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Raf-1 is not a major upstream regulator of MAP kinases in rat fibroblasts

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RCR cells are NRK clones in which Raf-1 production is blocked by the expression of an antisense RNA, and consequently they are refractory to transformation by various oncogenes. In RCR cells, MAP kinases (ERK1 and ERK2) were activated to an extent and in a time course similar to those of the original NRK cells, irrespective of whether the stimulus was oncogenic or non-oncogenic. Moreover, there was no significant elevation of ERK activities in oncogene-transformed NRK cells. These results indicate that Raf-1 kinase is not the major upstream activator of ERK's in NRK cells and that neither ERK1 nor ERK2 are likely to mediate oncogenic signals from Raf-1 kinase.

Raf-1; MAP kinase; Signal transduction; NRK cell

1. INTRODUCTION

Several distinct families of serine-threonine kinases constitute evolutionarily well-conserved signal pathways involved in growth and differentiation control of a wide variety of eukaryotes from unicellular to multicellular organisms. Among them are the mitogen-activated protein kinases (also called extracellular signal-regulated kinases or ERK's) and Raf-1 kinase. ERK's, existing as at least two isoforms called ERK1 (44 kDa) and ERK2 (42 kDa), are thought to play an important role in the regulation of the G_0/G_1 transition, meiosis and differentiation [1]. Raf-1 kinase (70–75 kDa), encoded by the cellular homologue of the v-*raf* oncogene [2], mediates growth and differentiation signals in mammals and flies [3].

Both ERK's and Raf-1 kinase in quiescent fibroblasts are rapidly activated in response to a wide variety of growth stimuli such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor, insulin, insulin-like growth factor I, and phorbol esters [4–10]. In some cells, MAP kinases are constitutively activated following *ras*, v-*raf*, or v-*src* transformation [11–14]. MAP kinase kinase (or MEK), a direct upstream regulator of ERK's, undergoes activation by Raf-1 in vitro [12,15]. Based on these data, it has been postulated that ERK's mediate signals originating from Raf-1 and may play a role in the transduction of oncogenic signals and/or in the maintenance of transformation. However, continuous elevation of ERK activity was failed to be detected in *ras*- and v-*raf*-transformed rat fibroblasts [14]. Moreover, a recent report with the baculovirus system suggests that Raf-1-dependent and independent pathways may exist for ERK activation [16]. Thus, quantitatively and qualitatively what role ERK's play in signal transduction mediated by c-*raf*-1 is highly controversial.

We have been investigating cascades involved in oncogenic signal transduction by employing NRK cell mutants [17] and NRK cell clones (RCR) blocked in Raf-1 kinase production by c-*raf*-1 antisense expression [18]. From the studies, we have concluded that the oncogenic signals originating from growth factors and a majority of oncogenes are transduced via a common pathway involving Raf-1 kinase as a key downstream mediator. These oncogenes include v-*erb*B, activated c-*erb*B-2, v-*fms*, v-*ras*, v-*mos*, v-*src*, SV40 T antigen, poliomavirus middle T antigen, human papillomavirus E6E7 and v-*fos*. This signal cascade is distinct from those for the regular anchorage-dependent mitogenic signals.

To clarify if Raf-1 is a major upstream activator of ERK's in vivo and if ERK's mediate oncogenic signals from Raf-1, we examined the activities of ERK's in RCR cells and in NRK cells transformed by EGF plus transforming growth factor-β (TGF-β) or various oncogenes. Here we show that Raf-1 is not the main upstream activator of ERK1 and ERK2 and that neither of them is likely to mediate the oncogenic signals.

