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Regulation of c-Jun N-Terminal Kinase by MEKK-2 and Mitogen-Activated Protein Kinase Kinase Kinases in Rheumatoid Arthritis

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The mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) is a critical regulator of collagenase-1 production in rheumatoid arthritis (RA). The MAPKs are regulated by upstream kinases, including MAPK kinases (MAPKKs) and MAPK kinase kinases (MAP3Ks). The present study was designed to evaluate the expression and regulation of the JNK pathway by MAP3K in arthritis. RT-PCR studies of MAP3K gene expression in RA and osteoarthritis synovial tissue demonstrated mitogen-activated protein kinase/ERK kinase kinase (MEKK) 1, MEKK2, apoptosis signal-regulating kinase-1, TGF-β activated kinase 1 (TAK1) gene expression while only trace amounts of MEKK3, MEKK4, and MLK3 mRNA were detected. Western blot analysis demonstrated immunoreactive MEKK2, TAK1, and trace amounts of MEKK3 but not MEKK1 or apoptosis signal-regulating kinase-1. Analysis of MAP3K mRNA in cultured fibroblast-like synoviocytes (FLS) showed that all of the MAP3Ks examined were expressed. Western blot analysis of FLS demonstrated that MEKK1, MEKK2, and TAK1 were readily detectable and were subsequently the focus of functional studies. In vitro kinase assays using MEKK2 immunoprecipitates demonstrated that IL-1 increased MEKK2-mediated phosphorylation of the key MAPKKs that activate JNK (MAPK kinase (MKK)4 and MKK7). Furthermore, MEKK2 immunoprecipitates activated c-Jun in an IL-1 dependent manner and this activity was inhibited by the selective JNK inhibitor SP600125. Of interest, MEKK1 immunoprecipitates from IL-1-stimulated FLS appeared to activate c-Jun through the JNK pathway and TAK1 activation of c-Jun was dependent on JNK, ERK, and p38. These data indicate that MEKK2 is a potent activator of the JNK pathway in FLS and that signal complexes including MEKK2, MKK4, MKK7, and/or JNK are potential therapeutic targets in RA. The Journal of Immunology, 2004, 172: 1612–1618.

Rheumatoid arthritis (RA) is a chronic inflammatory disease marked by synovial lining hyperplasia and sublining infiltration with mononuclear cells (1, 2). The invasive front of the synovium, sometimes called pannus, contributes to progressive cartilage and extracellular matrix destruction. Although many enzymes participate in this process, the matrix metalloproteinases (MMP) play a pivotal role (3, 4). MMP production in arthritis is, in turn, regulated by several signal transduction pathways, including the mitogen-activated protein kinases (MAPKs) (5).

All three MAPK families have been implicated in RA—extra-cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (6–8). The JNK pathway is of particular interest due to its ability to phosphorylate serine 63 and 73 on the c-Jun activation domain when cells are stressed by UV radiation, growth factors, or proinflammatory cytokines (9, 10). Because c-Jun is a component of AP-1, a transcription factor that initiates MMP gene expression, its activation by JNK can enhance MMP expression and subsequent joint destruction (11–13). Each MAPK is phosphorylated by upstream MAPK kinases (MAPKKK), which are dual specific kinases that can phosphorylate threonine and tyrosine residues (14). MAPKKKs are in turn activated by MAPKK kinases (MAPKKK or MAP3Ks), which are serine/threonine kinases. MAP3Ks and their downstream targets are activated by diverse extracellular stimuli and integrate these signals to direct an appropriate cellular response (15, 16).

