The Ras-Mitogen-activated Protein Kinase Pathway Is Critical for the Activation of Matrix Metalloproteinase Secretion and the Invasiveness in v-crk-transformed 3Y1

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Abstract

To search for the intracellular signaling pathway critical for the secretion of matrix metalloproteinase (MMP), we studied the effects of dominant negative Ras (S17N Ras) and dominant negative MEK1 (MEK1AA) expression in v-crk-transformed 3Y1. Expression of either S17N Ras or MEK1AA dramatically suppressed the augmented secretion of MMP-2 and MMP-9 in v-crk-transformed 3Y1. Similarly, a Ras farnesyltransferase inhibitor, manumycin A, and a MEK1 inhibitor, U0126, suppressed MMP secretion in a dose-dependent manner, whereas a PI3 kinase inhibitor, wortmannin, could not. In addition, the suppression of MMP secretion by S17N Ras showed good correlation with the inhibition of in vitro invasiveness of the cells. In contrast, expression of dominant negative C3G did not suppress MMP secretion, although it substantially blocked the c-Jun N-terminal kinase activation. Taken together, the Ras-MEK1 pathway, but not the C3G-JNK pathway, seems to play a key role in the activation of MMP secretion and, hence, the invasiveness of v-crk-transformed cells.

Introduction

Matrix metalloproteinases (MMPs) are a family of proteinases that implicated in multiple physiological and pathological processes (1). Among MMPs, MMP-2 and MMP-9 (gelatinases A and B) have drawn attention for their implication in tumor invasion and metastasis (2). Both MMP-2 and MMP-9 are secreted from the cells as inactive zymogens and activated by proteolytic cleavage (3). A membrane-type MMP, MT1-MMP, has been proposed as an activator of MMP-2 (4). In human cancer tissues, evidence accumulated suggests that MMPs are secreted from the stromal fibroblastic cells rather than the tumor cells themselves (5), yet the regulation of MMP secretion in the stromal cells remains largely unclear.

As a model, we have studied MMP secretion in fibroblasts transformed with oncogenes (6). As we reported, various oncogene products such as v-Src and v-erbB stimulated the secretion and proteolytic activation of MMP-2, suggesting the presence of a common signaling pathway critical for MMP secretion. To obtain more clues, we investigated the signaling pathway critical for the activation of MMP secretion in v-crk-transformed 3Y1 cells. v-Crk is a Mr 47,000 adapter protein, which contains the SH2 and SH3 domains, having been identified as a product of the oncogene encoded by the avian retrovirus CT10 (7). In v-crk-transformed cells, tyrosine phosphorylation of a limited number of cellular proteins is increased (7). In addition, v-Crk seems to tightly interact with multiple target molecules, including two guanine nucleotide exchange proteins, C3G and Sos, by its SH3 domain. The binding of Crk with Sos seems to activate the Ras-MAPK pathway similar to Grb2 (8, 9), whereas its binding to C3G activates JNK in a Ras-independent manner (10, 11). Thus, two signaling pathways are activated by the SH3-dependent mechanism in v-crk-transformed cells, yet their roles in tumor invasion remain to be clarified. In this study, we show that the Ras-MEK1-MAPK pathway is critical for the augmented secretion of MMP-2 and MMP-9, whereas the C3G-JNK pathway is dispensable for the activation of MMP secretion.

Materials and Methods

Cells and Plasmids. A rat fibroblast cell line, 3Y1, was cultured as described previously (12). v-crk plasmid ligated with the pMEXneo vector was transfected into 3Y1 or cotransfected with S17N ras ligated with pMAM-BS (Kaken Seiyaku), which contains the mouse mammary tumor virus promoter as described (13). Drug-resistant colonies were isolated, and cells expressing v-Crk and S17N Ras in the presence of dexamethasone (2 μM) were selected by immunoblotting (10, 13). The dominant negative C3G (11) and mek1AA were transiently introduced into cells by electroporation method (14). The mek1AA mutated at S218A and S222A by PCR was ligated into pTRE and pBabe-puro vectors.

Assay of MMPs by Zymography. The activities of MMPs in the conditioned media were assayed by zymography, as described previously (6).

Immunoblotting. Immunoblotting with anti-Crk antibody, anti-pan Ras antibody, anti-MMP-2 antibody, anti-MT1-MMP antibody (Fuji Yakuhin Kogyo), anti-PY20H antibody, and anti-active MAPK antibody was performed as described previously (14, 15).

