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Biochimica et Biophysica Acta 1310 (1996) 163–167

BBA Biochimica et Biophysica Acta

Regulation of heparin-binding EGF-like growth factor expression by phorbol ester in a human hepatoma-derived cell line

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Received 16 February 1995; revised 25 July 1995; accepted 21 August 1995

Abstract

Heparin-binding EGF-like growth factor (HB-EGF) is a recently identified potent mitogen for smooth muscle cells and fibroblasts. HB-EGF has been shown to be an EGF receptor ligand, and also to stimulate epithelial cell growth. A human hepatoma-derived cell line, Mahlavu, was analyzed for the production of HB-EGF mRNA and active HB-EGF protein. It was found that the cell line synthesized very low or undetectable basal level of HB-EGF mRNA. However, the addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) led to a rapid and transient rise in HB-EGF mRNA level. HB-EGF in Mahlavu cells appears to be regulated by a protein kinase C (PKC) pathway, since PKC inhibitors, H7, staurosporin, and calphostin C, abrogated the induction of HB-EGF mRNA by TPA. Unlike vascular smooth muscle cells, induction of HB-EGF gene transcription by TPA was blocked completely by incubation with cycloheximide, suggesting that protein synthesis may be a prerequisite for HB-EGF gene transcription in Mahlavu cells. Mahlavu cells were also found to release a bioactive HB-EGF-like protein into conditioned medium which stimulates DNA synthesis in EP170.7 cells. This activity was neutralized by an anti-HB-EGF antibody. These results indicate that HB-EGF gene transcription is regulated via a PKC pathway, resulting in secretion of active HB-EGF into the culture medium of hepatoma-derived Mahlavu cells.

Keywords: Heparin-binding EGF-like growth factor; Phorbol ester; Protein kinase C; Hepatoma-derived cell line

1. Introduction

Heparin-binding EGF-like growth factor (HB-EGF) is a recently identified 20–22 kDa glycoprotein of a member of epidermal growth factor (EGF) family, which was originally identified as a secreted growth stimulator for vascular smooth muscle cells by macrophages and macrophage-like cells [1–4]. HB-EGF is synthesized as a transmembrane precursor protein and processed into a secreted form composed of up to 86 amino acids [5]. Secreted HB-EGF is significantly bigger than EGF and transforming growth factor-α (TGF-α) and is structurally homologous to them [5]. HB-EGF is characterized by a highly hydrophilic heparin binding domain at the N-terminal, three disulfide loops with spacing characteristic of the EGF family [5], and shares about 40% homology with EGF and TGF-α at the C-terminal.

HB-EGF has been found to be expressed by vascular endothelial cells [6] and vascular smooth muscle cells [7], and is a component of wound fluid [8]. It has been shown that the HB-EGF transcript can be consistently detected in a number tissues, particularly lung, skeletal muscle, brain, and heart in humans, mice and rats. Relatively low transcript levels have also been detected in the livers of all three species [9].

It has been shown that EGF and TGF-α stimulate the growth of normal hepatocytes [10–13]. It has also been shown that TGF-α gene overexpression in transgenic mice induces liver neoplasia, implicating an involvement of TGF-α in hepatocarcinogenesis [14,15]. On the other hand, we have reported a potent hepatotrophic activity of HB-EGF, and expression of HB-EGF mRNA in nonparenchymal cells [16]. Furthermore, we have recently reported overexpression of HB-EGF mRNA and its polypeptide in human hepatocellular carcinoma tissue [17]. However, regulatory mechanism of HB-EGF production in human hepatoma cells has not been reported.

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SSDI 0167-4889(95)00149-2

We report here that the basal HB-EGF gene transcription in Mahlavu cells, a human hepatoma cell line, is very low, but is induced rapidly and transiently by 12-O-tetradecanoylphorbol-13-acetate (TPA). PKC inhibitors and cycloheximide abrogated this induction of HB-EGF gene transcription by TPA. Therefore, HB-EGF gene transcription may be regulated via a PKC pathway, and protein synthesis may be a prerequisite of transcriptional induction by TPA in Mahlavu cells.

