TGFβ1 Regulates Gene Expression Of Its Own Converting Enzyme Furin

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Abstract

TGFβ1 is known for its potent and diverse biological effects, including immune regulation, and cell growth and differentiation. We have recently shown that TGFβ1 precursor is processed by human furin COOH-terminal to the R-H-R-R^{278} cleavage site to generate authentic mature TGFβ1. In the present study, we demonstrate that steady-state furin mRNA levels are increased in rat synovial cells by 2 and 20 ng/ml TGFβ1. Stimulation with TGFβ1 results in a significant increase in furin mRNA levels, starting at 3 h with the peak effect observed at 12 h (2.5-fold increase ±0.4). TGFβ1 did not increase furin mRNA stability, and treatment of synovial cells with actinomycin D, before TGFβ1 addition prevented the increase in *fur* gene expression, suggesting that the observed regulation occurs at the level of gene transcription. Treatment of synovial and NRK-49F fibroblastic cells with exogenous TGFβ1 (5 ng/ml) or TGFβ2 (10 ng/ml) translates into an increase in pro-TGFβ1 processing as evidenced by the appearance of a 40-kD immunoreactive band corresponding to the TGFβ1 NH_2-terminal pro-region. Furin processing activity stimulated by TGFβ2 correlates with significant increase in extracellular mature and heat-activable TGFβ1 as determined by an isoform-specific ELISA assay. Taken together, these results demonstrate for the first time that TGFβ1 upregulates gene expression of its own converting enzyme, and that this expression is translated into augmented processing of the TGFβ1 precursor form. Such adaptive responsiveness of the TGFβ1 convertase may represent an important aspect of TGFβ1 bioavailibility in TGFβ1-related processes and pathological conditions. (*J. Clin. Invest.* 1997. 99:1974–1983.) Key words: transforming growth factor β • pro-protein convertase • inflammation • furin • gene regulation

Introduction

Approximately fifteen years ago, proteins that phenotypically transformed nonneoplastic rat kidney fibroblasts, and induced anchorage-independent growth of normal rat fibroblasts in soft agar, were identified and termed transforming growth factors (TGFβs) (1–2). Today, three TGFβ isoforms (TGFs β1, β2, and β3; encoded by separate genes) are known in the mammalian species (for reviews see references 3–5). TGFβ1 is the prototype of the TGFβ superfamily, which comprises activin/inhibins, bone morphogenic protein, and other members which share structural and functional similarities (6). Since the cloning of the TGFβs (7–9) and of their receptors (10–12), these ubiquitously expressed cytokines have attracted much attention because of their pleiotropic biological activities (for reviews see references 3–5, 13). For example, TGFβ is involved in embryogenesis, cell cycle arrest in late G1, wound healing, increased synthesis of extracellular matrix (ECM)[1] components, cell differentiation, tissue fibrosis, and the bifunctional modulation of hematopoietic cell growth. Accumulating evidence suggests that TGFβ is also a potent immunomodulator (for reviews see references 3–5). TGFβ has been documented to modulate Ig production (14) and to inhibit the generation/functions of cytotoxic T cells, NK cells, and of LAK cells. Following injury or immunologic challenge, TGFβ released from platelet stores (15) generates a chemotactic gradient for monocytes, neutrophils, and T lymphocytes. Once in the target organ, these cells (*a*) are exposed to increasing concentrations of TGFβ, (*b*) become activated, and (*c*) generate an inflammatory cascade by stimulating the release of even more TGFβ, other inflammatory cytokines, reactive intermediates, and prostaglandins. These activated leukocytes are eventually suppressed, and growth is inhibited by TGFβ via a strong feedback mechanism in favor of the resolution of the inflammatory process and tissue repair. In rheumatoid arthritis (RA), TGFβ acts as a chondroprotector by stimulating the synthesis of collagen and glycosaminoglycan by articular chondrocytes, which may counter the degradation of cartilage and joint destruction (16). TGFβ is also mitogenic for osteoblasts, and inhibits the formation of osteoclasts from bone marrow precursors (17), thus reducing bone loss in RA. Microgram amounts of TGFβ injected systemically for 1–2 wk protects against collagen-induced arthritis in rats, and antagonizes the evolution of both acute and chronic phases of polyarthritis induced by bacterial cell walls without discernible side effects (4, 18). Relevant proof of its antiinflammatory and immunosuppressive effects is provided from mice bearing a TGFβ1 null mutation. These mice develop systemic lupus erythematosus-like autoantibodies, and experience rapid wasting syndrome. Histological analysis revealed massive lymphoid infiltrates similar to those seen in pseudolymphoma of Sjögren's Syndrome (19, 20). Taken together, these findings identify TGFβ as a key molecule in the control of immunological and inflammatory reactions.

The active TGFβ1 molecule is defined as a 25-kD disulfide-

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC, pro-protein convertase; PKC, protein kinase C.

linked homodimer (15). Like many proteins (including polypeptide hormones, viral proteins, growth factors, and receptors) TGFβ1 is synthesized as a larger inactive precursor that must undergo proteolytic processing before releasing the bioactive product (21). Analysis of the TGFβ1 primary structure reveals that the 112-amino acid mature TGFβ1 is derived from the carboxy-terminal end of the 390-amino acid chain of the pre-pro–TGFβ1 (22). We have shown by in vitro and in vivo studies that TGFβ1 is efficiently processed by furin following an R-H-R-R^{278} sequence immediately before the amino-terminal Ala^{279} residue of the mature growth factor (23). Furthermore, the TGFβ1 processing defect in the furin-deficient LoVo cells can be corrected by coexpressing pro-TGFβ1 with the furin convertase. These studies identified furin as a relevant TGFβ1-converting enzyme.

Furin is the first member of a recently discovered family of mammalian processing enzymes collectively known as proprotein convertases or PCs (for reviews see references 24 and 25). Seven different PCs have now been identified. Some are mostly restricted to endocrine and neuroendocrine tissues (PC1/PC3 and PC2) (26, 27), or testicular spermatogenetic germ cells (PC4) (28), while others are widely expressed (paired basic amino acid cleavage enzyme [PACE4], PC5, and PC7) (29–31) or are ubiquitously expressed (furin) (32). PCs are Ca^{2+}-dependent serine proteases that are known to cleave within the precursor molecules, carboxyl-terminal to pairs of basic amino acids (e.g., R–R or K–R). Furin enzymatic activity has been extensively characterized, and has been shown to process more than 25 endogenous substrates which are soluble, like provon Willebrand factor (33), pronerve growth factor (34) or nonsoluble, like membrane-bound glycoproteins as the proinsulin receptor (35), and the HIV-1 glycoprotein gp160 (36), amongst others. These proteins are known to be routed via constitutive secretory pathway. Furin requires a R-X-K/R-R recognition motif for optimal processing, and is concentrated in the *trans*-Golgi network (37). The TGFβ1 precursor undergoes several posttranslational modifications in the Golgi apparatus that are required for proper folding, maturation, and secretion of the dimeric peptide (38).

