Basic Calcium Phosphate Crystals Induce Matrix Metalloproteinase-1 through the Ras/Mitogen-activated Protein Kinase/c-Fos/AP-1/Metalloproteinase 1 Pathway

IN Volvement of Transcription Factor Binding Sites AP-1 and PEA-3*

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Synovial fluid basic calcium phosphate (BCP) crystals are common in osteoarthritis and are associated with severe degenerative arthropathy. Besides stimulating synovial fibroblast-like cells to proliferate, BCP crystals are a potent inducer of human matrix metalloproteinases (hMMPs), which can speed up the articular joint tissue degeneration of osteoarthritis patients. Here, we report that transfections with hMMP1 luciferase reporter plasmids in fibroblast-like synoviocytes revealed that the induction of hMMP1 promoter by BCP crystals was mainly mediated through the −72AP-1 element. Elimination of the −72AP-1 element either by mutation or deletion abolished the induction of hMMP1 promoter activity by BCP crystals almost completely. Interestingly, a mutation at the −88PEA-3 site also abolished the induction of hMMP1 promoter. Further mutation at the −181AP-1 site resumed the induction, indicating that the −181AP-1 element had an effect opposite to the −72AP-1 element. The effect of −181AP-1 could be inactivated either by a mutation at this −181AP-1 site or by the −88PEA-3 element. In addition, dominant negative Ras, Raf, and MEK1/2 could block the induction of hMMP1, and a MEK1/2-specific inhibitor (UO126) could block the induction of hMMP1 and c-Fos by BCP crystals. Taken together, these data indicate that multiple elements, including at least AP-1 and PEA-3, are involved in the induction of hMMP1 gene expression by BCP crystals and that the induction follows the Ras/MAPK/c-Fos/AP-1/hMMP1 signaling pathway.

The calcium-containing crystal arthropathies that involve deposition of calcium pyrophosphate dihydrate (1) and basic calcium phosphate (BCP)1 (2) crystals are a group of clinically heterogeneous arthritides. The incidence of both of these calcium crystals in arthritis significantly increases with age. The etiology of these diseases is currently unknown. It has been found that BCP crystals exist in the joint fluid of up to 60% of osteoarthritis (OA) patients (3, 4). In addition, the presence of BCP crystals correlates strongly with radiographic evidence of cartilaginous degeneration and is associated with larger joint effusions when compared with joint fluid from osteoarthritic knees where BCP crystals are absent (3, 4). BCP crystals include hydroxyapatite, calcium phosphate, and calcium tricalcium phosphate and are more common in degenerative joints than in normal joints or joints affected with inflammatory forms of arthritis. Conversely, OA is both more common and more severe in patients with calcium-containing crystals. The most compelling argument favoring a role for BCP crystals in OA stems from their in vitro effects on cells derived from human articular tissues. In addition to stimulating fibroblast mitogenesis (5, 6), BCP crystals increase levels of MMP1, −8, −13, and stromelysin (6–8), which in turn may disturb the extracellular matrix deposition/degradation equilibrium.

OA is characterized by progressive deterioration and erosion of joint cartilage. Numerous studies have demonstrated significant involvement of MMPs in the pathologic degradation of the cartilage matrix (9–11). Collagenase is a subfamily of MMP enzymes that includes fibroblast collagenase (MMP1), neutrophil collagenase (MMP8), and collagenase-3 (MMP13). These enzymes play a major role in connective tissue remodeling, including degradation of native type II collagen, the major structural protein of cartilage. Human MMP1 (hMMP1) and hMMP13 are both present at significantly elevated levels in the synovial fluid and cartilage of patients with OA. Chondrocytes obtained from cartilage adjacent to OA lesions express higher levels of hMMP1 and hMMP13 in comparison with chondrocytes from more normal appearing cartilage located in the same joint (10). hMMP1 gene expression has been shown to be

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1 The abbreviations used are: BCP, basic calcium phosphate; OA, osteoarthritis; FLS, canine fibroblast-like synoviocyte; MMP, matrix metalloproteinase; hMMP, human MMP; MMP1luci, human matrix metalloproteinase-1 promoter/luciferase reporter plasmid; MAPK, mitogen-activated protein kinase; MEK1 and MEK2, mitogen-activated protein kinase kinase 1 and mitogen-activated protein kinase kinase 2; ERK1/2, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; MOPS, 4-morpholinepropanesulfonic acid; SRE, serum response element; PC, phosphocholate; IL-1β, recombinant human interleukin-1β; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; DEME, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

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stimated by native type I collagen, phospholipids, growth factors, and cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (12–14). Transcriptional activation of hMMP1 by phosphor 12-myristate 13-acetate (PMA) is mediated primarily through the −77AP-1 and −181AP-1 elements, a PEA-3 element, and a “TCC” motif (15, 16). Higher levels of induction can be seen with larger fragments of promoter, suggesting the presence of additional cis-acting elements upstream. The AP-1 site can be found in all of the three human collagenases and has been shown to be critical to the expression of human collagenases (16–18).

