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REQUIREMENT OF MULTIPLE SIGNALING PATHWAYS FOR THE AUGMENTED PRODUCTION OF HYALURONAN BY V-SRC

YUKO NAITO, NORIKO SUZUKI, PENGYU HUANG, HITOKI HASEGAWA, YASUYOSHI SOHARA, TAKASHI IWAMOTO and MICHINARI HAMAGUCHI*

Division of Cancer Biology, Nagoya University School of Medicine

ABSTRACT

Malignant transformation of cells is frequently associated with an augmented production of hyaluronan and the subsequent formation of a hyaluronan-matrix. In v-Src-transformed cells, hyaluronan directly activate cell motility in a tumor-specific manner. Despite its importance, the mechanism by which v-Src activates hyaluronan production remains unclear. Here we report that multiple signaling pathways are required for the augmented production of hyaluronan. Either the expression of a dominant negative Ras or the treatment of cells with manumycin A, a Ras farnesyltransferase inhibitor, was able to suppress hyaluronan production. In contrast, expression of MEK1EE, a constitutive form of MEK1, activated both hyaluronan synthase expression and hyaluronan production. AG-490, a Jak-2 inhibitor, or LY294002, a PI3K inhibitor, similarly suppressed the augmented production of hyarulonan. Taken together, our results suggest the involvement of multiple signaling pathways, including Ras-dependent and independent ones, in augmented hyaluronan production by v-Src.

Key Words: Hyaluronan, v-Src, Signaling, Ras, MEK1

INTRODUCTION

Hyaluronan (HA) is a nonsulfated high-molecular-mass glycosaminoglycan widely presenting in tissues as one of the major components of the extracellular matrix.[1)] HA is synthesized by three hyaluronan synthase isoforms, HAS1, HAS2, and HAS3,[1,2)] each of which has its own enzymatic properties and plays a distinctive role in HA matrix formation.[2)] A large body of evidence suggests the direct involvement of HA in tumor progression and metastasis.[1,3)] Overexpression of HAS2 gene in the human fibrosarocoma cell line activated anchorage-independent cell growth,[4)] while expression of HAS1 in mouse mammary carcinoma cells activated metastasis.[5)] In a lung cancer cell line producing large amounts of HA, the introduction of antisense against one of the HA receptors, CD44, into cells strongly inhibited cell invasion.[6)]

In cells transformed with v-*src*, an oncogene encoded by the Rous sarcoma virus, an accumulation of HA has long been observed.[7)] We showed that the transforming activity of v-Src had a clear correlation with its activity to stimulate HA production, and enabled HA to activate the cell motility of v-Src-transformed 3Y1 in a tumor-specific manner.[8)] Despite the importance of HA, exactly how the mechanism underlying HA production is regulated by v-Src remains

Corresponding author: Dr. Michinari Hamaguchi, Division of Cancer Biology, Nagoya University School of Medicine, Showaku, Nagoya 466-8550, Japan

largely unclear.

To obtain more clues to clarify that mechanism, we examined the signaling required for HA production and HAS gene expression in v-Src-transfomed cells. Here, we show the requirement of multiple signaling molecules including Ras for the augmented expression of HAS genes and the subsequent production of HA in v-Src transformed cells.

MATERIALS AND METHODS

Cell culture

Rat fibroblast cell lines, 3Y1, SR3Y1,[9] G2A3Y1,[10] S17NRas v-Src3Y1,[11] and MEK1EE 3Y1[12] were prepared as described previously.

Treatment of cells with pharmacological inhibitors

PD98059 (New England BioLabs), AG490 (Biomol Research Lab.), manumycin A (Sigma) and LY294002 (Calbiochem) were added to the medium according to the manufacturers' instructions.