Since furin is responsible for the proteolytic maturation of many pro-proteins, including growth factors such as TGFβ1 (23), it became interesting to evaluate the modulation of *fur* (*fes*/*fps* upstream region) gene expression. For example, we know that furin levels are differentially expressed in cell lines giving rise to proportional conversion of proinsulin into mature insulin (39). In mammals, high levels of *fur* transcripts were found in the liver and kidney, while lower levels were detected in the brain, spleen, and thymus, and even lower levels were found in the heart muscle, lung, and testis (32, 40). During embryogenesis in rat, in situ hybridization studies have also revealed differential spatial and temporal expression of the *fur* gene, with higher levels of furin mRNA detected in the heart and liver at stage e10, becoming more widely distributed during the later stages (41). This pattern of embryonic expression coincides with the time and localization at which the substrates pro-TGFβ1 (42) and proinsulin-like growth factor (43) are expressed. Although the regulation of *fur* gene expression by external stimuli is poorly understood, some evidence supports the role of cytokines in such regulation. The promoters regulating human *fur* gene expression have been cloned (44), and computer analysis of the published sequence (45) has revealed potential cytokine-related responsive elements such as AP-1, SP-1, C/EBPβ and USF/NF1 within the 5′ upstream region. In addition, it has been reported that *fur* gene expression can be upmodulated by PMA in the human lymphocytic cell line H9 (36). Here, we provide evidence for the first time that TGFβ1 upmodulates *fur* gene expression, which in turn increases pro-TGFβ1 maturation. Such process may permit significant adaptive responsiveness of the TGFβ1 convertase system. The involvement of this modulation in TGFβ-related pathological conditions (such as RA) is discussed.

Methods

Growth factors and chemical reagents. Recombinant human transforming growth factor beta-1 (TGFβ1) was a generous gift from Dr. Anthony F. Purchio (Oncogene Corp., Seattle, WA), murine tumor necrosis factor alpha (TNFα) was kindly provided by Genetech Inc. (South San Francisco, CA) and human recombinant interleukin-1 alpha (IL-1α) was supplied by Dr. Peter Lomedico (Hoffmann-La Roche, Nutley, NJ). Cycloheximide (CHX), collagenase type IV, and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Co. (St. Louis, MO). Actinomycin D was from Merck and Co. (Rahway, NJ).

Isolation and culture of rat synoviocytes. The isolation and culture of rat synovial cells is a modification of previously described methods (46). Briefly, the synovial membranes were isolated from the knees of healthy specific pathogen-free inbred Lewis (LEW) female rats (~ 100 g) (Harlan Sprague Dawley Inc., Indianapolis, IN) in sterile conditions, and were digested in a phosphate buffered saline (PBS) collagenase type IV (2 μg/ml) solution (Sigma) for 2 h in a humidified chamber containing 5% CO_2 at 37°C. The cells were then washed by centrifugation in sterile PBS. The synovial cells were allowed to adhere to sterile 100-mm petri dishes (Falcon Labware, Mississauga, Ont., Canada) containing D-MEM/F12 (Gibco BRL, Burlington, Ont., Canada), 20% fetal bovine serum (FBS) (Intergen Company, Rochester, NY) and 40 ng/ml garamycin (Shering Canada Inc., Pointe-Claire, Qué., Canada) for approximately 1 wk in the incubator. The synovial cells were then trypsinyzed and reseeded (1/4–1/6 dilution) for following passages, and gradually adapted to reduced concentrations of FBS (10%). The NRK-49F renal fibroblastic cell line obtained from ATCC (Rockville, MD) was cultured in DMEM (Gibco BRL), 20% FBS, and 40 ng/ml garamycin. Stimulations of exponentially growing cultures were performed in medium containing 5% FBS.

Synovial cell slide preparation. Similar to the previously described method (47), synovial cells were paraformaldehyde (PFA)-fixed onto poly-L-lysine coated slides. Briefly, 10-μl droplets of a suspension of rat synovial cells (20×10^7 cells/ml) in medium containing 5% FBS were allowed to settle onto poly-L-lysine glass slides in a moist chamber for 30 min. The slides were then transferred into a new dish filled with 4% PFA fixative for 20 min at room temperature. The cells were then washed in PBS, sequentially dehydrated in increasing concentrations of ethanol, air-dried, and stored at −80°C until in situ hybridization was performed.

In situ hybridization. Sense and antisense ^{35}S-labeled cRNA rat furin riboprobes were generated as previously described (48) after linearization of the plasmid with *Cla*I and *Xba*I, respectively. Briefly, 200 pmol of [^{35}S]UTP was dried down in a small RNase free Eppendorf tube. The radiolabeled riboprobes were prepared using an in vitro transcription kit (Promega Corp., Nepean, Ont. or Boehringer Mannheim Biochemicals, Laval, Qué. Canada) by resuspending the radioactive pellet in a total volume of 10 μl, containing 0.5 nM NTP-UTP (ATP, CTP, GTP), 1× transcription buffer, 20 U RNase inhibitor, 10 mM dithiothreitol (Sigma), 1 μg of rat furin linearized DNA template pSP72, and 1 μl of appropriate RNA polymerase T7. The reaction was carried out for 60–90 min at 37°C. The DNA template was then removed, and the cRNA riboprobe was purified. After decreasing riboprobe length to 300–400 nts by alkaline probe hydrolysis to allow better tissue penetration, hybridization was carried out for

24 h at 60°C in 30 μl of hybridization buffer containing 75% formamide, 10% dextran sulfate, 3× SSC, 50 mM $NaPO_4$, pH 7.4, 1× Denhart's solution, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sheared salmon sperm DNA, and 1 mM dithiothreitol. The coverslips were removed, and the slides were washed in 2× SSC, treated with RNase A (40 μg/ml) for 30 min at 37°C, and were then sequentially washed for additional 10-min time periods in 2×, 1×, and 0.5× SSC followed by a 1-h wash in 0.1× SSC at 60°C. The air-dried slides were dipped into emulsion, and exposure times varied from 15–30 d.

