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Molecular assembly of mitogen-activated protein kinase module in *ras*-transformed NIH3T3 cell line

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Abbreviations: ERK, extra-cellular signal regulated kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; LTR, long terminal repeat

Abstract

The *ras*, is a G-like protein that controls the mitogen-activated protein kinase (MAPK) pathway involved in control and differentiation of cell growth. MAPK is a key component of its signaling pathway and the aberrant activation may play an important role in the transformation process. To better understand roles of *ras* in the activation of MAPKs, we have established *ras* transformed NIH3T3 fibroblast cell line, and analyzed the MAPK module. The *ras* transformed cells formed numerous spikes at the edges of cells and showed loss of contact inhibition. The levels of ERK1/2 MAPKs as revealed by Western blot analysis were not significantly different between *ras* transformed and non-transformed cells. However, phosporylation of ERK MAPKs and the level of MEK were significantly increased although the heavily expressed level of Raf-1, an upstream component of MAPK pathway was unchanged in *ras* transformed NIH3T3 cells. The sedimentation profile of the MAPK module kinases in a glycerol gradient showed the presence of a rather homogenous species of multimeric forms of ERK1/2 and MEK as indicated by the narrow distribution peak areas. The broad sedimentation profile of the Raf-1 in a glycerol gradient may suggest possible heterologous protein complexes but the identification of interacting molecules still remains to be identified in order to understand the organization of the MAPK signal transduction pathway.

Keywords: *ras*-transformed cell line, MAPK module, ERK, MEK, Raf-1

Introduction

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases and well conserved in all eukaryotes (Pelech *et al*., 1992; Davis, 1993). The MAPKs are activated by series of phosphorylation cascade by upstream MAPK module kinases, MEK and Raf-1 (Blenis, 1993; Egan *et al*., 1993). The components of MAPKs module are rapidly and transiently activated by growth stimulatory signals in many types of cells, suggesting that they play important roles in cellular growth control. The activity of MAPKs is positively regulated by phosphorylation on both threonine and tyrosine residues (Anderson *et al*., 1990; Payne *et al*., 1991). Many transforming oncogenes encode mutant proteins whose cognate wild-type proto-oncogene products are components of signal transduction networks (Bos, 1989; Slamon *et al*., 1989; Bargmann *et al*., 1993). Transformation of cells induced by oncogenes is mostly mediated by modification of the protein (mainly phosphorylation) as well as an increase in the level of the protein (Slamon *et al*., 1989; Anderson *et al*., 1990; Tsutsumi *et al*., 1990; Blenis, 1993; Burgering *et al*., 1993; Janes *et al*., 1994). Of the oncogenes activating MAPK system, *ras* is one of the best known oncogene so far studied, and constitutive active mutants were found in many human cancers (Bos, 1989; Gallego *et al*., 1993). The *ras* family comprises a group of closely related transforming genes that are highly conserved in eukaryotes (Hall, 1992). The *ras* group includes H-*ras*, K-*ras*, and N-*ras* and the level of gene expression varies in different tissues and cell types. Cellular studies of Ras suggested that the transformation occurs through two stage processes as follows: 1) activation of ras mutant found in human tumors, *i.e.* transformation of primary fibroblasts supplemented with immortalizing oncogenes such as *myc* (Land *et al*., 1983), an adenovirus E1A (Ruley, 1983), mutant p53 or polyoma large T antigen gene (Linzer *et al*., 1979), 2) overexpressing normal Ras protein [transformation of NIH3T3 cells (Chang *et al*., 1982; Ricketts *et al*., 1988)], caused either by multiple copies of *ras* genes or by linking low numbers of the genes to retroviral long terminal repeat (LTR) elements. Thus the Ras-transformed NIH3T3 cells became one of the best models to study the biology of cancers cells.

So far, most studies have been focused on the modification (*i.e.* phosphorylation/dephosphorylation) or quantitative aspects of MAPK module in various types of cancer cells. However, the signal transduction dependent molecular assembly of the MAPK module components

was not characterized well. In this study, we have established *ras* transformed NIH3T3 cell line as a tumor model and have examined the level of expression and modification of MAPK modules. Furthermore, molecular assembly and disassembly of the MAPK module proteins in the NIH3T3 cells were investigated under conditions with or without induction by epidermal growth factor (EGF).

