Activation mechanism of c-Jun amino-terminal kinase in the course of endodermal differentiation of P19 embryonic carcinoma cells

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Abstract c-Jun amino-terminal kinase (JNK) is known to be activated and play critical roles during neural and endodermal differentiation of P19 embryonic carcinoma cells. In this study we demonstrated that of the two upstream protein kinases of JNK, only MKK4 activity was substantially enhanced in the endodermally differentiating P19 cells. This enhanced activity of MKK4 stemmed from the increased expression of MKK4 and its activation by phosphorylation. Activated MKK4 and JNK were localized in both nucleus and cytoplasm of the differentiating cells, while they were localized only in the nucleus in the undifferentiated cells, suggesting multiple roles of JNK in the course of the endodermal differentiation of P19 cells.

Key words: P19 embryonic carcinoma cell; Differentiation; c-Jun amino-terminal kinase; Mitogen-activated protein kinase kinase 4

1. Introduction

P19 is a murine embryonic carcinoma cell line that have been used as a model system for studying early embryonic development and differentiation. The P19 cells resemble the inner cell mass of early embryo and can differentiate into primitive endoderm-like cells, neuroectoderm-like cells or muscle-like cells depending on the culture conditions [1–3]. Treatment of aggregated P19 cells in a bacterial grade Petri dish with low concentrations (10 nM) of retinoic acid (RA) followed by plating onto a tissue culture plate leads to differentiation to primitive endoderm-like cells, while the treatment of aggregated P19 cells with higher concentrations (1 μM) of RA results in their differentiation into neurons and glias [1–3].

Studies on signaling systems responsible for RA-induced endodermal and neural differentiation of P19 cells have indicated that the c-Jun amino-terminal kinase (JNK) pathway plays an important role [4–6]. However there has been no study identifying mitogen-activated protein kinase kinase (MAPKK) responsible for activation of JNK during endodermal differentiation. Here, we present evidence indicating that JNK is activated by MAP kinase kinase 4 (MKK4) but not by MKK7 in the course of the differentiation. We further provide evidence indicating that the activated forms of JNK and MKK4 are localized in both the nucleus and the cytoplasm of the endodermally differentiating cells.

2. Materials and methods

2.1. Materials

MLK3 and cytokeratin Endo A cDNAs were generated from total RNA prepared from the differentiating P19 cells using reverse transcription polymerase chain reaction (PCR). JNK1 cDNA was described in [6]. MKK4, MKK7 and TAK1 cDNAs were described in [7]. Antibodies for JNK1 and MKK4 were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-MKK7 antibody was a generous gift from Dr. E. Nishida (Kyoto University, Kyoto, Japan). Phospho-specific JNK and MKK4 antibodies were purchased from New England Biolabs (Beverly, MA, USA). Cy3-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA, USA). Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were from Wako Pure Chemical (Osaka, Japan).

2.2. Methods

2.2.1. Cell culture and neural differentiation. P19 cells were cultured in αMEM (minimum essential medium) (Life Technologies, MD, USA) supplemented with 10% (v/v) fetal calf serum. To induce endodermal differentiation, cells were cultured in the presence of 10 nM RA in the bacteriological grade Petri dish for 2 days, replated onto a tissue culture grade dish and cultured for another 2 days in the absence of RA. The differentiation was confirmed by induction of cytokeratin Endo A mRNA (Fig. 2e). For osmotic shock, undifferentiated cells were incubated with medium supplemented with 0.7 M NaCl for 15 min.

2.2.2. Preparation of cell extract. Cells were lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% (v/v) Triton X-100, 50 mM NaF, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)). After 15 min, cell lysate was centrifuged at 4°C for 10 min at 12 000×*g*, and the supernatant was frozen immediately in liquid nitrogen and stored at −80°C until use.

2.2.3. Kinase assay. JNK and MKK kinase assays were carried out as described using glutathione-S-transferase (GST)-c-Jun and His-JNK3 [K55R] proteins as substrate respectively [6].

2.2.4. Northern blotting. Total RNA (10 μg) was separated through 1.2% (w/v) agarose/0.67 M formaldehyde gels, and transferred to a nylon membrane. Hybridization and washing conditions were as described [8].

2.2.5. Column chromatography. An aliquot of the sample (1 mg of protein) was desalted by gel filtration and subsequently applied to a Super-Q Toyopearl equilibrated with buffer A (20 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol). Then the bound proteins were eluted with linear gradient of NaCl (0–0.5 M) in buffer A.