Plasmids and probes. The rat cRNA riboprobe was generated from a 1228 nts cDNA clone obtained from a rat liver library λgt11, and was subcloned into pSP72 (Promega Corp.). This clone corresponds to the previously described rat furin cDNA (coding region 1111–2338) (49). The vector was linearized with *Xba*I, and the cRNA antisense riboprobe was 1244 nts. Radiolabeled riboprobes were prepared using [^{32}P]UTP (800 Ci/mmol; Amersham Canada Ltd., Oakville, Ont. Canada) according to the Ambion MAXIscript™ in vitro transcription kit (Ambion Inc., Austin, TX). Transcription mixtures were constituted of 50 μCi of [^{32}P]UTP, 10 mM DTT, 0.5 mM of ATP, CTP, and GTP, 1× transcription buffer, 12.5 U of RNase inhibitor, 1 μg of the appropriate linearized plasmid, and T7 RNA polymerase in a total volume of 20 μl. The reaction was carried out for 90–120 min at 37°C. 1 μl of RNase-free DNase 1 was then added for 15 min at 37°C to remove the DNA template, and the riboprobe was purified over a Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) spin column.

As a control of RNA loading and integrity, blots were hybridized with a 1.0-kb *Pst* I cDNA probe of the housekeeping gene glyceraldehyde-3–phosphate dehydrogenase (GAPDH; American Type Culture Collection). The GAPDH probe was labeled with a multiprime DNA labeling system by using [^{32}P]dCTP (specific activity > 3,000 Ci/mmol; Amersham Canada Ltd.).

Northern analysis. Total RNA was extracted from primary cultured synovial cells according to the previously described TRI-Reagent protocol (Molecular Research Center, Inc., Cincinnati, OH) (50). Aliquots of 5 μg of total RNA were run on a horizontal gel apparatus in 1% agarose gel containing 1× Mops and 6% formaldehyde submerged in 1× Mops buffer (pH 7.0). The samples were transferred onto a nylon membrane Hybond N^+ (Amersham Corp., Arlington Heights, IL) by overnight capillary action with 10× SSC. After blotting, the RNA was fixed with 0.05 N NaOH, and the membranes were stained in 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5). The membranes were then prehybridized for 2 h at 68°C with 1× hybridization buffer containing 120 mM Tris (pH 7.4), 600 mM NaCl, 8 mM EDTA (pH 8.0), 0.1% Na_4PP, 0.2% SDS, 625 μg/ml heparin, and 10% dextran sulfate. Hybridization began with the addition of the [^{32}P]UTP-labeled cRNA probe, and was carried out overnight in one part 2× hybridization buffer and one part deionized formamide. The membranes were sequentially washed in 2× SSC/1% SDS at room temperature, 2× SSC/1% SDS at 68°C, 0.1× SSC/0.2% SDS at 68°C and 0.1× SSC/0.1% SDS at 68°C.

For the cDNA GAPDH probe, prehybridization and hybridization were carried out in the same prehybridyzation buffer as that used for the cRNA riboprobe. The membranes were prehybridized for 4 h at 68°C, and hybridization was carried out overnight. The membranes were then washed once at room temperature for 20 min in 2× SSC, and once with 0.1× SSC/0.5% SDS at 68°C for 60 min, and were then rinsed off at room temperature in 0.1× SSC.

The membranes were then exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −80°C for times ranging from 2 h to 3 d. Signal intensity was quantitated by densitometry with a Pharmacia LKB Ultrascan XL (Pharmacia Biotech). Densitometric values are expressed as the ratio of furin/GAPDH densitometric quantification with control values set at 1.

Western blot analysis. Rat synoviocytes and NRK-49F fibroblastic cell line were incubated in the presence of medium, TGFβ1 (5 ng/ml), or TGFβ2 (10 ng/ml) for periods of 24–48 h. The cells were then trypsinyzed, washed once with PBS, and lysed in NP-40–containing lysis buffer under rotation for 45 min. 100 μg of total protein content were separated into 10% SDS-PAGE gels, transferred onto nitrocellulose membranes, and blocked and probed overnight with anti–LAP antibodies (1:1250 dilution; R & D Systems, Inc., Minneapolis, MN). Immunoreactive bands were revealed by ECL detection system (Amersham, Oakville, Ont., Canada) using monoclonal anti–goat horseradish peroxidase-labeled IgG.

TGFβ1-specific ELISA. Quantitative determination of bioactive TGFβ1 in cell culture supernates of TGFβ2-stimulated rat synoviocytes and NRK-49F cells were performed using an ELISA assay specific for mature and active TGFβ1 (R & D Systems, Minneapolis, MN). The ELISA assay was used according to the manufacturer's de-

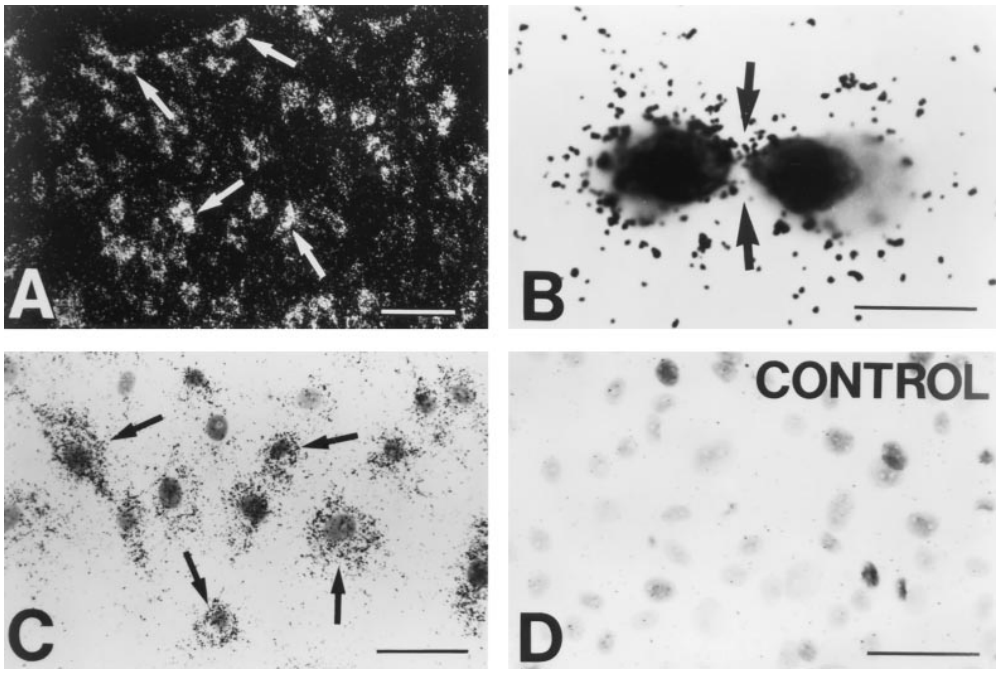

Figure 1. In situ hybridization of furin in cultured rat synoviocytes. Synovial cells were PFA-fixed onto poly-L-lysine–coated glass slides. Cells were stained with crysol violet, and then hybridized with rat furin cRNA antisense in *A* (116×), *B* (furin in dividing cells; 1940×), *C* (290×) and sense furin cRNA in panel *D* (control, 360×). Magnification bars represent 100 μm in *A*, 10 μm in *B* and 50 μm in *C* and *D*.

