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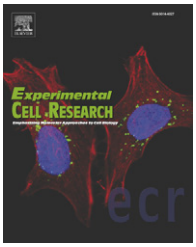

Research Article

# A role for AP-1 in matrix metalloproteinase production and invadopodia formation of v-Crk-transformed cells

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ABSTRACT

Both MMP-2 and MMP-9 play critical roles in tumor invasion, but their productions are differentially controlled. While the promoter region of *MMP-9* has the conserved proximal AP-1 binding site, that of the *MMP-2* has a noncanonical AP-1 site. To assess the role of AP-1 function, we examined the effects of dominant-negative Fos (ΔFos), BATF and siRNA against c-Jun on MMP production in v-Crk-transformed cells which have augmented production of MMP-2 and MMP-9. Suppression of AP-1 dependent transcription by conditional expression of dominant-negative Fos (ΔFos) and BATF substantially inhibited not only MMP-9 production but also MMP-2 production. The ChIP analysis showed the direct association of AP-1 and *MMP-2* promoter region. In addition, silencing of c-Jun expression by siRNA transfection suppressed MMP-2 and MMP-9 production and *in vitro* invasiveness. Furthermore, the invadopodia formation of v-Crk-transformed cells could be suppressed by BATF expression or c-Jun siRNA treatment. Taken together, AP-1 appears to play a critical role in the production of MMP-2 and MMP-9 and invadopodia formation of v-Crk-transformed cells.

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## Introduction

Matrix metalloproteinases (MMPs) are a family of neutral proteinases that play an important role for the destruction of extracellular matrix to be implicated in multiple patho-physiological processes [1]. Among MMPs, MMP-2 and MMP-9 (Gelatinase A and B) have drawn attention for their role in tumor invasion and metastasis [2,3]. MMP-2 and MMP-9 are secreted from the cells as inactive zymogens and activated by proteolytic cleavage [4,5]. Both MMP-2 and MMP-9 are accumulated and activated at the subcellular structures called invadopodia, actin-rich protrusions localized at cell–substratum contact points [6].

As a model to explore the mechanism by which MMP production is activated, we have studied signaling in cell lines that were transformed with oncogenes such as v-*src* and v-*crk* [7–9]. v-Crk, but not v-Src, appears to activate the production of MMP-2 and MMP-9, suggesting v-Crk-transformed cells as a good model to study the MMP production. v-Crk, a product of the oncogene encoded by the avian retrovirus CT10 [10], is an adapter protein containing the SH2 and SH3 domains. v-Crk tightly associates with multiple molecules including two guanine nucleotides exchange proteins, C3G and Sos. The binding of Crk with Sos activate the Ras–MAP kinase pathway [11,12] whereas its binding to C3G activates JNK in a Ras-independent manner [13,14]. We found the Ras–MEK–MAP kinase pathway, but not the C3G–JNK pathway, to be critical for the production of MMP-2 and MMP-9 [9]. Interestingly, v-Src also activates the Ras–MEK–MAP kinase pathway but stimulates only MMP-2 production [7,8].

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MAP kinase regulates gene expression by controlling the activities of both AP-1 and ETS transcription factors [15]. The promoter regions of the *MMP* genes including *MMP-9* are highly conserved with the presence of a proximal AP-1 binding site [16] which plays a major role in the *MMP* gene expression. AP-1 is a dimeric transcription factor composed primarily of the Jun (c-Jun, JunD, JunB) and Fos (c-Fos, FosB, Fra1, Fra2) classes of proteins. Members of the Jun family bind to DNA either as Jun/Jun homodimers or Jun/Fos heterodimers, whereas Fos proteins do not form homodimers [17].

In contrast to *MMP-9* and other *MMP*s, the promoter region of the *MMP-2* gene appears to lack the conserved proximal AP-1 site [16]. In hepatocellular cells, binding of the transcription factors such as YB-1, AP-2 and p53 to an enhancer element, RE-1, located at −1322 bp of MMP-2 promoter region, mediates transcription [18]. In astrocytoma cell lines, the proximal Sp-1 and AP-2 sites at −90 to −50 bp are sufficient for constitutive MMP-2 transcription [19]. In contrast to these observations, Bergman et al. [20] and Lee et al. [21] reported the binding of AP-1 family transcription factors to a noncanonical AP-1 site located at −1392 bp of *MMP-2* promoter region. It, therefore, remains to be clarified, whether AP-1 plays a critical role in MMP-2 production as well as MMP-9 production or simply acts as one of the regulatory factors.

