

c-Ras is required for the activation of the matrix metalloproteinases by concanavalin A in 3Y1 cells

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Abstract Concanavalin A (Con A) is known to trigger augmented secretion and proteolytic activation of the matrix metalloproteinases (MMPs) in fibroblasts. To study the signaling pathway critical for the activation of MMPs in fibroblasts, we examined the effects of dominant negative *ras* (S17N *ras*) expression under the control of conditionally inducible promoter in Con A-activated 3Y1 cells. We found that augmented secretion and proteolytic activation of MMP-2 and MMP-9 together with expression of MT1-MMP in Con A-activated 3Y1 were dramatically suppressed by S17N *ras* expression. These results strongly suggest that c-Ras plays a critical role in the augmented expression and proteolytic activation of MMPs in fibroblasts.

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Key words: Matrix metalloproteinase; MMP-2; MMP-9; *ras*; Concanavalin A; Signal transduction

1. Introduction

One of the critical steps for tumor invasion and metastasis is the destruction of extracellular matrix [1] that is catalyzed mainly by the matrix metalloproteinases (MMPs). MMPs are neutral proteinases that require Zn or Ca ions for their activity, and consist of a family including MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [2,3]. MMPs are secreted from cells as inactive zymogens, and subsequently activated by proteolytic cleavage [4]. A membrane-type MMP, MT1-MMP catalyzes proteolytic activation of MMP-2 [5].

In metastatic human cancer tissues, increased secretion and proteolytic activation of MMPs are often observed [6]. However, a large body of evidence has been accumulated that MMPs including MMP-2, MMP-9, stromelysin-3 and MT1-MMP in tumor tissues are secreted from the stromal fibroblastic cells rather than tumor cells themselves [7–9]. Thus, the regulatory mechanism of MMPs in the stromal fibroblastic cells of tumor is an important problem to be clarified.

To study the signaling pathway critical for the activation of MMPs in fibroblasts, we examined the effects of dominant negative *ras* (S17N *ras*) expression in concanavalin A (Con A)-activated 3Y1 cells. Con A, a tetravalent lectin, is known to trigger augmented secretion and proteolytic activation of MMPs in fibroblasts, hence is widely used for the study of MMP activation [10,11]. Since Con A treatment of cells is known to activate tyrosine phosphorylation and stimulate phosphatidylinositol 3-kinase [12,13], it may mimic the signaling mechanism that results in activation of MMPs. However,

the exact mechanism yet remains largely unclear. To examine whether c-Ras is involved in Con A-dependent signaling, we established a cell line in which S17N *ras* is conditionally expressed under the control of mouse mammary tumor virus (MMTV) promoter/enhancer. Expression of S17N *ras*, a mutant *ras* with Asn substitution for Ser at position 17 of Ras^H, was shown to yield a dominant inhibitory effect on endogenous Ras [14]. Here, we show that expression of S17N *ras* in 3Y1 inhibits both proteolytic activation and augmented secretion of MMP-2 and MMP-9 in Con A-activated cells. In addition, we show evidence that expression of MT1-MMP which catalyzes activation of MMP-2 is also activated by Con A and inhibited by S17N Ras. Our results strongly suggest that c-Ras plays a critical role in Con A-dependent signaling that results in induction and activation of MMPs in fibroblasts.

2. Materials and methods

2.1. Cell culture, plasmid construction and transfection

A rat fibroblast cell line, 3Y1, was cultured as previously reported [15]. S17N *ras*, a generous gift of Dr. L. Feig, was ligated into a vector pMAM2-BSD (Kaken Seiyaku) which has the MMTV promoter and transfected into 3Y1 cells as previously described [16]. Blastocidin (10 µg/ml)-resistant colonies were isolated and S17N Ras expressing clones in the presence of dexamethasone (2 µM) were selected by immunoblotting with anti-pan Ras antibody (Santa Cruz) as described previously [17].

2.2. Con A treatment of 3Y1

3Y1 and S17N *ras*-transfected 3Y1 were treated with Con A (5–20

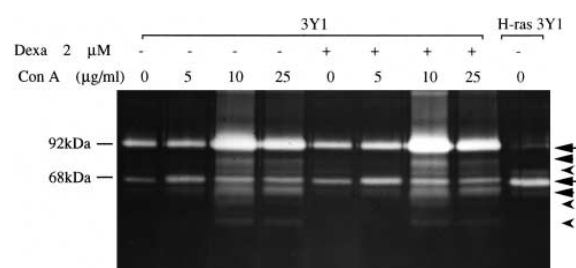


Fig. 1. Induction and activation of MMP-2 and MMP-9 by Con A treatment. 3Y1 cells were incubated with 0 or 2 µM dexamethasone for 24 h, and then treated with 0, 5, 10 or 25 µg/ml of Con A for 24 h in serum-deprived medium in the presence or absence of dexamethasone. Conditioned media were collected and subjected to gelatin zymography as described in Section 2. Arrows indicate 92- and 68-kDa zymogen forms of MMP-9 and MMP-2, respectively; large arrowheads, 84- and 63-kDa active forms of MMP-9 and MMP-2, respectively; small arrowheads, additional gelatinases observed in Con A-activated cells.