2. Materials and methods

2.1. Chemicals

12-O-Tetradecanoylphorbol-13-acetate (TPA), staurosporin, 1,2-dioctanoyl-*sn*-glycerol (DG), and cycloheximide were purchased from Sigma Chemicals (St. Louis, MO). [^{3}H]Thymidine, [^{32}P]dCTP and a multiprime DNA labeling kit were obtained from Amersham Japan, (Tokyo, Japan). 1-(5-Isoquinolinesulfonyl)2-2-methylpiperazine dihydrochloride (H7), and calphostin C [18], protein kinase C inhibitors, were obtained from Seikagaku Kougyo Corporation (Tokyo, Japan), and BIOMOL Research Laboratories (PA, USA), respectively.

2.2. Cell culture

Mahlavu cells [19] were cultured in Eagle's MEM (EMEM) with 10% FCS. For the analysis of HB-EGF gene expression, cells were seeded at a cell density of 2×10^5 cells/ml in 10-cm dishes and allowed to grow for 48 h in the presence of 10% FCS. Cells were washed with phosphate-buffered saline (PBS) three times, and the medium was changed to serum-free Cosmedium (Cosmo Bio., Tokyo, Japan) [20] containing variable doses of TPA. Following incubation for a variable number of hours, total RNA was isolated and subjected to Northern blot analysis. In order to investigate the effects of PKC inhibitors and cycloheximide on HB-EGF gene expression, cells were exposed to these substances under serum-free conditions for 2 h prior to incubation with TPA.

2.3. Northern blot analysis

Total RNA was isolated from cells according to the method of Chomczynski and Sacchi [21]. Twenty microgram of total RNA was fractionated on 1% agarose/formaldehyde gel in 1 × MOPS (3-[N-morpholino]propanesulfonic acid) buffer, and then transferred to a nylon membrane. The membrane was baked for 2 h at 80°C, and prehybridized in a solution of 50% formamide, 5 × SSPE (0.75 M NaCl, 25 mM $NaHPO_4$, 5 mM EDTA, pH 7.4), 0.5% (w/v) sodium dodecylsulfate (SDS), 5 × Denhardt's solution and 100 μg/ml salmon sperm DNA at 42°C. The membrane was hybridized overnight with a [^{32}P]dCTP-labeled human HB-EGF cDNA fragment spanning a portion of the coding and 3′-untranslated region [9] at 42°C. The membrane was washed twice with 2 × SSC and 0.1% SDS at room temperature, and twice with 0.1 × SSC and 0.1% SDS at 65°C for 15 min. Finally, the membrane was air-dried and exposed to a Kodak XAR film at −70°C.

2.4. Heparin affinity chromatography

TSK-heparin 5PW columns (8 × 7.5 mm, Toso, Japan) were equilibrated with 0.01 M Tris-HCl, 0.02 M NaCl (pH 7.4). Conditioned media were chromatographed on these columns by means of a fast-protein liquid chromatography (FPLC) system (Pharmacia LKB, Uppsala, Sweden). The columns were washed with 20 ml equilibration buffer, and bound protein was eluted with a 40-ml linear gradient of 0.2 M to 2 M NaCl in 0.01 M Tris-HCl (pH 7.4) at a flow rate of 1 ml/min. One millilitre fractions were collected, and a 10-μl aliquot from each fraction was tested for mitogenic activity on EP170.7 cells (a generous gift from Dr. Jackie Pierce, NIH, Bethesda) [22] as described below.