2.2.6. Indirect immunofluorescence. Cells were cultured on a poly-L-lysine-coated cover glass and then fixed for 15 min with 4% (w/v)

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paraformaldehyde. The fixed cells were permeabilized with 0.1% (v/v) Triton X-100, blocked for 30 min in phosphate-buffered saline (PBS) containing 2% (v/v) normal goat serum and subsequently incubated with the first antibody for 1 h at room temperature (RT) or overnight at 4°C. After extensive washing with PBS, the cells were incubated with the secondary antibody for 1 h at RT. After repeated washing with PBS the cells were examined by confocal microscopy with a Zeiss LSM410 microscope (Carl Zeiss, Jena, Germany).

3. Results and discussion

3.1. Analysis of upstream activators of JNK during differentiation of P19 cells

Although it was reported that JNK was activated during endodermal differentiation of P19 embryonal carcinoma cells, MAPKK responsible for the activation was not clear. Therefore, we first compared the activities of MKK4 and MKK7, which are known to be the physiological activators of JNK, of the endodermally differentiating cells with those of the undifferentiated or stress-treated cells. Cell lysate was prepared from undifferentiated, endodermally differentiating or stress-treated P19 cells and JNK1 was immunoprecipitated with specific antibody. The immune-complex kinase assay was performed with GST-c-Jun as substrate. We confirmed the previous observations by Jho et al. [4] of the activation of JNK1 during endodermal differentiation (Fig. 1a). The degree of the activation during endodermal differentiation was moderate compared with that induced by stress (Fig. 1a, lanes 2 and 3). To examine whether MKK4 and/or MKK7 were activated during differentiation, the immune-complex kinase assay was performed using a kinase-negative mutant of JNK3 (His-JNK3 [K55R]) as substrate. Interestingly, only MKK4 was substantially activated in endodermally differentiating cells, whereas both MKK4 and MKK7 were activated in response to stress (Fig. 1b,c). MKK4 itself is known to be activated through phosphorylation by upstream kinases. Concomitant with the activation of MKK4, an increase of phosphorylation of MKK4 endodermally differentiating cells was observed (Fig. 1d). These results indicated that MKK4, but not MKK7, was a major activator of JNK during endodermal differentiation. Wang et al. showed that MKK4 was constitutively activated in P19 cells stably transfected with Gα13 [Q226L] protein whose overexpression led to endodermal differentiation without RA, but they did not examine the activity of MKK7 [9]. Therefore, our studies provided the first evidence demonstrating that MKK4 but not MKK7 was a major upstream activator of JNK during RA-induced endodermal differentiation of P19 cells. In this context it should be noted that Hietakangas and coworkers showed that activation of JNK during hematopoietic differentiation of K562 cells was also mediated through activation of MKK4 but not MKK7 [10].