Assay of HA production

2×10^5 cells were seeded in 24-well plates and incubated for 24 or 48 hours. The amounts of HA in culture media were measured by a competitive ELISA-like assay as described previously.[5]

Immunoblotting

Analysis of tyrosine phosphorylated proteins and Ras by immunoblotting with specific antibodies was also described.[13,14] Anti-phosphotyrosine monoclonal (PY20H) and anti-pan Ras antibodies were purchased from Transduction Lab and Santa Cruz Biotechnology, respectively.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). 5 mg of total RNA was used in a first strand cDNA synthesized reaction using oligo$(dT)_{20}$ primer and ReverTra Ace (Toyobo) as indicated by the manufacturer. PCR amplification was performed using 1 µl of cDNA mixture in a 50-µl reaction volume including 0.5 µM of each primer, i. e., 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of AmpliTaq Gold (Applied Biosystems). Specific forward and reverse PCR primers used were: GAPDH ; 5'-TGATGCTGGTGCTGAGTATG-3' and 5'-GGTGGAAGAATGGGAGTTGC-3', HAS1 ; 5'-CTCTGGACTCTTGGGTCAGC-3' and 5'-ACCACAGGGCGTTGTATAGC-3', HAS2 ; 5'-TCGGAACCACACTGTTTGGAGTG-3' and 5'-CCAGATGTAAGTGACTGATTTGTCCC-3', HAS3 ; 5'-ACTCTGCATCGCTGCCTACC-3' and 5'-ACATGACTTCACGCTTGCCC-3'. The products were examined by agarose gel electrophoresis. Product intensities were calculated with NIH Imaging, and normalized by comparison to those of GAPDH. Values are means plus standard deviations of three independent experiments.

HABP staining

Cells were fixed with paraformaldehyde, permeabilized with Triton X-100 in PBS, and incubated with 5% FBS in TBST. For HABP staining, the fixed cells were incubated with biotinylated HA-binding protein (Seikagaku) followed by FITC-conjugated avidin D (Vector Lab.). Nuclei were visualized by 4',6-diamidiono-2-phenylindole (DAPI) staining.

RESULTS

HA production and HAS mRNA expression in src-transfected cells

We first assayed the levels of HA production in 3Y1 cell lines expressing wild-type v-Src,

v-Src G2A mutant or c-Src.[10] c-Src, the cellular counterpart of v-Src, is a tyrosine kinase which is inactive in cell transfomation. G2ASrc is a nonmyristoylated mutant of v-Src in which Gly at position 2 is substituted for Ala. G2ASrc is active in protein kinase but is so defective in membrane binding that lacks transforming activity. Cells (2×10^5 of each cell line) were cultured and HA that has accumulated in the conditioned media was assayed.[5] As shown in Fig. 1, v-Src-transformed 3Y1 (SR3Y1) produced large amounts of HA. Approximately 15 ng of HA/mg protein of the media was produced from SR3Y1 within 48 hours of culture. In contrast to SR3Y1, parental 3Y1 and 3Y1 expressing c-Src (c-Src3Y1) produced only trace amounts of HA estimated at around 0.4 ng of HA/mg protein of the media. G2ASrc-expressing 3Y1produced small amounts of HA amounting to 2.5 ng/mg protein of the media.

For these cell lines, we assayed the mRNA levels of three HAS isoforms by semi-quantitative RT-PCR analysis. As shown in Fig. 1B and C, steady-state levels of HAS mRNA, especially those of HAS1 and HAS2, were dramatically increased in v-Src-transformed cells. Relative amounts of HAS1 and HAS2 were sixfold and ninefold higher than those of 3Y1, respectively. In contrast, HAS3 amounts in SR3Y1 were only twofold higher than those in 3Y1. In c-Src3Y1, mRNA levels of three HASs all remained unchanged at levels similar to those of 3Y1. In G2A3Y1,