Our previous studies demonstrated that JNK is activated in RA synovium and that this pathway regulates collagenase-1 gene expression in cultured fibroblast-like synoviocytes (FLS) (13). The critical role for JNK in joint destruction was confirmed in the rat adjuvant arthritis model using a selective JNK inhibitor and in JNK2 knockout mice with passive collage-induced arthritis (17, 18). To further characterize the regulation of JNK in FLS, we recently demonstrated that JNK forms a signaling complex with MAPK kinase (MKK)4 and MKK7 (the JNK signalsome) that can activate c-Jun (19). In the present study, we evaluated the expression and regulation of MAP3Ks in RA synovium and FLS. Although the pathway is quite complex, MEKK2 emerges as an important MAP3K in the activation of the JNK pathway in arthritis and is a potential therapeutic target.

Materials and Methods

Synovial tissue (ST) samples

ST samples were obtained from patients with osteoarthritis (OA) and RA at the time of joint replacement as described previously (20). The diagnosis of RA conformed to the 1987 revised American College of Rheumatology criteria (21). The samples were either processed for cell culture or snap frozen and stored at −80°C. The studies were approved by the University of California, San Diego, Human Subjects Research Protection Program.
Fibroblast-like synoviocytes

STs were minced and incubated with 1 mg/ml collagenase in serum free DMEM (Life Technologies, Grand Island, NY) for 2 h at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% FCS (Life Technologies, endotoxin content <0.006 ng/ml), penicillin, streptomycin, and 1-glutamine in a humidified 5% CO2 atmosphere. After overnight culture, nonadherent cells were removed. Adherent cells were later trypsinized, split at a 1:3 ratio, and cultured in medium. Synoviocytes were used from passages 3 through 9 in these experiments when they are a homogenous population of FLS (<1% CD11b, <1% phagocytic, and <1% FcyRII positive) (20).

Abs and reagents

Affinity purified rabbit polyclonal MEKK1, MEKK2, and apoptosis-signal regulating kinase-1 (ASK1) Abs, goat polyclonal MEKK4 Abs, mouse monoclonal TGF-β activated kinase 1 (TAK1) and secondary Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal JNK Abs and anti-rabbit-HRP-conjugated Ab were purchased from New England Biolabs (Beverly, MA). Anti-MEKK3, anti-MKK4, anti-MKK7, anti-MLK3, appropriate secondary Abs, GST-MKK4, GST-MKK7, and GST-c-Jun were purchased from Upstate Biotechnology (Lake Placid, NY). IL-1β was purchased from R&D Systems (Minneapolis, MN). The JNK inhibitor SP600125, was provided by Celgene (San Diego, CA). The p38 inhibitor, SB203580, was purchased from Promega (Madison, WI) and the ERK inhibitor, apigenin, was purchased from Calbiochem (La Jolla, CA).

Reverse transcription and PCR

Total RNA was isolated from FLS and ST using RNA-STAT-60 (Tel-Test, Friendswood, TX) and then reverse-transcribed into cDNA using random hexamer primers from the Gene Amp kit (PerkinElmer, Branchburg, NJ). The total reaction volume was 50 µl with a final concentration of 1X buffer, 5.5 mM MgCl₂, 500 µM dNTP, 2.5 µM hexamer primers, 0.4 µM RNAse inhibitor, and 1.25 µM reverse transcriptase. Reverse transcription was performed for 30 min at 48°C. PCR, 50 pmol of forward and reverse primers for MEKK1, -2, -3, -4, ASK1, TAK1, MLK3, and GAPDH were used to amplify the cDNA (see Table I for primer sequences). DNA was amplified for 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in a total volume of 50 µl. The PCR products were run on a 1% agarose gel.

Immunohistochemistry

Immunohistochemistry was performed as previously described (22). Five micrometer cryosections of STs were cut from RA and OA patients, fixed in cold acetone for 10 min, and incubated with appropriate Abs overnight at 4°C. Isotype matched Abs served as negative controls. Endogenous peroxidase was depleted with 0.3% hydrogen peroxide and sections were then stained with biotinylated secondary Ab (Vector Laboratories, Burlingame, CA). The signal was developed using diaminobenzidine or 3-amino-9-ethylcarbazole and the sections were counterstained with hematoxylin.