Analysis of Ras-bound GDP/GTP. Analysis of Ras-bound GTP was performed as described (16). Briefly, cells were starved for 24 h in serum-free DMEM. Cells were washed, harvested in permeabilization buffer, and mixed with streptolysin O (final concentration, 0.4 units/ml). Then, labeled with 10 μCi of [α-32P]GTP (800 Ci/mmol; New England Nuclear) for 10 min, the guanine nucleotides bound to Ras were eluted and analyzed with TLC.

Invasion Assay by Matrigel. Cells were assayed for their invasiveness by a modified Boyden chamber method (6). Briefly, conditioned media obtained from 3Y1 were placed in the lower compartment of the chambers. Cells suspended in serum-free DMEM were seeded onto Matrigel-coated filters. After 12 h of incubation, cells invaded to the lower surface of the filters were fixed, stained, and counted.

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The abbreviations used are: MMP, matrix metalloproteinase; Crk, CT10 regulator of cellular transformation; C3G, Crk SH3-binding guanine nucleotide releasing protein; MAP, mitogen-activated protein; MAPK, MAP kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK/Erk kinase; JNK, c-Jun N-terminal kinase; GDP, guanosine 5’-diphosphate; GTP, guanosine 5’-triphosphate.

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Results and Discussion

We first examined the gelatinase activity in the conditioned media of 3Y1 and 3Y1 transfected with v-crk (v-Crk3Y1) by zymography, as described in “Materials and Methods.” Although 3Y1 secreted trace amounts of MMP-2 and MMP-9 only in zymogen forms, their activities secreted from v-Crk3Y1 increased up to five times higher than that of 3Y1 (Fig. 1A). In addition, the proteolytically activated form of MMP-2 appeared in the medium of v-Crk3Y1. To confirm these results, we isolated several independent clones of v-Crk3Y1, and all of them showed essentially similar results (Fig. 1A).

To study the role of Ras signaling in v-Crk-dependent activation of MMP secretion, we established v-Crk3Y1 cell lines transfected with conditionally inducible S17N ras. Expression of S17N Ras, a mutant Ras with Asn substitution for Ser at position 17 of Ras(17) was shown to yield a dominant inhibitory effect on endogenous Ras(17). Under the control of mouse mammary tumor virus promoter/enhancer, S17N ras was induced by treatment with dexamethasone (Fig. 1B). Because S17N Ras has a suppressive effect on cell growth, we used this conditionally inducible system to avoid the clonal difference of isolated clones. Among several clones isolated, two clones that responded well to the drug were used throughout the study. We confirmed that the Ras-bound GTP:GDP ratio was dramatically decreased by dexamethasone treatment in these cells (Fig. 1C). In contrast, dexamethasone treatment of S17N ras-transfected v-Crk3Y1 did not show gross inhibitory effect on tyrosine phosphorylation of cellular proteins (Fig. 1D). We found, however, that activation of MAPKs was strongly inhibited by the expression of S17N Ras (Fig. 1, E and F).

With these cell lines, we investigated the role of Ras signaling in MMP secretion. We found that expression of S17N Ras by treatment with dexamethasone dramatically suppressed the secretion of MMP-2 and MMP-9 in 3Y1 ras-transfected v-Crk3Y1 (Fig. 2A). In addition, the activated form of MMP-2 became undetectable by treatment with dexamethasone. In contrast, augmented secretion of MMPs, as well as proteolytic activation of MMP-2, was not suppressed by dexametha-
some treatment in the pMAM2-BSD-transfected cells (Fig. 2). Suppression of MMP secretion by S17N Ras was further confirmed by immunoblotting with anti-MMP-2 and anti-MMP-9 (Fig. 2, B and C). Interestingly, we found that intracellular levels of MMP-2 and MMP-9 were not grossly increased by v-Crk transformation or suppressed by S17N Ras expression (Fig. 2, D and E). In addition, intracellular levels of MT1-MMP, which catalyzes proteolytic activation of MMP-2, slightly increased by v-Crk transformation but did not decrease by S17N Ras expression (Fig. 2F). These results suggest that the Ras pathway is required for the secretion of MMPs but is not critical for the MT1-MMP production in v-Crk3Y1.