2. MATERIALS AND METHODS

2.1. *Cells and chemicals*

The NRK-49F cell line was obtained from the American Type Culture Collection. RCR4 was established after transfection of NRK with the rcr(−) plasmid encoding an antisense sequence to the 5'-end of c-*raf*-1 [18]. v-*erb*B, v-*fms*, v-*ras*, v-*fes*, f-*src*, v-*fos* or v-*raf*-transformed NRK cells were prepared by DNA transfection following G418 selection [17,18]. All the cells were maintained in Dulbecco's modified Eagle's medium (DMED) supplemented with 5% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 5% CO_2 at 35°C. TGF-β, EGF and PDGF (culture grade) were purchased

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from Nakarai Tesque Inc., Toyobo Inc. and R&D system, respectively. To stimulate growth, growth factors were added to the culture at final concentrations of 5 ng/ml for EGF, 5 ng/ml for PDGF or 1 ng/ml for TGF-β, unless indicated in the text.

2.2. *Preparation of cell lysates*

5×10^5 cells were seeded into a 60 mm-dish and incubated overnight. Following serum starvation for 48 h, the cells were stimulated with each growth factor for the indicated time, washed with ice-cold PBS and scraped in 0.5 ml of an ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5% Triton X-100, 50 mM 2-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2% aprotinin, 6 mM dithiothreitol and 1 mM sodium orthovanadate) [4]. The collected cells were homogenized and centrifuged at 15,000 rpm for 30 min with a microcentrifuge at 4°C. The supernatant was stored at −80°C until use.

2.3. *In-gel kinase assay*

For in-gel kinase assay [19], 5 μg of cell lysate was loaded on each lane and electrophoresed on a 9% SDS-polyacrylamide gel containing bovine brain myelin basic protein (Sigma) at a concentration of 0.5 mg/ml as a substrate. The gel was treated with 6 M guanidine-HCl and with a 0.04% Tween-40-containing buffer at 4°C, then incubated with 2.5 μCi [γ-^{32}P]ATP/ml and washed with 5% trichloroacetic acid/1% pyrophosphate. The gel was dried and exposed to a X-ray film for 15–48 h at −80°C.

2.4. *Western blot analysis*

Western blot analysis was carried out as described previously [18]. Briefly, 10 μg and 7 μg of cell lysates were used for Raf-1 and ERK detection, respectively. The lysates were boiled for 3 min and electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. The filters were pretreated with TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% non-fat dry milk and 1% BSA, and then probed with the anti-SP63 antibody that had been generated against the carboxyl-terminal 12 amino acids unique to Raf-1 [20] (prepared by Medical and Biological Laboratories Inc.) or the anti-ERK1 serum [21] which recognizes two proteins with approximate molecular weight of 42 and 44 kDa corresponding to ERK2 and ERK1, respectively .

3. RESULTS

3.1. *ERK activation is not accompanied with transformation*

Our previous studies have shown that the oncogenic signals originating from EGF plus TGF-β and a major-

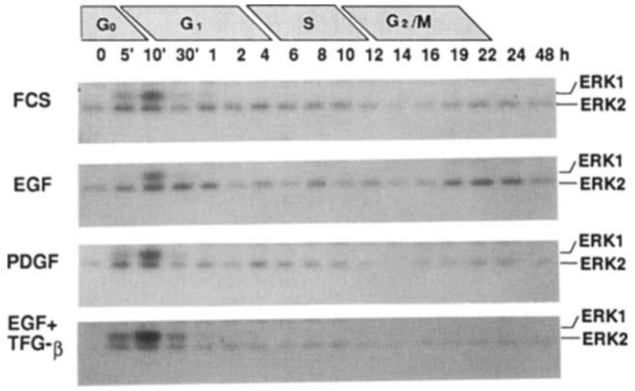

Fig. 1. Time courses of ERK activation in NRK cells. After 48 h serum starvation, NRK cells were stimulated with 5% FCS, EGF, PDGF, or EGF plus TGF-β. The lysis buffer was added to the culture at the time indicated in the figure. The lysates containing 5 μg protein were analyzed by in-gel kinase assay as described in section 2.

