. Sato, Kanazawa, Japan), as described previously (22). Briefly, point mutations were introduced by site-directed in vitro mutagenesis to replace the candidate Egr-1 binding sites using the QuikChange site-directed mutagenesis kit (Stratagene). The Egr-1 binding site at −219 (5′-G**GTGGGGGAG**G) was changed to 5′-G**GTG*CAC*GAG**G using the oligonucleotide 5′-GGAAT-AGTGACTGACGTGGAG**GTGCAC**GAGGTGGCTGGCC-CGGGCGAGG-3′ (Egr-1 binding sites in boldface, changed sequences in italics). Since the sequence contains an *Apa* I recognition site (5′-GCTCAC), the mutation in the TIMP-1 promoter was tested by both restriction analysis and DNA sequencing. The binding site at position −33 (5′-GGG**GCGGGGGTG**G) was altered to 5′-GGG**GCG*CCC*-GTG**G) using the complementary oligonucleotide 5′-GGTTT-CCGCACCCGCTGC**CAC*GGGC*GC**CCCCTAGCGTGGACA-TTATCCTC and tested by restriction analysis with *Nar* I (5′-GGCGCC) and by DNA sequencing. Using both oligonucleotides, a third promoter construct was made in an analogous way, carrying mutations at both Egr-1 binding sites (−33 and −219). CAT activity was measured in whole cell lysates after transient transfections of K4 cells using the CAT enzyme-linked immunosorbent assay (ELISA) kit from Roche Diagnostics. Transfection efficiencies were controlled by analyzing the fluorescence of green fluorescent protein (GFP) expressed from cotransfected pCMV–enhanced GFP (EGFP).

The second set of TIMP-1 promoter–reporter plasmids was constructed by ligating the *Sac* I–*Bgl* II TIMP promoter fragment of p1388-CAT into pGL3 basic vector (Promega, Mannheim, Germany). The Egr-1 binding site at position −33 was removed by deleting a 58-bp *Stu* I–*Eco*47 III restriction fragment. Egr-1–expressing T10 fibroblasts or T6 controls were cotransfected with the wild-type TIMP-1 promoter or with the promoter mutant and pRLO, respectively. As controls, pGL3 and pRLO were cotransfected. Forty-two hours after transfection, the cells were extracted using a Dual-Luciferase Reporter assay system (Promega). Both firefly and Renilla luciferase activity were measured using a luminometer (Berthold, Wildbad, Germany). The Renilla luciferase activity was used to correct for different efficacies of the individual transfections. Values are presented as the mean ± SD of 7 measurements of luciferase activity.

Transfection of fibroblasts. Immortalized fibroblasts were grown in 9-cm petri dishes to 50% confluence and were serum-starved overnight in medium containing 0.5% FCS. Then, cells were transfected using the cationic liposome transfection reagent DOTAP (Roche Diagnostics) with 5 μg of pCR–Egr-1–Neo DNA linearized by *Sca* I. Transfectants were selected by adding G418 (Invitrogen) to a 300 μg/ml final concentration. G418-resistant clones were selectively detached by 0.5 units/ml trypsin–EDTA and cultured in medium containing G418.

Immunoblotting analysis. Egr-1 expression was analyzed by immunoblotting (33). To reduce background levels of endogenous Egr-1 expression induced by FCS, cells were incubated overnight in 0.5% FCS medium. The cells were trypsinized and washed twice with ice-cold phosphate buffered saline (PBS). Cells (1×10^6) were resuspended in 50 μl lysis buffer containing 1% Nonidet P40 (NP40), 150 m*M* NaCl, 10 m*M* Tris HCl, pH 7, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 minutes. Cell debris was removed by centrifugation (22,000*g* for 20 minutes at 4°C) and the extract was separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using the Egr-1–specific antiserum C-19 (25 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Binding of the primary antibodies was detected with horseradish peroxidase–conjugated secondary antibodies (200 ng/ml; Dianova, Hamburg, Germany) and developed using the enhanced chemiluminescence method (ECL; Amersham Pharmacia Biotech, Freiburg, Germany).

RNA isolation and blot analysis. To reduce background levels of Egr-1 expression induced by FCS, cells were incubated overnight in 0.5% FCS medium. Cells (3×10^6) were trypsinized and washed twice with ice-cold PBS. Total RNA was extracted using the guanidinium isothiocyanate method described previously (21) or by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, 10–20 μg/sample was separated by gel electrophoresis in a 1% agarose gel containing 7% formaldehyde. Northern blotting was carried out using α^{32}P-dATP (Amersham Pharmacia Biotech)–radiolabeled complementary DNA (cDNA) probes, as described previously (21). For TIMP-1, the 780-bp *Eco* RI/*Eco* RI fragment of the pUC119–TIMP vector was used; for Egr-1, the 2-kb *Eco* RI fragment from the Z225 Egr plasmid was used (21). The blots were exposed to x-ray films and the signals were recorded by densitometry using GAPDH signals as the reference.

RNase protection assay (RPA). The probe set for the detection of TIMPs (TIMP probe set) contained probes for TIMPs 1, 2, 3, and 4. A fragment of the RPL32-4A gene (36) served as an internal loading control. RPAs (using 10 μg total RNA) were performed as described previously (37).

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. First-strand cDNA synthesis was performed using 2 μg of total RNA and oligo(dT) primers (Advantage RT-for-PCR Kit; Clontech, Heidelberg, Germany). The amount of cDNA in the samples was assessed by online real-time quantitative RT-PCR with the LightCycler System (Roche Diagnostics). For Egr-1, TIMP-1, and TIMP-4, RT-PCR primers were used as published (38–40). For TIMP-2 and TIMP-3, RT-PCR analysis–specific primer pairs were designed using the European Molecular Biology Laboratory (EMBL) gene library, and the DNA Strider (Dr. Christian Marck, Service de Biochemie et de Genetique Moleculaire, Gif-sur-Yvette, Cedex, France) and OLIGO software programs (Molecular Biology Insights, Cascade, CO).

The primers used were as follows: Egr-1 (38) 5′-ACAAGAAAGCAGACAAAAGTG and 5′-GGGAAGT-GGGCAGAAAGGATT; TIMP-1 (39) 5′-CTGATGGTGG-GTGGATGAGTAATGC and 5′-GTGGGTTGAGTGG-GGTGAGGAC; TIMP-2 (EMBL NM 003255; positions 334–733, annealing temperature 65°C) 5′-CGCTCGGCCTCC-TGCTGCT and 5′-AGGCTCTTCTGGGTGGTGCTCA; TIMP-3 (EMBL NM 000362; positions 1312–1738, annealing temperature 62°C) 5′-CAAGGTGGTGGGGAAGAAGC and 5′-CGGATGCAGGCGTAGTGTTT; TIMP-4 (40) 5′-ACAGCCAGAAGCAGTATC and 5′-CCAGAGGTCAGG-TGGTAA.