Western blot analysis

Cells were cultured in DMEM with 10% FCS in 100 mm dishes at 80% confluency. Cells were synchronized in DMEM by culturing with 0.1% FCS for 2 days and then recovering in serum-containing media for 24 h. Western blot analysis was performed with Abs according to manufacturer’s instructions. Briefly, the membranes were blocked with TBS plus 0.1% Tween 20 and 5% dry milk for 1 h at room temperature, followed by incubation with appropriate Ab at 4°C overnight. The membranes were washed three times and incubated with HRP conjugated secondary Ab for 1 h at RT. The proteins were visualized by chemiluminescence using Kodak X-AR film (Eastman Kodak, Rochester, NY).

Immunoprecipitation assay

RA and OA FLS were cultured in 100 mm diameter dishes. At 80% confluence, the cells were serum-starved in media containing DMEM supplemented with 0.1% FCS, penicillin, streptomycin, and 1-glutamine for 48 h. The cells were treated with IL-1 at a final concentration of 2 ng/ml for 15 min, washed once with cold PBS and harvested with lysis buffer. The lysate was centrifuged at 18,000 × g for 15 min and the supernatant was pre-cleared with appropriate sera. The lysates were incubated with Abs at a final concentration of 8 µg/ml for 4 h at 4°C. The lysates were then incubated overnight with 30 µl of 1:1 slurry of protein A or protein G-agarose beads at 4°C. The pellets were washed six times with washing buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol), incubated in 2 × nonreducing Laemmli sample buffer and heated for 3 min at 95°C. The samples were processed for SDS-PAGE and Western blot analysis.

In vitro kinase assay

FLS at 80% confluence were cultured in DMEM supplemented with 0.1% FCS for 4 h. The cells were treated with IL-1 or medium for 15 min, washed once with cold PBS and harvested with lysis buffer containing 1 mM DTT and 5 mM p-nitrophenylphosphate. The homogenate was centrifuged at 18,000 × g for 15 min and the supernatant was retained for immunoprecipitation. A total of 200 µg of protein were incubated with anti-MEKK1, anti-MEK2, anti-TAK1, or control IgG Abs at a final concentration of 8 µg/ml for 3 h at 4°C. The lysates were then incubated overnight with 30 µl of 1:1 slurry of protein A-agarose beads at 4°C. The next day, the beads were washed three times with lysis buffer containing DTT and 5 mM p-nitrophenylphosphate and then with complete kinase buffer (10 mM HEPES, pH 7.4, 25 mM MgCl₂, 20 mM β-glycerophosphate, 0.3 mM sodium orthovanadate, 2 mM DTT and 10 mM 5 mM p-nitrophenylphosphate, 10 µg/ml aprotinin, 1 µM peptatin A, and 1 mM PMSF and 20 µM ATP). After centrifugation, the pellet was incubated in a final volume of 30 µl of kinase reaction buffer (complete kinase buffer, 1 µCi of [γ-32P]ATP, 2 µg of substrate) for 30 min at 37°C. In some experiments, the pellet was preincubated with inhibitors of JNK (10 µM), ERK (20 µM), or p38 (10 µM). The reaction was stopped with 6× sample buffer and the samples were heated at 95°C for 3 min, fractionated using 10% SDS-PAGE. The gel was washed with water and fixed in 10% methanol/10% acetic acid solution for 30 min at RT and visualized by autoradiography. The density of target bands was analyzed using NIH Image (version 1.61; National Institutes of Health, Bethesda, MD).