Materials and Methods

Antibodies

Anti-MAPK rabbit polyclonal antibody was purchased from Stratagene [La Jolla, CA, USA). Phospho-specific MAPK antibody (raised against synthetic phospho-tyrosine peptide corresponding to residues 196 to 209 (DHTGFLTEY (P)VATRWC) of human ERK2] was purchased from New England Biolabs, Inc. (Beverly, MA, USA). Anti-MEK and anti-c-Raf-1 antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase conjugated secondary antibodies were also purchased from Transduction Laboratories.

Cell culture and *ras* transfection

NIH3T3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The NIH3T3 cells were transformed with a *Hind*III-*Eco*R1 fragment of pNRSac (Murray *et al.,* 1983) containing N-*ras*-oncogene. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units of penicillin, and 100 mg/ml of streptomycin as well as 0.7 mg/ml of G418 (Gibco/BRL. Gaithersburg, MD, USA). The cells were grown at 37°C in 5% CO_2 air mixture. Transformed cells were removed from foci by trypsin treatment and the stable cell line was established by selection with G418. The medium was replaced every three days for 3 months. For growth factor effect, cells were exposed to epidermal growth factor (EGF, 100 ng/ml) and incubated at 37°C for 15 min.

Extract preparation

Cells were rinsed twice with ice cold phosphate buffered saline (PBS) and harvested by scraping in ice-cold PBS. After addition of 0.5 ml of PBS, the cells were centrifuged down and resuspended in 300 µl of lysis buffer (70 mM β-glycerophosphate pH 7.2, 0.1 mM each meta- and ortho-vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5% Triton-X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 µg/ml each of pepstatin A, chymostatin, leupeptin, peptin, respectively). After incubation of the sample on ice for 30 min, the samples were sonicated for 20 s and unbroken cellular debris were removed by centrifugation at 23,000 g for 10 min. The supernatant was further cleared by a subsequent centrifugation. Samples were immediately aliquoted and frozen at -80°C. Protein concentrations were determined with Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

Western blot analysis

The lysates containing 40-100 µg of protein were subjected to 10% SDS-PAGE (Moon *et al*., 1999) and proteins were transferred onto a nitrocellulose membrane (Protran, Schleicher and Schuell Corporation, Dassel, Germany). Blots were incubated in the blocking solution (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 5% nonfat Carnation milk). Immunoblots were then washed with TBST and incubated for 2 h at room temperature or overnight at 4°C with the blocking solution containing appropriate amounts of primary antibodies. The blots were then washed and incubated with the blocking solution containing appropriate secondary antibody for 1 to 2 h. After washing, the blots were developed with Amersham ECL kit (Amersham International, Buckinghamshire, UK).

Glycerol gradient ultracentrifugation

For glycerol gradient experiments, protein was concentrated to 10 mg/ml by using Amicon centricon (Amicon, Inc., Bererly, MA, USA). One hundred and fifty µl of cell extracts (10 mg/ml) were used for glycerol gradient centrifugation. 10% and 30% glycerol (w/v) were prepared in modified H buffer (Elion *et al*., 1993) containing 150 mM NaCl. A linear 10 to 30% glycerol gradient in a total volume of 11.4 ml was generated at 4°C cold room in a SW41 pollyallomer tube with a Hoefer SG13 gradient maker and peristaltic pump. One hundred and fifty µl of whole cell extract in modified H buffer containing 150 mM NaCl was loaded onto a top of glyerol gradient. Molecular weight standard was loaded onto an identical 10-30% glycerol gradient. Gradients were centrifuged at 220,000 g at 4°C for 22 h in an SW41 rotor. Samples were removed with a 10 µl capillary pipet attached to narrow tubing to withdraw 570 µl aliquots from the bottom of each tube. One hundred and fifty µl of each aliquots of the fractions was used for immunoblot analysis.