The cDNA was amplified in the presence of SYBR Green I fluorescent dye. The quantification of GAPDH encoding messenger RNA (mRNA) by RT-PCR served as an internal control for each cDNA sample. TIMPs 1, 2, 3, and 4

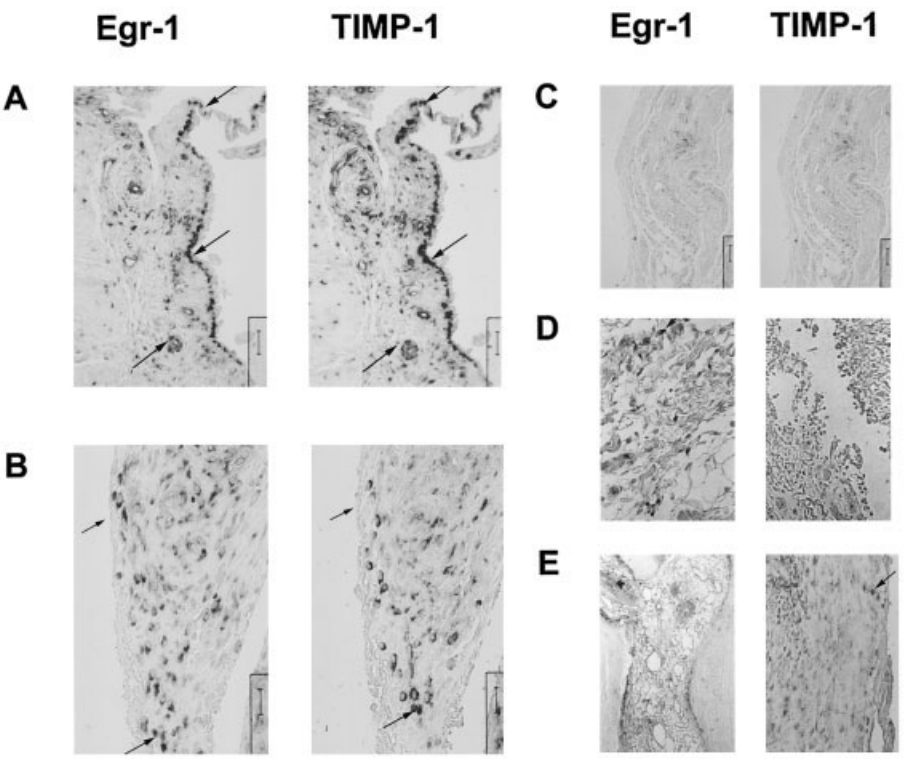

Figure 1. Coexpression of tissue inhibitor of metalloproteinases 1 (TIMP-1) and early growth response 1 (Egr-1) in synovial tissue. Serial sections of synovial tissue from a rheumatoid arthritis patient were analyzed by in situ hybridization using Egr-1 and TIMP-1 antisense (**A** and **B**) and sense (**C**) probes. Synovial fibroblasts coexpressing Egr-1 and TIMP-1 in the synovial lining (**A**) and in deeper regions (**B**) are shown by **arrows**. Specimens from a healthy donor (**D**) and from an osteoarthritis (OA) patient (**E**) were used as controls. In the synovial membrane of the healthy donor, only a few cells close to vessels expressed Egr-1; TIMP-1 signals were not detected (**D**). In OA synovial tissue, Egr-1 was detected in some cells close to cartilage, and TIMP-1–expressing cells were detected in deeper layers of the synovial membrane (**E**) (**arrow**). Bars within inset boxes = 1 μm.

encoding copy numbers in Egr-1–expressing clones were compared with the mock-transfected controls. The amounts of the respective TIMPs encoding mRNA in each sample were adjusted according to the copy numbers encoding GAPDH in the same sample. In addition, the external cytokine standards were used to equilibrate each analysis in a range of concentrations from 10^3 to 10^6 copies, as suggested by the manufacturer (Roche Diagnostics). For additional quality controls, melting profiles were recorded, and the PCR products were also separated on agarose gels to confirm the expected product sizes.

Gel retardation assays. Binding of Egr-1 to its specific recognition sequences was assayed by gel retardation as described (26). Complementary oligonucleotides (12.5 pmoles) carrying Egr-1 binding sites were annealed and labeled using 50 μCi α^{32}P-dATP (Amersham Pharmacia Biotech) and 5 units *Klenow* polymerase (Stratagene). Retardation assays were performed using the following oligonucleotides (31): Egr-1 binding site 5′-GGATCCAGCGGGGGCGAGCGGGGGCG-3′ annealed to 5′-TTCGCCCCCGCTCGCCCCCGCTGGATCC-3′; nonfunctional Egr-1 binding site 5′-GGATCCATCTTGGGCGATCTTGGGCG-3′ annealed to 5′-TTCGCCCAAGATCGCCCAAGATGGATCC-3′; human TIMP-1 promoter 5′-TGTCCACGCTAGGGGCGGGGGTGGCAGCGGGTGCTT-3′ annealed to 5′-TTGCACCCGCTGCCACCCCCGCCCCTAGCGTGGACA-3′ (corresponding to positions 1667–1700, GenBank accession no. Y09720). Sp-1 binding oligonucleotides were obtained from Santa Cruz Biotechnology. Nuclear extract was prepared from 5×10^7 Egr-1$^+$ L-929 fibroblasts (31). Cells were washed twice with PBS, resuspended in 400 μl of hypotonic buffer (10 m*M* HEPES, pH 7.9, 10 m*M* NaCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol [DTT], and 0.5 m*M* PMSF), and lysed by the addition of 25 μl of 10% NP40. Nuclei were extracted in 20 m*M* HEPES, pH 7.9, 0.4*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, and 1 m*M* PMSF with constant agitation at 4°C for 15 minutes. Extracts were stored as aliquots at −80°C. Binding to oligonucleotides was assayed in a volume of 18 μl containing 0.03 pmoles of α^{32}P-dATP–labeled oligonucleotide, 3 μg of double-stranded poly dldC competitor, and 0.5–1 μl of nuclear

