Activation of Nuclear Orphan Receptor NURR1 Transcription by NF-κB and Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein in Rheumatoid Arthritis Synovial Tissue

Alice N. McEvoy, Eithne A. Murphy, Tiia Ponnio, Orla M. Conneely, Barry Bresnihan, Oliver FitzGerald and Evelyn P. Murphy

*J Immunol* 2002; 168:2979-2987; doi: 10.4049/jimmunol.168.6.2979

http://www.jimmunol.org/content/168/6/2979

**References**

This article cites 52 articles, 10 of which you can access for free at: http://www.jimmunol.org/content/168/6/2979.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Activation of Nuclear Orphan Receptor NURR1 Transcription by NF-κB and Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein in Rheumatoid Arthritis Synovial Tissue1

Alice N. McEvoy,* Eithne A. Murphy,* Tiia Ponnio,† Orla M. Conneely,† Barry Bresnihan,* Oliver FitzGerald,* and Evelyn P. Murphy2*

Modulation of the NURR subfamily of nuclear receptors may be an important mechanism regulating pathways associated with inflammatory joint disease. We examined the signaling mechanisms through which inflammatory mediators, produced by rheumatoid arthritis (RA) synovial tissue, contribute to the regulation of the NURR subfamily. Markedly enhanced expression of NURR1 is observed in synovial tissue of patients with RA compared with normal subjects. Modulation by proinflammatory mediators in primary RA and normal synoviocytes shows that PGE₂, IL-1β, and TNF-α markedly enhance NURR1 mRNA and protein levels in contrast to other subfamily members, NUR77 and NOR-1. We have established that transcriptional activation of the NURR1 gene by IL-1β and TNF-α requires a proximal promoter region that contains a consensus NF-κB DNA-binding motif. IL-1β- and TNF-α-induced NF-κB binding to this site is due predominantly to p65-p50 heterodimer and p50 homodimer subunit protein complexes. We further demonstrate a direct CREB-1-dependent regulation by PGE₂ situated at promoter region −171/−163. Moreover, analyses confirm the presence of CREB-1 and NF-κB p50 and p65 subunit binding to the NURR1 promoter under basal conditions in freshly explanted RA synovial tissue. In summary, enhanced NF-κB- and CREB-1-binding activity on the NURR1 promoter by inflammatory mediators delineates novel mechanisms in the regulation of NURR1 transcription. PGE₂-, TNF-α-, and IL-1β-dependent stimulation of the NURR1 gene implies that NURR induction represents a point of convergence of at least two distinct signaling pathways, suggesting an important common role for this transcription factor in mediating multiple inflammatory signals. The Journal of Immunology, 2002, 168: 2979–2987.

1 Abbreviations used in this paper: RA, rheumatoid arthritis; bFGF, basic fibroblast factor; BP, blocking peptide; CRH, corticotropin-releasing hormone; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PKA, protein kinase A.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the Health Research Board of Ireland.

2 Address correspondence and reprint requests to Dr. Evelyn P. Murphy, Department of Rheumatology, Education and Research Center, St. Vincent’s University Hospital, Dublin 4, Ireland. E-mail address: evelyn.murphy@ucd.ie


Copyright © 2002 by The American Association of Immunologists

0022-1767/02/$02.00

Received for publication September 5, 2001. Accepted for publication January 15, 2002.

Inflammation and hyperplasia of the synovial membranes of rheumatoid arthritis (RA) is a delicate tissue lining the joint capsule; however, in RA, the synovium transforms into an aggressive, tumor-like structure called the pannus. Synovocytes (fibroblasts) and macrophages within the synovium orchestrate a self-perpetuating inflammatory response via the autocrine/paracrine actions of cytokines, growth factors, and PGs (PGE₂) (3). Macrophage-derived cytokines such as TNF-α and IL-1β are potent mitogens for proliferating synoviocytes in the vicinity of the affected cartilage that produce matrix-degrading molecules, including matrix metalloproteinases (MMPs) (4–6). It is the persistent invasive and destructive growth of synovial tissue that ultimately leads to joint erosion (2, 7, 8). Many of the inflammatory mediators and MMPs implicated in RA are regulated by inducible transcription factors. Transcription factors such as NF-κB, AP-1, and CREB are pivotal regulators of inflammatory responses. Several independent studies have implicated abnormal expression of these transcription factors in the modulation of gene expression known to regulate cellular proliferation, angiogenesis, cytokine, and MMP production in RA synovium (9, 10).