Immunofluorescence

For confocal immunofluorescence studies, FLS were cultured in eight-chamber poly t-lysine coated glass slides (Nunc, Naperville, IL) (16,000 cells/well) and stimulated with IL-1 for 15 min. Cells were then washed twice with PBS, fixed with 4% formaldehyde for 10 min, and permeabilized with 0.1% saponin for 10 min. The samples were blocked with 10% BSA/PBS for 1 h at room temperature and incubated with primary Ab to MEKK2, MKK4, or MKK7 diluted in 10 ng/ml BSA overnight at 4°C. Cells were then washed with 0.1% Tween 20/PBS and incubated with donkey anti-rabbit FITC conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA), chicken anti-goat Texas Red conjugate, or donkey anti-sheep CY5 conjugate (Molecular Biological Express, St. Louis, MO). Discrete punctate staining was observed in areas surrounding the nuclei, consistent with previous observations (23). The nuclei were stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The slides were mounted in Vectorshield and viewed using a fluorescent microscope (Nikon, Melville, NY).

Table I. Sequences of MAP3K primers (5' → 3')

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEKK1</td>
<td>TGGCGGCTTCTTTGGATCTTG</td>
</tr>
<tr>
<td>MEKK2</td>
<td>CAGATGTGTCATGAGCCCTTT</td>
</tr>
<tr>
<td>MEKK3</td>
<td>GAGGAAGATCGCCCTTTTT</td>
</tr>
<tr>
<td>MEKK4</td>
<td>TCTTAAGGTCCCTGTATCCTG</td>
</tr>
<tr>
<td>ASK1</td>
<td>AAGGCAAACCTCCAGGCG</td>
</tr>
<tr>
<td>TAK1</td>
<td>TCTGAGGTCCCTTTGTG</td>
</tr>
<tr>
<td>MLK3</td>
<td>CCAACCTTTCTCCCTTATG</td>
</tr>
</tbody>
</table>
Although the experiments were performed with paired Student’s *t* test unless otherwise stated. A comparison was considered statistically significant if *p* < 0.05.

### Results

#### MAP3K gene expression in ST

Because little is known about MAP3K expression in RA and OA STs, initial experiments were performed to determine mRNA and protein expression of MEKK1, MEKK2, MEKK3, MEKK4, ASK1, TAK1, and MLK3. Gene expression in RA and OA STs was determined using RT-PCR with MAP3K specific primers (n = 3 for RA and n = 3 for OA). MEKK1, MEKK2, ASK1, and TAK1 transcripts were readily detected in both RA and OA samples (see Fig. 1 for representative examples and Table II). No differences were observed between RA and OA samples. Con, Positive control.

Statistics were performed with paired Student’s *t* test unless otherwise stated. A comparison was considered statistically significant if *p* < 0.05.

### MAP3K protein expression in fibroblast-like synoviocytes

MAP3K gene and protein expression was then determined in cultured FLS derived from normal, OA, and RA ST. Using RT-PCR, we detected mRNA for all of the MAP3Ks analyzed (n = 3 for OA, n = 3 for RA, n = 3 for normal; see Fig. 4A for representative examples). No major differences in MAP3K expression were observed between synoviocytes from OA or RA synovium, although there appeared to be less MEKK2 in normal synoviocytes. As with ST, MEKK2 appeared to be particularly abundant. Western blot analysis was also performed on lysates from cultured FLS. MEKK1, MEKK2, and TAK1 were the most abundant (n = 3 for OA, n = 3 for RA, and n = 3 for normal, Fig. 4B and Table II). Trace amounts expression of MEKK3, ASK1, and MLK3 were demonstrated, and MEKK4 was not detected.