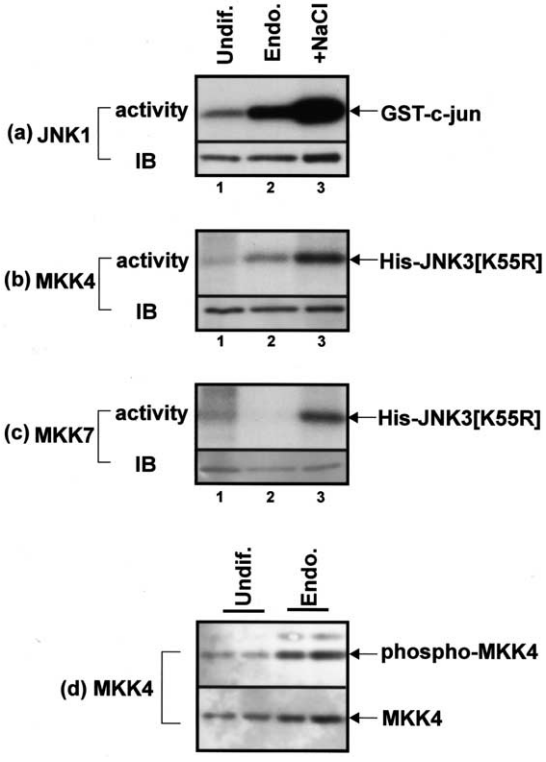

Fig. 1. Activation of JNK pathway during endodermal differentiation of P19 cells. a–c: Aliquots of cell extracts (500 μg protein for JNK assay and 50 μg protein for MKK assay) were immunoprecipitated with anti-JNK1, -MKK4 or -MKK7 antibody and the kinase activity was determined using GST-c-Jun (for JNK1) or His-JNK3 [K55R] (for MKK4 and MKK7) as substrate (each upper panel). In each experiment, samples prepared from the undifferentiated cells (indicated as 'Undif.'), endodermally differentiating cells (indicated as 'Endo.') and cells treated with 0.7 M NaCl for 15 min (indicated as '+NaCl') were analyzed. The protein levels of these kinases were monitored by immunoblot using the indicated antibodies (each lower panel). d: Aliquots of cell extracts (10 μg/lane) prepared from undifferentiated and endodermally differentiating cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with phospho-specific anti-MKK4 antibody (upper panels) and anti-MKK4 antibody (lower panels).

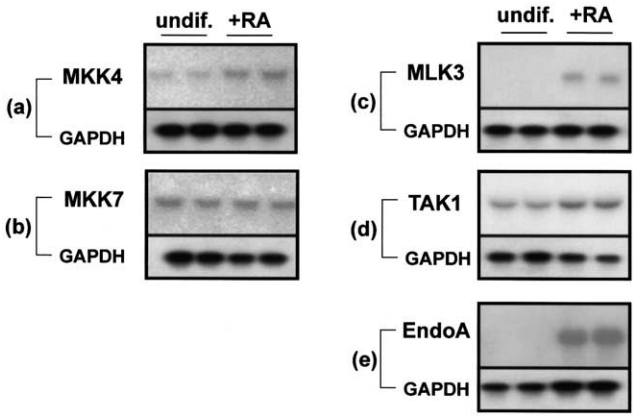

Fig. 2. Northern analysis of mRNAs of components of the JNK cascade during differentiation of P19 cells. Total RNA was prepared from the duplicated dishes of undifferentiated P19 cells and P19 cells during endodermal differentiation. RNA was separated on an agarose gel containing formaldehyde, transferred to nylon membrane and probed with ^{32}P-labeled cDNA as indicated (each upper panel). The membranes were deprobed and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to indicate equal loading of RNA samples (each lower panel).

3.2. Upregulation of the members of the JNK cascade during differentiation of P19 cells

While the activation of JNK in response to stress is transient, a persistent activation of JNK is observed during both neural and endodermal differentiation of P19 cells [5,6]. This suggests that the mechanism of activation of JNK during differentiation differs fundamentally from that caused by

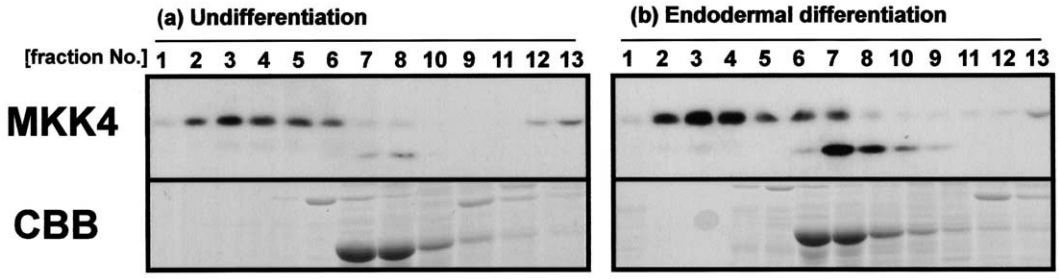

Fig. 3. MKK4 protein level during the endodermal differentiation of P19 cells. Whole cell extracts (1 mg protein) prepared from undifferentiated P19 cells (a) and endodermally differentiating P19 cells (b) were applied to an anion exchange column and eluted with a gradient of 0–0.5 M NaCl. Each fraction was subjected to SDS–PAGE and MKK4 protein was detected by immunoblotting (upper panels). Coomassie brilliant blue (CBB) staining of the same fractions is shown to demonstrate the amount of protein loaded (bottom panels).