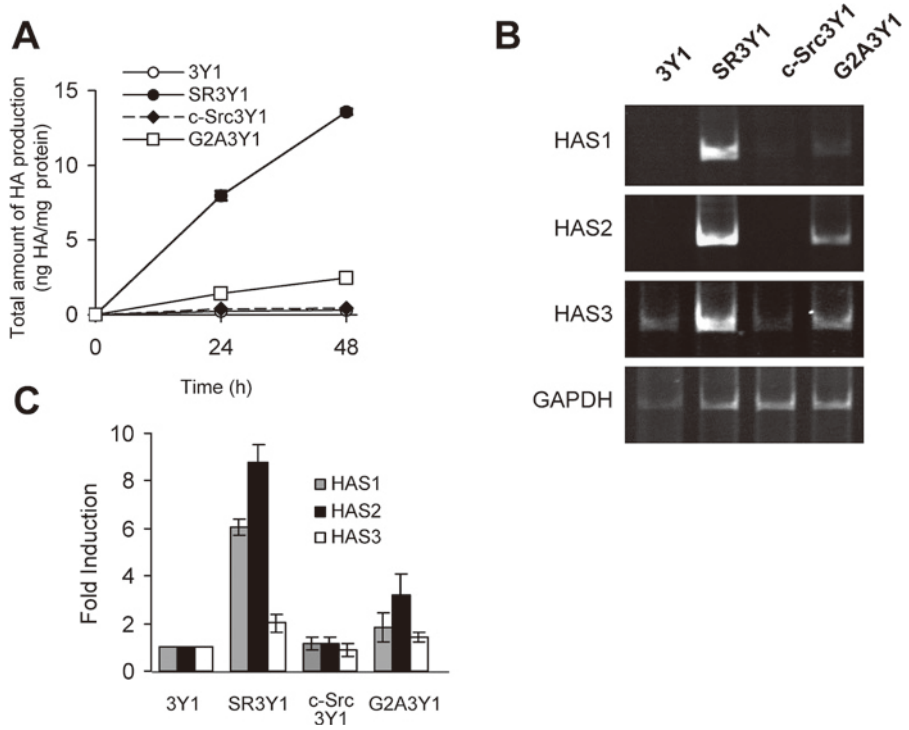

Fig. 1 HA production and HAS gene expression in Src mutant-transfected cells. (A) HA production of 3Y1, v-Src transformed 3Y1 (SR3Y1), c-Src transfected 3Y1 (c-Src3Y1) and non-myristoylated v-Src mutant transfected 3Y1 (G2A3Y1) were analyzed. 2×10^5 cells were seeded and incubated for the indicated time. HA in the conditioned media was assayed. (B) Semi-quantitative RT-PCR was performed as described in Materials and Methods to examine the mRNA levels of HAS genes in cells. (C) Results of semi-quantitative RT-PCR were normalized by comparison to the level of mRNA of GAPDH. Values are means plus standard deviations of three independent experiments.

mRNAs of three HAS increased slightly but remained clearly lower than those of SR3Y1. Approximately a twofold increase in HAS1 and a threefold increase in HAS2 were observed in G2A3Y1.

To confirm these observations, HA associated with these cells was examined by staining with FITC-labeled HA binding protein (HABP; Seikagaku Kogyo) (Fig. 2), which revealed that HA produced in SR3Y1 was, at least in part, associated with these cells. In contrast, cell-associated HA was not obvious in either 3Y1 and c-Src3Y1, or only weakly observed in G2A3Y1.

Ras signaling and HA production in v-Src transformed cells

To explore the signaling critical for HA production in SR3Y1, we first examined the effect of dominant negative Ras (S17NRas) expression in SR3Y1 on its HA production. S17NRas, a mutant Ras with an Asn substitution for Ser at position 17 of H-Ras, was found to exert a powerful inhibitory effect on endogenous Ras.[15] S17N*ras* ligated into pMAM2BSD vector was conditionally expressed in SR3Y1 (S17NRasSR3Y1) under the control of a mouse mammary tumor virus promoter/enhancer.[11] Treatment of S17NRasSR3Y1 with dexamethasone induced S17NRas expression, but did not interfere with the tyrosine phosphorylation of cellular proteins (Fig. 3A). In contrast, dexamethasone treatment effectively suppressed HA production and HAS expression in S17NRasSR3Y1 (Fig. 3B). It was somehow unexpected, however, to discover that dexamethasone treatment of parental SR3Y1 also suppressed HA production to some extent, whereas dexamethasone-treated S17NRasSR3Y1 was still able to induce low levels of HA

