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Stimulation of Hepatocyte Growth Factor Production in Human Fibroblasts by the Protein Phosphatase Inhibitor Okadaic Acid

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ABSTRACT. In this study, we examined whether the production of hepatocyte growth factor (HGF) in fibroblasts is regulated by protein phosphatase(s). Inhibitors of the enzymes okadaic acid and calyculin A were used for this purpose. Both inhibitors markedly stimulated HGF production in human skin fibroblasts in a dose-dependent manner. The effects of okadaic acid and calyculin A were maximal at 25–37.5 and 1.25 nM, respectively. Highly active HGF production in MRC-5 human embryonic lung fibroblasts was also promoted by both inhibitors. The effect of okadaic acid was accompanied by an up-regulation of HGF gene expression. The stimulating effect of okadaic acid on HGF production was synergistic with that of phorbol 12-myristate 13-acetate (PMA) and epidermal growth factor (EGF), whereas it was additive to the effect of cholera toxin. The protein kinase C (PKC) inhibitor GF 109203X inhibited the effect of PMA, but not of okadaic acid and EGF. The effect of okadaic acid as well as EGF was not inhibited, but rather enhanced in human skin fibroblasts pretreated for 24 hr with a high dose of PMA to deplete PKC, as compared with its effect in untreated cells. PD 98059, an inhibitor of mitogen-activated protein (MAP) kinase kinase, suppressed the effects of okadaic acid and EGF, but not those of cholera toxin and 8-bromo-adenosine 3′,5′-cyclic monophosphate (cAMP). These results suggest that HGF production in human skin fibroblasts is down-regulated by protein phosphatase(s) and that HGF production stimulated by okadaic acid is, at least in part, dependent on the activation of the MAP kinase cascade. BIOCHEM PHARMACOL **60**;10:1531–1537, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. HGF production; okadaic acid; calyculin A; protein phosphatase; MAP kinase; fibroblast

HGF,† also known as scatter factor, is a disulfide-linked heterodimer consisting of a heavy chain (about 60 kDa) and a light chain (about 35 kDa) [1, 2]. Although it was initially isolated as a mitogenic factor for adult rat hepatocytes in primary culture, HGF has multiple functions including mitogenic, motogenic, morphogenic, and tumor-inhibiting activities and acts on many kinds of epithelial cells, other than hepatocytes, and other types of cells [1, 2]. Nevertheless, there is mounting evidence that HGF is a vital factor for liver regeneration. First, HGF is the most potent mitogen for rat hepatocytes reported to date: it markedly stimulates the proliferation of the hepatocytes at less than one-tenth the molar concentrations of transforming growth factor-α and EGF [3]. Second, levels of HGF in the plasma and liver of rats either exposed to carbon tetrachloride or partially hepatectomized increase markedly prior to liver regeneration [4, 5]. These increases are accompanied by an elevation of the mRNA levels of this factor in the liver, spleen, and lung [6–9]. Third, rapid down-regulation of the high-affinity HGF receptor occurs in the plasma membranes of those rat livers [10]. Fourth, injection of HGF into normal or partially hepatectomized rats augments liver growth [11–13]. Enhancement of liver growth by HGF is also demonstrated in experiments using transgenic mice [14]. Fifth, continuous administration of neutralizing anti-HGF antibody to rats treated with carbon tetrachloride inhibits hepatocyte proliferation during liver regeneration [15]. Elevated HGF in plasma and liver after hepatic injury may play a role in promoting proliferation of the remaining hepatocytes [2].

HGF is mainly produced by mesenchymal cells such as fibroblasts and smooth muscle cells [1, 16]. HGF is induced by the activation of cAMP- and PKC-mediated pathways [17–19]. Its production is also stimulated by IL-1, tumor necrosis factor-α, interferon-γ, estrogen, a scatter factor-inducing factor, ascorbic acid, and growth factors such as EGF and basic fibroblast growth factor [20–26]. Thus, various serine/threonine protein kinases are involved in the signaling pathways for HGF induction. Protein phosphor-

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† *Abbreviations:* AP-1, activator protein-1; cAMP, adenosine 3′,5′-cyclic monophosphate; C/EBP, CCAAT/enhancer-binding protein; EGF, epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IL-1, interleukin 1; MAP kinase, mitogen-activated protein kinase; MEM, minimum essential medium; PKC, protein kinase C; and PMA, phorbol 12-myristate 13-acetate.