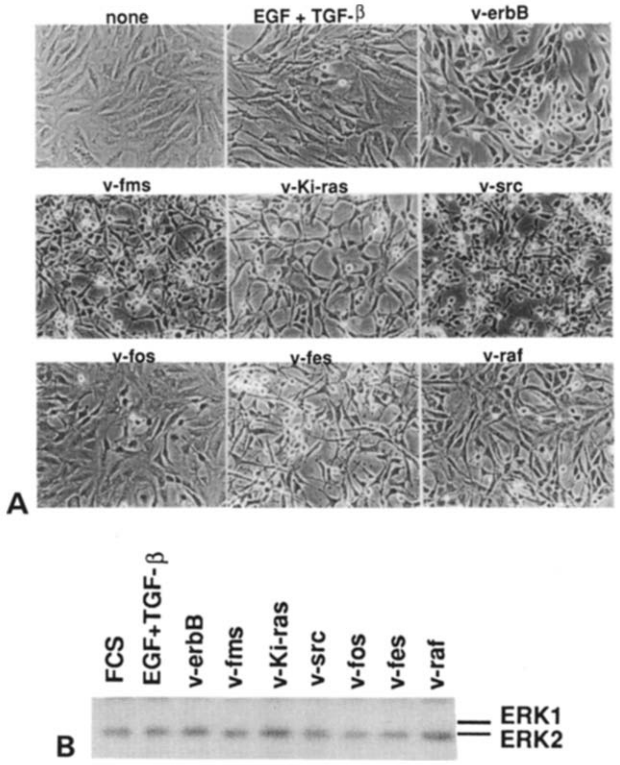

Fig. 2. (A) Morphologies of NRK cells transformed by growth factors and various oncogenes. NRK cells exponentially growing in growth medium, or exposed to EGF plus TGF-β for 48 h were indicated as *none*, or *EGF + TGF-β*. Transformants were generated by introduction of the oncogenes indicated in the figure. (B) ERK activity in transformed NRK cells. The cells in Fig. 2A were lysed, and lysates containing 5 μg protein were subjected to in-gel kinase assay.

ity of oncogenes are transduced through a common signal pathway and that Raf-1 is a downstream component of this pathway [17,18]. If ERK's are indeed major downstream effectors of Raf-1 kinase as suggested by the in vitro studies and play a key role in the oncogenic signal transduction, there must be a strong correlation between the activation of ERK's and the occurrence of transformation. We addressed this point by comparing the extent and time course of ERK activation in NRK cells following oncogenic or non-oncogenic growth stimulation. Growth-arrested NRK cells typically progress into S phase within 6 h and G_2 phase within 12 h, and return to G_1 by 20–22 h after growth stimulation (data not shown). Growth-arrested NRK cells were treated with serum, EGF or PDGF as a non-oncogenic stimulus or EGF plus TGF-β as an oncogenic stimulus, and the time course and extent of ERK activation were determined by the in-gel kinase assay. In good agreement with the previous reports, both ERK1 and ERK2 were activated by serum and the growth factors within 1 min after stimulation, peaked at 10 min and then fell gradually to the basal level (Figs. 1 and 4). The maximum activation of ERK2 was 2–3 times the basal level. On the contrary, ERK1 responded more clearly. The ERK1 activity was almost undetectable in growth-