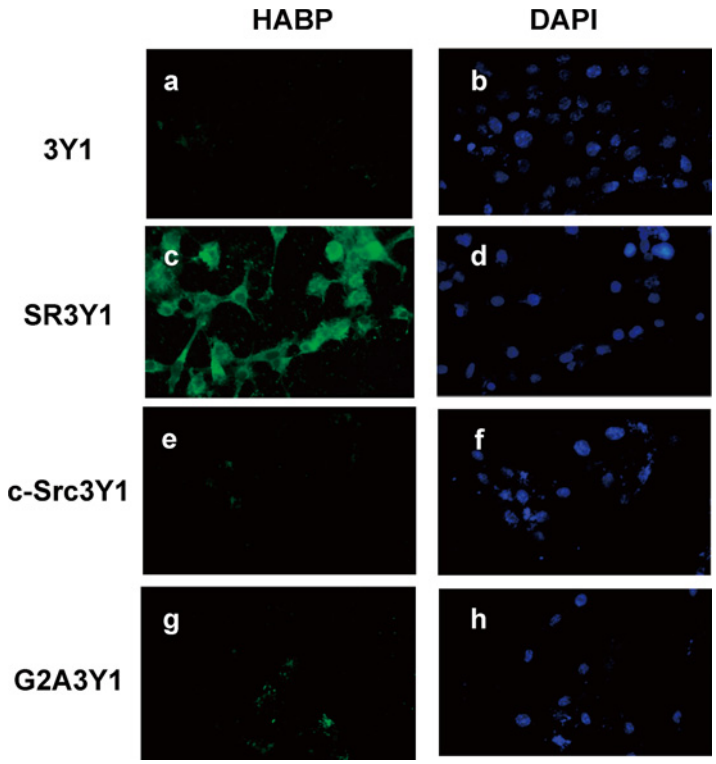

Fig. 2 HA matrix of 3Y1, SR3Y1, c-Src3Y1, and G2A3Y1 visualized by HABP staining. Cells were fixed and stained with biotinylated HABP (a, c, e, g) and counterstained with DAPI (b, d, f, h).

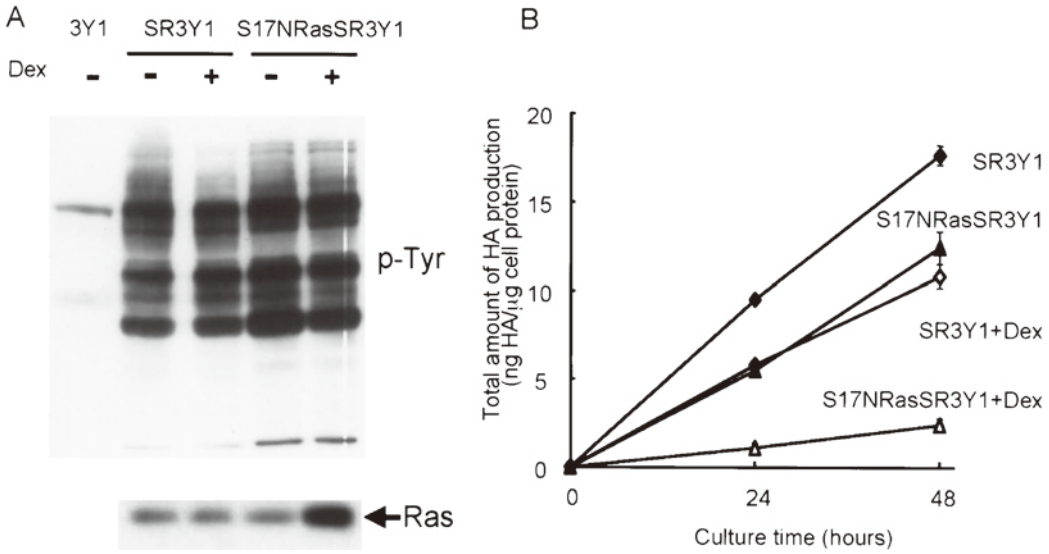

Fig. 3 Effect of dominant-negative Ras expression on HA production in SR3Y1. (A) Dominant-negative Ras was induced by treatment of cells with dexamethasone (Dex; final concentration; 2 mM). Cells were cultured for 48 hours in the presence or absence of Dex. Total cell lysates were analyzed by immunoblotting with anti-phosphotyrosine antibody (upper panel) or anti-pan-Ras antibody (lower panel). (B) 2×10^5 cells were seeded and incubated for the indicated time in the presence or absence of Dex. HA in conditioned media was assayed.

production.