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ylation is controlled by the activity of not only protein kinases, but also protein phosphatases. The studies presented here were designed to determine whether serine/threonine protein phosphatases are involved in the regulation of HGF production. We used two potent and specific inhibitors of serine/threonine protein phosphatase 1 and 2A, okadaic acid and calyculin A, for this purpose. Although all serine/threonine protein phosphatases have been subdivided into four major classes (protein phosphatase 1, 2A, 2B, and 2C), protein phosphatase 1, 2A, and 2C are the predominant types of protein phosphatase present in tissue extracts [27]. Both inhibitors markedly stimulated HGF production in fibroblasts, indicating a down-regulation of HGF production through protein phosphatase(s).

MATERIALS AND METHODS

Materials

Eagle's MEM was purchased from Nissui Pharmaceutical Co. Okadaic acid and calyculin A were obtained from Wako Pure Chemical Industries. PD 98059 and GF 109203X were from Calbiochem-Novabiochem, and [α-^{32}P]dCTP (~110 TBq/mmol) was from Amersham. Human HGF cDNA (*Bam*HI/*Kpn*I fragment, 2.2 kbp) was derived from plasmids originally obtained from Dr. Naomi Kitamura (Tokyo Institute of Technology) [28]. Other reagents were obtained from the described sources [18, 19].

Cell Culture

Normal human skin fibroblasts, isolated from a 4-month-old girl [18], were used between the 6th and 12th passages. MRC-5 human embryonic lung fibroblasts obtained from the American Type Culture Collection were used between 56 and 64 population doubling levels. All cells were grown as monolayers in MEM supplemented with 10% FBS and 2 mM L-glutamine at 37° in a humidified atmosphere of 5% CO_2 and 95% air, as described previously [18, 19].

Determination of HGF Levels in the Conditioned Media

The human skin fibroblasts and MRC-5 cells, trypsinized and suspended in the medium described in the previous section, were seeded in 24-well plates (Nunc) at a density of 1.8×10^4 cells/cm^2 (1 mL/well). After reaching confluence, the medium was replaced with MEM supplemented with 10% FBS and 2 mM L-glutamine (human skin fibroblasts) or MEM supplemented with 1% FBS and 2 mM L-glutamine (MRC-5 cells). Okadaic acid, calyculin A, and others were then added. The conditioned medium was collected after incubating the cells for various periods and was frozen at −30° for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [29], with a slight modification [22]. The standard curve for human HGF was linear within the range of 0.025 to 5.0 ng/mL. HGF levels were expressed as ng/mg cellular protein [18, 19]. Each experiment was performed at least 3 times and all results are expressed as the mean and SEM of several independent experiments. The statistical significance was analyzed by Student's *t*-test. *P* values less than 5% were regarded as significant.

Northern Blotting

The medium of confluent human skin fibroblasts and MRC-5 cells grown in 9-cm dishes (Nunc) was replaced with fresh MEM supplemented with 10% FBS and 2 mM L-glutamine and MEM supplemented with 1% FBS and 2 mM L-glutamine, respectively, and both cultures were incubated for 24 hr. Okadaic acid was then added without a medium change. After incubation for an appropriate period, total RNA was isolated from the cells using RNAzol B (TEL-TEST). Northern blotting was performed as described previously [18]. Briefly, total RNA (10 μg) was denatured with 2.2 M formaldehyde and 50% formamide, fractionated on 1% agarose gels containing 2.2 M formaldehyde, and transferred to a Biodyne nylon membrane. The membrane-bound RNA was hybridized to a ^{32}P-labeled human HGF cDNA probe. After being washed, the membrane was exposed to an imaging plate at room temperature, and the plate was analyzed using a bio-imaging analyzer, BAS-2000 (Fuji Photo Film Co.). The membranes were rehybridized with a GAPDH cDNA probe as an internal control. The human HGF and GAPDH cDNA fragments were labeled with [α-^{32}P]dCTP by the multiprime DNA labeling system (Amersham) according to the manufacturer's instructions.