The NURR subfamily belongs to a superfamily of structurally related transcription factors that control a variety of developmental and physiological processes (11, 12). This family of nuclear transcription factors can be divided into two major groups. The classical steroid receptors (e.g., estrogen, androgen, progesterone receptors) are ligand-activated transcription factors with characteristic structural features (13). Classical steroid receptors are involved in development, differentiation, and cell homeostasis by regulating the expression of specific target genes (12). Orphan receptors, which comprise the second group, have all the structural features of steroid receptors; however, by definition, the corresponding ligands and functions of these receptors are not known (14). NURR1 is an orphan member of this superfamily that is expressed constitutively in the developing and adult CNS (15). The protein exhibits a close structural relationship to the orphan receptors NUR77 and NOR-1 (16, 17). These three proteins comprise the NURR subfamily that binds to the same cis-acting consensus sequence (NBRE) to regulate target gene expression, and may function redundantly if expressed in the same cells (18–20). The three members of the subfamily play an important coordinate neuroendocrine-regulatory role at all levels of the hypothalamic-pituitary-adrenal axis (21, 22). Unlike most nuclear receptors, the NURR subfamily are products of immediate
early genes whose expression can be differentially induced in response to a variety of extracellular stimuli, including growth factors, neurotransmitters, and polypeptide hormones (21, 23–27). By coupling extracellular signals to nuclear receptor-regulated gene transcription, the induction of these nuclear receptors has been shown to be linked to changes in cellular phenotype. The NOR-1 gene was originally identified through its involvement in the chromosomal rearrangement associated with extraskeletal myxoid chondrosarcoma (28). Overexpression of NOR-1 may lead to alteration in chondrocyte differentiation and inappropriate activation of NURR1-dependent target genes and the extraskeletal myxoid chondrosarcoma phenotype (28–30). NURR1 induction by parathyroid hormone in bone cells suggests a role for this transcription factor in regulating bone metabolism (27). We have recently demonstrated involvement of NURR1 in the regulation of corticotropin-releasing hormone (CRH) expression and actions in human inflammatory joint disease (31, 32). Multipoint linkage analysis provides strong evidence that the CRH genetic locus is both linked to and associated with RA (33). Modulation of CRH receptor-mediated signaling may be an important mechanism regulating inflammatory events in human arthritis (32). We hypothesized that locally produced NURR1 may be a general mediator of an autocrine regulatory inflammatory cascade that serves to amplify the inflammatory response by increasing synovial CRH expression (31).

The aim of this study was to establish the signaling mechanisms through which inflammatory mediators contribute to synovial NURR1 expression. The expression pattern of NURR1 in inflamed synovium was examined and compared with normal synovial tissue. The pathological importance of the NURR transcription factors in inflammatory joint disease was assessed by determining the ability of the proinflammatory agonists, growth factors, and cytokines to regulate NURR transcript levels in normal and primary RA synoviocytes. We have defined highly conserved consensus sequences for the binding of both NF-κB subunits and CREB proteins in the NURR1 proximal promoter and examined the role of these sites in TNF-α, IL-1β, and PGE2-induced stimulation of NURR1 transcription. To analyze the relative contribution of the consensus sites to cytokine-mediated NURR1 transcription coupling and to further elucidate the mechanisms involved in these transduction pathways, we have generated a target gene construct containing proximal NURR1 promoter regions fused to a reporter gene. Using EMSA, we have elucidated CREB and NF-κB binding to the NURR1 promoter under basal conditions in freshly explanted RA synovial tissue. We discuss the role of proinflammatory mediators in inducing NURR1 transcription and consider the implications of these findings in the pathogenesis of RA.

Materials and Methods

Tissue collection

All synovial biopsies were obtained from the knee by arthroscopy following informed consent from patients diagnosed with RA (34). All patients attended the Early Arthritis Clinic at St. Vincent’s University Hospital (Dublin, Ireland). At the time of biopsy, all patients (n = 10) had active synovitis of recent onset (median 4 mo; range 0–12 mo). Patients may have been on nonsteroidal antiinflammatory medication but had received no disease-modifying agents, including prednisone, before the time of biopsy. Histologically normal synovium (n = 2) was obtained from patients undergoing lower limb amputation.

Synoviocyte cell culture

Synovial tissue obtained from the knee by arthroscopy was treated for 4 h with 1 mg/ml collagenase (type I; Worthington Biochemical, Freehold, NJ) in RPMI 1640 at 37°C in 5% CO2. Dissociated cells were plated in RPMI 1640 supplemented with 10% FCS (Life Technologies, Paisley, U.K.), penicillin (100 U/ml), and streptomycin (100 U/ml) (31). To eliminate non-adherent mononuclear cells from synovial cell preparations, the plated cells were cultured for at least 24 h. The cells were then washed extensively with PBS. Primary RA synoviocytes were used between the third and seventh passage for subsequent experiments (35). Primary synoviocyte cells were found to be morphologically homogeneous fibroblast-like cells and did not react with Abs to the macrophage/monocyte Ag CD14. The immortalized normal human K41 M synoviocyte cell line was grown, as described previously (36). All synoviocytes were grown in serum-free medium for 24 h before stimulation. Recombinant human TNF-α, IL-1β (Cabiobiochm, Darmstadt, Germany), TGF-β, platelet-derived growth factor (PDGF), basic fibroblast factor (bFGF) (R&D Systems, Minneapolis, MN), forskolin, and PGE2 (Sigma-Aldrich, Dorset, U.K.) were included, as indicated.