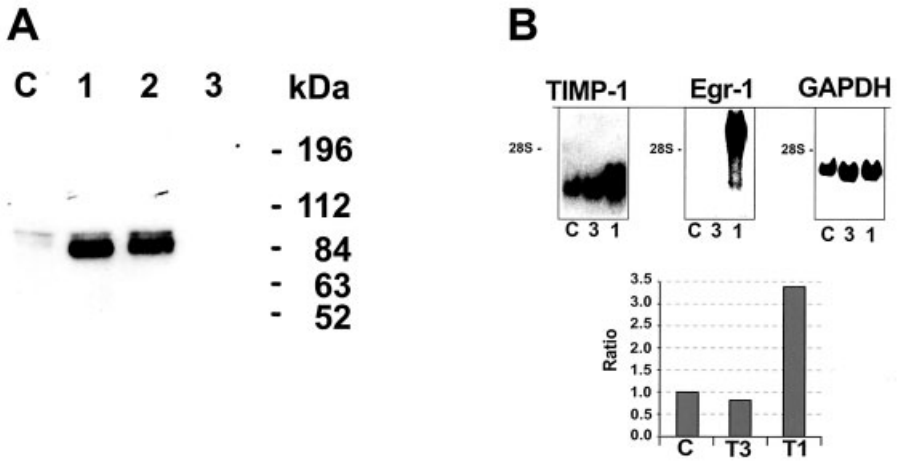

Figure 2. Expression of recombinant Egr-1 in transfectants. **A,** The pCR–Egr-1–Neo–transfected synovial fibroblasts were lysed after 12 hours of serum deprivation, and equal amounts of lysates were tested by Western blotting for Egr-1 expression. T1 (lane 1) and T2 (lane 2) showed prominent signals for the transfected Egr-1 protein, while in the untransfected cells (C) only the low background level of the endogenous Egr-1 was detectable. For the pCR–Egr-1–Neo–transfected and neomycin-resistant T3 Egr-1 protein, expression was not found (lane 3). This transfectant served as a mock control. **B,** Total RNA of the pCR–Egr-1–Neo transfectants and the untransfected cells (C) was analyzed by Northern blotting using a radiolabeled cDNA probe for TIMP-1. In the Egr-1–overexpressing clone T1, transcriptional induction of TIMP-1 could be detected when compared with the untransfected cells (C) and with the mock control clone T3 (top left). To determine quantity and quality of the RNA, a GAPDH cDNA fragment was rehybridized on the same blot (top right). A cDNA probe for Egr-1 was rehybridized on the same blot, confirming transfection with the Egr-1 expression vector (top middle). To specify the relative increase in TIMP-1 mRNA levels, the density of the TIMP-1 RNA signal of Northern blots was recorded using a video device, and signal intensities were equilibrated relative to the GAPDH signal. The Egr-1–overexpressing clone T1 revealed elevated TIMP-1 expression relative to the untransfected cells. In addition, the Egr-1–negative clone T3 displayed a lower TIMP-1 expression level, similar to that found with the untransfected cells (bottom). See Figure 1 for definitions.

extract in 31 m*M* KCl, 31 m*M* HEPES, pH 7.5, 6.25 m*M* $MgCl_2$, 0.06% NP40, 12.5% glycerol, 2.5 m*M* DTT, and 0.5 m*M* $ZnCl_2$ at room temperature for 30 minutes. For supershift assays, the Egr-1–specific antibody C-19 (Santa Cruz Biotechnology) was added at a final concentration of 20 μg/ml. DNA–protein complexes were separated by 4% PAGE using 0.5× Tris–borate–EDTA buffer at 150V at 4°C. Gels were fixed, dried, and exposed to x-ray films.

Detection of TIMP-1 by ELISA. Control cells and cells expressing Egr-1 were seeded in triplicate in 6-well plates at a starting density of 1.3×10^6 cells per well. Cells were inoculated for 24 hours in complete medium containing 10% FCS. Supernatants were aspirated and debris was removed by centrifugation (20,000*g* for 15 minutes at 4°C). Cells were washed twice with cold PBS and lysed in 100 μl lysis buffer containing 1% NP40, 150 m*M* NaCl, and 10 m*M* Tris HCl (pH 7). The lysates were cleared by centrifugation as described above. TIMP-1 in the supernatants and cell lysates was detected by ELISA (BioTrak, Amersham Pharmacia Biotech), which recorded the optical density at 450 nm. The mean and SD were computed from the respective raw data.

RESULTS

TIMP-1 and Egr-1 expression in synovial tissues of RA patients. Transcripts encoding TIMP-1 were detected by in situ hybridization in sections of synovial tissue from RA patients (Figure 1). Since the TIMP-1 staining pattern was highly reminiscent of the pattern previously found in Egr-1 transcripts (21) and because the human TIMP-1 promoter sequence contains 2 potential Egr-1–binding sites at positions −33 and −219 upstream of the transcription start, these findings suggested that Egr-1 regulates TIMP-1 expression in RA synovial fibroblasts. We therefore analyzed Egr-1 and TIMP-1 expression by in situ hybridization on serial sections of synovial tissue from different RA patients (Figure 1). The hybridization patterns of the Egr-1 and TIMP-1 probes indicated that both genes are coexpressed by a subset of cells located predominantly in the

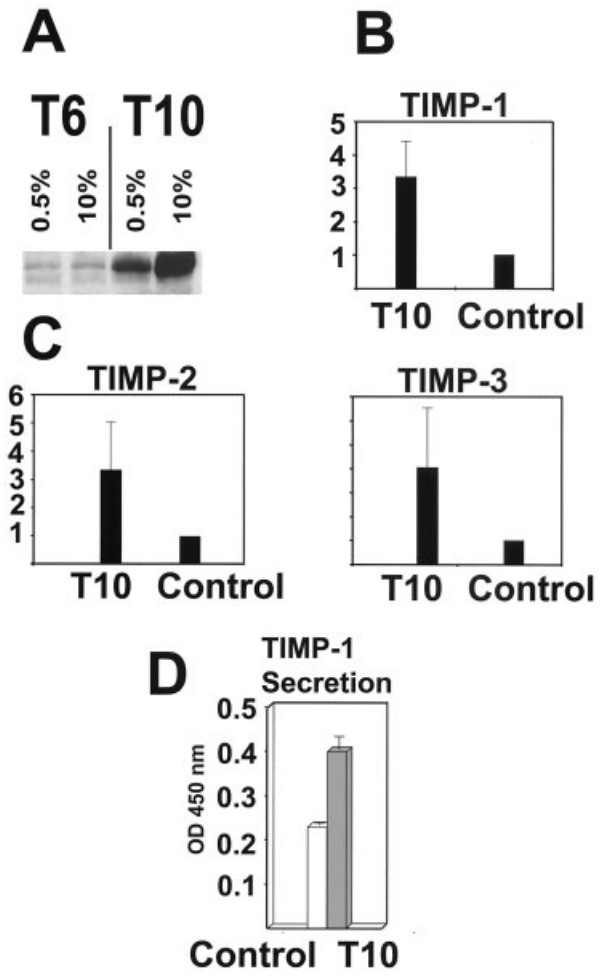

Figure 3. TIMP expression in Egr-1–expressing cells. **A,** Immortalized HSE.IM cells were stably transfected with Egr-1. Immunoblots of whole cell lysates of selected clones grown in medium containing either 0.5% or 10% fetal calf serum are shown: the mock-transfectant T6 and the Egr-1–overexpressing clone T10. **B,** RNA was extracted from Egr-1–expressing clone T10 and a mock-transfected control (T6). Then cDNA was generated and TIMP-1–encoding transcripts were enumerated by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using a light cycler. RT-PCR for GAPDH served as a control. PCR product quality was tested in all runs by melting curve analysis and gel electrophoresis. Egr-1–expressing clone T10 transcribed 3.5-fold more mRNA encoding TIMP-1 when compared with control cells T6. Bars show the mean and SD of 5 independent RT-PCR experiments. **C,** Increased expression of TIMP-2 and TIMP-3 in Egr-1 transfectant T10 by quantitative RT-PCR. Cells were analyzed using TIMP-2– and TIMP-3–specific primers. T10 transcribed 3.3-fold more mRNA encoding TIMP-2 when compared with control T6 and 4.1-fold more TIMP-3 mRNA. Bars show the mean and SD of 5 independent RT-PCR experiments. **D,** TIMP-1 secretion by mock transfected (T6, open column) and Egr-1–expressing cells (T10, shaded column) was analyzed by enzyme-linked immunosorbent assay using recombinant human TIMP-1 as controls. Egr-1–expressing T10 transfectants secreted 2-fold higher levels of TIMP-1 than the control clone T6. See Figure 1 for other definitions.