We are particularly interested in the effects of BCP crystals on the expression of the hMMP1 gene. Although it has been shown that BCP crystals stimulate the gene expression of hMMP1 in cultured fibroblasts (6, 7), the transcription regulation and signal transduction by which BCP crystals induce the gene expression of hMMP1 have been incompletely studied. Defining the mechanism of transcriptional induction of hMMP1 gene expression by the BCP crystals and the signaling pathway involved may provide insight into the pathophysiology of OA and crystal deposition disease, and the information may be useful for therapeutic agent design. Consequently, we undertook a study to determine the transcriptional requirements for augmented hMMP1 promoter activity in response to BCP crystals and to further define the signaling pathway involved.

**EXPERIMENTAL PROCEDURES**

BCP crystals were synthesized by a modification of published methods (6). Crystals were crushed and sieved to yield 10–20-μm aggregates that were sterilized and rendered pyrogen-free by heating at 200 °C for at least 90 min. Crystals were weighed and suspended by sonication in DMEM containing 10% FBS. FLSs used were isolated from Sprague-Dawley rats and adherent cells were cultivated in DMEM containing 10% FBS. FLSs were used to third to twelfth passage cells.

**Plasmids**—The PathDetect cis-reporting plasmids pAP1luci, pSRE-luci, pNF-κB plasmid, and pCRE-luci, which contain the luciferase reporter gene driven by the basic promoter element TATA box plus tandem repeats of the consensus binding sequence for the corresponding transcription factor, were obtained from Stratagene (La Jolla, CA). The PathDetect trans-reporting systems, each of which contains a pFR-luci reporter plasmid and a unique fusion trans-activator plasmid, were obtained from the same company. These trans-reporting systems are designed for the study of the in vivo effects of new gene products, growth factors and drug candidates on the activation of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and p38 MAPK. Each PathDetect trans-reporting system includes a unique fusion trans-activator plasmid that expresses a fusion protein. The fusion protein is a trans-acting, pathway-specific transcriptional activator. The fusion activator protein consists of the activation domain of either the c-Jun, Elk1, or CHOP transcriptional activator fused with the yeast GAL4 DNA binding domain. When a fusion trans-activator plasmid and the pFR-luci reporter plasmid are cotransfected into mammalian cells and the transfected cells are subsequently treated with external cellular stimuli, phosphorylation of the transcrption activation domain of the fusion trans-activation protein by JNK, MAPK, or p38 MAPK activated by the external cellular stimuli will induce transcription of the luciferase gene from the reporter plasmid pFR-luci. Expression levels of luciferase reflect the in vivo activation of these kinases and the corresponding signal transduction pathways. The plasmid pCMV-Raf/S621A expresses a dominant negative form of the Raf protein when transfected into mammalian cells, was from CLON-TECH (Palo Alto, CA). The plasmids pREP4-K57AMK1 and pREP4- K510AMK1, which express a dominant negative form of the MAPK kinase-1 (MEK1) or MAPK kinase-2 (MEK2) protein, were obtained from Dr. Jeffrey T. Holt (20). The pCMV-RhoN19, pCMV-RacN17, and pCMV-RosN17 expression plasmids express the dominant negative form of RhoA, Rac1, and Ras proteins when transfected into mammalian cells (21).

**Promoter Deletion Constructs**—The hMMP1 promoter/luciferase reporter plasmids, −4372hMMP1luci, −77AP-1 and −181AP-1, elements, a PEA-3 element, and a “TTC” motif (15, 16). Higher levels of induction can be seen with larger fragments of promoter, suggesting the presence of additional cis-acting elements upstream. The AP-1 site can be found in all of the three human collagenases and has been shown to be critical to the expression of human collagenases (16–18).
**RESULTS**

**BPC Crystals Induce the Promoter Activity of hMMP1 in a Dose-dependent Manner**—We have previously shown that BPC crystals induce significant accumulation of human MMP1 mRNA in human foreskin fibroblasts (6, 7, 19). Here, we show that the promoter activity of hMMP1 was induced in a dose-dependent manner by BPC crystals in FLSs (Fig. 1). This induction could be blocked by PC, a specific inhibitor of the biological effects of BCP and calcium pyrophosphate dihydrate crystals, indicating that the induction of hMMP1 promoter activity was BCP crystal-specific (19). The inductions of hMMP1 promoter by IL-1β and PMA are also shown in Fig. 1 for comparison. The modest changes in luciferase activity seen in BCP crystal-treated cells, when compared with those seen by