Transient transfection

Twenty-four hours before transfection, 1 × 105 synoviocyte cells were plated in 6 × 6-cm dishes and allowed to attach. Endotoxin-free DNA, 0.1 μg CMV-β-galactosidase (Promega, Madison, WI), or 0.1 μg transf-132/132 or -96/+373 NURR1-β-galactosidase was added to 35 μl Effective reagent (Qiagen, Cambridge, U.K.) in a volume of 900 μl RPMI 1640 (serum free) and incubated at room temperature for 30 min. RPMI 1640 (0.7 ml) was added to the transfection mixture, transfected onto the cells, and incubated for 6 h at 37°C in 5% CO2. An equal volume of medium was added and the cells were incubated overnight at 37°C in 5% CO2. The DNA-Effective-containing medium were replaced, and the cells were left untreated or treated with 10 ng/ml IL-1β, TNF-α, 1 μM PGE2, or 25 μM forskolin, and incubated for an additional 24 h. Protein concentrations were determined by the Bradford assay (Bio-Rad, Richmond, CA). β-galactosidase staining of cells growing in monolayer, cells were washed with cold PBS, fixed with cold 0.5% glutaraldehyde, and washed twice with PBS before incubation with staining solution (1 M MgCl2, 5 mM NaCl, 0.5 M HEPES (pH 7.3), 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide, and 2% 5-bromo-4-chloro-3-indoly-β-galactopyranoside) for 2 h at 37°C. β-Galactosidase levels in cell extracts were measured using 30 μl cell extract incubated for 1–4 h in 0.1 M sodium phosphate containing 2.5 mg/ml O-nitrophenyl-B-β-d-galactopyranoside, 0.3 mM MgCl2, and 15 mM 2-ME. The reaction was stopped by the addition of 1 M Na2CO3, and the OD of each reaction was read at 410 nm.

Northern blot analysis

Cell cultures grown in 25-cm2 tissue culture dishes were maintained in serum-free RPMI 1640 for 24 h before treatment. Total RNA was isolated using RNeasy (Qiagen) at specific times after treatment. RNA was quantitated by UV absorption, and 10 μg total RNA was electrophoresed on a standard Northern gel and transferred to nylon membrane (Bio-Rad). NURR1, NURR7, and NOR-1 cDNA probes, spanning the amino-terminal region to avoid cross-hybridization, were radiolabeled to a high sp. act. using [α-32P]dCTP and a random primer labeling system (Promega) (21, 31). Blots were exposed to film at −80°C using intensifying screens, and autoradiographic intensity was quantitated using an imaging densitometer.

Immunohistochemistry

Synovial tissue sections (7 μm) were placed on glass slides coated with 2% aminopropyl-triethoxy-silane. Synoviocytes grown on apyrogenic glass coverslips were treated with methanol for 15 min before staining. The primary Ab (1/100 dilution) for NURR1 (Santa Cruz Biotechnology, Santa Cruz, CA) was a rabbit polyclonal Ab mapping to the amino terminus of human and rat NURR1 (31). Following 2-h incubation with the primary Ab, a biotinylated secondary Ab (1/500; Vector Laboratories, Burlingame, CA) was spotted on sections, followed by the avidin-biotin-peroxidase complex (Vectorstain Elite ABC kit; Vector Laboratories). Immunofluorescence microscopy on K41 M synoviocytes was conducted using the similar procedures as that used for immunohistochemistry. Synoviocytes were incubated in diluted normal goat serum (Vector Laboratories). The primary polyclonal Ab for NURR1 was diluted 1/10 in 10% normal human serum and incubated on the sections for 90 min. Sections were washed in PBS and incubated for 30 min in biotinylated anti-rabbit secondary Ab (1/500; Vector Laboratories). A Cy3 or FITC fluorochrome-conjugated anti-biotin Ab (BN-34, 1:100; Sigma-Aldrich, St. Louis, MO) was added to bind the biotinylated anti-rabbit added previously. Following final washing in PBS, slides were mounted in DAko fluorescent mounting medium (DAko, Carpinteria, CA). For negative controls, isotype-matched nonimmune IgG were included and the primary Ab was preabsorbed with its specific synthetic peptide (blocking peptide (BP); 200 μg/ml; Santa Cruz Biotechnology).
Preparation of nuclear extracts and EMSAs

Nuclear protein extracts were prepared as previously described (21). Briefly, 1 x 10^6 primary synoviocytes or 100 mg synovial tissue were homogenized with 1 ml TBS (25 mM Tris-HCl (pH 7.4), 130 mM NaCl, and 5 mM KCl). The homogenate was centrifuged at 7,000 x g for 30 s and resuspended in 0.25 ml buffer A (10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, 0.1 mM EGTA, 0.5 mM PMSF, and 2 μg each of the protease inhibitors antipain, pepstatin A, and aprotinin per milliliter). Then 1.25 μl 10% Nonidet P-40 was added and mixed, and the cell suspension was incubated for 2 min on ice. The cells were centrifuged at low speed (1,700 rpm), and the supernatant was removed (cytosolic fraction). To the pellet, 0.125 ml buffer B (0.4 M NaCl, 10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl_2, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM PMSF) was added. The mixture was placed on ice for 2 h, with frequent agitation. Supernatants of the nuclear extracts were obtained by centrifugation at 15,000 x g for 5 min and then stored in aliquots at -80°C. The protein concentration was estimated with the Bradford protein assay kit (Bio-Rad).