Western Blotting

The medium of confluent human skin fibroblasts grown in 6-well plates (Nunc) was replaced with fresh MEM supplemented with 10% FBS and 2 mM L-glutamine, and the cultures were incubated for 15 hr. Cells were then treated with or without PD 98059 for 2 hr followed with or without a 15-min okadaic acid treatment. Cells were washed once with ice-cold PBS, scraped into PBS, and washed 3 more times with PBS. Cells were then lysed by adding 100 μL of SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue). Lysates were briefly sonicated, boiled for 10 min, and centrifuged. Protein in extracts was quantified using Coomassie protein assay reagent (Bio-Rad). Equivalent protein aliquots were separated by 10% SDS–PAGE, transferred electrophoretically to Immobilon-P transfer membranes (Millipore), and probed with phosphospecific anti-MAP kinase antibody (New England Biolabs), which detects p42 and p44 MAP kinase only when catalytically activated by phosphorylation at tyrosine-204. Blots were incubated with horseradish peroxidase-conjugated secondary antibody (New England Biolabs) and developed with the enhanced chemiluminescence method (Wako Pure Chemical Industries).

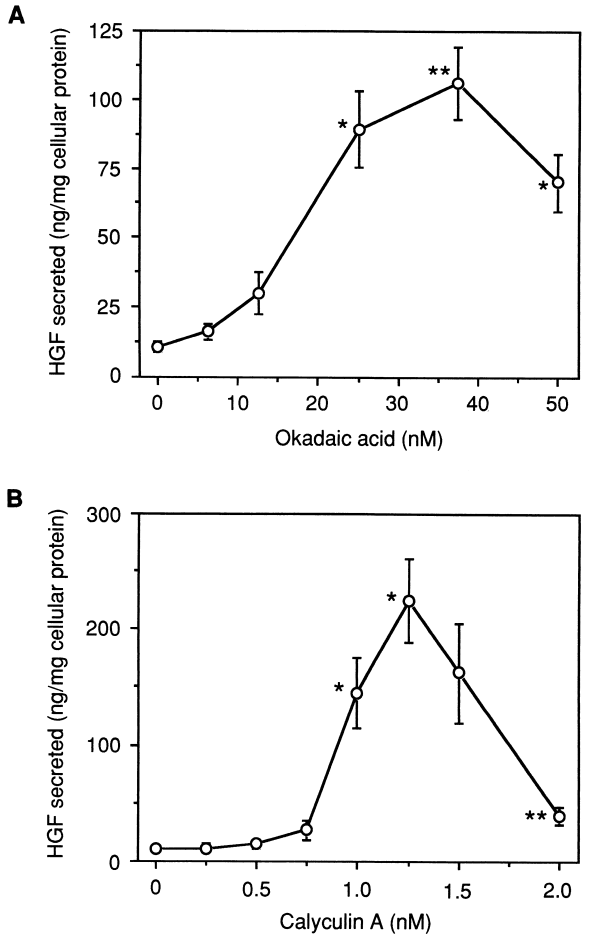

FIG. 1. Dose–response of okadaic acid- and calyculin A-induced HGF production in human skin fibroblasts. The confluent cells were incubated for 72 hr with the indicated concentrations of okadaic acid (A) and calyculin A (B). The HGF secreted into the medium was measured by an ELISA. The data are the means ± SEM of four (A) or three (B) independent experiments. Values that are significantly different from those of the control are indicated by: *$P < 0.05$; **$P < 0.01$.