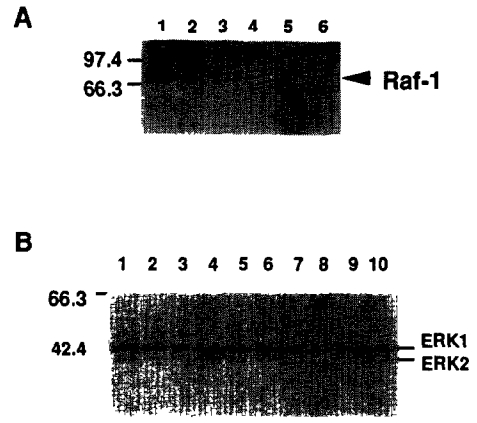

Fig. 3. After 48 h serum starvation, NRK and RCR cells were stimulated to grow with 5% FCS, EGF, PDGF, or EGF plus TGF-β for 10 min. (A) Expression of Raf-1. The level of Raf-1 protein was examined by Western blot analysis as described in section 2. Lanes: (1) NRK with no stimulation; (2) RCR4 with no stimulation; (3) RCR4 with FCS; (4) RCR4 with EGF; (5) RCR with PDGF; (6) RCR4 with EGF plus TGF-β. (B) Expression of ERK's. The levels of ERK1 and ERK2 proteins were determined by Western blot analysis. Lanes: (1) NRK with no stimulation; (2) RCR4 with no stimulation; (3) NRK with FCS; (4) RCR4 with FCS; (5) NRK with EGF; (6) RCR4 with EGF; (7) NRK with PDGF; (8) RCR4 with PDGF; (9) NRK with EGF plus TGF-β; (10) RCR4 with EGF plus TGF-β.

arrested cells, elevated to a level comparable to that of ERK2, and then fell to an undetectable level within 2 h. Upon treatment with EGF plus TGF-β, NRK cells underwent transformation within 48 h (Fig. 2A). However, the time course and the extents of the activation of ERK1 and ERK2 by the oncogenic stimulus were similar to those by non-oncogenic stimuli, suggesting that there is no particular association of ERK activation with the transformation process.

3.2. *ERK activity is not elevated in transformed NRK cells*

The lack of the association of ERK activation with transformation was also observed in fully transformed NRK cells. Cell lysates were prepared from NRK cells transformed by EGF plus TGF-β, v-*erb*B, v-*fms*, v-Ki-*ras*, v-*src*, v-*fes*, v-*fos* or v-*raf* (Fig. 2A), and their ERK activities were determined by the in-gel kinase assay. As shown in Fig. 2B, the ERK2 activity was detectable but not significantly different between the fully transformed and untransformed cells. On the other hand, the ERK1 activity was undetectable in both types of cells. Strikingly, neither ERK1 nor ERK2 was activated in v-*raf*-transformed cells. The activation of ERK1 and ERK2 by non-oncogenic as well as oncogenic growth stimuli and no significant elevation of the ERK activity in the fully transformed, particularly v-*raf*-transformed, cells indicate that Raf-1 kinase is not the major activator of ERK1 and ERK2 and that ERK1 and ERK2 are unlikely to mediate oncogenic signals originating from growth factors and oncogenes.

3.3. *ERK activity is not influenced by the presence or absence of Raf-1 kinase*

To obtain further evidence, the ERK1 and ERK2 activities in RCR cells that was blocked in Raf-1 production were assayed following stimulation with serum, EGF, PDGF or EGF plus TGF-β.

The levels of the Raf-1, ERK1 and ERK2 proteins during the stimulation were unchanged in NRK and RCR cells, as verified by Western blotting with specific antibodies that detected Raf-1 or ERK1 and ERK2 [20,21]. No Raf-1 band was detectable in RCR4 cells, irrespective of the presence or absence of growth stimulation with serum, EGF, PDGF or EGF plus TGF-β (Fig. 3A). The levels of ERK1 and ERK2 were not influenced either (Fig. 3B).

RCR4 cells were stimulated with serum, EGF, PDGF or EGF plus TGF-β for 1 to 30 min, and assayed for ERK activity (Fig. 4). ERK1 and ERK2 in the RCR cells were activated with a time course and to a extent both very similar, if not identical, to those in NRK cells. Virtually identical results were obtained with other RCR cells (RCR1, -2, -3, -5, -6) (data not shown). Thus, in NRK cells, ERK1 and ERK2 were activated to the same extent and with the same time course, irrespective of whether the stimuli were oncogenic or non-oncogenic, and their activation was unchanged with or without Raf-1 kinase. From these results, we conclude that in NRK cells Raf-1 is not the main upstream regulator of ERK1 and ERK2 and that neither ERK1 nor ERK2 is likely to mediate the oncogenic signal.