For the EMSAs, 1 μg nuclear extract was incubated for 20 min in the presence of 20 nM HEPES (pH 7.9), 5 mM MgCl_2, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 8% Ficoll, 600 mM KCl, 500 ng/μl poly(dioxygeninosinic-deoxycytidylic) acid, 50 mM DTT, and [α-32P]dCTP-labeled double-stranded oligonucleotide. The following oligonucleotides were used in the mobility shift assays: κB wild type, 5'-TAGGAGGAAATCCCAT-3'; κB mutant, 5'-TTTAGGTTATCCCAT-3'; CRE wild type, 5'-TCGTGAAGTCAGGT-3'; and CRE mutant, 5'-TCGTGTAAGTCAGGT-3' (the underlined sequences indicate consensus sequence of the binding sites). The oligonucleotide probes were annealed with counter oligonucleotides, end labeled with [α-32P]dCTP using DNA polymerase I (Klenow fragment) and then purified by Sephadex G-50 column chromatography. The DNA-protein samples were electrophoresed at 11 V/cm for 2.5 h through a 5.0% non-denaturing polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer. After gel electrophoresis, the gel was dried and exposed to x-ray film (Kodak X-Omat; Kodak, Rochester, NY) with intensifying screens for 24 h at -80°C.

The specificity of the protein-DNA complexes was tested by immunodepletion and competition assays. For immunodepletion assays (super-shift experiments), Abs to NF-κB/C, p50, NF-κB/p65, or CREB-1 (Santa Cruz Biotechnology) were incubated with nuclear extracts for 20 min at room temperature before EMSA. For competition studies, the EMSA reaction was performed with excess concentrations of nonradiolabeled oligonucleotide probe, as indicated.

Statistical analysis

Data are expressed as mean values ± SEM. Comparisons between normal synovium and synovium obtained from patients diagnosed with RA were made using the Student’s t test for unpaired values. Comparisons between treatments were made using Student’s t test for paired values.

Results

Expression of NURR1 in normal and RA synovial tissue

Specific NURR1 immunostaining was extensive in all samples from patients with RA (n = 6) and significantly less in samples from normal patients (n = 2) (Fig. 1). In normal synovial tissue, the localization of NURR1 was most intense in the synovial vasculature, including endothelial cells (Fig. 1A). Occasional synoviocytes in the hypocellular normal synovial lining layer and sublining regions were also NURR1 positive. In RA, strong NURR1 staining was observed in synoviocyte and macrophage cells of the synovial lining layer and the subsynovial regions (Fig. 1C). Synovial vascular endothelium was also a site of increased NURR1 staining in all inflamed tissue studied. Importantly, NURR1 staining in RA cells was predominately nuclear compared with dominant cytoplasmic localization in normal synovium (Fig. 1, B and D).

PGE_2, IL-1β, and TNF-α promote NURR1 expression in RA and normal synoviocytes

Cytokine-stimulated release of resorptive agents such as MMPs and PGE_2 by synoviocytes occurs in association with a change from fibroblast-like to stellate morphology (37). Our immunohistochemical staining analysis indicates that TNF-α (Fig. 2A), IL-1β, and PGE_2 (data not shown) induction of NURR1 also occurs in parallel with a transformation of RA synoviocytes (n = 3) from the fibroblast-like to stellate shape. Similar to the nuclear staining seen in vivo (Fig. 1), NURR1 is primarily localized to the nucleus in primary RA synoviocytes under both basal and stimulated conditions.

We extended this analysis to investigate the relative effects of TNF-α, IL-1β, and PGE_2 on the induction of NURR1 gene expression in RA synoviocytes (n = 3) by Northern blot analysis (Fig. 2, B and C). Basal NURR1 mRNA levels in primary RA synoviocytes varied between synovial tissue samples, reflecting differences in the grade of tissue inflammation and patient to patient variability. Importantly, induction of NURR1 mRNA levels by all inflammatory mediators tested peaked at 1–2 h and returned to basal levels within 4 h of treatment. TNF-α (6.87 ± 2.1-fold) and IL-1β (5.55 ± 1.9-fold) significantly up-regulated NURR1 mRNA (p < 0.05); consistently, PGE_2 had the most potent and
sustained effect (31.3 ± 12.6-fold) in stimulating NURR1 mRNA levels in these cells. Coadministration of inflammatory stimuli augmented the effects on NURR1 transcription additively.

To elucidate the relative contribution of the three NURR subfamily members in the mediation of cytokine-induced inflammatory responses in synovioum, we compared the induction of NURR1 (Fig. 2B), NUR77, and NOR-1 gene expression in primary RA synoviocytes (Fig. 2C). NUR77 and NOR-1 transcript levels were modestly and differentially regulated by each of the inflammatory agonists studied. Inflammatory treatment resulted in a rapid but modest induction of NUR77 and NOR-1 mRNA levels that also peaked at 1 h (Fig. 2C) and declined thereafter. In contrast, NURR1 mRNA levels were significantly altered, indicating that this is the major cytokine-regulated member of the NURR subfamily.