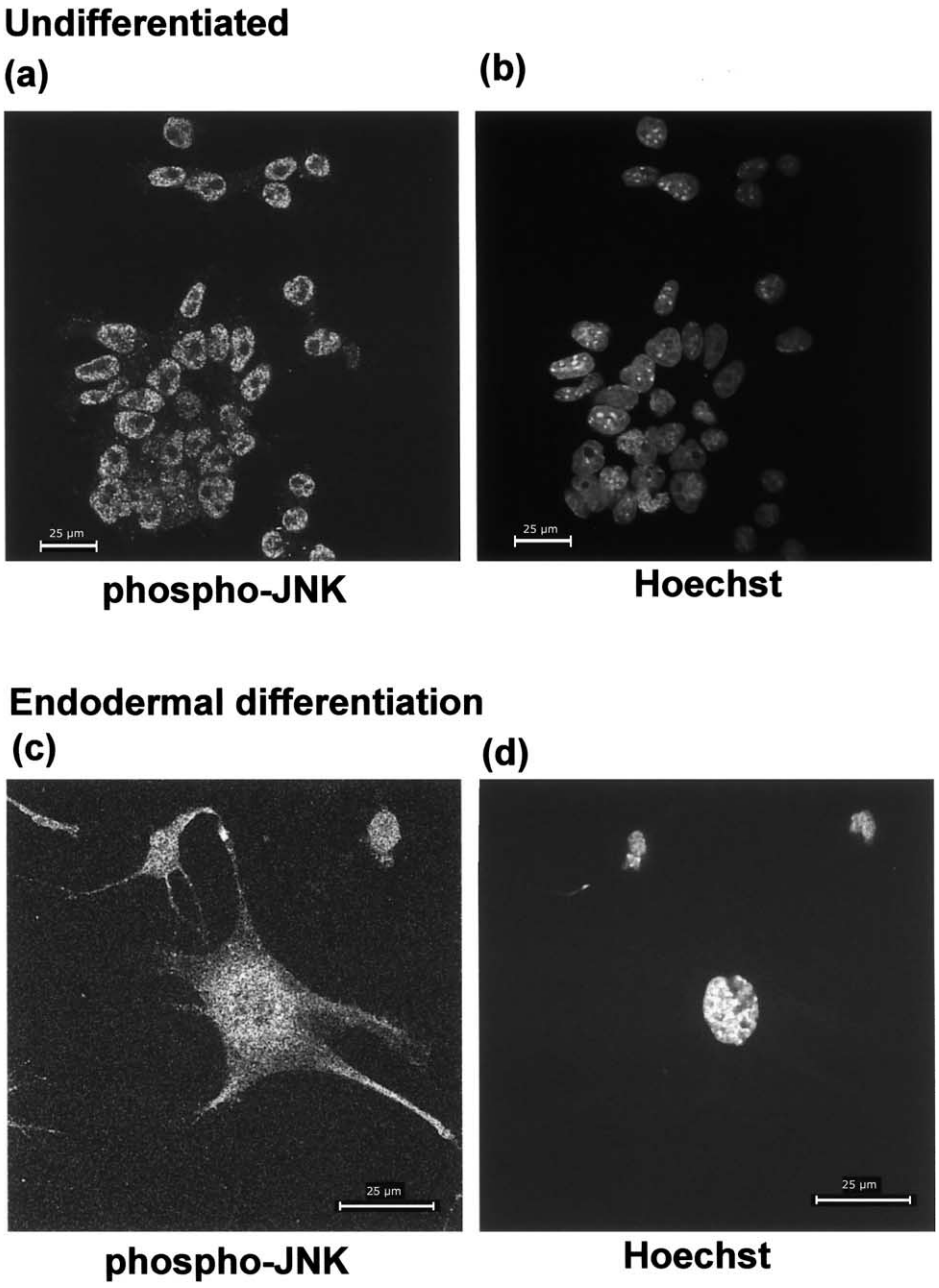

Fig. 4. Subcellular localization of activated JNK protein in the differentiating P19 cells. Undifferentiated P19 cells (a,b) and endodermally differentiated cells (c,d) were fixed with paraformaldehyde and activated JNK protein was detected using the phospho-specific JNK antibody followed by incubation with Cy3-conjugated goat anti-rabbit IgG antibody (a,c). Hoechst 33258 dye was used to show the nuclei (b,d).