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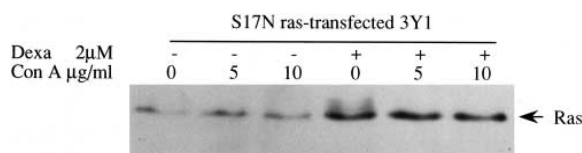


Fig. 2. S17N Ras expression in transfected 3Y1 treated with Con A. S17N *ras*-transfected cells incubated with or without dexamethasone for 24 h were treated with 0, 5 or 10 μ g/ml of Con A in the presence or absence of dexamethasone for an additional 24 h. Cells were collected and examined by immunoblotting with anti-pan Ras antibody.

μ g/ml) in serum-deprived medium for 24 h, and conditioned media were collected for zymography.

2.3. Assay of gelatin-degrading MMPs by zymography

The activities of MMPs in the conditioned media were assayed by zymography as previously described [18].

2.4. Western blotting

Immunoblotting with anti-pan Ras and anti-MT1-MMP (Fuji Yakuhin Kogyo) was performed as described previously [19].

3. Results and discussion

We first examined the effect of Con A treatment on MMP secreted from 3Y1 by zymography. Without treatment, 3Y1 cells secreted only zymogen forms of MMP-2 and MMP-9 of 68 and 92 kDa, respectively, as judged by immunoprecipitation and immunoblotting with specific antibodies (data not shown). We found that Con A treatment dramatically activated both secretion and proteolytic activation of MMP-2 and MMP-9 in a dose-dependent manner. As shown in Fig. 1, secretion of 68- and 92-kDa bands was elevated, and 63- and 84-kDa bands together with additional smaller bands appeared by Con A treatment. As previously reported [6],

these 63- and 84-kDa bands were active forms of MMP-2 and MMP-9. We confirmed that treatment of 3Y1 with dexamethasone which was used in the following study showed no inhibitory effect on augmented secretion and proteolytic activation of MMPs by Con A.

In contrast to Con A treatment, we found that only MMP-2, but not MMP-9, was activated in H-*ras* transformed 3Y1. Gum et al. [20] reported that transformation of ovarian adenocarcinoma cell line, OVCAR-3, by H-*ras* resulted in the activation of MMP-9 secretion. Although our results were not consonant with those of Gum et al., this may simply reflect the difference of cell lines used for the study. At any rate, our results suggest that the signaling pathway for the activation of MMP-9 may differ in part from that of MMP-2.

To study the role of Ras in Con A-dependent activation of MMPs, we established 3Y1 cells transfected with conditionally inducible S17N *ras*. Among several clones we isolated, one that responded well to the dexamethasone treatment was used throughout the study. Under the control of MMTV promoter/enhancer, S17N *ras* was induced by treatment with dexamethasone (Fig. 2). We confirmed that Con A treatment had no inhibitory effect on dexamethasone-dependent expression of S17N *ras*. We found Con A treatment slightly activated tyrosine phosphorylation of cellular proteins as previously reported [12,13], but S17N *ras* expression has no clear effect on overall tyrosine phosphorylation of both untreated and Con A-treated cells (data not shown).

We next examined the effect of S17N *ras* expression on Con A-dependent secretion and activation of MMPs by zymography (Fig. 3A). Without S17N *ras* expression, transfected cells responded well to Con A treatment and showed enhanced secretion and proteolytic activation of both MMP-2 and MMP-9. In contrast, S17N *ras* expression by dexamethasone treatment dramatically suppressed both secretion and proteolytic activation of MMP-2 and MMP-9. We next examined