synovial lining layer (Figure 1A) but also are coexpressed in deeper regions (Figure 1B). Since synovial tissues from osteoarthritis (OA) patients or healthy donors revealed neither prominent Egr-1 nor TIMP-1 signals (Figures 1D and E), the in situ hybridizations suggested that Egr-1 regulates TIMP-1 expression in synoviocytes of RA patients. This hypothesis is supported by our previous results showing that Egr-1 is constitutively expressed in the RA synovium at high levels in synovial fibroblasts but not in CD68+ monocytes (21–23). To prove our hypothesis, we transfected human synovial fibroblast lines lacking high levels of Egr-1 activity with an Egr-1 expression vector and analyzed them for TIMP-1 expression.

TIMP-1 expression in Egr-1–transfected synovial fibroblast cell lines. Two different synovial fibroblast cell lines immortalized with SV40–T-ag K4IM (33) and HSE.IM (41) were used to generate stable Egr-1 transfectants. Egr-1 protein expression was tested by immunoblotting, and representative examples with at least a 10-fold increase in Egr-1 expression levels are shown in Figure 2A. Since the K4IM transfectant T3 did not express Egr-1, it was used as a mock transfection control in all further experiments.

We compared the TIMP-1 expression levels between Egr-1^+ K4IM transfectants, T3 control cells, and untransfected parental fibroblast lines by Northern blotting. As shown by the representative example in Figure 2B for the clones T1 (Egr-1^+) and T3 (Egr-1^-), Egr-1 expression correlated with enhanced transcription levels of the endogenous TIMP-1 gene. A quantitative analysis using GAPDH as an internal standard revealed a 3.5-fold induction of TIMP-1 mRNA in T1 (Figure 2B). Analyzing other Egr-1 transfectants of K4IM, we found ~2–3.5-fold higher levels of TIMP-1 mRNA when compared with the respective controls (data not shown). Since transfectant T3 neither expressed Egr-1 nor enhanced levels of TIMP-1 mRNA, the induced transcription of the endogenous TIMP-1 gene correlated strongly with Egr-1 expression.

In addition to the experiments with K4IM cells, we generated stable Egr-1 transfectants using the immortalized HSE.IM synovial fibroblast line and compared TIMP-1 expression in Egr-1–expressing cells (T10) with an Egr-1^- control (T6) by quantitative RT-PCR (Figure 3A). The overexpression of Egr-1 in T10 cells correlated with a 3.5-fold induction of TIMP-1 mRNA (Figure 3A). These results were further corroborated by transfecting the fibrosarcoma line HT1080 transiently with the Egr-1 expression vector, revealing a

3-fold induction of TIMP-1 mRNA by quantitative RT-PCR (data not shown).

We also found increased levels of TIMP-2 and TIMP-3 transcripts in extracts of Egr-1–expressing HSE.IM T10 transfectants but not in Egr-1$^-$ T6 controls (Figure 3B). In contrast, TIMP-4 levels remained unchanged (data not shown). Therefore, these results strongly suggest that enhanced TIMP-1 expression depends on Egr-1 activity and is biased neither by the host cell line nor by SV40–T-ag expressed in the immortalized K4 and HSE cell lines.

Since active TIMP-1 is secreted to the extracellular space, TIMP-1 protein levels in the supernatants of the Egr-1 transfectants were tested by ELISA. For Egr-1$^+$ fibroblasts, TIMP-1 protein was detected at concentrations >50 ng/ml, whereas control cells inoculated at the same density and under the same conditions expressed TIMP-1 at 50% lower levels (Figure 3C).

Egr-1 binding sites in the TIMP-1 promoter. To analyze whether the inducible TIMP-1 expression was directly mediated by Egr-1, we investigated the binding of Egr-1 to the TIMP-1 promoter with gel retardation experiments. Nuclear extracts were isolated from Egr-1$^+$ fibroblast lines and compared with nuclear extracts from cells expressing Egr-1 only at basal levels. The inspection of the putative Egr-1 binding site (5′-GGGCGGGGG-TGGC-3′) also revealed a binding site for the basic transcription factor Sp-1 (RGCGGG), suggesting that Sp-1 activity could interfere with Egr-1 binding. Therefore, we addressed the question of Egr-1 binding and competition with Sp-1 for a common binding sequence, by adding 20-fold and 120-fold molar excesses of unlabeled oligonucleotide containing a cognate Sp-1 binding site.

In nuclear extracts from control cells, 2 signals were detected that disappeared when unlabeled Sp-1–specific oligonucleotides were added as competitors in excess (Figure 4, lanes 1, 6, and 7). Extracts from Egr-1$^+$ cells showed an additional signal (lane 2) that was still visible after adding a 20-fold or 120-fold molar excess of the unlabeled Sp-1 competitor oligonucleotide (Figure 4, lanes 3 and 4). This signal, generated with Egr-1$^+$ extracts, probably represents a specific binding of Egr-1 on the Egr-1 binding site in the TIMP-1 promoter sequence, since this band disappeared in the presence of the Egr-1–specific antibody C-19 (lane 5). Therefore, the gel retardation experiments showed that Egr-1 binds specifically to a recognition sequence located within the TIMP-1 promoter 33-bp upstream of the TIMP-1 transcription start site.