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**Northern Blot Analysis**—FLSs were placed in 100-mm plates at 7.5 × 10⁵ cells/plate and grown until 90% confluent (24–36 h). The cells were then washed with phosphate-buffered saline and subsequently incubated in DMEM containing 0.5% heat-inactivated FBS for 24 h. At the onset of the experiments, media were replaced by serum-free DMEM, by serum-free DMEM containing MEK1-specific inhibitor PD98059 (100 μM), or by serum-free DMEM containing PC (1 mM) and preincubated for 15 min. The cells were then treated with BCP crystals (50 μg/ml) for 30 min. The total cellular RNA was harvested using RNAzol reagent (Tel-Test, Inc., Friendswood, TX) and quantified by optical density. Total RNA samples (15 μg/lane) were subjected to Northern blot analysis. Briefly, after electrophoresis in MOPS electrophoresis buffer, the RNA was transferred in 10× SSC buffer by capillary action using a sponce. The RNA was fixed to the nylon membrane by UV light exposure with a Stratagene UV linker (Stratagene, La Jolla, CA). The membrane was then prehybridized 2–4 h in 5× SSPE, 5× Denhardt’s reagent, 0.5% SDS, 100 μg/ml denatured salmon sperm carrier DNA (Invitrogen), and 50% formamide. The filter was hybridized with random-primed 32P-labeled c-fos probe (Invitrogen). After overnight hybridization at 42°C in 6× SSPE, 0.5% SDS 100 μg/ml denatured salmon sperm carrier DNA, and 50% formamide, the membrane was washed twice in 1× SSC with 0.1% SDS at room temperature and then washed twice at 55°C using 0.1× SSC and 0.1% SDS. The filter was autoradiographed with Kodak X-OMAT AR film for 24–72 h at ~70°C. The c-fos probe was a 1.3-kilobase pSol v-fos fragment from the pfos-1/ plasmid supplied by I. Verma (Salk Institute, San Diego, CA).
The induction of the promoter activity of hMMP1 by BCP crystals was blocked by the dominant negative RasN17 and RafS620A proteins. FLSs were cotransfected with 1.2 µg of −4372hMMP1luci plasmid (A) together with 0.3 µg of one of the four plasmids, pCMV-RasN17, pCMV-RafS621A, pCMV-RhoN17, or pCMV plus 0.3 µg of β-galactosidase plasmid as an internal control using LipofectAMINE in Opti-MEM I. The transfected cells were subsequently treated with BCP crystals (50 µg/ml). Twenty-four h later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. In a separate experiment (B), FLSs were cotransfected with 1.2 µg of −4372hMMP1luci together with either 50 ng of pCMV or pCMV-RafS621A plus 0.3 µg of β-galactosidase plasmid and subsequently treated with BCP crystals. Coexpression of the dominant negative forms of RhoA or Rac1 has either no effect or only a partial effect on induction of hMMP1luci (A, bar groups 1 and 3). In contrast, expression of the dominant negative RasN17 completely abrogated the BCP induction of the reporter activity of hMMP1luci (A, bar group 2). The induction of hMMP1luci by BCP crystals was also abrogated by the dominant negative protein RafS621A (B). EGF was used as positive control. Six independent transfections, each run in triplicate, were performed, and the results were normalized to β-galactosidase activity and expressed as the means ± S.E. Relative promoter activity was calculated by arbitrarily setting the activities of the samples without BCP treatment as 100.

of the following four plasmids: pCMV-RasN17, pCMV-RacN17, pCMV-RhoN17, or the empty vector pCMV. The transfected cells were subsequently treated with BCP crystals. As shown in Fig. 3A, cotransfection of the dominant negative form of RasN17 completely abrogated the induction of hMMP1 promoter by BCP crystals. In contrast, the expression of the dominant negative RhoAN19 proteins had no effect on the induction of hMMP1 promoter activity by BCP crystals. The dominant negative Rac1 protein could partially block the induction of hMMP1 promoter activity by BCP crystals. Taken together, these results indicated that BCP crystals induced the hMMP1 promoter activity primarily via a Ras-dependent pathway.