Results

Morphologies of NIH3T3 cells and *ras*-transformed cells

A stable NIH3T3 cell line transformed with *ra*s oncogene was obtained by transformation of *ras* gene followed by neomycin selection. The *ras*-transformed cells were characterized by the loss of contact inhibition and spikes budding out of the cells (Figure 1B). In order to confirm

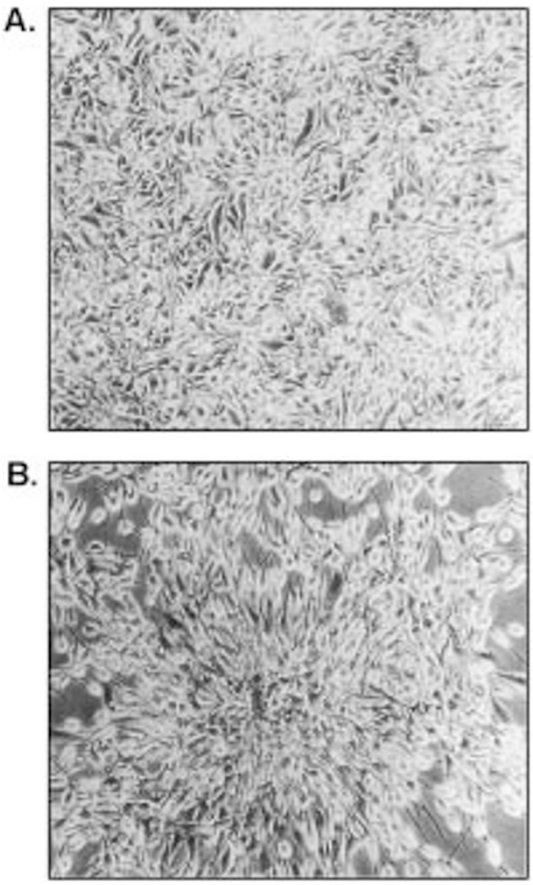

Figure 1. *ras* dependent transformation of NIH3T3 cells. (**A**) Non-transformed cells, (**B**) *ras*-transformed cells. NIH 3T3 or *ras* transformed NIH3T3 cells were maintained in DMEM containing 10% FCS and G418 as described in "Materials and Methods", and viewed by phase-contrast microscopy and photographed. Neo stands for cells harboring control neomycin gene and Ras represents *ras*-transformed cells. Magnification, $\times$ 400.

whether the phenotypic changes were caused by *ras* oncogene, PCR was performed and found that the *ras* gene was uniquely detected in *ras*-transformed cells. This typical transformation model was used for assessing activation of MAPK module kinases. The *ras*-transformed cells showed an ability to form colonies in soft agar whereas no growth was observed by non-transformed cells (Ahn *et al*., 1995).

Status of MAPK module in *ras*-transformed cells

To examine whether the MAPK modules were activated either by mitogenic stimuli or transformation signal generated by r*as,* we first analyzed levels of total and phosphorylated form of ERK1/2 MAP kinases. The NIH3T3 cells transformed with neomycin resistant gene followed by neomycin selection was used as a negative control. The levels of ERK1/2 as revealed by Western blot analysis were not different between Neo and Ras-transformed cells (Figure 2A). However, phosporylation of

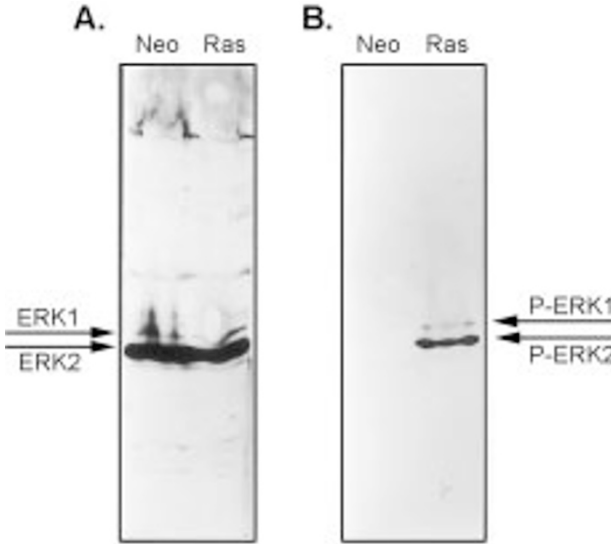

Figure 2. Expression and activation of MAPK in NIH3T3 cells transformed with *ras* proto-oncogene. NIH3T3 cells were cultured in DMEM containing 10% FCS and G418. Cell extracts were prepared as described in "Materials and Methods". ERKs (**A**) and phospho-ERK (**B**) proteins were detected by Western blot analysis using anti-ERK and anti-phospho-ERK antibodies respectively. Each Neo and Ras stands for cells harboring control *neo* gene and *ras* gene.

