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SUPPRESSION OF SERUM-INDUCED c-*jun* EXPRESSION BY ACTIVATED Ki-*ras* IN HUMAN COLON CANCER CELLS

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Summary Through gene-targeting, we have established human colon cancer cell lines, HK2-6 and HKe-3, with and without activated Ki-*ras*, respectively, derived from a human colon cancer cell line HCT116, and we have reported that activated Ki-*ras* is involved in the deregulation of c-*myc* expression. To further examine the relation between Ki-*ras*-mediated signals and other immediate early genes, c-*jun* was analyzed on these cells stimulated by serum. Rapid and strong induction of c-*jun* was observed in HKe-3, but not in HCT116 or HK2-6. To elucidate the regulatory mechanisms of c-*jun* expression by Ki-*ras*, protein kinase C (PKC) and c-Raf were examined at serum-starved and serum-stimulated conditions. Phosphorylations of c-Raf were same among these cells, however, the cytosolic PKC activity in HKe-3 was two times higher than that in HCT116 on serum-starved and serum-stimulated conditions. These results suggested that serum responsiveness of c-*jun* may be suppressed by activated Ki-*ras* through PKC rather than c-Raf pathway in colon cancer cells.

Key Words serum responsiveness, activated Ki-*ras*, colon cancer, c-*jun*, protein kinase C, c-Raf

INTRODUCTION

We previously demonstrated that activated Ki-*ras* is involved in the deregulation of c-*myc* in human colon cancer cell lines using the gene-targeting system (Shirasawa *et al.*, 1993). These results raised the possibility that activated Ki-*ras* will be involved in the deregulation of other immediate early genes, such as c-*jun*, which is involved in growth, differentiation, and apoptosis (Kitabayashi *et al.*, 1991; Verheij *et al.*, 1996).

c-Jun belongs to AP-1 family, whose expression is rapidly stimulated by

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serum, forming homodimers and/or heterodimers with other AP-1 families (Angel *et al.*, 1988). c-Jun was activated by phosphorylation on serine resides 63 and 73 through Ha-*ras*-mediated signals in fibroblasts (Smeal *et al.*, 1992). To elucidate whether activated Ki-*ras* is involved in the regulation of c-*jun*, expressions of c-*jun* mRNA and c-Jun were investigated on the colon cancer cells disrupted at activated Ki-*ras* (HKe-3) and its parental cells (HCT116). Activities of PKC and phosphorylation of c-Raf, which are downstream targets of Ras, were also examined on these cells.

MATERIALS AND METHODS

Cell culture. Colon cancer cell line HCT116 [American Tissue Culture Collection (ATCC) CCL 247] was obtained from ATCC. HCT116 has a mutation of Ki-*ras* at codon 13. HK2-6 and HKe-3 are clones derived from HCT116, with and without activated Ki-*ras*, respectively, through gene-targeting (Shirasawa *et al.*, 1993). These cell lines were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 10% CO_2.

RNA preparation. Cultured cells (5×10^6) from each cell line were lysed with 5 ml of the denaturing solution containing 1% sodium dodecyl sulfate (SDS), 10 mM sodium acetate (pH 4.5). The lysate was transferred to a 15 ml polypropylene tube, and 5 ml of water saturated phenol was added. The solution was shaken vigorously at 70°C for 7 min, and then shaken in dry-iced alcohol for 3 min, followed by centrifugation at 3,000 rpm for 20 min. The upper aqueous phase was transferred to a fresh tube, subsequently extracted by 25/24/1 phenol/chloroform/isoamyl alcohol once, then by 24/1 chloroform/isoamyl alcohol. The aqueous phase was precipitated with 2.5 volumes of ethanol and 1/10 volumes of sodium acetate (pH 5.2). The precipitant RNAs were centrifuged at 15,000 rpm at 4°C, then the pellet was suspended in diethyl pyrocarbonate treated water.

Northern blot analysis. Fifteen micrograms of total RNA isolated from each cell line were electrophoresed in 1% agarose gel containing 20 mM MOPS, 5 mM sodium acetate (pH 4.5), 1 mM EDTA, and 2.2 M formaldehyde at 140 V for 3 hr followed by transfer to nylon membrane (Hybond N+, Amersham). The filters were hybridized with ^{32}P-labeled DNA probes in solution containing 50% of deionized formamide, 5×SSPE, 10×Denhardts (0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin), 1% SDS, and 100 μg/ml sonicated salmon sperm DNA at 42°C overnight. The filters were washed in solution containing 2×SSPE/0.1% SDS at room temperature for 10 min, and then washed with 2×SSPE/0.1% SDS at 55°C. The washed filters were exposed to Bio-Image-Analyzer (BAS2000, FUJIX, Kanagawa, Japan) for the radioactivity of each band, and then the filters were exposed to Kodak XAR-5 film for 2–8 days. c-*jun* probe was generated by PCR using primer set, 5′-GCAGAGTCCCGGAGCGAACT-3′

and 5′-CACTGTCTGAGGCTCCTCCT-3′ (Angel *et al.*, 1988; Hattori *et al.*, 1988).