2.5. Growth factor assay

Growth factor activity was monitored by measuring [^{3}H]thymidine incorporation into the DNA of exponentially growing EP170.7 cells. Cells were grown in RPMI 1640 medium supplemented with 15% FCS, 5% interleukin-3 (IL-3) containing medium [23], and penicillin (100 U/ml)/streptomycin (100 μU/ml). After washing with RPMI 1640, EP 170.7 cells were plated at a density of 2×10^4 cells/well in 96-well plates. Appropriate FPLC column fraction aliquots were added in a total volume of 200 μl. Thirty-six hours later, 1 μCi of [^{3}H]thymidine was added in a volume of 10 μl PBS. The cells were harvested after 4 h and the incorporated [^{3}H]thymidine counts were determined by the 1205 Betaplate system (Pharmacia LKB). Recombinant HB-EGF protein (Scios Nova, Mountain View, CA) was used as a standard to estimate the HB-EGF protein content in samples.

2.6. Neutralization of growth factor activity

An anti-human HB-EGF neutralizing polyclonal antibody (no. 197) was directed against a 75-amino acid form of recombinant human HB-EGF and raised in goats by Dr. Deborah Damm (Scios Nova). The antibody (no. 197) specifically neutralizes human HB-EGF, but not human EGF, TGF-α, amphiregulin or β-cellulin [24]. In order to neutralize HB-EGF obtained from Mahlavu cell-conditioned media, appropriate amounts of anti-HB-EGF antibody no. 197, and 10 ml of fraction 22 in Fig. 4A (mitogenic activity peak) were added directly to an EP170.7 cell plate. Incorporation of [^{3}H]thymidine into DNA was measured as described above. Normal goat IgG was used in control incubations.

3. Results

3.1. Induction of HB-EGF mRNA by TPA in Mahlavu cells

Northern blot analysis shows a very low or undetectable level of HB-EGF transcripts; however, incubation of Mahlavu cells with the tumor promoter TPA resulted in a drastic increase in HB-EGF signal that are approx. 2.5 kb, similar to the major HB-EGF message expressed in cultured human macrophages and U-937 cells (Fig. 1A). This induction was transient, occurring within 2 h following initiation of TPA exposure, returning to basal levels at 6 h (Fig. 1B).

3.2. TPA-Induced HB-EGF gene expression was abrogated by protein kinase inhibitors

Since TPA is known to activate protein kinase C (PKC), it was reasonable to speculate that HB-EGF mRNA induction by TPA might be regulated via a PKC pathway. DG, a potent activator of PKC, also stimulated HB-EGF gene expression (Fig. 1C). Then, the effect of staurosporin (a general protein kinase inhibitor) and H7 and calphostin C (PKC inhibitors) on TPA-induced HB-EGF gene expression was investigated. As shown in Fig. 2, 50 nM staurosporin, 50 μM H7 or 0.5 μM calphostin C completely inhibited TPA-induced HB-EGF gene expression. These results suggest that PKC might be involved in the mechanism of HB-EGF mRNA induction by TPA.