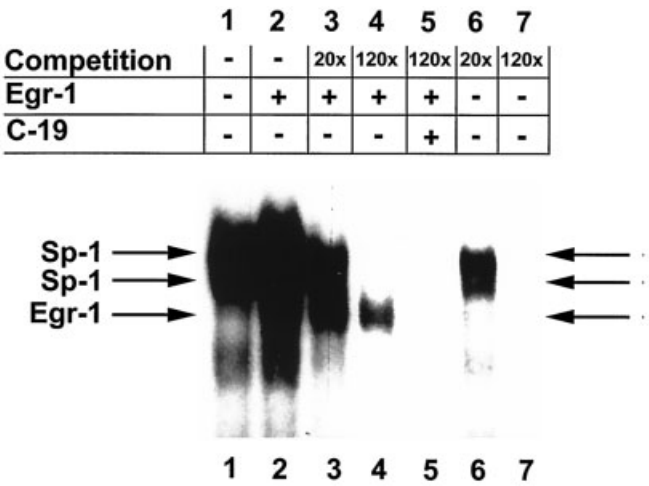

Figure 4. DNA binding activity of Egr-1 protein to TIMP-1 promoter. Electrophoretic mobility shift assay was performed using a radiolabeled oligonucleotide from the TIMP-1 promoter containing a putative Egr-1 binding motif. Nuclear extracts were isolated from serum-induced Egr-1–expressing cells (lanes 2, 3, 4, and 5) and compared with noninduced cells (lanes 1, 6, and 7). To compete for Sp-1 binding, a 20- or 120-fold molar excess of unlabeled Sp-1 binding oligonucleotides (lanes 3, 4, 6, and 7) was added. Binding of the Egr-1 protein to the TIMP-1 promoter was disrupted by an Egr-1–specific antibody, proving the identity of the protein (lane 5). See Figure 1 for definitions.

Functional analysis of Egr-1 binding sites in the TIMP-1 promoter. To confirm specific binding of Egr-1 to the recognition sequence in the TIMP-1 promoter in vivo, we performed reporter gene assays. Transient transfection of Egr-1–expressing HSE.IM T10 and of T6 control cells using a TIMP-1 promoter–luciferase construct showed a 3-fold induction of TIMP-1 promoter activity by Egr-1. Deletion of the Egr-1 binding site at position −33 again reduced the TIMP-1 promoter activity to background levels (Figure 5A). In a second set of experiments, we assessed the expression of a CAT reporter gene driven by the human TIMP-1 promoter. The function of the Egr-1 binding site at position −33 was analyzed by changing the overlapping Egr-1/Sp-1 binding sites from 5′-GGG**GCGGGGGTG**G to 5′-GGG**GCGCCCGTG**G, removing the recognition sequences for both Egr-1 (G**GCGGGGGTG**G) and Sp-1 (**GGGGCGG**). Since another potential Egr-1 binding site was found at position −219 (5′-G**GTGGGGGAG**G), this site was changed by in vitro mutagenesis in a second TIMP-1–CAT reporter construct to 5′-G**GTGCACGAG**G. In a third reporter plasmid, we combined both Egr-1 binding site mutants.

The activity of all 3 mutated TIMP-1 promoters

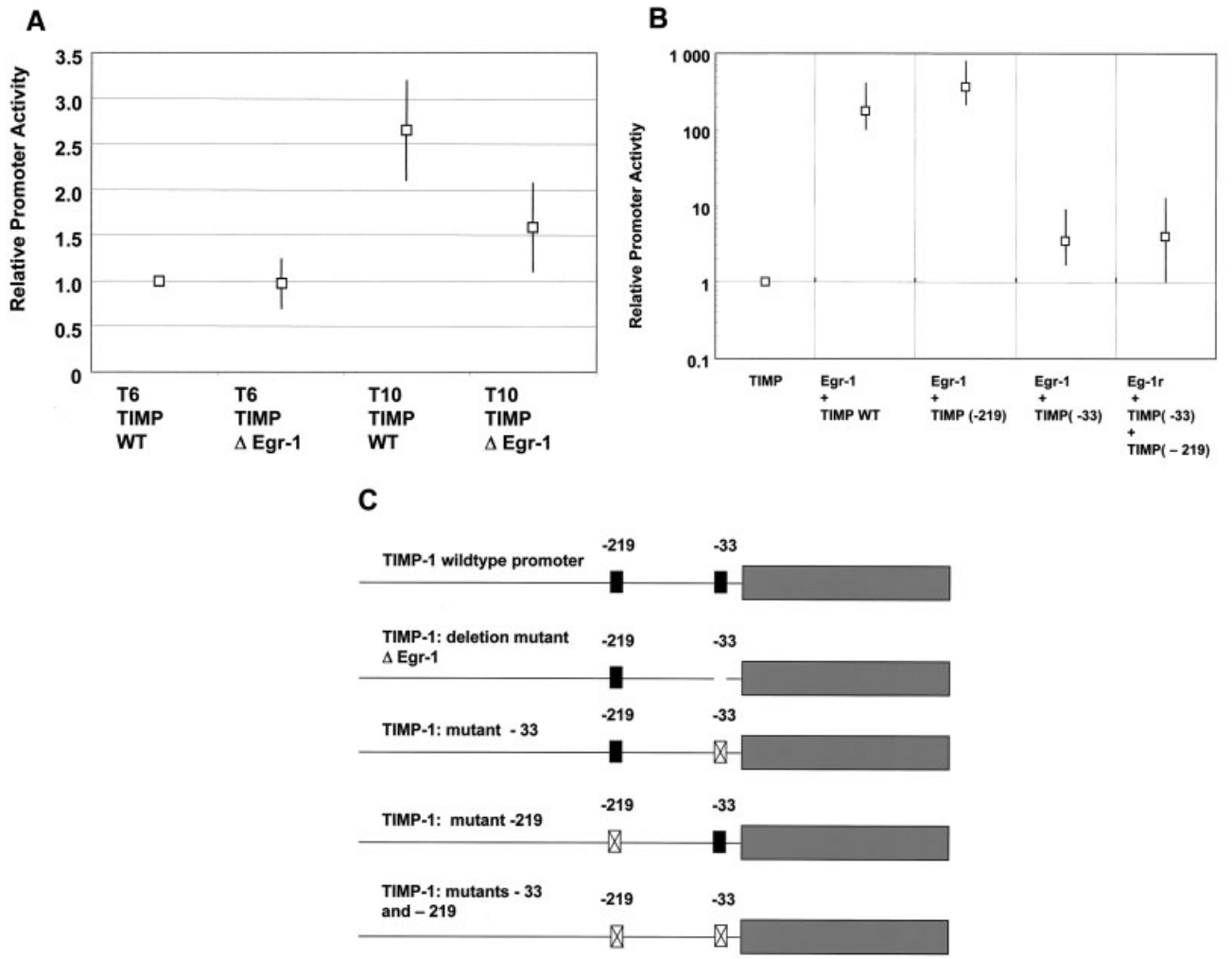

Figure 5. Functional analysis of the TIMP-1 promoter. **A,** Egr-1–expressing T10 transfectants and Egr-1$^-$ T6 control fibroblasts were transfected with the wild-type (WT) TIMP-1 promoter cloned into pGL3 basic or the corresponding plasmid lacking the Egr-1 binding site at position −33. The induction index of the TIMP-1 promoter activity by Egr-1 is shown in comparison to controls. The data represent the mean ± SD of 2 independent experiments. **B,** TIMP-1 promoter activity analyzed with plasmid p1338-CAT and the respective mutants lacking the Egr-1 recognition sequences. Plasmids were cotransfected with the Egr-1 expression vector pEF–Egr-1 together with pCMV–enhanced green fluorescent protein into K3IM or K4IM fibroblasts (−33). **C,** Schematic representation of the Egr-1 binding site deletion and point mutants. See Figure 1 for definitions.