RESULTS

Human skin fibroblasts were incubated for 72 hr with okadaic acid and calyculin A, and the amount of HGF secreted into the media was then measured by an HGF ELISA. Both okadaic acid and calyculin A increased HGF production in a dose-dependent manner (Fig. 1, A and B). The effect of okadaic acid was maximal at the concentrations of 25–37.5 nM, showing about 9-fold stimulation. Calyculin A was effective at concentrations much lower than okadaic acid. Maximal secretion by the cells exposed to calyculin A was observed at 1.25 nM and was about 20 times that by the untreated cells. MRC-5 human embryonic lung fibroblasts are known as highly active HGF-producing cells. Both okadaic acid and calyculin A also stimulated HGF production in MRC-5 cells: 407 ± 11, 469 ± 61, and 184 ± 29 (mean ± SEM) ng HGF secreted during 48-hr incubation per mg protein for 9 nM okadaic acid-treated, 1.5 nM calyculin A-treated, and control cells, respectively.

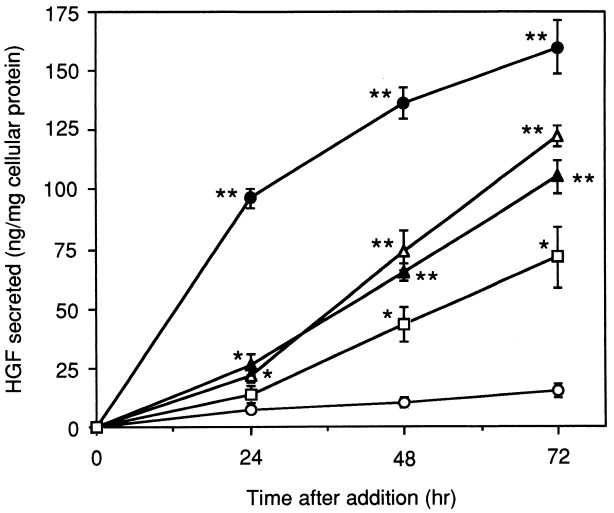

FIG. 2. Time–course of okadaic acid-stimulated HGF production in human skin fibroblasts. The confluent cells were incubated for the indicated periods without (○) or with 25 nM okadaic acid (△), 1 mM 8-bromo-cAMP (□), 7.5 nM PMA (●), and 10 ng/mL of EGF (▲). The HGF secreted into the medium was measured by an ELISA. The data are the means ± SEM of three independent experiments. Values that are significantly different from those of the control are indicated by: *$P < 0.05$; **$P < 0.01$.

The time–course of HGF production in human skin fibroblasts treated with okadaic acid was compared with that in cells treated with other HGF inducers (Fig. 2). The stimulating effect of okadaic acid was minimal at 24 hr, but marked at 48 and 72 hr. This time–course was similar to that of cells treated with EGF or 8-bromo-cAMP, but contrasts with that of PMA-stimulated HGF secretion, the rate of which was most marked during the first 24 hr.

Figure 3 shows the HGF mRNA levels in human skin fibroblasts incubated for given periods with okadaic acid. The level did not significantly increase before 24 hr after addition of okadaic acid. HGF mRNA levels in cells treated with okadaic acid for 24 and 48 hr were 400–500% of that of control cultures incubated in medium only. The lack of up-regulation of HGF mRNA expression before 24 hr coincides with the minimal increase in HGF secretion

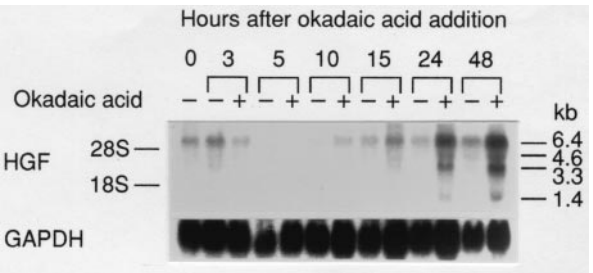

FIG. 3. Up-regulation of HGF gene expression by okadaic acid in human skin fibroblasts. The confluent cells were incubated with or without 37.5 nM okadaic acid for the indicated periods. Total RNA was isolated and Northern blotted using ^{32}P-labeled cDNA probes for human HGF, and then for human GAPDH.