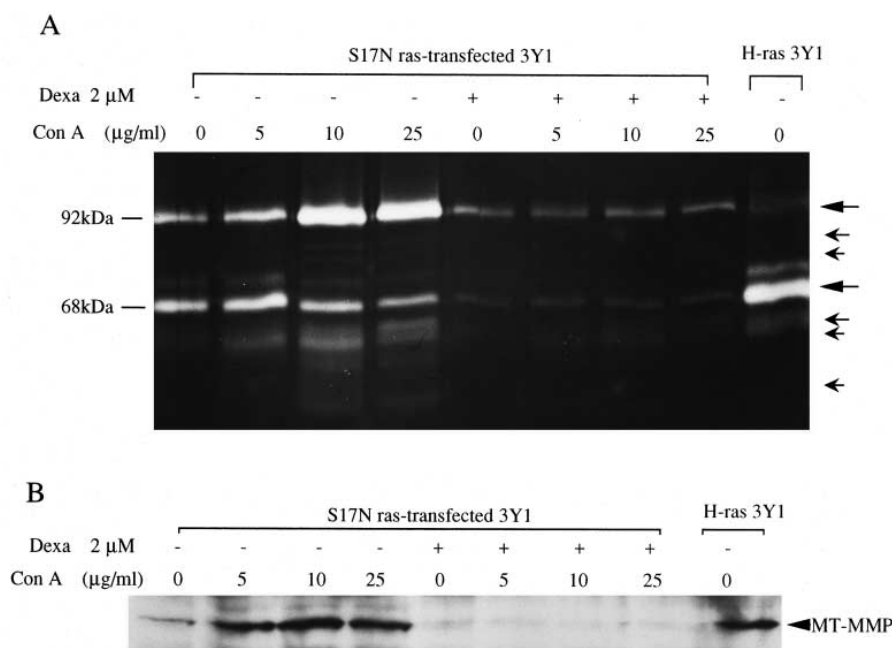


Fig. 3. Effects of S17N *ras* expression on induction and activation of MMPs by Con A treatment. S17N *ras*-transfected 3Y1 cells preincubated with 0 or 2 μ M dexamethasone for 24 h were treated with 0, 5, 10 or 25 μ g/ml of Con A for 24 h in serum-deprived medium in the presence or absence of dexamethasone. A: After treatment, conditioned media of cells were collected and subjected to zymography. B: After treatment, cells were subjected to immunoblotting with anti-MT1-MMP antibody.

the expression of MT1-MMP that catalyzes proteolytic activation of MMP-2 by immunoblotting with anti-MT1-MMP (Fig. 3B). While MT1-MMP expression was strongly activated by Con A treatment in cells without dexamethasone, its expression was drastically suppressed by dexamethasone treatment. Thus, these results strongly suggest that c-Ras plays a critical role not only in augmented secretion and proteolytic activation of MMP-2 and MMP-9 but also in expression of MT1-MMP which catalyzes MMP-2 activation. Our results also suggest that these MMPs share similar signaling pathways for their expression.

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References

- [1] L.A. Liotta, *Cancer Res.* 46 (1986) 1–7.
- [2] L.M. Matrisian, *Trends Genet.* 6 (1990) 121–125.
- [3] J.F. Woessner, *FASEB J.* 5 (1991) 2145–2154.
- [4] W.G. Stetler-Stevenson, H.C. Krutzsch, M.P. Wachter, I.M.K. Margulies, L.A. Liotta, *J. Biol. Chem.* 264 (1989) 1353–1356.
- [5] H. Sato, T. Takino, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, M. Seiki, *Nature* 370 (1994) 61–65.
- [6] M. Kataoka, S. Yamagata, S. Akiyama, T. Watanabe, H. Takagi, A.A. Thant, K. Iida, S. Saga, J. Kishi, M. Hamaguchi, *Int. J. Oncol.* 8 (1996) 773–779.
- [7] C. Pyke, E. Ralfkiaer, P. Huhtala, T. Hurskainen, K. Dano, K. Tryggvason, *Cancer Res.* 52 (1992) 1336–1341.
- [8] H. Ohtani, T. Nagai, H. Nagura, *Jpn. J. Cancer Res.* 86 (1995) 833–839.
- [9] A. Okada, B. Jean-Pierre, R. Nicolas, C. Marie-Pierre, R. Marie-Christine, C. Pierre, B. Paul, *Proc. Natl. Acad. Sci. USA* 92 (1995) 2730–2734.
- [10] S. Hurum, J. Sodek, J.E. Aubin, *Biochem. Biophys. Res. Commun.* 107 (1982) 357–366.
- [11] C.M. Overall, J. Sodek, *J. Biol. Chem.* 265 (1990) 21141–21151.
- [12] S. Ohta, T. Inazu, T. Taniguchi, G. Nakagawara, H. Yamamura, *Eur. J. Biochem.* 206 (1992) 895–900.
- [13] T. Matsuo, K. Hazeki, O. Hazeki, K. Toshiaki, M. Ui, *Biochem. J.* 315 (1996) 505–512.
- [14] A.L. Feig, M.G. Cooper, *Mol. Cel. Biol.* 8 (1988) 3235–3243.
- [15] M. Hamaguchi, C. Grandori, H. Hanafusa, *Mol. Cel. Biol.* 8 (1988) 3035–3042.
- [16] M. Hamaguchi, H. Hanafusa, *Proc. Natl. Acad. Sci. USA* 84 (1987) 2312–2316.
- [17] M. Hamaguchi, N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, Y. Nagai, *EMBO J.* 12 (1993) 307–314.
- [18] M. Hamaguchi, S. Yamagata, A.A. Thant, H. Xiao, H. Iwata, T. Mazaki, H. Hanafusa, *Oncogene* 10 (1995) 1037–1043.
- [19] M. Hamaguchi, H. Xiao, Y. Uehara, Y. Ohnishi, Y. Nagai, *Oncogene* 8 (1993) 559–564.
- [20] R. Gum, E. Lengyel, J. Juarez, J.-H. Chen, H. Sato, M. Seiki, D. Boyd, *J. Biol. Chem.* 271 (1996) 10672–10680.