was then compared with the wild-type TIMP-1 promoter in cotransfection experiments using the pEF–Egr-1 expression vector as a source for Egr-1 and K4.IM synovial fibroblasts as a host. Transfection frequencies were controlled by analyzing the expression of GFP produced from a CMV–EGFP expression vector that was also included in the cotransfection experiments. The results from 2 independent transfection assays showed a >100-fold induction of TIMP promoter activity by cotransfected Egr-1, which was almost completely abolished when the binding site at position −33 was mutated (Figure 5B). The mutation of the G**GTGGGGGAG**G sequence at position −219 alone did not alter the TIMP-1 promoter activity, whereas the combination of both mutations in the TIMP-1 promoter reduced CAT expression levels as drastically as was found for the reporter plasmid carrying only the −33 mutation. We therefore concluded from the electrophoretic mobility shift and promoter activity assays that the TIMP-1 promoter carries a single Egr-1 binding site at position −33 that is recognized by the Egr-1 protein in vivo as well as in vitro.

Regulation of other TIMP genes by Egr-1. Since the quantitative RT-PCR experiments also revealed that

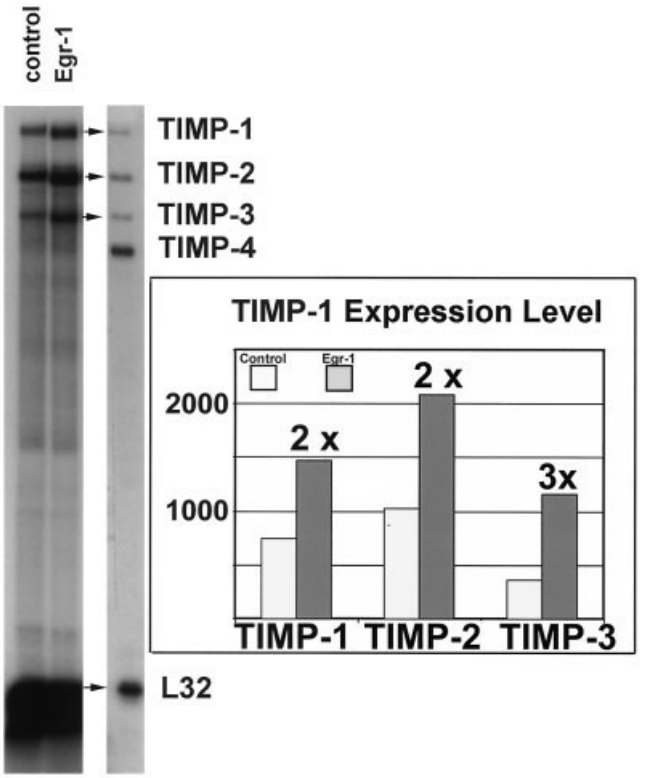

Figure 6. Detection of steady-state mRNA amounts encoding TIMP gene products by RNase protection assay. In extracts from Egr-1$^+$ clone T10, higher signals encoding TIMPs 1, 2, and 3 were detected when compared with control clone T4. TIMP-4 mRNA was not detected by RNase protection assay (left). The signal intensities were recorded by a video device, and the induction index was computed (right). See Figure 1 for definitions.

the TIMP-2 and TIMP-3 genes might be regulated by Egr-1, we quantified expression of all TIMP genes simultaneously by analyzing their mRNA levels in Egr-1–expressing T10 cells versus control cells, by RPA (Figure 6). Basal levels of TIMPs 1, 2, and 3 mRNA were found in HSE.IM control cells, whereas TIMP-4 was not expressed. As expected from the results shown earlier in this study, Egr-1–transfected cells transcribed ~2 times more TIMP-1 mRNA than the controls, and enhanced expression was also detected for TIMP-2 (3.4-fold) and TIMP-3 (4-fold). These results corroborate the quantitative RT-PCR data shown above (Figures 3B and C). TIMP-4 mRNA was detected by quantitative RT-PCR at very low copy numbers, and a thermocycler end point RT-PCR showed only very dim TIMP-4 PCR signals (results not shown). A difference of TIMP-4–encoding transcript amounts between Egr-1$^+$ cells and controls was not seen (data not shown).

DISCUSSION

The transcription factor Egr-1 is overexpressed in the synovial fibroblasts of RA patients when compared with those of OA patients or healthy controls (13,21,22). Therefore, Egr-1 may serve as an activation marker in RA synovial fibroblasts. The metabolic and functional consequences of Egr-1 overexpression in synovial fibroblasts are presently unclear. By analyzing the expression of Egr-1 and TIMP-1 by in situ hybridization in synovial sections from RA patients, we found very similar expression of Egr-1 and TIMP-1 in both synovial lining cells and deeper regions. To prove that the high levels of TIMP-1 expression were a result of the Egr-1 transcription factor activity, we established Egr-1–overexpressing transfectants from human synovial fibroblast cell lines immortalized with SV40–T-ag (33,41). We used this approach because these cells show normal expression levels of Egr-1, which are not as high as the levels found in synovial fibroblasts from RA patients. In addition, these cells retained the expression of many other characteristic synovial fibroblast markers (33). Analysis of the transfectants revealed that cells expressing high amounts of Egr-1 also contained 2–3.5 times more TIMP-1 mRNA and extruded higher levels of TIMP-1 protein.

By electrophoretic mobility shift and TIMP-1 promoter activity assays, we then demonstrated that the Egr-1 protein binds directly to a recognition site present at position −33 in the TIMP-1 promoter that overlaps with a binding site for the basic transcription factor Sp-1. Since Sp-1 is expressed constitutively in many different cell types, Sp-1 activity may be responsible for basal-level transcription of the TIMP-1 gene. In cells expressing high levels of Egr-1, such as synovial fibroblasts, Egr-1 may replace Sp-1 binding to TIMP-1 to further up-regulate TIMP-1 transcription. Similar regulatory mechanisms for basal and induced transcription have already been described for other genes carrying overlapping binding sites for Egr-1 and Sp-1 in their promoter regions (28,42,43). Our studies suggest that the expression of the TIMP-2 and TIMP-3 genes might be controlled in a similar manner as those genes. The TIMP-4 gene was not induced in Egr-1–expressing cells.