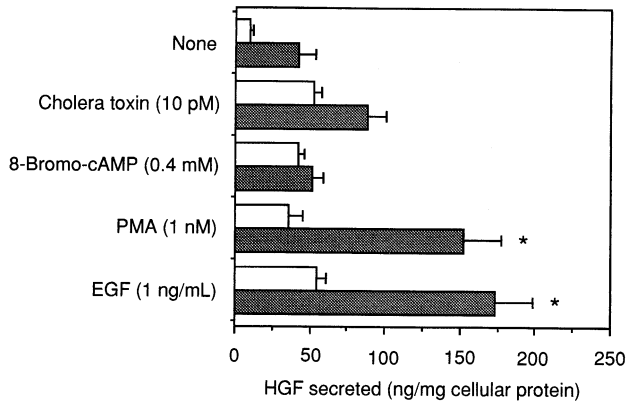

FIG. 4. Synergistic induction of HGF by okadaic acid and some HGF inducers in human skin fibroblasts. The confluent cells were incubated for 72 hr with the indicated concentrations of HGF inducers in the presence (shaded columns) and absence (open columns) of 12.5 nM okadaic acid. The HGF secreted into the medium was measured by an ELISA. The data are the means of three independent experiments. Bars indicate SEM. Values that are significantly different from those for the HGF inducer alone are indicated by: *$P < 0.05$.

during the first 24 hr. Okadaic acid also up-regulated HGF gene expression in MRC-5 cells (data not shown).

HGF production in human skin fibroblasts is stimulated by the activation of cAMP- and PKC-mediated pathways and various growth factors which are known to stimulate the MAP kinase cascade [17–19, 26, 30]. Since all of these pathways involve serine/threonine protein kinases and okadaic acid is an inhibitor of serine/threonine protein phosphatases, a combination of okadaic acid with the HGF inducers may synergistically accumulate the phosphorylated form of a target protein(s) and enhance HGF production. The results of such a combination experiment are shown in Fig. 4. The effect of okadaic acid was synergistic with that of EGF or the PKC-activating phorbol ester PMA, but was only additive to the effect of cholera toxin. Whether induction of HGF by okadaic acid is dependent on the activation of PKC or on the action of growth factors was then examined. The specific PKC inhibitor GF 109203X did not inhibit HGF induction produced by either okadaic acid or EGF, whereas it strongly inhibited the effect of PMA (Table 1). The effect of okadaic acid, like that of EGF, was not diminished, but rather augmented in the cells pretreated with a high concentration (0.5 μM) of PMA to deplete PKC (Table 1). The results in Table 1 suggest that the activation of PKC is not involved in HGF production induced by okadaic acid. Table 1 also shows the effect of PD 98059, an inhibitor of MAP kinase kinase [31], on HGF production stimulated by okadaic acid. HGF induction evoked by okadaic acid was significantly inhibited by PD 98059, although its inhibition was 43%, which is less than the 68% and 64% inhibitions, respectively, of EGF- and PMA-induced HGF production. The dose of PD 98059 used inhibited phosphorylation of MAP kinase induced by okadaic acid to the level below that in control cells cultured in medium only (Fig. 5). PD 98059 did not suppress HGF induction by cholera toxin (Table 1). Thus, the response of okadaic acid-induced HGF production to various treatments and inhibitors, including the MAP kinase kinase inhibitor, resembles that of EGF-induced HGF production, and thus the activation of the MAP kinase cascade may be involved, at least in part, in this induction.