Next, we cotransfected FLSs with the −4372hMMP1luci plasmid together with the plasmid pCMV-RafS621A or with the empty vector pCMV and subsequently treated the transfected cells with BCP crystals. Results showed that the BCP crystal-mediated induction of the promoter activity of MMP1 was completely abrogated by the dominant negative protein RafS621A (Fig. 3B). The induction of the promoter activity of MMP1 by EGF and the effect of the dominant negative protein RafS621A on the induction were also shown in Fig. 3B for comparison.

**BCP Crystals Activate the Promoter Activity of hMMP1 Primarily through a Ras/Raf/MAPK/c-fos/AP-1/MMP1 Signaling Pathway**

The downstream factors along the Ras/Raf signaling pathway are MEK1 and MEK2. Recently, we have found that BCP crystals activated the phosphorylation of extracellular signal-regulated kinases (ERK1/2) in human foreskin fibroblast (25). To further define the signaling pathway, we cotransfected FLSs with the pFR-luc reporter plasmid together with the fusion trans-activator plasmids, pFA2-Jun, pFA2-Etk1, and pFA-CHOP, or the negative control plasmid pPC-dbd separately. The transfected cells were subsequently treated with BCP crystals. Twenty-four h later, cells were lysed, and lysates were assayed for luciferase activity. It can be seen that BCP crystals only activated the MAPK pathway and not the p38 MAPK or the JNK pathway (bar groups 1–4). The induction of the reporter activity of pFR-luci, when FLSs were cotransfected with the pFA2-Etk1 plasmid, by BCP crystals was about 2-fold (bar group 5) after normalization against the reporter activity of the negative control sample (bar group 1). Three independent transfections, each run in triplicate, were performed. Results were expressed as the means ± S.E. Relative promoter activity was calculated by arbitrarily setting the activities of the samples without BCP treatment as 100.
BCP crystals. As shown in Fig. 4A, BCP crystals only activated the MAPK pathway and neither the p38 MAPK nor the JNK pathways. BCP crystals treatment decreased the reporter activity of the pFR-luci ~30% compared with the untreated control when FLSs were cotransfected with the pFR-luci plasmid plus either pFA2-c-Jun, pFA-CHOP, or the negative control pFC2-dbplasmids (Fig. 4A, bar groups 1–3). In contrast, BCP crystal treatment increased the reporter activity of the pFR-luci ~30% compared with the untreated control when FLSs were cotransfected with the pFR-luci plus the pFA2-E1K1 plasmid (Fig. 4A, bar group 4). The induction of the reporter activity of pFR-luci by BCP crystals when FLSs were cotransfected with the pFA2-E1K1 plasmid was about 2-fold (Fig. 4A, bar group 5) after normalization against the reporter activity from the negative control sample (FLSs were cotransfected with the pFC2-dbplasmid). EGF was used as a positive control. It can be seen that EGF activated the MAPK and p38 MAPK pathways but not the JNK pathway (Fig. 4A), which is consistent with reports from the literature (28, 29).

Next, we looked at the ability of MEK1/2-specific inhibitor UO126 to inhibit the induction of the hMMP1 promoter, the c-fos promoter, and the pAP1luci reporter by BCP crystals. FLSs were separately transfected with ~4372hMMP1luci, c-Fosluci, or pAP1luci and subsequently treated with BCP crystals or BCP crystal plus specific MEK1/2 inhibitor UO126 (10 μM). As shown in Fig. 4B, the specific MEK1/2 inhibitor UO126 could block the induction by BCP crystals of all of the three promoter activities.

Previously, we have shown that the induction of the hMMP1 message by BCP crystals was blocked by the MEK1/2-specific inhibitor UO126 at a concentration of 10 μM and the MEK1-specific inhibitor PD98059 at a concentration of 50 μM (30). The specific MEK1 inhibitor PD98059 inhibits the activation of MEK1 (IC50 = 5 μM) much more effectively than MEK2 (IC50 = 50 μM). The fact that the induction of the hMMP1 message by BCP crystals could only be blocked by the MEK1-specific inhibitor PD98059 at a high concentration suggests that both MEK1 and MEK2 may play a role in mediating the induction of MMP1 by BCP crystals. Our results from cotransfection assays indicated that the dominant negative MEK1 plus MEK2 proteins (Fig. 4C) could indeed inhibit the induction of MMP1 promoter activity by BCP crystals.

MEK1-specific Inhibitor PD98059 Blocked the Induction of Endogenous c-fos Message by BCP Crystals—We next examined the c-fos mRNA levels after the treatment of FLSs with BCP crystals, BCP crystals plus MEK1-specific inhibitor PD98059, or BCP crystals plus PC. Northern blot (Fig. 5) showed that BCP crystals induced the c-fos message substantially and that the induction was totally blocked by the MEK1-specific inhibitor PD98059 (lanes 1–3). The induction by BCP crystals was also totally blocked by the BCP crystal-specific inhibitor PC, as expected (lane 4).