To assess the spectrum of stimuli that activate NURR1 in these cells, we examined the ability of growth factors to regulate NURR1 expression (Fig. 3). In contrast to the prominent effects of PGE2 primary synoviocytes stimulated with bFGF and PDGF modestly up-regulated (up to 3.1- to 3.6-fold) and TGF-β had little effect (1.1-fold) on NURR1 mRNA levels. Immunohistochemical studies of NURR1 expression in primary synoviocytes confirmed that treatment with PDGF and bFGF induced a moderate increase in endogenous NURR1 protein (Fig. 3B).

Because proinflammatory cytokines and PGE2 consistently appeared to be the more potent inducers of NURR1, we further tested the ability of these mediators to regulate NURR1 expression in synoviocytes from nonarthritic joints. A human synoviocyte line (K41 M) from a healthy donor has been established and well characterized (36). K41 M synoviocytes maintain the ability to respond to TNF-α and PGE2 stimulation; however, these cells fail to respond to IL-1β due to loss in expression of IL-1R (36). Similar to the temporal responses observed in primary RA synoviocytes, a rapid and transient increase in NURR1 mRNA levels was observed following TNF-α and PGE2 stimulation. In accordance with pathways shown to date to regulate NURR1, forskolin activation of cAMP/protein kinase A (PKA) pathways rapidly induced NURR1 in these cells (Fig. 4A). Immunohistochemical studies of NURR1 expression in K41 M synoviocytes revealed a similar pattern of staining to that seen in primary RA synoviocytes (Fig. 2). Treatment with TNF-α and forskolin induced a rapid and marked increase in endogenous NURR1 protein (Fig. 4B). The specificity of NURR1 staining was verified by the significant reduction in staining seen when the NURR1 Ab was preincubated with an excess of Ag (BP).

**Inflammatory mediators enhance the transcriptional activity of the NURR1 promoter in primary RA and normal synoviocytes**

To determine whether inflammatory mediators are capable of regulating the expression of the NURR1 promoter, we generated β-galactosidase reporter plasmids containing proximal promoter regions of the NURR1 gene and tested these in transfection experiments (Fig. 5A). Transcriptional regulation of the NURR1 promoter was measured by transient transfection in primary RA synoviocytes. Individual RA synoviocyte cell lines were transiently transfected with each construct and incubated with or without cytokine. These experiments showed that in primary RA synoviocytes NURR1 basal activity for both the −1329/+132 and −396/+373 promoter regions were extremely high and variable. The autocrine action of PGE2 produced by primary RA synoviocytes (3) may explain the high transcriptional activity of the NURR1 promoter.

Having established that the K41 M cells produce endogenous NURR1 and respond to TNF-α and PGE2 in a manner similar to primary RA synoviocytes, we assayed the transcriptional activity of the NURR1 promoter constructs using this synoviocyte cell line (Fig. 5A). Consistent with the results of Northern blot analysis, TNF-α (4 ± 1.9-fold), PGE2 (9.8 ± 1.7-fold), and forskolin (6.2 ± 1.6) significantly (p < 0.01) enhanced the transcriptional activity of the −1329/+132 NURR1 promoter region.

Similar to the larger NURR1 promoter region, the −396/+373 region was efficiently induced by PGE2 and forskolin, but did not respond significantly to TNF-α treatment (Fig. 5A). These results suggest that cis-acting elements mapping between −1329/−396 appear to be required for the TNF-α-mediated activation, while sequences within the proximal promoter −396 region can confer...
PGE\textsubscript{2} and forskolin responsiveness to the NURR1 promoter. It is well established that IL-1\textsubscript{a} and TNF-\textalpha mediate transcription coupling through NF-\kappaB, and PGE\textsubscript{2} may signal by activation of CREB-dependent pathways. Examination of the 5\textquotesingle flanking region of the NURR1 gene (38, 39) reveals potential consensus sequences for the binding of both NF-\kappaB (\(585/-576\)) and CREB (\(171/-163\)) (Fig. 5B). Moreover, the CRE and NF-\kappaB consensus sequences are highly conserved across the human and mouse NURR1 promoter sequence (Fig. 5B). This very high evolutionary conservation suggests that these binding sites may have critical functional roles.

**IL-1\textbeta and TNF-\textalpha induce NF-\kappaB-binding activity to the NURR1 promoter**

We have identified a potential consensus NF-\kappaB binding site located within the \(-585/-576\)-bp region of the NURR1 promoter (Fig. 5B). Because of the role of this region in the inducibility of the promoter by TNF-\kappaB, we predicted that NF-\kappaB activation may be necessary for cytokine-mediated effects on NURR1 expression in synovial tissue. To test the ability of this site to bind NF-\kappaB, we designed a synthetic oligonucleotide that included the \(-585/-576\) NURR1 \kappaB site and used EMSA to examine DNA-binding properties of NF-\kappaB in primary RA synoviocytes treated with IL-1\textbeta and TNF-\textalpha for 1 h (Fig. 6).