Serum stimulation. Cells (5×10^6) of each line were cultured in DMEM containing 0.1% FCS for 40 hr followed by adding serum to the final concentration of 10%. Cells were harvested at each time point; 0 min (before), 15 min, 30 min, 60 min, 180 min, amd 360 min. The extracted RNAs were subjected to Northern blot analysis.

Western blot analysis. Proteins were extracted from HCT116 and HKe-3 at serum-starved and serum-stimulated conditions. Fifteen micrograms of total lysates were electrophoresed in 8% SDS/PAGE at a constant current of 18 mA for 90 min followed by transfer to PVDF membrane (NCI). Western blotting were performed by ECL system (Amarsham) using anti c-Jun monoclonal antibody (Transduction Laboratories) and anti Raf-1 polyclonal antibody (Santa Cruz). Phosphorylated status of c-Raf proteins were determined by their mobility difference in SDS/PAGE as reported (Morrison *et al.*, 1988).

Assay for protein kinase C. Cultured cells were rinsed with cold PBS and scraped with 0.5 ml of a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/ml of aprotinin and leupeptin, followed by homogenization in pre-cooled dounce homogenizer. After removing cellular debris by centrifuging for 2 min, the supernatant were partially purified by DEAE cellulose column. The eluates were used for measuring activity of protein kinase C according to the manufacturer's protocol (GIBCO BRL). Total protein concentrations were determined by the method of Bradford *et al.* (1976).

RESULTS

First, we examined expressions of immediate genes at exponentially growing state in HCT116, HK2-6 and HKe-3. However, no remarkable difference was observed except c-*myc* expression among these cells (Shirasawa *et al.*, 1993; data not shown).

Next, we analyzed c-*jun* expression at serum-starved-, serum-stimulated conditions. HCT116, HK2-6, and HKe-3 were stimulated by serum following serum starvation for 40 hr. HKe-3 clearly showed c-*jun* mRNA expression induced by serum at 30 min and the maximum expression was observed at 60 min (Fig. 1). Expression of c-*jun* in HKe-3 was gradually decreased after 60 min, and it came back to the basal level at 360 min. Whereas, in HCT116 and HK2-6 having activated Ki-*ras*, little serum-induced expression of c-*jun* were observed (Fig. 1; data not shown). The expression of c-*jun* in HKe-3 was as 10–15 times as much as that observed in HCT116.

To confirm expression of c-Jun protein, Western blotting was done on HCT116 and HKe-3 at various conditions using anti-c-Jun antibody. At exponentially growing- and serum-starved-conditions, c-Jun expression of HCT116 was lower than that of HKe-3, suggesting that basal level of c-Jun in HCT116 was suppressed on these conditions (Fig. 2; data not shown). Total amount of c-Jun in

HCT116 was increased at 30 min after serum stimulation, however, no evidential phosphorylation was observed (Fig. 2). HKe-3 clearly showed c-Jun production at 30 min and its mobility was shifted due to its phosphorylation (Fig. 2). These results suggested that mRNA- and protein-expression of c-*jun* were correlated with each other.

To elucidate the relation between the differential c-Jun expression and activated Ki-*ras*-mediated signaling pathway, phosphorylation of c-Raf, one of the

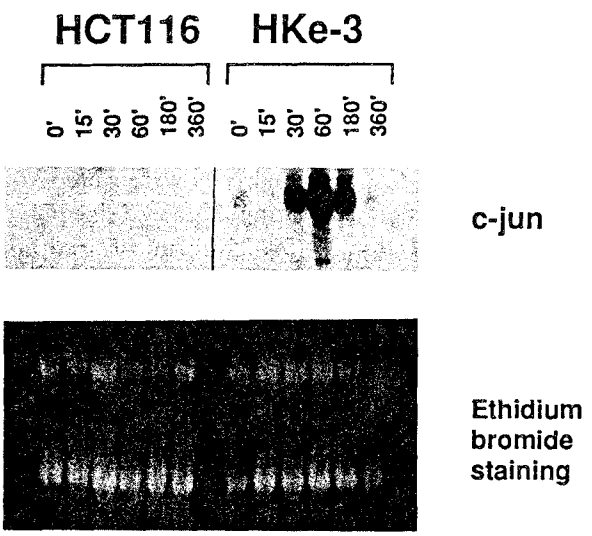

Fig. 1. Time course of c-*jun* mRNA expression induced by serum in HCT116 and HKe-3. The expression of c-*jun* and ethidium bromide staining as control were indicated.