The −83/+67 Fragment Is Sufficient to Mediate BCP Crystal-Dependent Induction of hMMP1 Promoter Activity. Progressive 5′ deletion plasmids of hMMP1luci together with β-galactosidase control plasmid were transiently cotransfected into FLSs and subsequently treated with BCP crystals. Twenty-four h later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. The hMMP-1 promoter activities of all deletion fragments were induced similarly by BCP crystal treatment (A and B) except for the 61 bp deletion plasmid (B, last bar group). Four to six independent transfections, each run in triplicate, were performed, and the results were normalized to β-galactosidase activity and expressed as the means ± S.E. RLU, relative light units.

PD98059, or BCP crystals plus PC. Northern blot (Fig. 5) showed that BCP crystals induced the c-fos message substantially and that the induction was totally blocked by the MEK1-specific inhibitor PD98059 (lanes 1–3). The induction by BCP crystals was also totally blocked by the BCP crystal-specific inhibitor PC, as expected (lane 4).

The −83/+67 Fragment Is Sufficient to Mediate BCP Crystal Induction of the Promoter Activity of hMMP1—To determine the region of the hMMP1 promoter required for its induction by BCP crystals, progressive 5′ deletions of hMMP1luci plasmids were transiently cotransfected into FLSs together with a β-galactosidase control plasmid. The cells were subsequently treated with BCP crystals and assayed for luciferase and β-galactosidase activities. As shown in Fig. 6, the hMMP1 promoter activities of all deletion fragments (down to −83 bp) were induced similarly by BCP crystal treatment, resulting in ~2–3-fold induction. In contrast, luciferase activity in FLSs transiently transfected with the −61luci reporter driven by 61 bp of the hMMP1 promoter was not altered in response to BCP crystals. These data suggested that the sequence residing between −83 and −61 bp was critical for the induction of hMMP1 promoter activity by BCP crystals.

−72AP-1 Is Responsible for, at Least in Part, the BCP Crystal Induction of the Promoter Activity of hMMP1—The −72AP-1 site within the hMMP1 promoter is crucial to both the basal and induced activity of hMMP1 promoter (16, 17). The promoter mapping studies described above revealed that the induction of hMMP1 transcription by BCP crystals was probably mediated through the same AP-1 element within the hMMP1 promoter. To further confirm that the induction of hMMP1 transcription by BCP crystal was mediated primarily thought the −72AP-1 site, additional reporter derivatives were constructed (see Fig. 7) and tested. As shown in Fig. 8, elimination of the −72AP-1 site within the −83luci reporter plasmid through mutation abolished the induction almost completely (compare the last two bar groups).
The MMP1 promoter contains another AP-1 site at −181 and a PEA-3 site at −88. We decided to investigate whether the −181AP-1 and the −88PEA-3 played a direct role in the induction of transcription of the hMMP1 promoter by BCP crystals. We constructed five deletion constructs of −192luci, −104luci, and −83luci and corresponding mutant constructs −192M181AP1, −104M88PEA3, and −83M72AP1 were transiently cotransfected into FLSs together with β-galactosidase control plasmid and subsequently treated with BCP crystals. Twenty-four hours later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. Results from using these constructs indicated that a single mutation in the sequences of the −181AP1 within the −192MMP1 promoter or the −88PEA3 within the −104MMP1 promoter had no significant effect on the induction of hMMP1 promoter activity by BCP crystals (Fig. 8, bar groups 1–4).