It should be noted that expression of S17N Ras did not grossly inhibit tyrosine phosphorylation of cellular proteins, including p130Cas and paxillin (data not shown), but suppressed MMP secretion. These results indicate that tyrosine phosphorylation of cellular proteins such as p130Cas is not sufficient but requires downstream signaling via Ras for the activation of MMP secretion.

We next studied whether suppression of MMP secretion by S17N Ras inhibited the invasiveness of cells by the modified Boyden Chamber method, as described in “Materials and Methods.” Cells were seeded onto the upper chambers, and cells that penetrated the filters after 12 h of incubation were fixed, stained, and counted. Without dexamethasone treatment, S17N ras-transfected v-Crk3Y1 could penetrate through the reconstituted membrane to the level similar to v-Crk3Y1, whereas 3Y1 could not (Fig. 2G). In contrast, S17N ras-transfected v-Crk3Y1 significantly lost the invasiveness by treatment with dexamethasone, whereas v-Crk3Y1 transfected with control vector maintained the invasiveness after dexamethasone treatment.

We next studied the role of MEK1, a downstream effector for Ras, in MMP secretion. In the earlier studies, Ras was found to mediate its effects on cellular proliferation by activation of a linear cascade of kinases: Raf, MEK, and MAPK (ERK1/2; Ref. 18). Activation of MAPKs in turn regulates the activities of nuclear transcription factors, including Elk-1. However, a large body of evidence accumulated recently suggests that Ras signaling involves a complex array of signaling pathways such as PI3 kinase, RacGDS, SEK-JNK, p120GAP, RIN1, NF1 GAP, and AF6 (18). In addition to their signaling pathways such as PI3 kinase, RalGDS, SEK-JNK, MAPKs (ERK1/2), or anti-Erk2 antibodies (D). Conditioned media of the cells were subjected to zymography (E). Similarly, cells transfected with dominant negative C3G (15 μg) were incubated with serum-free medium. Cells were harvested and subjected to immunoblotting with anti-Myc monoclonal (9E10; A), anti-MEK1 (B), anti-phospho-MAPKs (C), or anti-Erk2 antibodies (D). Conditioned media of the cells were subjected to zymography (E). Similarly, cells transfected with dominant negative C3G (15 μg) were incubated with serum-free medium. Cells were harvested and subjected to immunoblotting with anti-C3G antibody (F). anti-phospho-stress-activated protein kinase/JNK (G), or anti-stress-activated protein kinase/JNK antibody (H). The conditioned media were subjected to zymography (I), v-Crk, v-Crk-transformed 3Y1; Vec., v-Crk3Y1 transfected with pTRE vector alone; pTRE-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pTRE vector; pBabe-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pBabe vector; v-Crk-C3G (Clone 1 and Clone 2), two independent clones of v-Crk3Y1 transfected with dominant negative C3G.

As a step to identify the critical downstream effector for the activation of MMP secretion, we examined the effect of dominant negative MEK1 (MEK1AA) expression in v-Crk3Y1. In this experiment, two types of vector, pTRE and pBabe, were used to confirm the effect of MEK1AA. Myc-epitope-tagged mek1AA gene was ligated into these vectors, namely, pBabe and pTRE clones. The vectors were transiently transfected into v-Crk3Y1, and expression and dominant negative C3G expression in v-Crk3Y1 cells were subjected to transient transfection with mek1AA (15 μg) by electroporation method. The cells were incubated with serum-free medium for 24 h. After incubation, cells were harvested and subjected to immunoblotting with anti-Myc monoclonal (9E10; A), anti-MEK1 (B), anti-phospho-MAPKs (C), or anti-Erk2 antibodies (D). Conditioned media of the cells were subjected to zymography (E). Similarly, cells transfected with dominant negative C3G (15 μg) were incubated with serum-free medium. Cells were harvested and subjected to immunoblotting with anti-C3G antibody (F). anti-phospho-stress-activated protein kinase/JNK (G), or anti-stress-activated protein kinase/JNK antibody (H). The conditioned media were subjected to zymography (I), v-Crk, v-Crk-transformed 3Y1; Vec., v-Crk3Y1 transfected with pTRE vector alone; pTRE-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pTRE vector; pBabe-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pBabe vector; v-Crk-C3G (Clone 1 and Clone 2), two independent clones of v-Crk3Y1 transfected with dominant negative C3G.