In RA, it has been postulated that an imbalance of expression between the MMPs and their inhibitors may contribute to the degradation of extracellular matrix and cartilage (18,19). In synovial fibroblasts of RA patients, the enhanced expression of MMPs, including MMP-1 and MMP-3 as well as their inhibitor TIMP-1, is well documented by in situ hybridization, with the

highest level of expression predominantly at sites of synovial attachment to cartilage (16,17). In the synovial fluid of RA patients, high levels of TIMPs 1, 2, and 3 were found (44), and the balance between TIMP-1 and MMP-3 activity was shown to regulate the conversion of progelatinase B to its active form. Since the transcription factor activity of Egr-1 seems to control the levels of several TIMP genes, the regulation of Egr-1 expression may function as a molecular switch that is part of the complex process that regulates MMP activities in the inflamed joint. In contrast, in rodent endothelial cells Egr-1 was shown to activate membrane type 1 MMP (MT1-MMP) expression, thereby promoting matrix destruction and facilitating invasive growth of these cells (45). However, it is known that the regulation of MT1-MMP expression differs in rodents and humans, suggesting species-specific gene regulation. The role of Egr-1 in the regulation of MT1-MMPs or other members of the MMP family in human cells remains to be determined.

The protective effect of TIMP-1 on the integrity of the extracellular matrix is, beyond its relevance to RA, of importance for metastatic tumor growth as well. The tumorigenic growth of cells transformed by c-Ha-ras was inhibited by recombinant TIMP-1, whereas in murine fibroblasts (NIH-3T3), transfection with antisense TIMP-1 RNA induced an oncogenic phenotype (46,47). Interestingly, the tumorigenic growth of NIH-3T3 fibroblasts transformed by v-sis could also be inhibited by constitutive expression of Egr-1, suggesting that Egr-1 has a tumor-suppressing function similar to the Wilms' tumor suppressor gene (48). Therefore, we considered the possibility that Egr-1 might have a function similar to the Wilms' tumor suppressor gene, which is a transcription factor that is structurally related to Egr-1 and has an almost identical zinc finger DNA binding domain compared with Egr-1. Our results indicate that one of the tumor-suppressing functions of Egr-1 can be attributed to the inhibition of MMP-1 activity by raised TIMP-1 levels. In addition, TIMP-1 was shown to inhibit chemotaxis of endothelial cells and thereby angiogenesis (49). Moreover, elevated TIMP-1 and TIMP-2 expression is associated with fibrosis (50). Since Egr-1 transcripts were detected in the subsynovial hyperplastic RA tissue, elevated TIMP-1 expression in RA synovial fibroblasts may modulate in situ vascularization of the synovium as well as synovial fibrosis.

Recent studies show that the invasive behavior of RA synoviocytes could be inhibited by overexpression of recombinant TIMP-1, and overexpression of TIMP-3 almost completely blocked the invasion of the fibroblasts into a Matrigel matrix in a transwell system (51). In contrast, application of an adenovirus-based expression system carrying the TIMP-1 gene by intravenous injection into DBA/1 mice immunized with bovine type II collagen resulted in earlier onset of inflammation, enhanced cartilage destruction, and bone erosion (52). This finding indicates that TIMP-1 or TIMP-3 may induce different metabolic responses, depending on the cell types and localization of their expression. Additional experiments must determine the exact function of TIMPs at articular sites.

Our results suggest that the transcription factor Egr-1 regulates the expression of TIMP-1 in synovial fibroblasts of RA patients by direct interaction with the TIMP-1 promoter. Moreover, our data indicate that Egr-1 is involved in regulating the transcription of the TIMP-2 and TIMP-3 genes but not the TIMP-4 gene. However, the benefit of MMP inhibition by activation of TIMP-1 in RA synovial fibroblasts may be achieved only at the risk of enhanced inflammation.

ACKNOWLEDGMENTS

The authors thank Beate Fischer, Michaela Weis-Klemm, Anita Hack, and Eva Wussler for excellent technical assistance and support.

REFERENCES

1. Geiler T, Kriegsmann J, Keyszer GM, Gay RE, Gay S. A new model for rheumatoid arthritis generated by engraftment of rheumatoid synovial tissue and normal human cartilage into SCID mice. Arthritis Rheum 1994;37:1664–71.
2. Muller-Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, et al. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. Am J Pathol 1996;149:1607–15.
3. Aupperle KR, Boyle DL, Hendrix M, Seftor EA, Zvaifler NJ, Barbosa M, et al. Regulation of synoviocyte proliferation, apoptosis, and invasion by the p53 tumor suppressor gene. Am J Pathol 1998;152:1091–8.
4. Kulka JP, Bocking D, Ropes MW, Bauer W. Early joint lesions of rheumatoid arthritis. Arch Pathol Lab Med 1955;59:129–50.
5. Gay S, Gay RE, Muller-Ladner U. Structure and function of synoviocytes. In: Koopman WJ, editor. Arthritis and allied conditions. Philadelphia: Williams & Wilkins; 1997. p. 243–54.
6. Muller-Ladner U, Kriegsmann J, Gay RE, Gay S. Oncogenes in rheumatoid arthritis. Rheum Dis Clin North Am 1995;21:675–90.
7. Burmester GR, Locher P, Koch B, Winchester RJ, Dimitriu-Bona A, Kalden JR, et al. The tissue architecture of synovial membranes in inflammatory and non-inflammatory joint diseases. I. The localization of the major synovial cell populations as detected by monoclonal reagents directed towards Ia and monocyte-macrophage antigens. Rheumatol Int 1983;3:173–81.
8. Bucala R, Ritchlin C, Winchester R, Cerami A. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. J Exp Med 1991;173:569–74.
9. Ritchlin C, Dwyer E, Bucala R, Winchester R. Sustained and

distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. Scand J Immunol 1994;40:292–8.
10. Lipsky PE, Davis LS, Cush JJ, Oppenheimer-Marks N. The role of cytokines in the pathogenesis of rheumatoid arthritis. Springer Semin Immunopathol 1989;11:123–62.
11. Houssiau FA. Cytokines in rheumatoid arthritis. Clin Rheumatol 1995;14 Suppl 2:10–3.
12. Franz JK, Kolb SA, Hummel KM, Lahrtz F, Neidhart M, Aicher WK, et al. Interleukin-16, produced by synovial fibroblasts, mediates chemoattraction for CD4+ T lymphocytes in rheumatoid arthritis. Eur J Immunol 1998;28:2661–71.
13. Trabandt A, Aicher WK, Gay RE, Sukhatme VP, Fassbender HG, Gay S. Spontaneous expression of immediately-early response genes c-fos and egr-1 in collagenase-producing rheumatoid synovial fibroblasts. Rheumatol Int 1992;12:53–9.
14. Krane SM, Conca W, Stephenson ML, Amento EP, Goldring MB. Mechanisms of matrix degradation in rheumatoid arthritis. Ann N Y Acad Sci 1990;580:340–54.
15. McCachren SS, Haynes BF, Niedel JE. Localization of collagenase mRNA in rheumatoid arthritis synovium by in situ hybridization histochemistry. J Clin Immunol 1990;10:19–27.
16. Gravallese EM, Darling JM, Ladd AL, Katz JN, Glimcher LH. In situ hybridization studies of stromelysin and collagenase messenger RNA expression in rheumatoid synovium. Arthritis Rheum 1991;34:1076–84.
17. McCachren SS. Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. Arthritis Rheum 1991;34:1085–93.
18. MacNaul KL, Chartrain N, Lark M, Tocci MJ, Hutchinson NI. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts: synergistic effects of interleukin-1 and tumor necrosis factor-α on stromelysin expression. J Biol Chem 1990;265: 17238–45.
19. Martel-Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP. Excess of metalloproteases over tissue inhibitor of metalloprotease may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. Lab Invest 1994;70:807–15.
20. Firestein GS, Paine MM, Littman BH. Gene expression (collagenase, tissue inhibitor of metalloproteinases, complement, and HLA–DR) in rheumatoid arthritis and osteoarthritis synovium: quantitative analysis and effect of intraarticular corticosteroids. Arthritis Rheum 1991;34:1094–105.
21. Aicher WK, Heer AH, Trabandt A, Bridges SL Jr, Schroeder HW Jr, Stransky G, et al. Overexpression of zinc-finger transcription factor Z-225/Egr-1 in synoviocytes from rheumatoid arthritis patients. J Immunol 1994;152:5940–8.
22. Aicher WK, Dinkel A, Grimbacher B, Haas C, Seydlitz-Kurzbach EV, Peter HH, et al. Serum response elements activate and cAMP responsive elements inhibit expression of transcription factor Egr-1 in synovial fibroblasts of rheumatoid arthritis patients. Int Immunol 1999;11:47–61.
23. Kuchen S, Pap T, Kunzler P, Gay RE, Gay S, Aicher WK. Zinc finger transcription factor Egr-1 induces the expression of collagenase inhibitor TIMP-1 in rheumatoid arthritis synovial fibroblasts [abstract]. Arthritis Rheum 1999;42 Suppl 9:S247.
24. Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, et al. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 1988;53:37–43.
25. Herschman HR. Primary response genes induced by growth factors and tumor promoters. Annu Rev Biochem 1991;60: 281–319.
26. Dinkel A, Warnatz K, Ledermann B, Rolink A, Zipfel PF, Burki K, et al. The transcription factor early growth response 1 (Egr-1) advances differentiation of pre-B and immature B cells. J Exp Med 1998;188:2215–24.
27. Miyazaki T. Two distinct steps during thymocyte maturation from CD4−CD8− to CD4+CD8+ distinguished in the early growth response (Egr)-1 transgenic mice with a recombinase-activating gene-deficient background. J Exp Med 1997;186:877–85.
28. Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG, Tsai-Morris CH. A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene 1987;1:343–55.
29. Swirnoff AH, Milbrandt J. DNA-binding specificity of NGFI-A and related zinc finger transcription factors. Mol Cell Biol 1995; 15:2275–87.
30. Shingu T, Bornstein P. Overlapping Egr-1 and Sp1 sites function in the regulation of the transcription of the mouse thrombospondin 1 gene. J Biol Chem 1994;269:32551–7.
31. Dinkel A, Aicher WK, Haas C, Zipfel PF, Peter HH, Eibel H. Transcription factor Egr-1 activity down-regulates Fas and CD23 expression in B cells. J Immunol 1997;159:2678–84.
32. Cao XM, Koski RA, Gashler A, McKiernan M, Morris CF, Gaffney R, et al. Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. Mol Cell Biol 1990;10:1931–9.
33. Haas C, Aicher WK, Dinkel A, Peter HH, Eibel H. Characterization of SV40 T antigen immortalized human synovial fibroblasts: maintained expression patterns of EGR-1, HLA-DR and some surface receptors. Rheumatol Int 1997;16:241–7.
34. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
35. Kriegsmann J, Keyszer GM, Geiler T, Lagoo AS, Lagoo-Deenadayalan S, Gay RE, et al. Expression of E-selectin messenger RNA and protein in rheumatoid arthritis. Arthritis Rheum 1995;38: 750–4.
36. Dudov KP, Perry RP. The gene family encoding the mouse ribosomal protein L32 contains a uniquely expressed intron-containing gene and an unmutated processed gene. Cell 1984;37: 457–64.
37. Pagenstecher A, Stalder AK, Campbell IL. RNase protection assays for the simultaneous and semiquantitative analysis of multiple murine matrix metalloproteinase (MMP) and MMP inhibitor mRNAs. J Immunol Methods 1997;206:1–9.
38. Grimbacher B, Aicher WK, Peter HH, Eibel H. Measurement of transcription factor c-fos and EGR-1 mRNA transcription levels in synovial tissue by quantitative RT-PCR. Rheumatol Int 1997;17: 109–12.
39. Ishiguro N, Ito T, Kurokouchi K, Iwahori Y, Nagaya I, Hasegawa Y, et al. mRNA expression of matrix metalloproteinases and tissue inhibitors of metalloproteinase in interface tissue around implants in loosening total hip arthroplasty. J Biomed Mater Res 1996;32: 611–7.
40. Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R, et al. TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis: a prevailing nondegradative lung microenvironment? Am J Physiol Lung Cell Mol Physiol 2000;279:L562–74.
41. Parak WJ, Dannöhl S, George M, Schuler MK, Schaumburger J, Gaub HE, et al. Metabolic activation stimulates acid production in synovial fibroblasts. J Rheumatol 2000;27:2312–22.
42. Ackerman SL, Minden AG, Williams GT, Bobonis C, Yeung CY. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc Natl Acad Sci U S A 1991;88:7523–7.
43. Skerka C, Decker EL, Zipfel PF. A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. J Biol Chem 1995;270:22500–6.

44. Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, et al. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis 2000;59:455–61.
45. Haas TL, Stitelman D, Davis SJ, Apte SS, Madri JA. Egr-1 mediates extracellular matrix-driven transcription of membrane type 1 matrix metalloproteinase in endothelium. J Biol Chem 1999;274:22679–85.
46. Alvarez OA, Carmichael DF, DeClerck YA. Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases. J Natl Cancer Inst 1990;82:589–95.
47. Khokha R, Waterhouse P, Yagel S, Lala PK, Overall CM, Norton G, et al. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science 1989;243:947–50.
48. Huang RP, Darland T, Okamura D, Mercola D, Adamson ED. Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. Oncogene 1994;9:1367–77.
49. Johnson MD, Kim HR, Chesler L, Tsao-Wu G, Bouck N, Polverini PJ. Inhibition of angiogenesis by tissue inhibitor of metalloproteinase. J Cell Physiol 1994;160:194–202.
50. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 2000;1477:267–83.
51. Van der Laan WH, Goossens PH, Pieterman EJ, Quax PHA, TeKoppele JM, Breedveld FC, et al. More profound inhibitory effect of TIMP-3 gene transfer than TIMP-1 gene transfer on synovial fibroblast invasion [abstract]. Arthritis Rheum 2000;43 Suppl 9:S170.
52. Jorgensen C, Apparailly F, Baker AH, Lisignoli G, Facchini A, Sany J. Paradoxical effect of TIMP-1 gene transfer in collagen induced arthritis [abstract]. Arthritis Rheum 2000;43 Suppl 9:S232.