The −181AP-1 Element Has an Effect Opposite to the −72AP-1 Element, and the −88PEA-3 Site Is Critical for the Induction of hMMP1 Promoter by BCP Crystals—It has been reported that the −181AP-1 element has a different effect on the induction of hMMP1 by phorbol ester in the context of 321 bp of the hMMP1 promoter compared with the −72AP-1 element (31). It has also been reported that the cooperation between −88PEA-3 and −72AP-1 is required for the maximal phorbol ester induction of the hMMP1 promoter activity (16, 32, 33). Although a single mutation at the −181AP-1 and −88PEA-3 sequences had no significant effect on the induction of hMMP1 promoter activity by BCP crystals in the context of the −192MMP1 and −104MMP1 promoters, we decided to investigate the role of the −181AP1 and the −88PEA3 sequences further. We constructed several additional mutants of the −192luci promoter/reporter plasmid (see Fig. 7). To our surprise, mutation at the −88PEA3 site within the −192MMP1 promoter decreased the induction of the −192luci by BCP crystals substantially (Fig. 9A, bar group 2). This result indicated that the −88PEA3 site was critical for the induction of the hMMP1 promoter by BCP crystals, at least in the context of the −192MMP1 promoter. However, the results from transfections using the −104luci and −104−M88PEA3luci plasmids suggested that PEA-3 was not important in the induction of hMMP1 by BCP crystals directly (Fig. 8, bar groups 3 and 4). When double mutations were introduced (−192M181AP1−72AP1luci) at both the −181AP-1 and −72 AP-1 sites, the induction of the −192MMP1 promoter by BCP crystals was knocked out almost completely, but residual induction could still be seen (1.3-fold compared with the 2-fold induction of the −192luci (Fig. 9A, bar groups 5 and 1)). Although this result suggested that the −88PEA3 site might be directly involved in the induction by BCP crystals, it could not explain how the induction of −192luci by BCP crystals was almost completely abolished when the −88PEA3 site was mutated.

When double mutations were introduced (−192M181AP1−88PEA3luci) at both the −181AP-1 and −88PEA3-3 sites, leaving only the −72AP-1 site intact, the induction by BCP crystals increased from about 2-fold for the wild type promoter, −192luci, to about 3-fold for the mutant promoter, −192M181AP1−88PEA3luci (Fig. 9A, bar groups 4 and 1). These results provided additional evidence that the induction of the hMMP1 promoter by BCP crystals was mainly mediated through the −72AP-1 site, but more importantly, these results indicated that the −181AP-1 site had an effect opposite to that of the −72AP-1 site. The −181AP-1 actually inhibited the induction of hMMP1 promoter activity by BCP crystals mediated through the −72AP-1 site. The inhibitory effect of the −181AP-1 could be inactivated either by a mutation at the −181AP-1 site or by the intact −88PEA3 element.

The reporter activities of the same constructs induced by PMA are shown in Fig. 9B for comparison. It is clear that the mechanism of transcriptional induction of the hMMP1 promoter by BCP crystals is different from the one by PMA.

**DISCUSSION**

The present study demonstrates that BCP crystal-induced hMMP1 promoter activation is associated with AP-1, PEA-3, and SRF transcription factors and follows the Ras/Raf/MAPK/c-fos/AP-1/MMP1 signaling pathway. The induction of

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**Fig. 7.** Diagram of mutant constructs of −192hMMP1 promoter/luciferase reporter constructs.

**Fig. 8.** The −72AP-1 is responsible, at least in part, for induction of the hMMP1 promoter activity by BCP crystals. 5′ deletion plasmids of hMMP1luci, −192luci, −104luci, and −83luci and corresponding mutant constructs −192M181AP1, −104M88PEA3, and −83M72AP1 were transiently cotransfected into FLSs together with β-galactosidase control plasmid and subsequently treated with BCP crystals. Twenty-four hours later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. Results from using these constructs indicated that a single mutation in the sequences of the −181AP1 within the −192MMP1 promoter or the −88PEA3 within the −104MMP1 promoter had no significant effect on the induction of hMMP1 promoter activity by BCP crystals (Fig. 8, bar groups 1–4).
The same constructs were transiently cotransfected with group 1 and group 4 to about 3-fold. When double mutations were introduced to both the 72AP-1 sites, the induction by BCP crystals was similar to the induction by PMA, and the mutation at the 72AP-1 site intact, the induction of hMMP1 promoter was about 60% of the induction by BCP crystals. However, when double mutations were introduced to both the 181AP-1 and 72AP-1 sites, the induction by BCP crystals was similar to the induction of 192M-72AP-1 (bar groups 3 and 5). Mutation at the 181AP-1 and 88PEA-3 sites, leaving only the 72AP-1 site intact, increased the induction by BCP crystals from about 2-fold for the wild type promoter (bar group 1) to about 3-fold (bar group 4). B, the induction by PMA. The same constructs were transiently cotransfected into FLSs together with β-galactosidase control plasmid, and subsequently treated with BCP crystals. Twenty-four h later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. Mutation at the 88PEA-3 site within the 192-hp hMMP1 promoter decreased the induction of the −192luci by BCP crystals substantially (bar group 2). Mutation at the −72AP-1 site abolished almost 60% of the induction by BCP crystals (bar group 3). However, when double mutations were introduced to both the 181AP-1 and −72AP-1 sites, the induction by BCP crystals was similar to the induction of −192M-72AP-1 (bar groups 3 and 5). Mutation at both the 181AP-1 and −88PEA-3 sites, leaving only the −72AP-1 site intact, increased the induction by BCP crystals from about 2-fold for the wild type promoter (bar group 1) to about 3-fold (bar group 4). B, the induction by PMA. The same constructs were transiently cotransfected into FLSs, together with β-galactosidase control plasmid, and subsequently treated with PMA at a concentration of 200 nm. Twenty-four h later, cells were lysed, and lysates were assayed for luciferase and β-galactosidase activities. Four to six independent transfections, each run in triplicate, were performed, and the results were normalized to β-galactosidase activity and expressed as the means ± S.E.