DISCUSSION

The data presented in this report demonstrate that low concentrations of the specific protein phosphatase 1 and 2A inhibitors okadaic acid and calyculin A markedly induced HGF production not only in human skin fibroblasts but also in MRC-5 cells, a high HGF producer. These results suggest that HGF production is negatively regulated by protein phosphatase(s). Since protein phosphatase 2A is much more sensitive to okadaic acid than protein phosphatase 1 and okadaic acid concentrations of up to 50 nM dose dependently inhibit protein phosphatase 2A but not protein phosphatase 1 in NIH3T3 cells in culture [27, 32], the effect can most likely be attributed to protein phosphatase 2A. However, the possibility may not be ruled out that these long-term treatments with low concentrations of the

TABLE 1. Effects of the PKC inhibitor GF 109203X, PKC down-regulation with PMA, and the MAP kinase kinase inhibitor PD 98059 on HGF production stimulated by okadaic acid and other HGF inducers in human skin fibroblasts

HGF inducer	HGF secreted (ng/mg cellular protein)					
	GF 109203X		PKC down-regulation		PD 98059	
	−	+	−	+	−	+
None	10.2 ± 1.5	8.3 ± 1.5	16.4 ± 1.6	38.8 ± 5.5*	15.0 ± 1.6	5.8 ± 0.6*
Okadaic acid	79.0 ± 3.6	78.1 ± 2.7	106.8 ± 10.7	191.3 ± 5.5†	96.1 ± 4.4	54.9 ± 6.0†
Cholera toxin	53.3 ± 9.3	52.9 ± 12.9	61.7 ± 3.3	93.9 ± 8.7*	72.3 ± 4.7	98.5 ± 8.5
PMA	195.5 ± 19.0	55.6 ± 9.5*	213.6 ± 19.8	34.5 ± 5.0†	187.1 ± 25.3	66.5 ± 13.9*
EGF	111.6 ± 14.6	93.5 ± 12.7	117.7 ± 6.6	254.9 ± 18.8†	105.3 ± 11.7	34.2 ± 2.5*

The confluent cells were preincubated for 1 hr with or without 0.3 μM GF 109203X and for 2 hr with or without 25 μM PD 98059. The cells were then incubated for 72 hr with or without okadaic acid (25 nM), cholera toxin (10 pM), PMA (7.5 nM), and EGF (10 ng/mL) in the presence and absence of GF 109203X or PD 98059. For PKC down-regulation, the confluent cells were pretreated for 24 hr with or without 0.5 μM PMA. The cells were then washed and incubated with the indicated additives for an additional 72 hr in the presence or absence of 0.5 μM PMA. The HGF secreted into the medium was measured by an ELISA. The data are the means ± SEM of three independent experiments. Values that are significantly different from those for HGF inducer alone or those of cultures untreated with 0.5 μM PMA are indicated by: *$P < 0.05$; †$P < 0.01$.

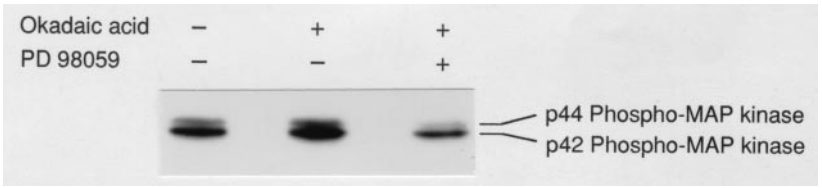

FIG. 5. Effect of PD 98059 on MAP kinase phosphorylation by okadaic acid in human skin fibroblasts. The confluent cells were preincubated for 2 hr with or without 25 μM PD 98059 and then incubated for 15 min with or without 25 nM okadaic acid. Equivalent protein aliquots (25 μg) of cell extracts were separated by SDS–PAGE, transferred to Immobilon-P membranes, and probed with phosphospecific anti-MAP kinase antibody.

inhibitors can cause unbalancings of protein phosphatase and protein kinase equilibria in cells and that the effects are thus indirectly related to phosphatase inhibition.