### Signaling complexes containing MAP3Ks in FLS

Our studies of MAP3Ks were primarily initiated to identify the potential upstream regulators of the JNK signaling, which contains JNK, MKK4, and MKK7 (19). We considered whether larger complexes that contain MAP3Ks and the JNK signalosome might exist. Lysates of RA and OA FLS were immunoprecipitated with anti-MEKK1, MEKK2, MEKK3, MEKK4, ASK1, and TAK1 Abs. MEKK2 and TAK1, along with traces of MEKK3, were observed. Representative examples are shown.
MAP3Ks are associated with MKKs. As shown in Fig. 5, MKK4 and MKK7 coprecipitated with MEKK1, MEKK2, and TAK1 (n = 3 for RA and n = 3 for OA). No differences were observed between RA and OA FLS. Colocalization of MEKK2, MKK4, and MKK7 was confirmed by immunofluorescence and confocal microscopy (Fig. 6 for individual and merged images). The kinases were distributed evenly in the cytoplasm, although a portion was detected in the nucleus of FLS. Therefore, MAP3Ks can form complexes in FLS with MAPKKs that could potentially facilitate signal transduction to the components of the JNK pathway. Because MAP3Ks associate with MKK4 and MKK7 in FLS, we considered whether JNK might also be present in these complexes. FLS lysates were immunoprecipitated with anti-MEKK1, -MEKK2, or -TAK1 Ab and analyzed by Western blot with anti-JNK Ab. Fig. 5 shows JNK coprecipitated with MEKK1, MEKK2, and TAK1 (n = 3 for RA and n = 3 for OA).

FIGURE 3. Immunohistochemistry for MEKK2 in RA tissues. A representative example of immunohistochemistry to detect MEKK2 is shown for RA ST. MEKK2 was detected in RA synovium, especially in the synovial lining. A serial section stained with an isotype matched control Ab (IgG) was negative.

FIGURE 4. MAP3K gene and protein expression in FLS. A, Total RNA was isolated from RA, OA, and normal FLS, and RT-PCR was performed using MAP3K-specific primers. Representative examples of MAP3K gene expression are shown. No differences in expression were observed between the three groups; B, Total protein from RA (n = 3), OA (n = 3), and normal (n = 3) FLS were extracted and Western blot performed. Immunoreactive MEKK1, MEKK2, and TAK1 were most abundant in FLS. No differences were noted between RA and OA; and a trend toward lower levels of MEKK2 was noted in normal FLS. Con, Positive control.

FIGURE 5. Signaling complexes containing MAP3Ks in FLS. Immunoprecipitation experiments were performed to detect proteins associated with MEKK1, MEKK2, and TAK1. RA and OA FLS were stimulated with medium or 2 ng/ml IL-1 for 15 min. Total protein was extracted and immunoprecipitated with Abs to MEKK1, MEKK2, TAK1, or an irrelevant control Ab (IgG). Western blot analysis was performed on immunoprecipitates to detect MKK4, MKK7, or JNK. Representative examples are shown. Con, Positive control.

FIGURE 6. Colocalization of MKK4, MKK7, and JNK in FLS. Immunofluorescence microscopy was performed to confirm whether the MEKK2, MKK4, and MKK7 colocalize in FLS. Cultured FLS were stimulated with 2 ng/ml IL-1 for 15 min and localization of MKK4, MKK7, and JNK was studied with a mixture of Abs. A representative cell with MEKK2, MKK4, and MKK7 is shown demonstrating cytoplasmic and, to a lesser extent, nuclear staining. A merged view is also shown where the white pixels identify colocalization of all three kinases. Purple pixels show colocalization of MKK4 and MKK7 without MEKK2.
MAP3K function in FLS

To determine the ability of cytokines to activate the three readily detectable MAP3Ks in FLS, in vitro kinase assays were performed for MEKK1, MEKK2, and TAK1. Each MAP3K was immunoprecipitated from control and IL-1-activated FLS using specific Abs. In vitro kinase assays were then performed with the immunoprecipitates to determine their ability to phosphorylate GST-MKK4 and GST-MKK7. Modest basal kinase activity was detected for MEKK1, MEKK2, and TAK1 in FLS. When cells were stimulated with IL-1, GST-MKK4, and GST-MKK7 phosphorylation by MEKK2 were significantly increased (see Fig. 7A; n = 4, p < 0.05). However, phosphorylation of MKK4 by MEKK1 and TAK1 immunoprecipitates was not significantly increased by 15 min of IL-1 incubation. MKK7 activation by MEKK1 and TAK1 was not increased by IL-1, and a trend toward increased TAK1-induced MKK7 phosphorylation did not reach statistical significance. (See Fig. 7B; n = 4, p > 0.10). These data suggest that MEKK2 can participate in IL-1-induced phosphorylation of two key components of the JNK signalsome (MKK4 and MKK7). Although MEKK1 and TAK1 were able to phosphorylate MKK4 and MKK7, IL-1-mediated induction was inconsistent.