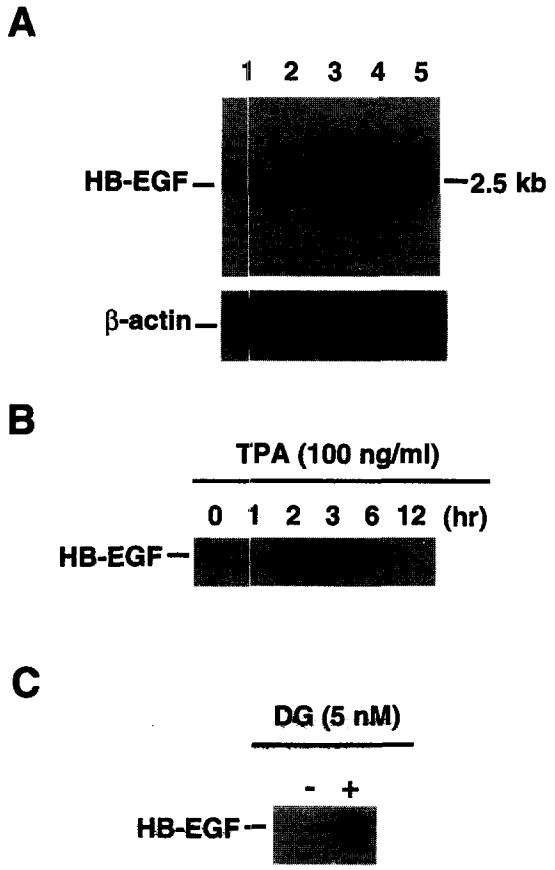

Fig. 1. Induction of HB-EGF gene expression by TPA in Mahlavu cells. A: HB-EGF mRNA in Mahlavu cells incubated with incremental doses of TPA for 3 hr under serum-free conditions. Lane 1: without addition of TPA; and lanes 2, 3, 4, and 5: incubated with 1, 10, 100, and 1000 ng/ml of TPA, respectively. Rehybridization with β-actin cDNA [31] represented an internal standard. B: HB-EGF mRNA in Mahlavu cells incubated with 100 ng/ml TPA up to 12 h. C, HB-EGF mRNA in Mahlavu cells incubated with 5 μM DG for 3 h.

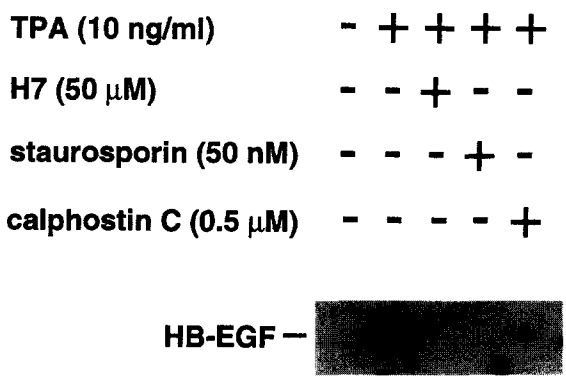

Fig. 2. Effect of protein kinase inhibitors on TPA-induced HB-EGF gene expression in Mahlavu cells. Cells were exposed to H7 (50 mM), or staurosporin (50 nM) or calphostin C (0.5 μM) for 2 h prior to incubation with TPA (10 ng/ml). As to calphostin C, cells were incubated with this agent under ordinary fluorescent lighting.

3.3. Effect of cycloheximide on HB-EGF mRNA induction by TPA

HB-EGF mRNA level is induced in smooth muscle cells within 1 h following exposure to TPA [6]. In contrast, HB-EGF mRNA induction by TPA was not seen within 1 h following TPA exposure in Mahlavu cells, suggesting that the mechanism of HB-EGF gene induction by TPA in Mahlavu cells might be different than in smooth muscle cells, and that PKC might not be the primary regulatory pathway. An inhibitor of protein synthesis, cycloheximide, inhibited HB-EGF mRNA induction by TPA (Fig. 3). These results indicate that some protein synthesis might be required for the induction of HB-EGF gene transcription in Mahlavu cells.

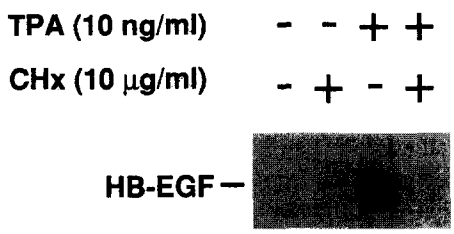

Fig. 3. Effect of cycloheximide on TPA-induced HB-EGF gene expression in Mahlavu cells. Cells were exposed to cycloheximide for 2 h prior to incubation with TPA (10 ng/ml).