tailed protocol. The amount of TGFβ1 detected by the ELISA assay parallels the amounts measured in a standard TGFβ bioassay using mink lung epithelial cells (Mv1Lu).

Results

In situ hybridization of furin in rat synoviocytes

To assess the expression of furin mRNA in synovial cells, low passages rat synovial cells were PFA-fixed onto poly-L-lysine-coated glass slides, stained with crysol violet, and were hybridized with either rat cRNA sense or antisense riboprobes. Results expressed in Fig. 1 (*A* and *C*) demonstrated the baseline endogenous expression of rat furin mRNA in cultured synovial cells as illustrated by the presence of autoradiographic grains in the cytoplasm. It has been demonstrated that the synovial lining is composed of macrophage-like and fibroblast-like synoviocytes (51). In our synoviocyte preparation, the furin mRNA positive cells were typically large (25–30 μm) with diffuse light-staining nuclei, typical features of the fibroblast-like synoviocytes (52). Notice that within this population, furin mRNA was heterogeneously expressed. In *B*, we observed the presence of furin mRNA in a dividing synovial cell, indicating that the expression of furin mRNA is not restricted to one stage of the cell cycle. As a control (*D*), hybridization was carried out with a rat furin mRNA sense riboprobe in the same fashion as in the previous three panels. No specific in situ hybridization was observed in this condition. Based on our previous observation that TGFβ1 is efficiently processed by furin (23), and the fact that synovial cells play a major role in RA (53 and references therein), this result establishes cultured rat synovial cells as a good model for the study of furin expression in inflammation.

Effect of cytokines and PMA on furin mRNA accumulation

Since TGFβ1, IL-1α, and TNFα are major constituents of the inflammatory cascade, and are cytokines found in a variety of acute and inflammatory reactions including RA (54), it was of interest to evaluate whether these mediators could regulate cellular levels of furin convertase mRNA. For this evaluation, we performed Northern blot analysis on total cellular RNA obtained from rat synovial cells cultured in the absence or the presence of hTGFβ1 (2 ng/ml), mTNFα (20 ng/ml), and hIL-1α (2 ng/ml) for incubation periods of 1, 3, 6, and 18 h. Autoradiography of the membranes revealed that synovial cells constitutively express furin mRNA as a 4.4 kb signal upon probing

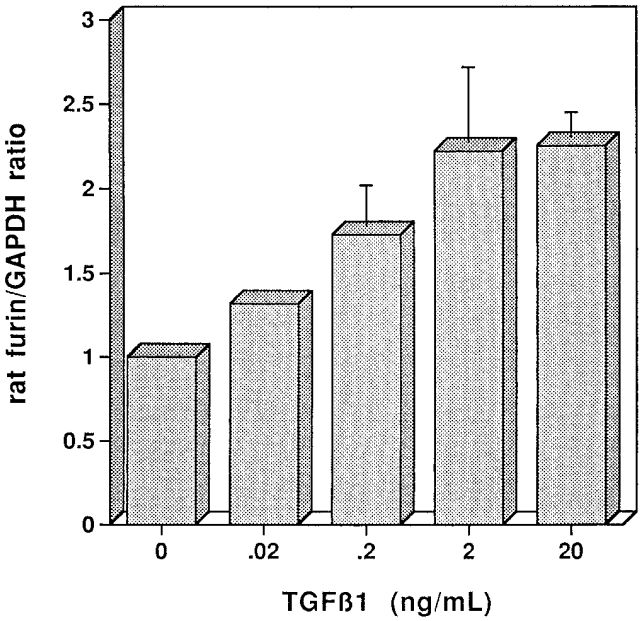

Figure 3. Concentration-dependent effect of TGFβ1 on rat furin mRNA accumulation. Synoviocytes were cultured in the presence of 0–20 ng/ml of TGFβ1 for an incubation period of 18 h. The cells were lysed, and total RNA was extracted. Equal amounts of RNA (5 μg/lane) were separated by gel electrophoresis, blotted onto a nylon membrane, and hybridized with rat furin cRNA riboprobe and cDNA GAPDH probes. Data are expressed as the mean±SEM, $n = 3$.

with a rat furin riboprobe (Fig. 2). Accumulation of furin mRNA was augmented 2.3-fold at time 18 h of stimulation in the presence of TGFβ1, as compared to control. This accumulation did not represent a general increase in cellular gene expression, since the level of GAPDH expression was unchanged. In contrast, treatment of synoviocytes with TNFα and IL-1α did not upregulate furin mRNA at 18 h or at earlier 1-, 3-, and 6-h time points (data not shown). Therefore, among the cytokines tested, only TGFβ1 specifically increased the accumulation of rat furin mRNA.