The \(-585/-576\) NURR1 \kappaB probe efficiently and specifically bound two inducible nuclear protein complexes (complexes 1 and 2) present in TNF-\kappaB- and IL-1\textbeta-treated primary RA synoviocytes but absent in unstimulated cells (Fig. 6A, NT). As observed during a previous study of NF-\kappaB regulation of cyclooxygenase-2 and MMP-1 expression by IL-1\textbeta (40, 41), we also detected two additional bands (complexes 3 and 4) not significantly regulated by IL-1\textbeta or TNF-\kappaB also bind to the \kappaB probe. The identity of the proteins in these complexes and their significance are not known. Competition electrophoretic mobility shift experiments using 50-fold molar excess of unlabeled oligonucleotide revealed all complexes were specifically competed with self oligonucleotide.
NF-κB supershift and reduction in complexes 1 and 2, while addition of DNA fragment was specific and competitive. Recognition site (H9260/H9260). To determine the levels of NF-κB in RA synovium, we transfected K41 M cells with various binding activity was detected in all RA samples (H9252). Stimulation of the cells with PGE2 resulted in significant binding of protein to this DNA fragment (Fig. 7A). The increased DNA binding could be effectively competed with 50-fold molar excess of CREwt, but not with a mutated consensus sequence (CREmt). Supershift assays using anti-CREB-1 antisera resulted in a shift of both the constitutive and PGE2-induced protein complex (Fig. 7A). Thus, these results confirm that the CRE binding site plays an important role in the induction of this promoter by PGE2 pathways and also contributes to the basal activity of the NURR1 promoter in synoviocyte cells.

Constitutively CREB-1 binding to the NURR1 promoter in RA synoviocytes and synovial tissue

Using EMSA and nuclear extracts from primary RA synoviocytes (n = 3), we analyzed protein binding to the NURR1 CRE consensus binding site (CREwt), which contains the −171/−163 nucleotide sequence of the NURR1 promoter. Results shown in Fig. 7 demonstrate that in unstimulated RA synoviocytes, nuclear proteins formed a DNA-protein complex that was specifically competed with self oligonucleotide (CREwt), but not with an oligonucleotide containing a mutated recognition site (CREmt).

High DNA-binding activity of NF-κB to the NURR1 promoter in RA synovium

To determine the levels of NF-κB binding to the NURR1 promoter in RA synovial tissue, equal amounts of fresh nuclear extracts obtained from RA synovium were analyzed by EMSA using labeled −585/−576 NURR1 κB probe (Fig. 6B). A marked and variable binding activity was detected in all RA samples (n = 6), indicating constitutive binding of NF-κB to the NURR1 promoter in RA synovium. The DNA-protein complexes detected correspond to the NF-κB/p50- and NF-κB/p65-binding activity observed in TNF-α-stimulated RA synoviocytes (Fig. 6B). To further confirm the specificity of NF-κB subunit binding, we performed cold competition with self −585/−576 κB probe and immunodepletion assays (Fig. 6B).

 Constitutively CREB-1 binding to the NURR1 promoter in RA synoviocytes and synovial tissue

Using EMSA and nuclear extracts from primary RA synoviocytes (n = 3), we analyzed protein binding to the NURR1 CRE consensus binding site (CREwt), which contains the −171/−163 nucleotide sequence of the NURR1 promoter. Results shown in Fig. 7 demonstrate that in unstimulated RA synoviocytes, nuclear proteins formed a DNA-protein complex that was specifically competed with self oligonucleotide (CREwt), but not with an oligonucleotide containing a mutated recognition site (CREmt). Stimulation of the cells with PGE2 resulted in significantly increased binding of protein to this DNA fragment (Fig. 7A). The increased DNA binding could be effectively competed with 50-fold molar excess of CREwt, but not with a mutated consensus sequence (CREmt). Supershift assays using anti-CREB-1 antisera resulted in a shift of both the constitutive and PGE2-inducible protein complex (Fig. 7A). Thus, these results confirm that the CRE binding site plays an important role in the induction of this promoter by PGE2 pathways and also contributes to the basal activity of the NURR1 promoter in synoviocyte cells.

To determine the extent of CREB binding to the NURR1 promoter in RA synovium (n = 6), equal amounts of fresh nuclear extracts obtained from RA synovium were analyzed by EMSA using the labeled −170/−163 NURR1 CRE probe (Fig. 7B). A significant and variable protein-binding activity was detected in all...
RA samples \( (n = 6) \), indicating that CREB-1 binding to the NURR1 promoter is constitutive in RA synovium. The DNA-protein complexes detected correspond to the CREB-1-binding activity observed in PGE\(_2\)-stimulated RA synoviocytes (Fig. 7). To further confirm the specificity of CREB-1 binding, we performed cold competition with self oligonucleotide-mutated oligonucleotide and CREB-1 immunodepletion assays (Fig. 7).