To assess the role of AP-1 in v-Crk-dependent production of MMPs, we employed three different methods; conditional expression of dominant-negative Fos which has a dominant inhibitory effect on AP-1, conditional expression of BATF and treatment with siRNA against c-Jun. Here, we show that inhibition of the transcriptional function of AP-1 activity dramatically decreases not only the production of MMP-9 but also that of MMP-2. Furthermore, we show that AP-1 directly interacts with the promoter region of *MMP-2* gene by the ChIP assay. In addition, we show that the suppression of AP-1 activity shows good correlation with the inhibition of invadopodia formation and *in vitro* invasiveness of v-Crk-transformed cells.

## Materials and methods

### *Cell culture and plasmids*

A rat fibroblast cell line, 3Y1 and v-Crk-transformed 3Y1 cells (v-Crk3Y1) were cultured as previously described [9]. cDNA of BATF or HA-tagged ΔFos was cloned into the PMAM2-BSD (Kaken Seiyaku) vector and then transfected to v-Crk3Y1 cells. Drug resistant colonies were isolated and cell lines in which either BATF or HA-ΔFos was conditionally induced by treatment with 2 μM dexamethasone were established.

### *Luciferase reporter constructs*

To measure AP-1-dependent transcriptional activity, AP-1 consensus site was subcloned into the pGL3-Promoter vector (Promega). The *MMP-2* promoter fragments of 2161, 1780, 1659, 1380 and 865 bp 5′ relative to the translational start site were amplified by PCR and subcloned into the luciferase expression vector pGL3-Basic (Promega). Rat genomic DNA was used as a template for PCR to generate a series of reporter constructs. These constructs were designated Luc 2161, Luc 1780, Luc 1659, Luc 1380 and Luc 865.

### *Luciferase activity assay*

$1 \times 10^5$ cells were seeded in 6-well plates and treated or untreated with 2 μM dexamethasone for 24 h. Cells were transfected with AP-1 reporter plasmid and PRL-TK control plasmid, and 24 h later, cells were harvested for luciferase assay. Luciferase activity was measured and normalized using the dual luciferase kit (Promega). To measure the luciferase activity after siRNA transfection, v-Crk3Y1 cells were transfected with siRNAs and 24 h later, cells were transfected with AP-1 reporter plasmid and PRL-TK control plasmid. 24 h later, cells were harvested for luciferase assay. Three independent transfections were performed to measure the average values and standard deviations.

### *RT-PCR analysis*

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. First strand cDNA synthesis was performed with 1 μg of total RNA using a ReverTra Ace kit (TOYOBO). The primer sequences we used were as follows: MMP-2; 5′-AGATCTTCTTCTTCAAGGACCGGTT-3′ and 5′-GGCTGGTC-AGTGGCTTGGGGTA-3′ (224 bp), MMP-9; 5′-AGGCTACAGCTTTGC-TGCCCC-3′ and 5′-GCTGCTTCTGAAGCATCAGCA-3′ (193 bp), GAPDH; 5′-TGAT-GCTGGTGCTGAGTATG-3′ and 5′-GGTGGAAGAATGGGAGT-TGC-3′ (618 bp). The reaction mixture was subjected to 30 cycles of PCR amplification for MMP-2 and MMP-9, and 25 cycles for GAPDH. PCR products were analyzed on a 6% TBE gel by ethidium bromide staining.

### *Northern-blot analysis*

Total RNA was isolated with RNeasy mini kit (Qiagen) and 10 μg of total RNA were resolved by electrophoresis in a formaldehyde-agarose gel and then blotted onto nylon membranes (Amersham Pharmacia Biotech). The blots were hybridized with [α-$^{32}$P] dCTP-labelled MMP probe or with [α-$^{32}$P] dCTP-labelled GAPDH probe.

### *Assay of gelatin-degrading MMPs by zymography*

The production of MMPs was assayed by zymography as described previously [7]. Briefly, cells were incubated with serum-free media for 12 h and then the conditioned media were clarified by centrifugation and subjected to electrophoresis with SDS-polyacrylamide gels copolymerized with gelatin. Gels were washed, incubated with reaction buffer (50 mmol/L Tris–HCL (pH 7.4), 0.02% $NaN_3$, 10 mmol/$CaCl_2$), and stained with Coomassie Blue.

### *Immunoblotting*

Immunoblotting with specific antibodies was performed as described previously [22].

### *Invasion assay*

Cells were assayed for their invasiveness by modified Boyden chamber method as described [23]. Briefly, cells were suspended in serum-free DMEM, and seeded onto Matrigel-coated filters. After 6 h of incubation, cells that had invaded to the lower surface of the filter were fixed, stained, and quantified by counting three randomly

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