Requirement of MEK1 signaling in HA production

To confirm the role of Ras signaling, we next examined the role of MEK1, a major downstream effecter of Ras, in HA production. HA production, HA matrix formation and HAS expression were examined in cells expressing MEK1EE (MEK1EE3Y1), a constitutive active form of MEK1 in which Ser218 and Ser222 were replaced to Glu.[12] As shown in Fig. 4, MEK1EE3Y1 had augmented the production of HA and HAS mRNA, and a clear formation of HA matrix was observed around the cells. It should be noted, however, that the mRNA levels of three HAS isoforms in MEK1EE3Y1 were higher than those of SR3Y1.

Requirement of multiple signaling in HA production

To confirm and extend these observations, we next examined the role of other signaling molecules by use of various pharmacological inhibitors, since MEK1/MAPK has been proven to be merely an element of Ras signaling. SR3Y1 was incubated with either DMSO alone, PD98059 (a MEK1 inhibitor),[16] AG-490 (a Jak-2 inhibitor),[17] manumycin A (a Ras farnesyltransferase inhibitor)[18] or LY294002 (a PI3K inhibitor).[6] After incubation, HA secreted in the conditioned media was assayed. As shown in Fig. 5, treatment of SR3Y1 with manumycin A clearly suppressed HA production, suggesting again the role of Ras signaling in that production. In contrast, around 70% of HA production still continued in the presence of PD98059, suggesting the involvement of MEK1-independent signaling in producing HA. Indeed, LY294002 most strongly suppressed HA production. Similarly, AG-490 was able to suppress HA production to a level similar to that of manumycin A. These results suggest the involvement of PI3k and Jak2 kinase in the production of HA.