ERK1/2 MAPKs were observed in the *ras*-transformed NIH3T3 cells (Figure 2B).

To know the status of upstream MAPK module kinases, we checked expression levels of MEK and Raf-1 kinases. Here, the Raf-1 was heavily expressed in both the neomycin resistant gene-transformed control and the *ras*-transformed NIH3T3 cells and did not show any significant differences in the levels of expression (Figure 3A). Interestingly, the level of MEK1 was distinctly observed only in the *ras*-transformed cell line (Figure 3B).

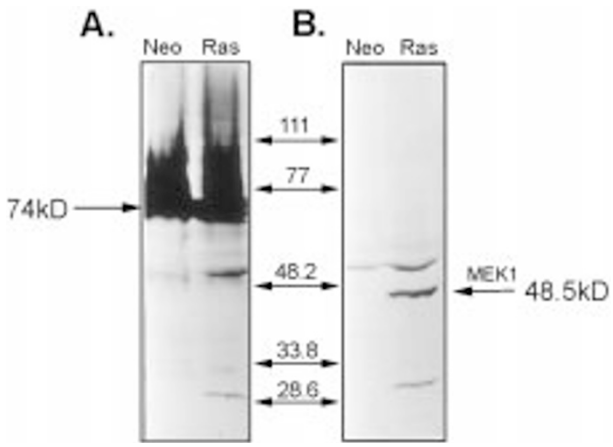

Figure 3. Expression of MAPK module kinases in *ras* transformed NIH3T3 cells. The Raf-1 (**A**) and MEK (**B**) proteins were detected by Western blot analysis by using anti-Raf-1 or anti-MEK antibodies, respectively. Cell growth conditions are same as described in Figure 2.

Effect of transformation on the molecular assembly of MAPK modules

To better understand how Ras affects MAPK module components (MEK, Raf-1, and ERKs) for transformation of the cells, a possible selective high affinity association of these proteins was carried out by the glycerol gradient ultacentrifugation. This centrifugation method allows the separation and estimation of relative molecular sizes of protein entities present in the mixture of protein solution of the cell lysates but it is not an equilibrium sedimentation method and it only measures molecular entity in a very high affinity association such as in the intermolecular association of homodimer, homo-tetramers whose association constant exceeds 10-9. In this approach, we could estimate the molecular weight of the native proteins and possibly other protein complexes. As shown in Figure 4A, the ERK1/2 MAPKs is mostly appeared at the peak of molecular weight around 80 kD and it suggests that the ERK1/2 (41 or 44 kD proteins) may present as dimer or interact with other proteins with a high affinity in similar sized ERKs. However, the distribution of 80 kD molecules in a few fractions that were not significantly changed when cells were treated by EGF. The dimeric complex may be stable and may not dissociated after EGF signal transduction. Interestingly, the Raf-1 proteins were distributed broadly by the glycerol gradients (Figure 5A). However, the MEK protein appeared at the narrow ranges of the glycerol gradient, and peaked at the molecular weight size of 150 kD. The pattern of distribution profiles of both Raf-1 and MEK were not significantly changed in the EGF induced cells (compare Figure 5A and 5B).