Fig. 3. Effects of dominant negative MEK1AA expression and dominant negative C3G expression on MMP secretion in v-Crk3Y1. v-Crk3Y1 cells were subjected to transient transfection with mek1AA (15 μg) by electroporation method. The cells were incubated with serum-free medium for 24 h. After incubation, cells were harvested and subjected to immunoblotting with anti-Myc monoclonal (9E10; A), anti-MEK1 (B), anti-phospho-MAPKs (C), or anti-Erk2 antibodies (D). Conditioned media of the cells were subjected to zymography (E). Similarly, cells transfected with dominant negative C3G (15 μg) were incubated with serum-free medium. Cells were harvested and subjected to immunoblotting with anti-C3G antibody (F). anti-phospho-stress-activated protein kinase/JNK (G), or anti-stress-activated protein kinase/JNK antibody (H). The conditioned media were subjected to zymography (I), v-Crk, v-Crk-transformed 3Y1; Vec., v-Crk3Y1 transfected with pTRE vector alone; pTRE-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pTRE vector; pBabe-MEK1AA, v-Crk3Y1 transfected with mek1AA ligated with pBabe vector; v-Crk-C3G (Clone 1 and Clone 2), two independent clones of v-Crk3Y1 transfected with dominant negative C3G.
v-Crk activates MMP secretion via Ras-MEK1 pathway

vectors and introduced into v-Crk3Y1 by electroporation, as described in “Materials and Methods” (Fig. 3, A and B). As shown in Fig. 3C, v-Crk3Y1 had activated MAPK as judged by anti-phospho-MAPK antibody, and this activation of MAPK was suppressed by the expression of MEK1AA. We found that secretion of both MMP-2 and MMP-9 was dramatically suppressed in meklAA-transfected v-Crk3Y1, whereas control vector-transfected v-Crk3Y1 showed no change in MMP secretion (Fig. 3E). These results suggest that MEK1 plays a critical role in augmented secretion of MMPs in v-Crk3Y1.

We next examined the role of the C3G-JNK pathway for the augmented MMP secretion in v-Crk3Y1 by use of a dominant negative form of C3G. Dominant negative C3G was transiently introduced into v-Crk3Y1, and its expression was monitored by immunoblotting (Fig. 3F). As reported previously (11), v-Crk3Y1 had activated JNK detected by anti-phospho-JNK, and this JNK activation was strongly suppressed by the expression of dominant negative C3G (Fig. 3G). We found that augmented secretion of MMP-2 and MMP-9, as well as proteolytic activation of MMP-2, was not blocked by the expression of dominant negative C3G (Fig. 3I), suggesting that the C3G-JNK pathway is dispensable for the activation of MMP secretion in v-Crk3Y1.

To confirm these observations, we next examined the effects of various inhibitors on MMP secretion in v-Crk3Y1. When v-Crk3Y1 was pretreated with the indicated doses of manumycin A, a potent inhibitor of Ras farnesyltransferase (19), secretion of MMP-2 and MMP-9 was suppressed in a dose-dependent manner (Fig. 4A). Under these conditions, both the expression of v-Crk and the tyrosine phosphorylation of cellular proteins in v-Crk3Y1 were not perturbed by the drug treatment (data not shown). Similarly, v-Crk3Y1 treated with U0126, a potent inhibitor of MEK1, showed suppression in the secretion of MMP-2 and MMP-9 in a dose-dependent manner (Fig. 4B), whereas v-Crk expression and cellular protein phosphorylation were not inhibited (data not shown). In contrast to these inhibitors, wortmannin, a potent inhibitor of PI3 kinase that is another downstream effector for Ras, did not show suppression on MMP secretion in v-Crk3Y1 (Fig. 4C).

In summary, we showed for the first time that v-Crk, an adaptor-type oncogene product, activated MMP secretion in a Ras-MEK1-dependent manner. The activation of MMP secretion via the Ras pathway showed good correlation with the invasiveness of the cells, suggesting the critical role of the Ras pathway in tumor invasion. In contrast, suppression of the C3G-JNK-pathway by a dominant negative form of C3G did not inhibit MMP secretion, indicating the C3G-JNK-pathway is dispensable for the activation of MMP secretion and, hence, for the invasion of v-crk-transformed cells. Interestingly, the Ras-MEK1 signaling pathway was not critical for the production of MT1-MMP in 3Y1 transformed with v-crk. A more extensive search for the downstream factor(s) that directly regulates the secretion of MMPs is an important issue to be elucidated.

References

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