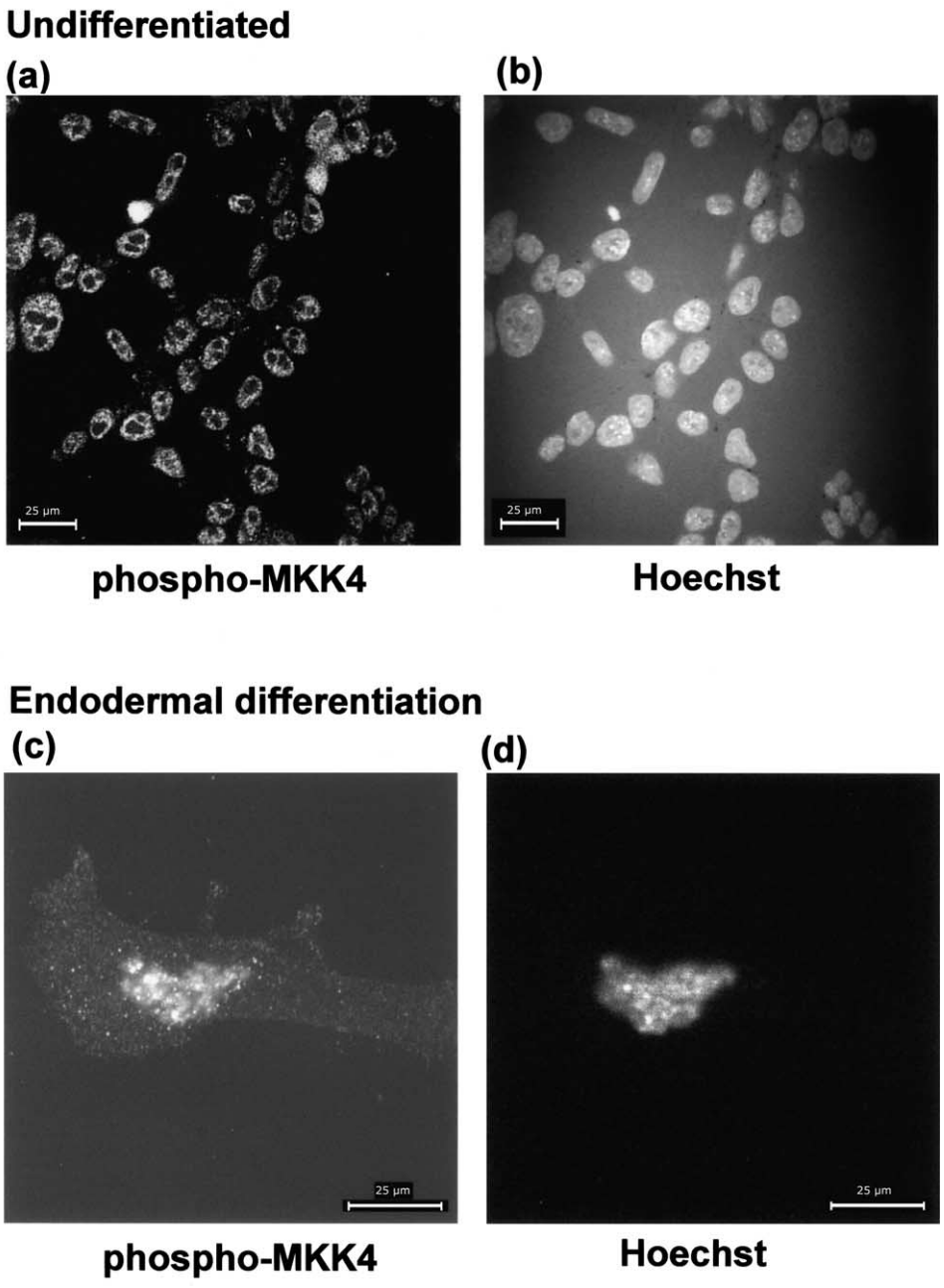

Fig. 5. Subcellular localization of activated MKK4 proteins in the differentiating P19 cells. Undifferentiated P19 cells (a,b) and P19 cells during endodermal differentiation (c,d) were fixed with paraformaldehyde and activated MKK4 was detected using the phospho-specific MKK4 antibody followed by incubation with Cy3-conjugated goat anti-rabbit IgG antibody (a,c). Hoechst 33258 dye was used to show the nuclei (b,d).

stress. We therefore examined whether mRNA of an upstream activator was induced in the course of the differentiation by Northern analysis. Total RNA was prepared from undifferentiated or differentiating cells and probed with cDNAs encoding components of the JNK cascade. MKK4 mRNA was increased during endodermal differentiation while expression of MKK7 mRNA did not change (Fig. 2a,b). These data agree well with the observation that MKK4, but not MKK7, was activated during differentiation. No change in the expression levels of the mRNAs for MEK1, which is an upstream MAPKK of ERK, and MKK3 and MKK6, which are upstream MAPKKs of p38, was observed during differentiation (unpublished data).