**Discussion**

Inflammatory diseases, including RA, display an elaborate network of signaling pathways that control angiogenesis, cell influx and activation, inflammatory mediator release, and activity, resulting in tissue proliferation and degradation (10). Remarkably, these pathways are highly diverse and yet display the capacity to activate, in a cell- and stimulus-specific manner, an extraordinarily specific cohort of genes implicated in the disease process (9). The mechanism by which stimulus-specific gene expression is achieved remains unclear, but may possibly be due to integration at the level of transcription factor activity. This study has provided substantial and novel evidence to support the conclusion that NURR1 induction represents a point of convergence of at least two distinct signaling pathways, signifying an important common role for this transcription factor in mediating multiple inflammatory signals.

Our findings revealed that RA synovial tissue produces markedly increased levels of NURR1 compared with normal synovial tissue. To elucidate the regulatory mechanisms underlying peripheral NURR1 gene expression, we focused on the ability of proinflammatory mediators associated with joint inflammation and destruction to stimulate synovial NURR1 synthesis. Endogenous expression of the NURR1 mRNA was confirmed by in vitro studies of normal and primary RA synoviocytes. We have demonstrated that steady state NURR1 mRNA expression in synoviocytes was increased by IL-1\(\beta\), TNF-\(\alpha\), and PGE\(_2\), and that the NURR1 promoter responds to these same immunological stimuli in a manner similar to the response of endogenous NURR1 expression. In accordance with pathways shown to date to regulate NURR1 expression, we demonstrated a direct CREB-1-dependent regulation by PGE\(_2\). Further analysis reveals that IL-1\(\beta\)- and TNF-\(\alpha\)-induced activation of NF-\(\kappa\)B binding to the NURR1 promoter and that NF-\(\kappa\)B subunit binding to this site are due primarily to p65-p50 heterodimer and p50 homodimer protein complexes. Finally, our experiments have provided in vivo evidence of CREB-1 and both NF-\(\kappa\)B/p50 and NF-\(\kappa\)B/p65 subunit binding to the NURR1 promoter under basal conditions in freshly explanted RA synovial tissue, providing direct evidence for the involvement of NURR1 in inflammatory pathways that are central to the pathogenesis of RA.