Functional analysis of the MAP3K-JNK signalsome complex

We have previously shown that the JNK signalsome from activated FLS can phosphorylate GST-c-Jun in vitro (19). Because our studies of MAP3K complexes suggest that MEKK1, MEKK2, and TAK1 associate with the JNK signalsome components, we determined if the MAP3K immunoprecipitates can phosphorylate c-Jun. RA FLS were serum starved for 48 h and stimulated with medium or IL-1 for 15 min. The lysates were immunoprecipitated with anti-MEKK1, -MEKK2, or -TAK1 Abs and evaluated in a kinase assay using GST-c-Jun as the substrate. IL-1 stimulation induced GST-c-Jun phosphorylation activity in all three of the immunoprecipitates, MEKK1 (n = 4, p = 0.025), MEKK2 (n = 5, p = 0.01), and TAK1 (n = 5, p = 0.01, Fig. 8, A and B).

Because JNK is a likely component of the signaling complex that activates c-Jun, kinase assays were then performed in the presence of SP600125, which is a selective JNK inhibitor. SP600125 significantly inhibited GST-c-Jun phosphorylation by the MEKK2 complex, indicating that it requires JNK (See Fig. 7A; n = 3, p < 0.05). Of interest, SP600125 also blocked the ability of the MEKK1 complex to phosphorylate GST-c-Jun even though these MAP3Ks did not appear to increase MKK4 or MKK7 activation in IL-1-stimulated

![FIGURE 7](image-url)  
**FIGURE 7.** IL-1-mediated activation of MKK4 and MKK7 by MEKK2. MAP3K function was determined using in vitro kinase assays. Serum starved cultured RA FLS were stimulated with medium or 2 ng/ml IL-1 for 15 min, and total protein was extracted and immunoprecipitated with Abs to MEKK1, MEKK2, or TAK1. A, The ability of the immunoprecipitate to phosphorylate GST-MKK4 or GST-MKK7 was determined (n = 4). Low levels of constitutive activation were observed in unstimulated cells. IL-1 increased MEKK2-mediated phosphorylation of GST-MKK4 and GST-MKK7. Incubation of IL-1 for 15 min did not significantly increase MEKK1- and TAK1-induced phosphorylation of the MKks, although there was a trend toward increased MKK7 activation by TAK1. Representative experiments are shown. B, Fold change in kinase activity of MEKK1, MEKK2, and TAK1 immunoprecipitates to phosphorylate GST-MKK4 or GST-MKK7 upon incubation with IL-1 (*, p < 0.05 for IL-1 compared with medium).
FLS (n = 3, p < 0.04). Neither the ERK inhibitor nor the p38 inhibitor significantly decreased the ability of the MEKK1 complex to phosphorylate GST-c-Jun. Therefore, MEKK1 appears to act primarily through the JNK pathway. MEKK1 can potentially accomplish this through an alternate MAPKK pathway in FLS or by directly phosphorylating JNK as previously described in other cell types (23). Inhibitors of all three MAP kinase pathways could decrease phosphorylation of GST-c-Jun by the TAK1 complex (Fig. 8A). Together these data suggest that 1) MEKK1 and MEKK2 complex with and activate JNK signalosome components (MKK4, MKK7, and/or JNK) that can subsequently phosphorylate c-Jun; and 2) TAK1 can activate c-Jun through multiple MAPK pathways.