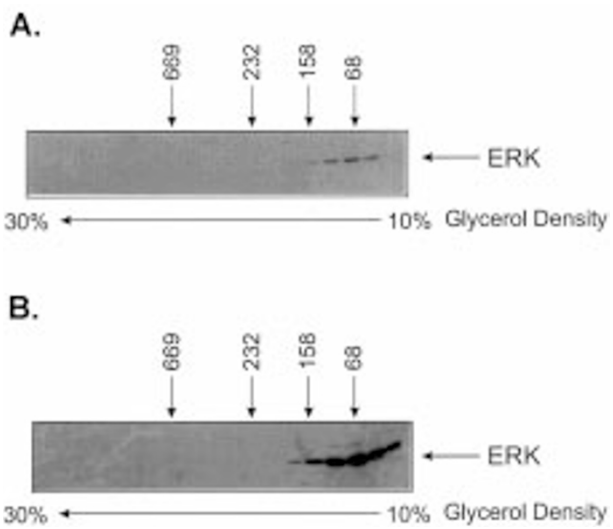

Figure 4. Effect of EGF in the glycerol gradient profile of ERK MAPK. One hundred and fifty microliter of cell extracts (10 mg/ml) were subjected to 10-30% glycerol gradient (w/v) in H buffer containing 150 mM NaCl and protease inhibitors (see Materials and Methods). Molecular weight standards were loaded onto an indentical 10-30% glycerol gradient and used as native molecular weight size markers. Samples were separated by ultracentrifugation and fractionated as described in "Materials and Methods". Each aliquots of the fractions (150 μl) was used for immunoblot analysis. The ERK proteins were detected by anti-ERK polyclonal antibody. NIH3T3 Cells were grown in DMEM containing 10% FCS without (**A**) and with (**B**) EGF. For mitogen stimulation, 100 ng/ml of EGF were treated before harvesting the cells. Numbers on the top of panel represent the peaks of molecular weight standards in a glycerol gradient. 669 kD, Thyroglobulin; 232 kD, catalase; 158 kD, aldolase; 68 kD, albumin.

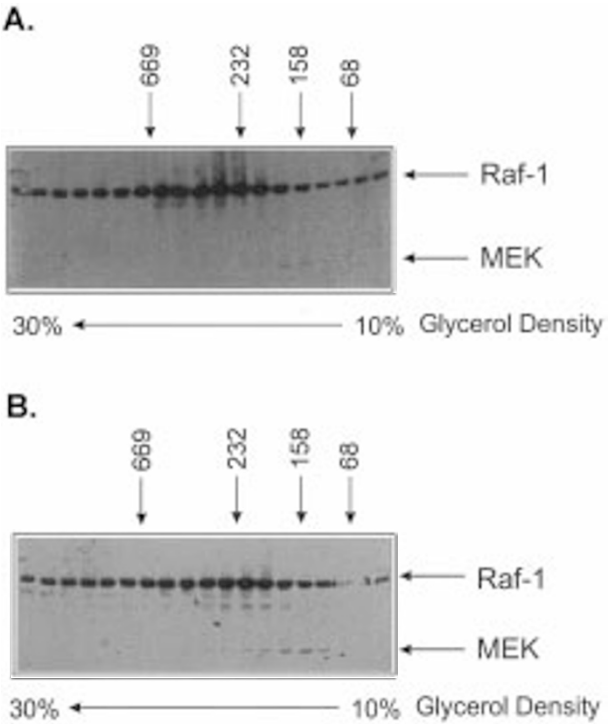

Figure 5. The effect of EGF in the glycerol gradient profile of Raf-1 and MEK1 kinases. Experimental protocols were performed same as described in Figure 4. Each panel **A** and **B** represent non-induced or induced sample by EGF, respectively. The Raf-1 and MEK proteins were detected by Western blot analysis using the mixture of anti-Raf-1 and anti-MEK antibodies. Numbers on the top of panel represent the peaks of molecular weight standards in a glycerol gradient as described in Figure 4.

Discussion

Mitogen-activated protein kinases (MAPKs), also described as extra-cellular signal regulated kinases (ERKs) belong to a group of proteins known as serine/threonine kinases and its activational status is a determining factor for signal transduction (Blenis, 1993; Burgering *et al.*, 1993; Egan *et al.*, 1993; Pages *et al.*, 1993; Cobb *et al.,* 1995). The MAPK pathway is activated by various external stimuli including growth factors and neurotransmitters (Burgering *et al.*, 1993; Egan *et al.,* 1993; Stevenson *et al.*, 1994). Ras is also one of the central components of the ERK MAPK signaling pathway, and its constitutively active mutations were found in numerous human cancers (Bos, 1989). Activation of *ras* is characterized by activation of downstream MAPK mo-

dule kinases through series of phosphorylation cascade, Raf-1 → MEK → ERK. Understanding of how *ras* interact with MAPK kinases will help to design and develop novel approaches for cancer treatment by blocking the excess growth stimulation that might be caused by Ras (Levitzki *et al*., 1995).