Increased angiogenesis and IL-1\(\beta\)- and TNF-\(\alpha\)-dependent induction of adhesion molecule expression on synovial membrane endothelial cells are the earliest histopathologic features of RA (42, 43). IL-1\(\beta\) and TNF-\(\alpha\) are present at high levels in RA synovium, and their blood concentration correlates with the severity of disease (44). RA synovial tissues also release large quantities of PGs, mainly PGE\(_2\), prostacyclin, and thromboxane. PGE\(_2\) mediates both the inflammatory and destructive features of RA, and has been shown to stimulate production of vascular endothelial growth factor by synoviocytes through the cAMP/PKA signaling pathway (4, 35, 45). Growth factors that have been demonstrated in RA...
synovium and implicated in angiogenesis and the pathogenesis of progressive joint damage include bFGF, TGF-β, and PDGF (2). These peptide growth factors enhance fibroblast migration, proliferation, extracellular matrix protein synthesis, and degradation, all of which play an important role in RA synovitis. The pathological importance of the NURR transcription factors was verified by establishing the ability of these proinflammatory agonists, produced locally in RA, to stimulate NURR1 transcription in synoviocytes. TNF-α, IL-1β, and PGE2 induced a rapid and marked increase in endogenous NURR1 mRNA levels in normal and primary RA synoviocytes. Consistently, PGE2 had the most potent and sustained effect in stimulating NURR1 mRNA in these cells. Immunohistochemical analysis of stimulated primary RA synoviocytes shows a predominant nuclear localization of NURR1. Similar nuclear staining is seen in RA compared with normal synovial tissue and may highlight an important transcriptional regulatory role for NURR1 in vivo. We recently demonstrated the involvement of NURR1 in the regulation of immune CRH expression and peripheral CRH actions (31). We further established that CRH signaling plays a role in the vascular changes associated with joint inflammation in human arthritis (32). Localization of NURR1 in synovial endothelial cells suggests NURR1 may play a transcriptional regulatory role central to synovial tissue homeostasis. Our observation of enhanced NURR1 expression in the synovial lining layer, subsynovial synoviocytes, and vascular endothelial cells of RA synovial tissue confirms that NURR1 is expressed primarily in cells believed to be at the leading edge of invasive tumor-like synovium (pannus) into adjacent cartilage and bone (2, 8). Mediators of joint destruction in RA originate primarily in synoviocytes and macrophage cells, which are the dominant cell populations at the cartilage-pannus junction (2, 5, 46). Accumulating evidence suggests that partial transformation of RA synoviocytes increases the invasive potential of RA synovium (46). Cytokine-stimulated release of resorptive agents, such as MMPs and PGE2, by synoviocytes occurs in association with a change from fibroblast-like to stellate morphology (37). Our analysis indicates that cytokine induction of NURR1 also occurred in parallel with a transformation of synoviocytes from a fibroblast-like to stellate shape. These findings suggest that increased NURR1 gene expression may be a component of a programmed change in gene expression that occurs when synoviocytes are activated to secrete inducible proinflammatory mediators of bone resorption. Several independent studies have implicated abnormal expression of nuclear immediate early genes, such as c-fos and c-jun, in the modulation of gene expression known to regulate cellular proliferation and invasive activity in RA synovium (9). Recent studies have demonstrated the involvement of CREB in synovial cell hyperplasia in patients with RA (47, 48). In this study, we observed constitutive nuclear CREB-1 binding to the NURR1 promoter in primary RA synoviocytes and freshly explanted RA synovial tissue. Furthermore, we report that this specific CREB-1 binding can be induced by PGE2 and mimicked through activation of cAMP/PKA pathways by forskolin in RA synoviocytes. These findings support earlier observations that the stimulatory effect of PGE2 on gene transcription is mediated through cAMP/PKA-dependent activation and binding of CREB transcription factors in RA synoviocytes (35). The elucidation that PGE2 is the most potent regulator of NURR1 promoter activity, together with the demonstration that NURR1 mRNA is robustly induced by PGE2, suggest that NURR1 may play an important transcriptional regulatory role in mediating synovial PGE2 action. We have previously demonstrated that PGE2 robustly enhances the transcription activity of the human CRH promoter and increases levels of CRH mRNA in primary synoviocytes (31). NURR1 can up-regulate the expression of the CRH gene by interacting with specific cis-acting sequences in its proximal promoter region (21, 31). Thus, PGE2 may regulate synovial CRH expression through its ability to enhance the expression of NURR1. The data provided in this study confirm that the temporal increases in NURR1 expression by PGE2 preclude the effects of PGE2 on CRH gene regulation in primary RA synoviocytes (31). Furthermore, preliminary data from our laboratory indicate that CRH-dependent induction of PGE2 in RA synovial tissue may be mediated by NURR1, suggesting a central role for NURR1 in the positive potentiation of an autocrine PGE2 regulatory loop in human inflammatory arthritis (A. McEvoy, unpublished observations). NF-κB activity is mediated by a family of transcription factor subunits that bind DNA as hetero- or homodimers (49). Activation of NF-κB transcription through the induction of proinflammatory cytokines, chemokines, adhesion molecules, and MMPs is a central event in inflammatory diseases (9, 40, 41). In many cell types, NF-κB subunits are present in the cytoplasm in an inactive form, bound to inhibitory proteins such as IκB (49). A variety of extracellular stimuli, including TNF-α and IL-1β, induces ubiquitin-dependent degradation of the inhibitory proteins and a rapid nuclear translocation of NF-κB (50). In both RA and animal models of inflammatory arthritis, NF-κB p50 and p65 subunits are overexpressed and are highly activated (51). NF-κB p50/p65 hetero- and homodimers are intimately involved in activation of many inflammatory genes regulated by IL-1β and TNF-α (49). The NF-κB binding site in the NURR1 promoter is positionally conserved across species, suggesting this sequence has important regulatory functions. Consistent with these observations, we observed high DNA-binding activity of NF-κB p50/p65 heterodimer and p50 homodimer subunits to the human NURR1 promoter in the synovial tissue of RA patients. Furthermore, we demonstrated that TNF-α and IL-1β rapidly increase both NF-κB p50/p65 and p50/p50 subunit binding to the NURR1 promoter in primary RA synoviocytes. The abundance of the NF-κB/p50 subunit binding to the NURR1 κB consensus sequence is of particular interest because in RA synoviocytes, cytokine-induced MMP-1 expression is primarily activated by NF-κB/p50 homodimers (41). More importantly, the recent construction of NF-κB/p50-deficient mice has established that the NF-κB/p50 subunit is essential for the development of local joint inflammation and destruction in models of collagen-induced arthritis (52). Our data demonstrate that both TNF-α and IL-1β use the NF-κB signaling pathway to stimulate NURR1 expression in RA synovial cells. The control of NURR1 gene expression through an NF-κB-dependent mechanism provides important new insights for NURR1 modulation by proinflammatory mediators. The vast clinical potential of nuclear receptors as drug targets has already been proven in the case of steroid receptors, in particular those for estrogens, androgens, and glucocorticoids. In the case of NURR1, the identification of molecular signaling pathways both regulating NURR1 and regulated by NURR1 may provide new approaches for intervention using the transcription factor as a molecular target of drug therapy. Clinical trials and animal studies evaluating the potential of TNF-blocking strategies and other anticytokine agents in the treatment of inflammatory arthritis disease activity suggest that multiple agents within the cytokine cascade may need to be targeted to prevent disease progression and joint destruction. The findings outlined in this study demonstrate that NURR1 induction occurs downstream to multiple inflammatory mediators and may therefore be an effective target for anticytokine therapy in human inflammatory arthritis.
Acknowledgments

We thank Drs. David Kane and Leanne Stafford for synovial biopsy collection and Martina Gogarty for tissue sectioning. We are very grateful to Dr. H. Eibl (Clinical Research Unit for Rheumatology, University Hospital, Freiburg, Germany) for providing the K41 M cell line.

References