Discussion
Joint destruction in patients with RA is mediated by a variety of enzymes, especially the MMPs (1). This extensive family of enzymes includes collagenases, stromelysins, and gelatinases that can, in combination, degrade essentially all components of the articular extracellular matrix (24, 25). Signaling mechanisms such as the MAP kinases have been implicated in MMP gene regulation and joint destruction in arthritis (13, 18, 26, 27). All three MAPK families (ERK, JNK, and p38) are activated in the RA ST and can participate in this process (5). However, the hierarchy of MAPKs varies depending on the extracellular stimulus, the specific enzyme, and the cell type (16). Because the FLS in the intimal lining are a primary source of MMP production in RA (28, 29), this cell type has been a focus of arthritis-related studies. JNK, in particular, plays an especially important role in cytokine-mediated c-Jun activation and collagenase-1 gene expression in FLS (13, 17). Previous experiments to define the upstream kinases that interact with JNK have identified two MAP3Ks, MKK4, and MKK7, that form a functional signaling complex with JNK in cultured FLS (i.e., the JNK signalosome) (19).

The present studies were designed to determine the kinases upstream from the MAP3Ks that can regulate the activation of JNK in synoviocytes. The MAP3K system is extraordinarily diverse and complex, and multiple members, including MEKK-1, -2, -3, -4, ASK1, TAK1, and MLK3, can activate JNK in other cell types (16). To narrow the focus of our experiments, initial qualitative protein and mRNA expression studies were performed to prioritize the MAP3Ks in ST and FLS. Using RA and OA synovia, gene expression was readily detected by PCR for a subset of MAP3Ks (MEKK1, MEKK2, ASK1, and TAK1). Western blot analysis demonstrated immunoreactive MEKK2 and TAK1 as well as limited amounts of MEKK3 protein, but not MEKK1 or ASK1. Immunohistochemistry confirmed and only MEKK2 was abundant in frozen sections of ST. The protein and RNA analysis did not rule out the presence of small amounts of the other kinases, but instead served to focus our subsequent functional analysis. To complement our studies on intact synovium, we also examined the patterns of MAP3K production cultured FLS. MAP3K expression in these cells was less restricted than intact tissue, and most of the kinases were detected by PCR and/or Western blot analysis. As with synovium, however, MEKK2 appeared to be especially abundant. MEKK1 and TAK1 were also readily detected using the same methods.

Our expression studies suggested that MEKK2, as well as MEKK1 and TAK1, might be important upstream kinases in ST and FLS. These kinases are, of course, not unique to ST. MEKK2 is a ubiquitous MAP3K that is expressed in human peripheral blood lymphocytes, brain, heart, muscle, spleen, kidney, and liver (30). MEKK1, which is the best-characterized member of the MAP3K family, is present in multiple tissues, including spleen, heart, brain, lung, and kidney (31). MEKK1 has also been detected in several normal cell types such as mast cells, (32) and tumor lines like Jurkat (33). The third MAP3K of particular interest in the FLS, TAK1, is expressed in human kidney, skeletal muscle, spleen, thymus, and ovary (34). However, TAK1 levels are low in peripheral blood leukocytes, liver, and heart (35).

Overall, the expression data in synovial samples supported further studies on the role of MEKK2 as a candidate MAP3K for the regulation of JNK in FLS. Immunoprecipitation studies showed that MEKK2 associates with MKK4, MKK7, and JNK. Although these data suggest that MEKK2 is part of a JNK signalosome, it is also possible that distinct MEKK2/MKK4/JNK or MEKK2/MKK7/JNK complexes reside within the cell. In cotransfection studies, MEKK2, MKK7, and JNK1 overexpression in COS-1 leads to the formation of a complex containing all three kinases (36). In other studies, COS-1 cells transfected with MKK4 and MEKK2 phosphorylated GST-JNK suggesting formation of a single or multiple complexes between MEKK2, MKK4, and JNK (36, 37). However, no previous MAP3K studies have identified kinase complex formation in primary cells or without using genetic constructs to increase endogenous production. Our observation that MKK4 and MKK7 coprecipitate in FLS suggests that the MEKK2 (and possibly other MAP3Ks) can interact directly with the JNK signalosome.