pAP1luci by BCP crystals suggests that the transcription factor AP-1 is involved in the BCP crystal-induced transcription of hMMP1. This result is consistent with our previous findings that BCP crystals enhance the binding of proteins to an oligonucleotide containing the consensus binding sequence for AP-1 transcription factor (26). The involvement of the AP-1 transcription factor is further confirmed by deletion and mutation studies. Deletion or mutation of the −72AP-1 site within the hMMP1 promoter results in decreased responsiveness to BCP crystals. In contrast to the −72AP-1 element, a single mutation at the −181AP-1 element has no observable effect on the BCP crystal induction of hMMP1 in the context of 192 bp of the hMMP1 promoter. These results suggest that the two AP-1 sites have different effects on the transcription of hMMP1 induced by BCP crystals.

Cooperation between the −88PEA-3 and the −72AP-1 is required for phorbol ester induction of hMMP1 promoter activity (16, 32, 33), and cooperation between PEA-3 and AP-1 has also been reported for many other promoters (34–37). In contrast to the induction of hMMP1 promoter by phorbol ester, our results here indicate that cooperation between −88PEA-3 and the −72AP-1 is not required for the BCP crystal-induced transcription of hMMP1. However, mutation at the −88PEA-3 site abolishes the BCP crystal-dependent induction of hMMP1 in the context of the −192hMMP1 promoter almost completely, indicating that the −88PEA-3 plays a critical role in the BCP crystal-induced transcription of hMMP1. Further mutation at both the −88PEA-3 and the −181AP-1 sites (−192M181AP1−88PEA3luci) not only recovers the induction; it actually increases the induction of hMMP1 to about 3-fold from 2-fold for −192luci and 1.3-fold for −192−M88PEA3luci. One possible explanation for this observation is that the −181AP-1 has an opposite effect on the transcription of the hMMP1 gene induced by BCP crystals compared with the −72AP-1. The −181AP-1 has an inhibitory effect on the −72AP-1-mediated induction of hMMP1 by BCP crystals. The inhibitory effect of the −181AP-1 can be inactivated or inhibited either by a mutation at the −181AP-1 site or by the intact −88PEA-3 site through an unknown mechanism. Mutation at the −88PEA3 site releases the inactivation effect of the −88PEA3 element on the −181AP1 element so that the induction of −192luci mediated through the −72AP-1 site is inhibited.

The induction of the hMMP1 promoter by PMA has been extensively studied (16, 31–33). Both the −181AP-1 and −72AP-1 sites are involved in mediating the induction of hMMP1 promoter by PMA, and the mutation at the −72AP-1 site is not sufficient to block the induction (31). Our results (Fig. 9B) are consistent with the previous findings, but more importantly, our results clearly show that the mechanisms of induction of hMMP1 promoter by BCP crystals and by PMA are different (Fig. 9, compare A with B) although all of the three sites, −181AP-1, −88PEA3, and −72AP-1, within the hMMP1 promoter are involved in both cases.

Surprisingly, the reporter activity of pNF-κBluci was not altered by BCP crystal treatment, although the binding of protein to NF-κB consensus binding sequence has been shown to be enhanced by BCP crystal treatment (26). A possible explanation is that additional factors and/or an alteration of bound NF-κB family members may be necessary to achieve transcription at the NF-κB sites. Another explanation is that the sensitivity of the reporter assays using synthetic reporter plasmids is low compared with other assays, such as RNA protection and gel shift assays. For example, although both of the CRE and SER sites are shown to be involved in the induction of c-fos promoter using RNA protection assays, we found that only the pSEREluci reporter gene was slightly induced by BCP crystals (Fig. 2). Another example is that Hata et al. (38) have found that although protein kinase C-γ enhances binding of proteins at the TRE site, it is unable to stimulate transcription from multiple TREs linked to the chloramphenicol acetyltransferase reporter gene.