4. DISCUSSION

We have shown that in rat fibroblasts Raf-1 kinase is not the main upstream activator of ERK1 and ERK2 and that neither ERK1 nor ERK2 is likely to be a downstream mediator of the oncogenic signal from Raf-1. Our results do not support, if not contradict, the recent in vitro biochemical data and the functional data in

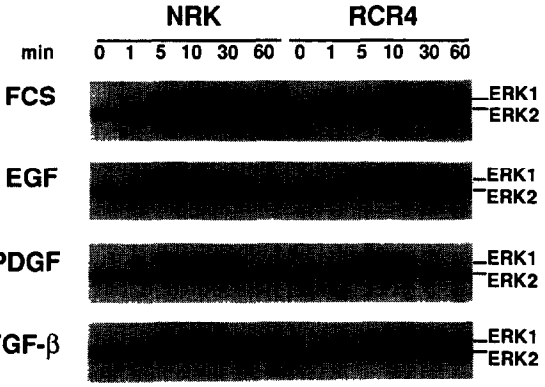

Fig. 4. ERK activity in RCR4 cells after growth factor stimulation. After 48 h serum starvation, NRK and RCR4 cells were stimulated with 5% FCS, EGF, PDGF, or EGF plus TGF-β. The lysis buffer was added to the culture at the time indicated in the figure. The lysates containing 5 μg protein were subjected to in-gel kinase assay.

yeast, both indicating that mammalian Raf-1 kinase is capable of activating MAP kinase kinase (MEK) and, consequently, ERK's [12,15,22]. In general, biochemical reactions seen with partially purified enzymes and functional interactions obtained under overexpression in yeast often magnify events that only rarely occur under physiological conditions. This may be the case for ERK activation by Raf-1.

If Raf-1 were not the major activator of ERK's in vivo, then what would the activator be? In yeast, protein kinases homologous to ERK's and MEK1 constitute signal cascades for the regulation of pheromone-induced sexual development and growth-associated budding [23]. In budding yeast STE7 and FUS3 constitute the pheromone signal cascade whereas MKK1/MKK2 and MPK1 constitute a cascade for the regulation of osmosis during budding. STE7 and MKK1/MKK2 are highly homologous not only mutually but also with MEK1 of mammals. Similarly, FUS3 and MPK1 are highly homologous not only mutually but also with ERK1/ERK2 of mammals. In these cascades, STE7 and MKK1/MKK2 are regulated by STE11 and BCK1, respectively. STE11 and BCK1 are mutually homologous but not with Raf-1 kinase. Thus, in these signal cascades, the MAP kinase kinase family is regulated by another kinase family dissimilar to Raf-1. Recently a kinase similar to STE11 has been identified in mice as an activator of MEK1 [24]. These data taken together with ours suggest that in close similarity to the yeast cascades, MEK1 may mainly be regulated by a STE11-like kinase(s).

It has been reported that the ERK activity is constitutively elevated in *ras*- and *raf*-transformed NIH 3T3 or Swiss 3T3 cells and in *gip*2- and *src*-transformed Rat 1a fibroblasts, but not in *ras*- and *raf*-transformed Rat 1a cells [11,12,13,25]. We failed to observe an elevation of the ERK activity in transformed, even v-*raf*-transformed, rat NRK cells. Such inconsistency supports our conclusion that ERK's do not play a crucial role in oncogenic signal transduction. The reason for the activation of ERK's in certain cell types by certain oncogenes is unclear but could be subtle differences in some phosphorylation dephosphorylation metabolism brought about by transformation.

Finally, what would the immediate downstream mediator of oncogenic signals from Raf-1 kinase be? In flies, a signal cascade comprised of a PDGF receptor-like receptor called torso and D-raf kinase plays a central role in growth and differentiation regulation [26]. Recently a gene (*dsor1*) encoding a MEK1-like kinase has been identified as an extragenic suppresser of a Raf-1 kinase mutant fly [27]. In light of our data, this finding suggests that a downstream effector of Raf-1 kinase might not be MEK1 but a kinase similar to MEK1. We and others recently identified MEK2, a MEK1 homologue, from rat and mouse [28,29]. This might be a candidate for a Raf-1 effector.

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