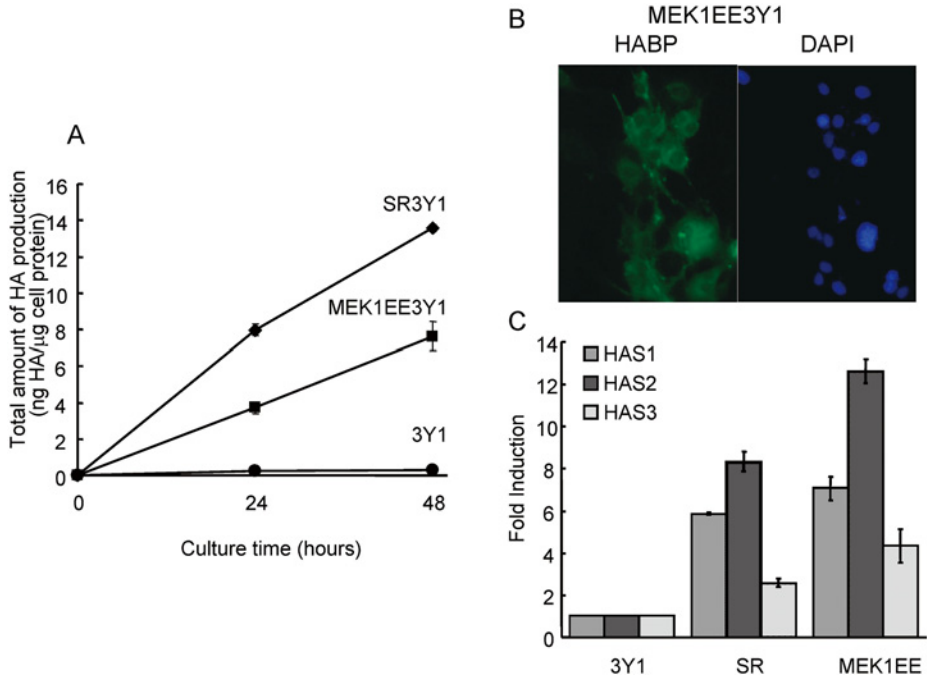

Fig. 4 HA production and HAS gene expression in MEK1EE3Y1. (A) HA production of MEK1EE3Y1 was analyzed. 2×10^5 cells were seeded and incubated for the indicated time. HA in the conditioned media was assayed. (B) HA matrix of MEK1EE3Y1 was visualized by HABP staining. Cells were counterstained with DAPI. (C) Semi-quantitative RT-PCR was performed to examine mRNA levels of HAS genes in MEK1EE3Y1. Results of semi-quantitative RT-PCR were normalized by comparison to the level of mRNA of GAPDH. Values are means plus standard deviations of three independent experiments.

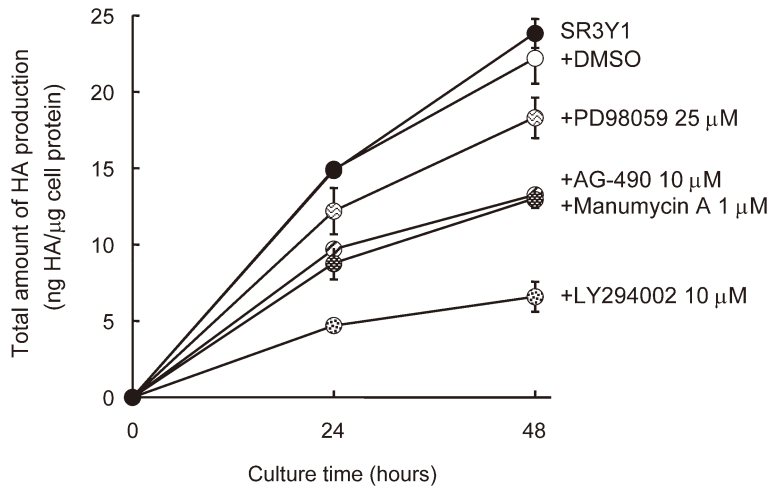

Fig. 5 Effect of inhibitors on HA production. SR3Y1 was incubated with either DMSO alone, PD98059 (25 μM), AG-490 (10 μM), manumycin A (1 μM), or LY294002 (10 μM) for the indicated times. After incubation, HA in the conditioned media was assayed. Values are means plus standard deviations of three independent experiments.

DISCUSSION

A large body of evidence has been accumulated demonstrating that unrestrained activation of the intracellular signaling pathways plays a critical role in the generation and malignant conversion of tumors. The precise mechanisms of tumorigenesis by the activation of multiple signaling pathways are, however, still largely under investigation. Among multiple signaling molecules, Ras is the most well characterized, and numerous studies have been emphasizing its potent role in tumorigenesis. Most of these studies were, however, focusing on its role in the uncontrolled growth of cells. In this study, we showed that Ras signaling also plays an important role in the tumor-specific production of HA. Among multiple downstream signaling pathways of Ras, MEK1 is the most well characterized. Experiments with MEK1EE showed that its constitutive activation did indeed activate both HA production and HA matrix formation. However, treatment of cells with PD98059 did not strongly suppress HA production, suggesting that in SR3Y1 that production is not solely determined by Ras-MEK signaling, but requires other Ras-independent and Ras-dependent signaling, including Jak and PI3K. It is interesting to note that the mRNA levels of three HAS genes in MEK1EE were higher than those of SR3Y1, whereas HA production in MEK1EE was lower than that in SR3Y1. These results suggest that the production of HA is not simply controlled at the transcriptional level of HAS genes but may involve post-transcriptional processes. Studies that may clarify this discrepancy, including the modification of HAS protein by the v-Src kinase, remain to be done. For such studies, the preparation of good antibodies that recognize each HAS will be required.

In conclusion, we demonstrated for the first time that the augmented production of HA in SR3Y1 involves multiple signaling, including Ras-MEK1, PI3K and Jak kinase. Additional studies, including the identification of downstream effectors of PI3K and Jak, should result in further clarification.

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