Based on the previous observation that PMA upregulated furin mRNA in H9 lymphocyte cell line (36), we have also evaluated the effect of PMA (10^{-7} M) on rat furin mRNA ac-

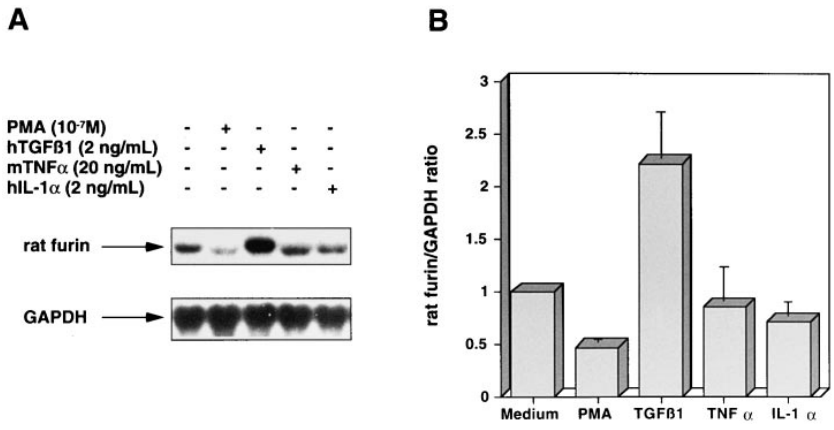

Figure 2. Effect of PMA, IL-1α, TNFα, and TGFβ1 on furin mRNA accumulation. Synoviocytes were cultured in the absence or in the presence of PMA (10^{-7} M), hIL-1α (2 ng/ml), mTNFα (20 ng/ml) and hTGFβ1 (2 ng/ml) for an incubation period of 18 h. The cells were then lysed, and total RNA was extracted and analyzed by Northern blot. (*A*) Autoradiogram of a typical experiment using cRNA riboprobe for rat furin and a cDNA probe for GAPDH. (*B*) Densitometry ratios of rat furin/GAPDH. Data are expressed as the mean±SEM, $n = 2$.

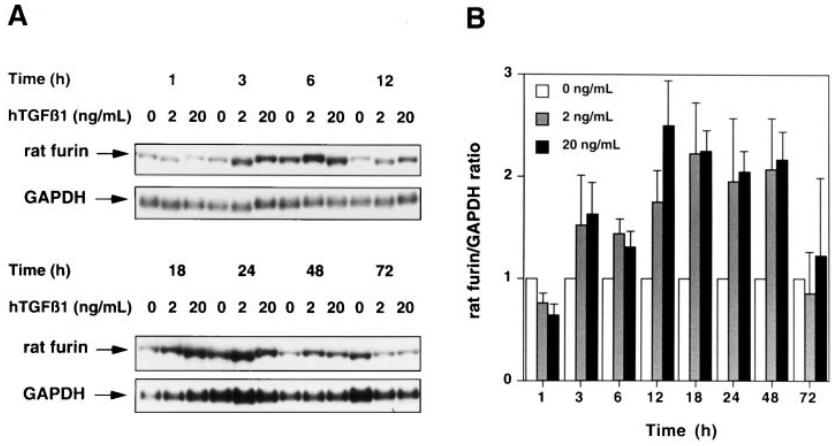

Figure 4. Kinetics of rat furin mRNA accumulation induced by TGFβ1. Synoviocytes were cultured with 0, 2, or 20 ng/ml of TGFβ1 for different periods of time ranging from 1–72 h. The cells were then lysed, and total RNA was isolated and prepared for Northern blot analysis. (*A*) Autoradiogram of a typical experiment using cRNA riboprobe for rat furin, and a cDNA probe for GAPDH. (*B*) Densitometry ratios of rat furin/GAPDH. Data are expressed as the mean±SEM, $n = 2$.

cumulation. In contrast to TGFβ1, PMA stimulation of synovial cells for 18 h does not increase furin mRNA levels. Significant upregulation, however, was observed at shorter time points (data not shown).

Concentration-dependent and kinetics of TGFβ1-induced regulation of furin mRNA accumulation

Synoviocytes incubated for 18 h with 0–20 ng/ml of TGFβ1 showed a gradual augmentation in furin mRNA levels, with detectable effects seen at 0.2 ng/ml TGFβ1, and maximal augmentation (2.25-fold) observed at 2 and 20 ng/ml (Fig. 3). Such maximal accumulation was seen using concentrations of TGFβ1 reported to be present in synovial effusions of RA patients (54, 55). In a time-course study, furin mRNA accumulation was increased at 3 h with maximal effect (2.5-fold increase) at 12 h poststimulation. This effect was sustained for up to 48 h of stimulation, declining thereafter (Fig. 4).

Mechanisms of TGFβ1-induced rat furin mRNA accumulation

mRNA $t_{1/2}$. To determine whether the augmented furin mRNA accumulation by TGFβ1 resulted from a transcriptional or posttranscriptional mechanism, we first examined whether TGFβ1 modulated furin mRNA stability. For this, synovial cells were incubated in the presence or absence of TGFβ1 for 18 h, new mRNA synthesis was abolished by the addition of 5 μg/ml actinomycin D, and the disappearance of furin mRNA was measured at different time points as indicated in Fig. 5. In control cells (without TGFβ1), the levels of furin mRNA progressively declined during actinomycin D treatment with a calculated half-life of 9.8±2.4 h. Treatment with TGFβ1 resulted in a similar decay rate with a half-life of 10.1±2.4 h. These demonstrate that the accumulation of furin mRNA induced by TGFβ1 is not because of changes in stability.

Inhibition of transcription. To ascertain whether TGFβ1-increased rat furin mRNA accumulation via transcriptional activation of the *fur* gene, we performed experiments in which synoviocytes were pretreated with or without actinomycin D (5 μg/ml) for 10 min before the addition of either medium or TGFβ1 (20 ng/ml). Incubation was allowed for 12 h. As shown in Fig. 6, pretreatment with actinomycin D completely abolished the TGFβ1-increased accumulation of rat furin mRNA. Similar results were obtained with a 24-h incubation period in the presence of TGFβ1 (data not shown). Taken together, these results suggest that in synovial cells, increased furin mRNA expression by TGFβ1 occurs at the transcription level.

Inhibition of protein synthesis. We verified whether de novo protein synthesis was required for TGFβ1-induced increase of the *fur* gene expression by using the protein synthesis inhibitor cycloheximide. Synoviocytes were pretreated with or without cycloheximide (10 μg/ml) for 30 min before addition of either