Recently several MAPKKKs that phosphorylate and activate MKK4 have been reported [11–18]. These include the MEKK family (MEKK1, MEKK2, MEKK3 and MEKK4/MTK1), the MLK family (MLK1, MLK2, MLK3 and DLK), TAK1 and ASK1. Kanungo et al. reported that MEKK4 mediated endodermal differentiation of P19 cells [19]. We therefore examined whether the MAKKK mRNAs were induced in the course of the differentiation. We could not detect any change of MEKK1, MEKK3, MEKK4 and ASK1 mRNA levels (unpublished data). Of the MAPKKKs we examined, the expression levels of both MLK3 and TAK1 mRNAs were increased during endodermal differentiation (Fig. 2c,d). mRNAs of these kinases were also induced during

neural differentiation suggesting that induction of these proteins is commonly required for the differentiation to proceed (Akiyama et al., submitted).

Although the mRNA of MKK4 was increased in the course of the endodermal differentiation, we could not detect changes in their protein levels by Western blotting if we used the whole cell extract for analysis (Fig. 1b). We then partially purified MKK4 protein from the cell extracts by anion exchange chromatography and subjected each fraction of the eluates to Western blotting. By this method, we observed a marked increase in the MKK4 protein in the endodermally differentiating cells (Fig. 3b). Whole cell extract may contain so many interfering proteins that it is not possible to recognize MKK4 by antibody efficiently on the polyvinylidene difluoride (PVDF) membrane. Partial purification of the cell extract decreased the number of proteins transferred to the membrane and may improve the efficiency of interaction between antibody and its target protein. The expressions of these proteins were not induced by addition of 0.7 M NaCl for 15 min (unpublished data). These results suggested that the enhanced activity of MKK4 during endodermal differentiation stemmed from not only increased phosphorylation of MKK4 but also increased expression of MKK4 protein.

3.3. Subcellular localization of activated JNK and MKK4 in differentiating P19 cells

It is well established that JNK is activated and translocated into the nucleus in response to stress [20]. Therefore, we examined the subcellular localization of the activated JNK and MKK4 in the differentiating P19 cells using phospho-specific JNK and MKK4 antibodies. Confocal microscopic observation showed that both the phospho-JNK and phospho-MKK4 existed exclusively in the nucleus in undifferentiated P19 cells (Figs. 4a and 5a). In the endodermally differentiating cells the activated JNK and MKK4 were localized both in the nucleus and the cytoplasm (Figs. 4c and 5c). Activated JNK and MKK4 were also localized both in the nucleus and the cytoplasm in neurally differentiating cells (Akiyama et al., submitted). These observations may suggest that the cytosolic localization of the active form of JNK and MKK4 is uniquely required for cell differentiation. In this context activated JNK in the cytosol may phosphorylate cytoskeletal proteins in endodermally differentiating cells. Cytokeratin Endo A (type II keratin, known as cytokeratin 8) and Endo B (type I keratin, known as cytokeratin 18) could be putative targets of JNK in endodermally differentiating cells, since they have serine and threonine residues followed by proline, which conforms to the optimal consensus for phosphorylation by JNK. In fact, it has recently been reported that JNK was implicated in the phosphorylation of Endo A on Ser-73, a serine residue followed by proline, upon stimulation of the pro-apoptotic cytokine receptor Fas/CD95/Apo1 in HT-29 cells [21]. These results raise the possibility that cytokeratin Endo A may be one of the targets of JNK during endodermal differentiation of P19 cells.

In neurally differentiating P19 cells, JNK and MKK4 existed as high molecular weight complex including scaffold protein JSAP-1 (JNK/SAPK interacting protein-1) (Akiyama et al., submitted). Interestingly both JNK1 and MKK4 proteins existed as monomeric form in endodermally differentiating cells suggesting that detailed mechanism of regulation of JNK by MKK4 may differ between two directions of differentiation (unpublished observation).

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