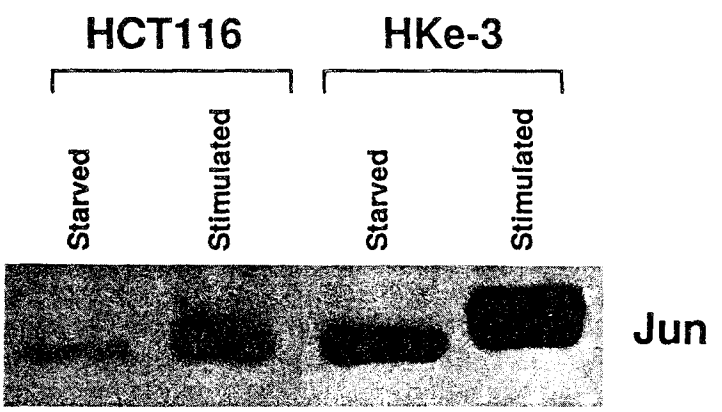

Fig. 2. c-Jun expression and its phosphorylation at serum-starved and serum-stimulated (30 min) conditions.

downstream targets of Ras, was examined through a mobility difference between hyper-phosphorylated and hypo-phosphorylated form on SDS-PAGE. Same samples used for c-Jun Western blotting were examined for phosphorylation of c-Raf. Hyperphosphorylated and hypophosphorylated components of c-Raf were indicated by arrows (Fig. 3). On serum stimulation, phosphorylated c-Raf were increased in both HCT116 and HKe-3. No difference of c-Raf was observed among these cells quantitatively and qualitatively at serum-starved- and serum-stimulated-conditions (Fig. 3). These results, together, suggested that c-Raf signaling pathway would not be involved in the differential expression of c-*jun* mRNA and c-Jun protein in these colon cancer cells.

PKC, which is proposed as another component functioning downstream of Ras, was examined between these cells (Hunter, 1991). When PKC is stimulated by biological active substances such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), it is activated and rapidly translocates from cytosol to membrane (Nishizuka, 1992; Chida *et al.*, 1986a). The activities of cytosolic PKC in HCT116 and HKe-3 at serum-starved condition were 125, 284 pmol/min/mg protein, respectively. The activity in HCT116 was almost twofold lower than that in HKe-3 before serum stimulation (Fig. 4). After serum stimulation, PKC activities in both HCT116 and HKe-3 were rapidly decreased about 40% within 15 min. The decrease of PKC activity in HCT116 was also 2–3-times lower than in HKe-3. The decrease of PKC activity in cytosolic fraction implies translocation of PKC from cytosol to membrane and the decrease in the cytosol fraction was almost equal to the increase

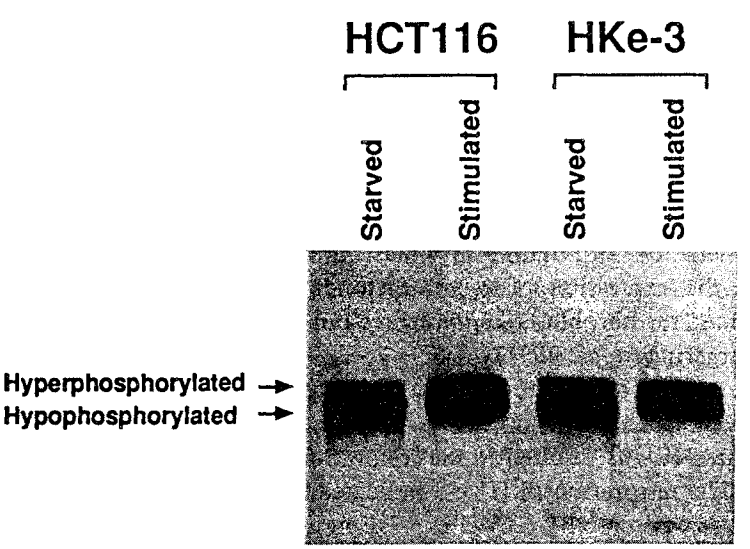

Fig. 3. Western blot analysis of c-Raf protein at serum-starved and serum-stimulated (30 min) conditions.