To understand the role of *ras* in the cellular transformation, we have established *ras* transformed NIH3T3 cells. The *ras* transformed cells grow fast and changed its morphology characterized by formation of numerous spikes at the edges of cells. Furthermore, the cells were polarized to aggregate. The phenotypic change of *ras* transformed cells was confirmed by amplifying the gene using PCR. To understand roles of Ras in the activation of MAPK cascade, we checked status of MAPK module kinases by Western blot analysis of *ras* transformed or control NIH3T3 cells. In this experiment, we have found a significant activation of ERK1/2 MAPKs in the *ras* transformed cells, which is evident by phosphorylation of the protein (Figure 2B). The phospho-ERK MAPKs were not significantly observed in normal NIH3T3 cells although similar levels of the ERK proteins were found in both *ras* transformed and non-transformed cells (Figure 2A and 2B). Therefore, Ras dependent activation of MAPKs may be caused by phosphorylation of the proteins rather than the increase in the levels of ERK proteins. To investigate the status of other upstream MAPK module kinases in the *ras* transformed cells, we performed Western blot analysis of the Raf-1 and MEK proteins. Although the Raf-1 and ERK proteins did not change its protein levels significantly, MEK protein was highly induced in Ras transformed cells. The increased level of MEK in Ras transformed NIH3T3 cells suggests that the MEK protein may be overexpressed or stabilized in Ras transformed cell. There is no report suggesting the overexpression of MEK is directly related to tumorigenesis. However, several reports show that the overexpression of wild-type MEK1 markedly enhanced the transforming efficiency of Mos (Okazaki *et al.,* 1995), and MEK1 could confer both tumorigenic and metastatic potential upon NIH3T3 cells Ras-independently (Welch *et al.,* 2000). The maintenance of the high level of MEK depends on Ras transformation. Therefore, the high level of MEK dependent Ras could be important for the activation of the MAP kinase signaling cascade to involve transformation of the cells. But, further investigations are required to know whether the high level of MEK is acquired by gene expression or protein stability in Ras transformed cell.

Previous studies suggested possibilities of the presence of the complex between MAPK module kinases (Seger *et al*., 1992; Matsuda *et al*., 1993). Here, we investigated the presence of high affinity complexes among MAPK module kinases (Raf-1, MEK, and ERKs) in the NIH 3T3 cells. Furthermore, we also checked any changes in the status of the complex (es) by modulating the MAPK signal transduction. Here, we used EGF which is a well known initiator of MAPK signal transduction (Seger *et al.,* 1992; Burgering *et al*., 1993). In 10-30% glycerol gradient, the ERK1/2 MAPK kinases were mostly sedimented at the position of molecular weight about 80 kD (Figure 4A). This suggests that the ERK1/2 (41 or 44 kD proteins) may present as homo-dimer or interact with other proteins with high affinity in similar sized ERKs.

Our gradient centrifugation results of the Raf-1 protein distributed broadly on a glycerol gradient suggest that the Raf-1 may likely be involved in the formation of the high molecular weight complex(es) or the nonspecific association with many of the common cellular proteins. The Raf-1 protein are known to translocate to the membrane where numerous proteins, for example, cytoskeletal proteins are present for maintenance of cellular structure (Daum *et al*., 1994) and is known to interact with other Ras independent signaling molecules for activation of MAPKs (Burgering *et al*., 1993; Marquardt *et al*., 1994; Dent *et al*., 1995; Yao *et al.,* 1995). The MEK protein located at the peak of 150 kD suggest that the protein (48 kD) may exist as multimeric forms or may present as a complex with other proteins, ERK or others. Here, distribution profiles of Raf-1 and MEK proteins were not significantly changed under a condition induced by EGF (compare Figures 5A and 5B). Several possibilities are suggested for reasons of not being able to detect the changes. First, the signal mediated molecular assembly of the proteins may be a transient event (Marshall, 1995), therefore, the change was not sensitively detected in our assay system. Secondly, it is also possible that quantity of proteins used for switching protein-protein interaction may be relatively small compared to the total levels of the proteins.

It was reported that the MAPK module kinase proteins were used for other cellular functions except Ras-dependent MAPK signaling for cell growth control (Burgering *et al*., 1993; Dent *et al*., 1995; Yao *et al*., 1995).

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