Concerning the molecular mechanism of okadaic acid-induced HGF production, the following lines of evidence suggest that the activation of the MAP kinase cascade is involved. First, HGF is induced by growth factors, including EGF, platelet-derived growth factor, and fibroblast growth factor, which activate MAP kinase in fibroblasts [26, 30]. Second, it has been reported that okadaic acid activates MAP kinase in several cell lines, including normal human skin fibroblasts [33–35] as confirmed in Fig. 5. Several studies also indicate that protein phosphatase 2A plays a negative role in the regulation of MAP kinase activity [34, 36]. The synergistic induction of HGF by the combination of okadaic acid and EGF (Fig. 4) may result from the inhibition of MAP kinase inactivation and the activation of MAP kinase, both of which lead to the accumulation of phosphorylated, activated MAP kinase. Third, the effect of okadaic acid was suppressed by the specific MAP kinase kinase inhibitor PD 98059 (Table 1). Although our conclusion relies on pharmacological inhibition, PD 98059 appears to have little effect on other protein kinase systems such as protein kinase A, PKC, and tyrosine kinases at the concentrations used [31]. In fact, it had no inhibitory effect on cholera toxin- or 8-bromo-cAMP-induced HGF production (Table 1). Despite a complete inhibition by PD 98059 of the increase in MAP kinase phosphorylation (Fig. 5), its inhibition of okadaic acid-induced HGF production was weaker than those of EGF- and PMA-induced HGF production (Table 1). These results suggest that an activation of another pathway(s) other than the MAP kinase cascade is also involved in the effect of okadaic acid. Although the PKC-activating phorbol ester PMA stimulated HGF production that was inhibited by PD 98059 (Table 1), this inhibition may be related to the PMA-induced activation of MAP kinase [30, 37] rather than PKC activation. Likewise, PKC activation is not involved in the effect of okadaic acid, because okadaic acid-induced HGF production was not inhibited by the PKC inhibitor and was also detected in the PKC-depleted cells (Table 1).

The signaling pathway for HGF induction downstream of the MAP kinase activation is not known. It is noteworthy, however, that okadaic acid has induced the activation of AP-1, which is abrogated by PD 98059 in a papilloma producing mouse keratinocytes [38], although it has not been confirmed whether the induction of HGF in the cells treated with PMA or EGF is mediated by AP-1. It has been suggested that the MAP kinase-mediated phosphorylation of JunD and FosB is critical to this AP-1 activation [38]. Another candidate downstream of the MAP kinase activation is the C/EBP family of transcription factors. Jiang and Zarnegar [39] have shown that up-regulation of the mouse HGF gene by PMA and some cytokines, such as EGF and IL-1, is mediated through the induction of DNA-binding activities of C/EBPβ (also known as NF-IL6) and C/EBPδ and their protein levels. Phosphorylation of threonine-235 of human C/EBPβ by MAP kinase has been documented to cause its activation [40]. Thus, it seems likely that an activation and/or induction of these transcription factors is involved in the signaling pathway for okadaic acid-induced HGF production downstream of the MAP kinase activation. There was a lag of more than 15 hr before up-regulation of HGF mRNA expression in okadaic acid-treated cells (Fig. 3). This long time lag suggests that okadaic acid stimulates HGF production by inducing the synthesis of some mediator.

There have been several instances in which the inhibition of protein phosphatase 1 and 2A induced the production of cytokines. Examples are induction of IL-1 and tumor necrosis factor-α by okadaic acid in human monocytes and macrophages [41, 42]. These effects of okadaic acid have been shown to be mediated through the activation of AP-1 and nuclear factor-κB (NF-κB) [41, 42]. Okadaic acid also stimulates nerve growth factor production in rat astroglial cells that is mediated by induction of IL-1 secretion [43]. IL-1, however, may not be involved in okadaic acid-induced HGF production, because exogenous IL-1 stimulates HGF production much less potently than okadaic acid in the human skin fibroblasts [22, 25].

In conclusion, this study demonstrated that the protein phosphatase 1 and 2A inhibitors okadaic acid and calyculin A markedly stimulated HGF production in fibroblasts, indicating that the serine/threonine protein phosphatase(s) plays a negative modulatory role in the regulation of HGF production. Our results suggest that okadaic acid-induced HGF production is mediated, at least in part, by the activation of the MAP kinase cascade. The activity of protein phosphatase 1 and 2A in mammalian cells has been shown to change in response to various extracellular signals [44]. These changes in HGF-producing cells might modulate HGF production.

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