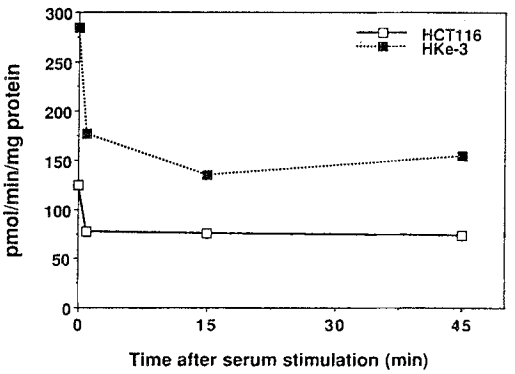

Fig. 4. Time course of cytosolic PKC activity in HCT116 and HKe-3 at serum-starved and serum-stimulated conditions.

in the membrane fraction, where PKC functions (Chida *et al.*, 1986a,b). The difference of PKC activities between the cells may affect the biological responses to serum stimulation.

DISCUSSION

The proto-oncogene c-*jun* shows numerous cellular phenotypes such as oncogenic transformation, differentiation, and the response to toxic agents (Smeal *et al.*, 1991; Kitabayashi *et al.*, 1991; Verheij *et al.*, 1996). The diversity of functions by c-Jun will be due to its combinatorial association with other members of transcriptional factors that exhibited distinct DNA binding specificity (Nakabeppu *et al.*, 1988; Hai and Curren, 1991). The transcriptional response of the c-*jun* is regulated post-translationally by protein phosphorylations (Angel *et al.*, 1988). Analysis of c-Jun phosphorylation suggests that c-Jun is activated by phosphorylation on serine resides through Ha-*ras* in fibroblasts (Smeal *et al.*, 1992; Binetruy *et al.*, 1991).

However, the relation between activated Ki-*ras* and c-*jun* expression was not fully understood. In NIH3T3 fibroblast, transient expression of activated Ha-*ras* induced the expression of c-*jun* (Sistonen *et al.*, 1989). Whereas, in Ha-*ras* transformed rat fibroblasts, c-*jun* expression was induced little by serum stimulation (Burgering *et al.*, 1991).

The present report introduces a novel system to comparatively investigate the role of activated Ki-*ras* in colon cancer cells. This system showed that elimination of activated Ki-*ras* resulted in the restoration of serum responsiveness of c-*jun* in HKe-3. The restoration in HKe-3 indicated that HCT116 have serum responsiveness of c-*jun* potentially. Lower expression of c-Jun was also observed in HCT116 on exponentially growing- and serum-starved-conditions (Fig. 2; data not shown). These observations indicated that in colon cancer cells with activated Ki-*ras*, the

expression of c-*jun* mRNA and c-Jun protein would be suppressed. These phenomenon also indicated that intracellular changes of molecules involved in the regulation of c-*jun* expression occurred in the colon cnacer cells having activated Ki-*ras* (Satake *et al.*, 1989; Nishizuka, 1986; Wolfmann and Macara, 1987; Wolfmann *et al.* 1987).

c-Raf and PKC, candidate molecules downstream of Ras, were also investigated. c-Raf phosphorylation, which is thought to be active form, was not different among those cells (Fig. 3), indicating that activation of c-Raf could not explain the differential expression of c-*jun* in these cells. While the activity of PKC in HCT116 was almost twofold lower than that in HKe-3 before and after serum stimulation (Fig. 4). After serum-stimulation, cytosolic PKC activities in both HCT116 and HKe-3 were rapidly decreased about 40% within 15 min. The extent of decrease in cytosolic PKC activities was also twofold lower in HCT116 than in HKe-3. The decrease of PKC activity in the cytosolic fraction implies the translocation of cytosolic PKC to the membrane and the decrease was almost equal to the increase in the membrane fraction during the first 2 hr after stimulation (Chida *et al.*, 1986a). The membrane fraction of PKC was biologically active and was essential for cellular responses such as cell proliferation and differentiation (Nishizuka, 1992). Therefore, 2-3-fold difference in PKC activities between HCT116 and HKe-3, when the cells were stimulated by serum, may affect the serum responsiveness of c-*jun*.

To confirm that PKC could mediate signals to c-Jun in these cells, TPA, which was a potent stimulator of PKC, was added to these cells. TPA induced c-Jun expression in HKe-3 like serum stimulation, but not in HCT116 or HK2-6 (data not shown). These observations suggested that PKC would be involved in the responsiveness of c-*jun* in the colon cancer cells and that the differential expression of c-*jun* between HCT116 and HKe-3 may be caused through the differential PKC activities.

However, they might just reflect the result of cellular phenotypes because PKC activities were controlled in complex mechanisms (Nishizuka, 1986). Kinase activity of Mitogen Activating Protein Kinases, including ERK, JNK (SAPK), and p38 should be determined, leading to the elucidation of Ki-*ras*-mediated signal transductions in colon cancer cells. This system will be useful for understanding the functions of Ki-*ras*, c-*myc*, c-*jun*, and other genes involved in colorectal tumorigenesis.

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