Functional studies then demonstrated that MEKK2 in resting FLS can phosphorylate MKK4 as well as MKK7 and that kinase activity could be further increased by IL-1. The MEKK2 complex in FLS can directly phosphorylate c-Jun, suggesting that MEKK2 participates in a discrete signaling unit. Similar results were previously reported with phosphorylation of GST-c-Jun by MEKK2-transfected HEK293 cells (38) and COS-1 cells (36). The direct role of JNK in the MEKK/MKK/JNK complex was confirmed using a selective JNK inhibitor to block c-Jun phosphorylation.

Although MEKK1 expression was less prominent in FLS and ST, it is also a potent activator of the JNK pathway. Several studies demonstrate that MEKK1 overexpression in NIH 3T3 or COS-1 cells activates MKK4 and MKK7 (39–41). In FLS, MEKK1 also coprecipitates with and phosphorylates MKK4 and MKK7. Surprisingly, IL-1 did not significantly increase MEKK1 kinase activity using GST-MKK4 or GST-MKK7 after 15 min, although later time points were not formally evaluated to determine whether late MKK phosphorylation occurs. This contrasts with previous observations that MEKK1 is required for JNK activation by various proinflammatory stimuli such as TNF-α, IL-1, dsRNA, and LPS in some cell types (37). However, functional studies in FLS confirmed MEKK-mediated JNK activation by IL-1 because 1) the MEKK1 immunoprecipitates phosphorylate GST-c-Jun; and 2) S600125 blocked this activity. Therefore, MEKK1 might use an alternative MAPKK pathway to increase JNK activation in FLS after IL-1 stimulation. More likely, MEKK1 can directly bind and phosphorylate JNK (23, 42) thereby allowing JNK to phosphorylate c-Jun without requiring the JNK signalosome or other MAPKs.

The third MAP3K implicated in JNK activation in FLS is TAK1, which is known to interact with JNK as well as p38 via MKK4, MKK7, MKK3, and MKK6 (43, 44). Like MEKK1, TAK1 associates with and phosphorylates MKK4 and MKK7 in FLS. TAK1 immunocomplexes from IL-1-stimulated cells can phosphorylate GST-c-Jun. Studies with selective MAPK inhibitors in the kinase assays suggest that TAK1 function is complex and is dependent on JNK, ERK, and p38.

Taken together, these data suggest that the several MAP3Ks can activate JNK in FLS through distinct mechanisms. MEKK2 forms a complex with MKK4 and MKK7 that is activated by IL-1, contains JNK, and can phosphorylate c-Jun. MEKK1, in contrast, can associate with MKK4 and MKK7 but its IL-1-inducible function appears to be independent of these MAPKs. The ability of MEKK1 complexes to
phosphorylate c-Jun after IL-1 stimulation is probably related to its capacity to directly interact with JNK.

The novelty of this work stems, in part, from methodical use of tissue from nonneoplastic human diseases and primary cells to characterize the physical and functional associations of critical MAP3Ks. The presence of signaling units that are IL-1 inducible and can regulate pathways required for genes involved in joint destruction was demonstrated. Based on expression and functional studies, MEKK2 emerges as an especially important kinase that is both highly expressed and tightly linked to JNK in synoviocytes. TAK1 and MEKK1 also appear to activate JNK, albeit through complex mechanisms. Targeted therapies directed toward the formation of signaling complexes or the activity of upstream kinases can potentially suppress c-Jun activation and subsequent MMP expression in inflammatory arthritis.

References
3. Firestein, G. S. 1996. Invasive fibroblast-like synoviocytes in rheumatoid arthri-
cellular signal-regulated kinase, c-jun N-terminal kinase, and p38 mitogen-
12. Geng, Y., J. Valbracht, and M. Lotz. 1996. Selective activation of the mitogen-
vated protein kinase conservation of a three-kinase module from yeast to human. Physiol. Rev. 79:143.

MAP KINASE REGULATION IN ARTHRITIS