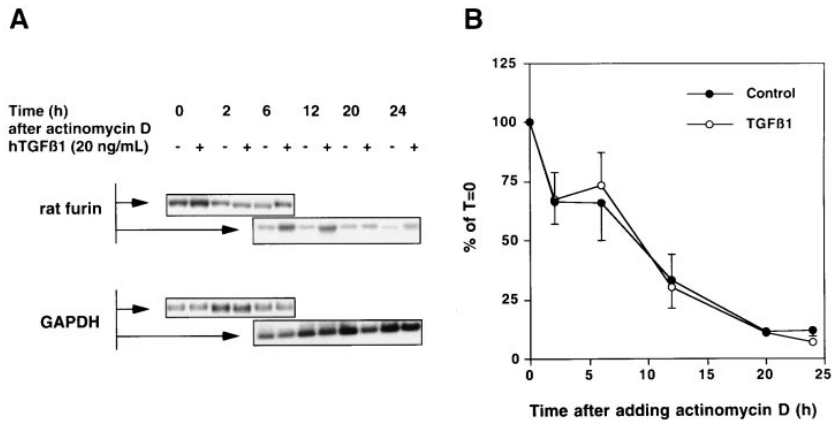

Figure 5. Densitometric evaluation of furin mRNA half-life ($t_{1/2}$) in control and TGFβ1-treated cells. Synoviocytes were incubated for 18 h with medium (*open circles*) or TGFβ1 (20 ng/ml) (*closed circles*), and the levels of furin mRNA were determined before (0 h) and 2, 6, 12, and 20 h after the addition of actinomycin D (5 μg/ml). Cells were lysed at the indicated times, and total RNA was extracted and analyzed by Northern blot. (*A*) Autoradiogram of two typical experiments using cRNA riboprobe for rat furin, and a cDNA probe for GAPDH. (*B*) Percentages of remaining rat furin mRNA relative to time 0, corrected for corresponding GAPDH values. Calculated $t_{1/2}$ was 9.8±2.4 and 10.1±2.4 h for medium and TGFβ1-stimulated synoviocytes, respectively. Data are expressed as the mean±SEM, $n = 4$.

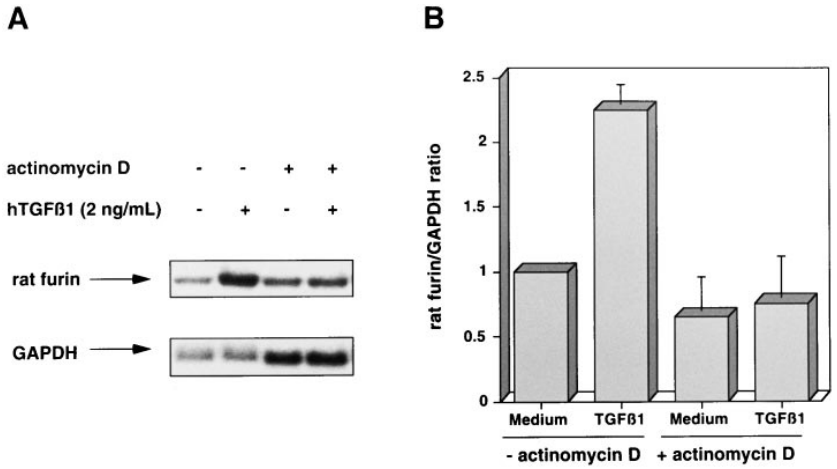

Figure 6. Effect of actinomycin D pretreatment on TGFβ1-induced furin gene transcription. Synoviocytes were pretreated for 10 min with medium or Actinomycin D (5 μg/ml) to block novel RNA synthesis. Medium or TGFβ1 (20 ng/ml) was then added. After 12 h of incubation, cells were lysed, and total RNA was extracted and analyzed by Northern blot. (*A*) Autoradiogram of a typical experiment using cRNA riboprobe for rat furin and a cDNA probe for GAPDH. (*B*) Densitometry ratios of rat furin/GAPDH. Data are expressed as the mean±SEM, $n = 4$.

medium or TGFβ1 (20 ng/ml). Incubation was allowed for 24 h. As shown in Fig. 7, the TGFβ1-increased accumulation of rat furin mRNA was also abrogated by pretreatment with cycloheximide. Similar results were obtained in a 12-h incubation with TGFβ1 (data not shown). This suggests that new protein synthesis is required for the effect of TGFβ1 on furin mRNA accumulation.

Effect of TGFβ1-induced increase in fur gene expression on pro-TGFβ1 maturation

We next asked whether the increase in furin mRNA accumulation induced by TGFβ1 correlates with increased proteolytic conversion of endogenous pro-TGFβ1. For this inquiry, we performed 24- and 48-h TGFβ1 (5 ng/ml) stimulation protocols with low passaged rat synovial cells and the NRK-49F renal fibroblastic cell line. TGFβ1-related digestion products in cell lysates were analyzed by electrophoresis in reducing 10% SDS-PAGE gels followed by immunoblotting. In unstimulated synovial cell lysates, we observed one major band with an apparent molecular weight of 50 kD corresponding to the intact precursor pro-TGFβ1 (56) (Fig. 8 *A*, lane *1*). Cell lysates from synoviocytes stimulated with TGFβ1 for 48 h show the appearance of a second band with an apparent molecular weight of 40 kD, which corresponds to TGFβ1 NH_2-terminal pro-region as detected using anti–latency associated peptide (LAP) antibodies (Fig. 8 *A*, lane *2*). Densitometric quantification of immunoreactive pro-region over precursor bands revealed a 2.6-fold increase in pro-TGFβ1 processing in synovial cells. Similar data were obtained with TGFβ2 stimulation (data not shown). Furthermore, a 2.1- and 2.9-fold increase in pro TGFβ1 processing was also revealed using the NRK-49F renal fibroblastic cell line following 24 and 48 h stimulations with TGFβ1 respectively (Fig. 8 *B*, lanes *1–4*). Thus, TGFβ1-increased endogenous *fur* gene expression correlates with endogenous pro-TGFβ1 proteolytic processing activity.

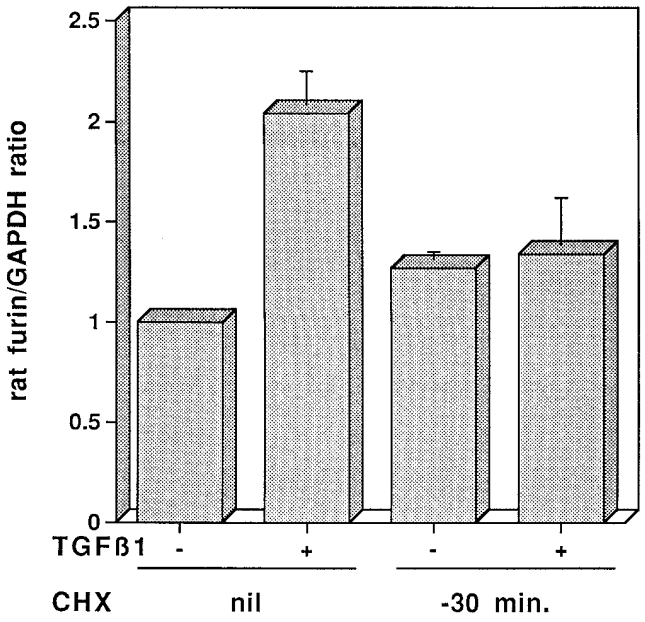

Figure 7. Effect of cycloheximide pretreatment on TGFβ1-induced rat furin mRNA accumulation. Synoviocytes were either untreated (*nil*) or pretreated with cycloheximide (*CHX*) (10 μg/ml) for 30 min before stimulation with media or TGFβ1 for 24 h. Cells were then lysed, and total RNA was extracted and analyzed by Northern blot. Histograms represent densitometry ratios of rat furin/GAPDH. Results are expressed as the mean±SEM, $n = 2–5$.