The activation of the c-fos gene in response to a variety of mitogenic agents, including serum and growth factors, is mediated through the Ras/MAPK pathway (24, 39). Transcriptional activation of the c-fos gene is a synergistic process in which multiple c-fos promoter elements are targeted to effect maximal activation. The serum response element in the c-fos gene is necessary and sufficient for rapid induction of the c-fos gene by serum, growth factors, and PMA (40, 41). Our results here demonstrate the activation of SRF and c-fos by BCP crystals, suggesting the involvement of SRF in the activation of c-fos gene expression, which in turn forms the AP-1 transcription factor with Jun family members and activates the hMMP1 by binding to the −72AP-1 element. Although the CRE site in the c-fos promoter has been shown to be necessary for a maximal response to BCP crystals, our results showed that the reporter of pCREluci did not respond to BCP crystal treat-

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ment. These results suggest that cooperation of CRE with other sites may be required for BCP crystal-induced gene expression.

Ras can stimulate multiple signaling pathways (42–43). One of these involves the sequential activation of Raf, MEK1/2, and ERK1/2 (45–48). ERK1/2, in turn, increase the synthesis and/or activity of several transcription factor family members (49–51). Here, we have shown that the induction of hMMP1 gene expression by BCP crystals is primarily Ras-dependent and follows the Ras/Raf/MEK1/2/ERK1/2/c-fos/AP-1/hMMP1 pathway. By using dominant negative mutant constructs, we have shown that both dominant negative forms of Ras (RasN17) and Raf (RafS621A) completely blocked the transcriptional activation of hMMP1 by BCP crystals.

Interestingly, expression of a dominant negative Rac1 (RacN17) could partially block the BCP crystal-mediated induction of hMMP1 promoter, suggesting that Rac1 also plays a role in the signal transduction involved in the induction of hMMP1. Rac1 has been identified as a crucial mediator of Ras-dependent cellular responses (52). It has been shown that Rac1 is important in the activation of JNK- and p38 MAPK-regulating pathways parallel to the ERK cascade (21, 53). Therefore, it is conceivable that these signaling pathways can also lead to the activation of hMMP1. However, the facts that BCP crystals did not activate JNK or p38 MAPK (as judged by the results from assays using the PathDetect trans-reporting system) and that the dominant Ras blocked the induction completely exclude the two pathways cited above. One possible alternative signaling pathway is Ras/Rac1/ERK/hMMP1; i.e. there may exist two signaling cascades, both of which are initiated by Ras, in the induction of hMMP1 gene transcription by BCP crystals (Fig. 10). This model of signaling is similar to the model of signaling in the polyoma-virus middle-T antigen-mediated activation of the SRE proposed by Urich et al. (54). Recently, Kheradmand et al. (55) have demonstrated that activation of Rac1 is involved in the induction of hMMP1 gene expression by cell shape change. We have repeatedly observed cell shape change (shrinkage) upon exposing cells to BCP crystals. However, whether this observed cell shape change contributes to the induction of hMMP1 by BCP crystals via a Rac1-dependent pathway is unclear and currently under investigation at our laboratory.

It has been shown that crystal treatment of human fibroblasts results in translocation of the protein kinase C enzyme from the cytosolic to the membrane fraction of the cell, an indicator of protein kinase C activation (26). Down-regulation of protein kinase C activity using the phosphor ester PMA inhibits the BCP crystal-mediated mitogenesis and induction of c-Fos and c-Myc in 3T3 cells (56). However, how protein kinase C fits in the Ras/Raf/MEK1/2/ERK1/2/c-fos/AP-1/hMMP1 signaling pathway is puzzling and is currently also under investi-
47. O'Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994) Cell 78, 137–147
48. Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994) Nature 370, 386–389
49. Rivera, V. M., Miranti, C. K., Misra, R. P., Ginty, D. D., Chen, R. H., Blenis, J., and Greenberg, M. E. (1993) Mol. Cell. Biol. 13, 6260–6273
50. Johansen, F. E., and Prywes, R. (1995) Biochim. Biophys. Acta 1242, 1–10
51. Treisman, R. (1995) EMBO J. 14, 4905–4913
52. Qiu, R. G., Chen, J., Kirn, D., McCormick, F., and Symons, M. (1995) Nature 374, 457–459
53. Ichijo, H. (1999) Oncogene 18, 6087–6093
54. Urich, M., Senften, M., Shaw, P. E., and Ballmer-Hofer, K. (1997) Oncogene 14, 1235–1241
55. Kheradmand, F., Werner, E., Tremble, P., Symons, M., and Werb, Z. (1998) Science 280, 898–902
56. Mitchell, P. G., Pledger, W. J., and Cheung, H. S. (1989) J. Biol. Chem. 264, 14071–14077
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