Relationship between fur gene expression and mature TGFβ1 production

We then evaluated if the increase in pro-TGFβ1 processing stimulated by TGFβ2 was also extended to an increase in the production of mature and heat-activable TGFβ1 in cell supernates. In these experiments, we used the TGFβ2 isoform since it also increased *fur* mRNA levels (unpublished observation) and it did not interfere with the measurement of TGFβ1 in the TGFβ1 isoform-specific ELISA assay. In fact, this assay detects only mature and bioactive TGFβ1 based on its binding to type II TGFβ receptors (which do not bind the pro- or latent form). Then, TGFβ1 is revealed by isoform-specific antibodies. Rat synovial and NRK-49F fibroblastic cells were incubated 24 and 48 h in the presence or absence of TGFβ2 (10 ng/ml), cell culture supernatants were collected and heat-activated, and the amounts of TGFβ1 were measured. As shown in Table I, a 3.0-4.1-fold increase in the amounts of mature and activable TGFβ1 was detected upon stimulation of synovial cells with TGFβ2. Similar observations were made with the fibroblastic cell line NRK-49F (data not shown). Thus, growth

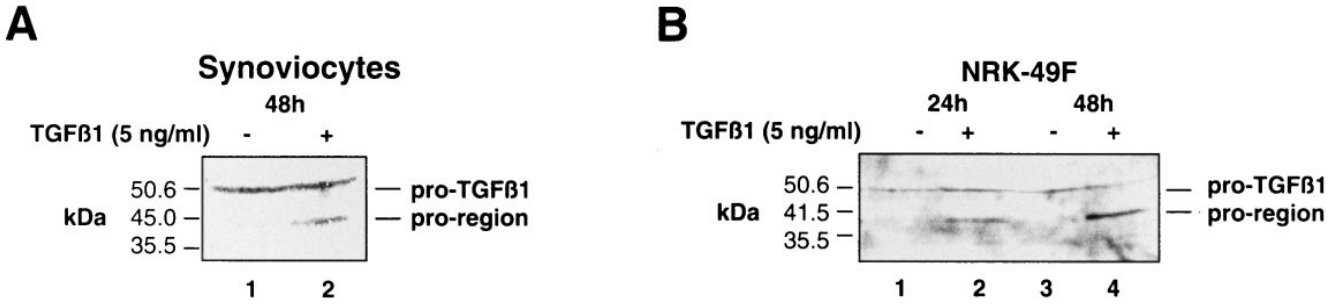

Figure 8. Effect of TGFβ1-stimulation on the maturation of intracellular pro-TGFβ1 in rat synovial cells. (*A*) Rat synovial cells in exponential growth phase were incubated with either medium or 5 ng/ml TGFβ1 for 48 h, and were then lysed. 100 μg of protein content was then separated in 10% reducing SDS-PAGE gels. Immunoblotting was performed using an anti–LAP antibody (1:1250). Lane *1*, unstimulated synovial cells; lane *2*, TGFβ1-stimulated cells for 48 h. (*B*) Rat NRK-49F fibroblastic cells in exponential growth phase were stimulated with either medium or 5 ng/ml TGFβ1 for periods of 24 and 48 h, and were then lysed. 100 μg of protein content was then separated in a 10% reducing SDS-PAGE gel. Immunoblotting was performed using an anti–LAP antibody (1:1250). Lane *1*, unstimulated NRK-49F cells, 24 h; lane *2*, TGFβ1-stimulated cells, 24 h; lane *3*, unstimulated NRK-49F cells, 48 h and lane *6*, TGFβ1-stimulated cells, 48 h.

factor-increased endogenous *fur* gene expression correlates with increased mature and bioactivable TGFβ1 production.

Discussion

In this report we provide evidence that the *fur* gene is expressed in synovial cells, and that its expression is selectively upregulated by growth factor TGFβ1, which in turn is also a furin cleavage product (23). We also show for the first time that the modulation in *fur* gene expression results in increased pro-TGFβ1 processing, the first enzymatic step leading to the production of bioactive TGFβ1. Although there are no previous reports on the regulation of *fur* expression by growth factors or other physiological agents, it has been documented that the phorbol ester PMA can upregulate *fur* gene expression in the human lymphocytic cell line H9 (36). In our system using primary cultured rat synoviocytes, the kinetics of TGFβ1 and PMA stimulation on furin mRNA accumulation exhibit significant differences. Whereas, TGFβ1 had maximal effect at longer time points (between 12–48 h stimulation), PMA had different phase kinetics by maximally increasing furin mRNA levels at a shorter stimulation time point (6 h), and no increase was detected after 18 h. It is widely established that phorbol esters such as PMA bind directly to and activate protein kinase C (57). PMA stimulation data and the work of others using the lymphoid H9 cell line (36) suggest that PKC stimulation might be part of the mechanism by which the *fur* gene is regulated. TGFβ1 on the other hand, indirectly activates PKC by initiating a phosphorylation cascade through binding to its specific type I and II receptors (58). This cascade results in the activation of phosphatidyl choline phospholipase C (PC-PLC), which hydrolyzes PC to generate diacylglycerol (DAG), the endogenous PKC activator (59). In several systems, TGFβ and PMA act through similar mechanisms via protein kinase C by inducing its translocation to the plasma membrane (60). This renders PKC sensitive to DAG, and causes activation of the protooncogenes c-*jun* and c-*fos* which form the AP-1 transcription complex (61). It is therefore possible that TGFβ1-stimulated increase in furin steady-state mRNA levels is a PKC-mediated event. Efforts are underway to identify the implication of PKC in this process.

Table I. Measure of Bioactive TGFβ1 from TGFβ2-stimulated Synoviocytes

	TGFβ1 in supernates (pg/ml)			
	24 h		48 h	
Stimulation protocol	Unstimulated	TGFβ2-stimulated	Unstimulated	TGFβ2-stimulated
Experiment				
1	300.8	888.8	424.3	1277.6
2	ND	ND	261.2	808.8
3	155.0	631.8	ND	ND

Rat synovial cells were incubated in the presence or the absence of TGFβ2 (10 ng/ml) for periods of 24 and 48 h. Cell supernates were then collected, and a sample was heat-activated (80°C, 5 min) and used to quantitate mature and bioactive TGFβ1 as described in Methods. Data is presented as TGFβ1 pg/ml of cell culture medium. ND = not determined.

The synovial membrane is a thin, 1–3-cell–deep lining which surrounds the intraarticular cavity, providing the cartilage with oxygen, nutrients, and glycosaminoglycans (such as hyaluronic acid) and giving the synovial fluid its characteristic viscosity and lubricant properties. The synovial lining is composed of two structurally distinct cell types (type A; macrophage-like synovial cells, and type B; fibroblast-like synovial cells) which undergo dramatic changes in RA and are directly implicated in the inflammatory process and cytokine networks of the knee joint (51). Macrophage-like synoviocytes are typically small, bipolar, and characteristically possess a dense-staining nucleus of 5–10 μm, whereas fibroblast-like synoviocytes are larger with a pale diffuse nucleus of 15–25 μm (52). It is expected that each of these cell types should express furin mRNA, since furin gene expression has been demonstrated to be ubiquitous (32). In situ hybridization of synoviocyte cultures demonstrated that furin mRNA levels are ubiquitously expressed, however, cells with morphological features characteristic of fibroblast-like synoviocytes express much higher furin mRNA levels. Based on our morphological cellular analy-

sis, we cannot rule out the possibility that macrophage-like cells also express furin mRNA, since it is well known that this population, which is eventually lost in vitro during the early passages of cell culture, is a major constituent of the arthritic joints. Previous studies have shown that macrophage-like synovial cells preferentially express intracellular adhesion molecule (ICAM-1) (62) and gelatinase B (63), whereas their fibroblast-like counterparts mainly express vascular cell adhesion molecule (VCAM-1) (62) and stromelysins (64). This functional diversity could now be extended to *fur* gene expression in the synovial lining. Since fibroblast-like synovial cells produce most of the synovial lining-derived TGFβ (65), and since furin has been found to activate stromelysin-3, an enzyme which destroys the antiproteolytic functions of proteinase inhibitors (66), the expression of furin in this population could have important physiological implications in ECM composition and dynamics.

Actinomycin D pretreatment had no effect on TGFβ1-induced furin mRNA regulation, which suggests that increased transcriptional activity of the *fur* gene is the mechanism responsible. The promoters regulating the expression of the *fur* gene have been cloned (44). Promoters P1A and P1B resemble housekeeping gene promoters in that they are very GC-rich and contain several SP1 sites. Promoter P1, on the other hand, has both TATA and CCAAT elements in the proximal promoter region, and was reported to be *trans*-activated by transcription factor C/EBPβ. TGFβ has been shown to regulate transcriptionally a number of genes. For example, TGFβ1 auto-induces the activity of the TGFβ1 gene through induction of the AP-1 complex (67). Also, CTF/NF-1 and USF (a ubiquitous factor of the basic helix-loop-helix family) transcription factors act in cooperation for the induction of the plasminogen activator inhibitor gene expression by TGFβ (68). Computer sequence analysis revealed that several regulatory elements for transcription factors such as AP-1, C/EBPβ, and USF, are found within the promoter of the *fur* gene (44, 45). It is therefore possible that TGFβ1 enhances the expression of the *fur* gene in synoviocytes through regulation of AP-1, C/EBPβ, and/or USF transcription factors. Protein synthesis is necessary for the transcriptional activation of the *fur* gene, suggesting that induction rather than activation (phosphorylation/dephosphorylation) of existing transcription factors could be the primary mechanism of TGFβ1 action. Supporting this, gene expression of the transcription factors C/EBPβ (69) and AP-1 were found to be upregulated by TGFβ (67).

It has been widely documented through the literature that TGFβ1 is autostimulatory, increasing the expression of its own gene and corresponding protein (67, 70). Here, we report that the augmentation in *fur* gene expression is associated with an increase in endogenous pro-TGFβ1 processing and elevated production of mature TGFβ1 present in heat-activable latent form (noncovalent reassociation with its pro-region). As expected from previous reports and the known low abundance of physiological levels of the furin protein (34, 71, 72), we were unable to measure the endogenous levels of either the zymogen or the 90-kD active form, and cannot comment as to whether the increase in mRNA results in elevated furin (active form). Nevertheless, the observed upmodulation of pro-TGFβ1 processing represents a potentially important regulatory step of the amplification loop involved in TGFβ1 autoregulation. In fact, this upmodulation will likely increase the intensity and duration of TGFβ1 biological functions in microenvironments that favor the activation of the latent TGFβ1 form, such as inflammatory sites (73). In this context, the recent availability of furin inhibitors will help to define the exact role of furin in TGFβ1-related processes (74).

Rheumatic diseases are characterized by irreversible ECM protein and cartilage degeneration because of the overexpression and activation of matrix metalloproteinases such as stromelysin-3 (75). The regulation in furin enzymatic activity may have implications in the dynamics of ECM degradation and synthesis homeostasis. For example, TGFβ is known to increase expression of several ECM proteins such as collagen and proteoglycans in a variety of cell types (76). TGFβ1 protects the ECM components from excessive degradation (as observed in RA) by decreasing the expression of metalloproteinases such as collagenase (77), transin/stromelysin (78), and plasminogen activator (79), and also by stimulating expression of protease inhibitors such as plasminogen activator inhibitor (PAI) (79) and tissue inhibitor of metalloproteinases (TIMP) (77). Recently, our laboratory has linked furin as one of the endoproteases responsible for TGFβ1 endoproteolytic processing (23). On the other side of the spectrum, furin has also been involved in the processing and activation of matrix metalloproteinase stromelysin-3, which has been shown to destroy the antiproteolytic function of α1 proteinase inhibitor (66, 67). Thus, furin seems to have a dual action on the outcome of the ECM proteins. By proteolytically processing TGFβ1 (23), furin can enhance production and conservation of the ECM via elevated concentrations of biologically available mature TGFβ1. Furin can also activate matrix metalloproteinases such as stromelysin-3 (67), and can ensure the integrity of the ECM. In this context, the increase in furin expression by TGFβ1 may therefore augment ECM turnover. Efforts are underway to assess the pattern of expression of furin in the articular joints of collagen-induced arthritis in rats.

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