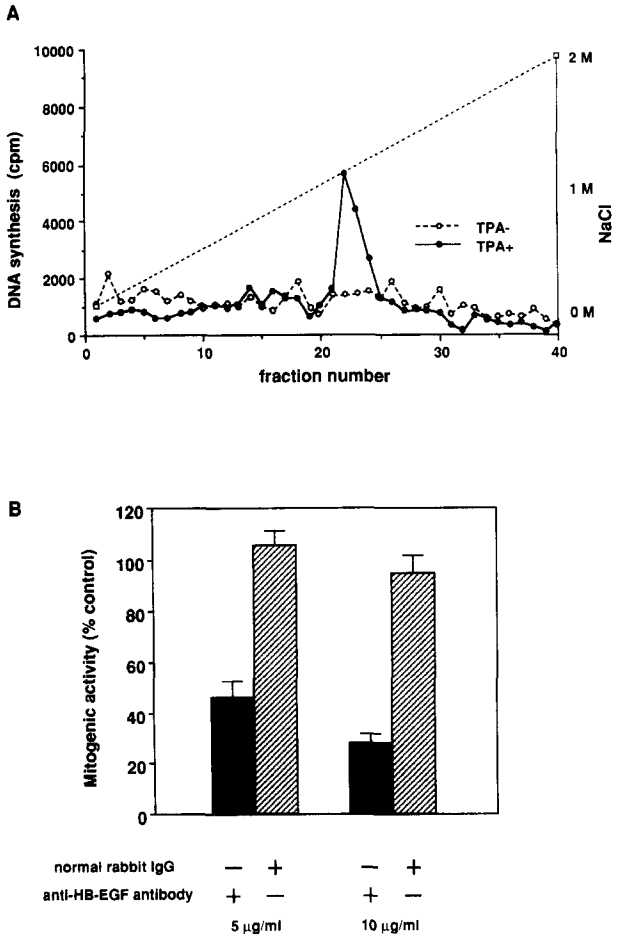

Fig. 4. HB-EGF activity in conditioned media from Mahlavu cell cultures. A: Conditioned media from Mahlavu cell cultures were incubated with or without TPA (10 ng/ml) for 24 h, and subjected to a TSK-heparin 5PW affinity column. HB-EGF activity in the conditioned medium was determined as described in Section 2. B: HB-EGF activity of fraction 22 from A was neutralized by an anti-human HB-EGF polyclonal antibody in a dose-dependent manner, as assayed by mitogenic activity for EP170.7 cells. Values are expressed as mean ± S.E. of triplicate experimental samples.

3.4. Identification of Mahlavu cell-derived HB-EGF in the conditioned medium

Although HB-EGF mRNA was readily detectable in Mahlavu cells, it was important to demonstrate that these cells also produce HB-EGF protein, since it is known that growth factor mRNA is not always translated into protein in some cells [25]. HB-EGF-like protein in Mahlavu cell-derived conditioned media was assessed by a combination of TSK-heparin affinity chromatography and an EP170.7 cell assay. HB-EGF-like activity (identified by increased DNA synthesis in EP170.7 cells) was undetectable in conditioned medium from Mahlavu cells not exposed to TPA (Fig. 4A). Although incubation with TPA for 6 h was not enough to elevate HB-EGF-like activity to be a detectable level (data not shown), detectable HB-EGF-like activity accumulated in conditioned medium after 24 h. Mahlavu cell-derived presumed HB-EGF was eluted from a TSK-heparin column with 1.0 M to 1.2 M NaCl (Fig. 4A). Anti-HB-EGF antibody no. 197 was then added prior to the EP170.7 cell assay. We found that the EP170.7 cell mitogenic activity was neutralized by anti-HB-EGF antibody in a dose-dependent manner. Ten micrograms per millilitre of the antibodies inhibited approx. 80% of the mitogenic activity (Fig. 4B). These results demonstrate that Mahlavu cells release an active form of HB-EGF protein into their culture media in the presence of TPA.

4. Discussion

HB-EGF, a member of the epidermal growth factor (EGF) family, is a potent mitogen for fibroblasts, smooth muscle cells [1], and epithelial cells including keratinocytes and hepatocytes [16]. HB-EGF appears to act on target cells through EGF receptor, and may play a role in the regulation of epithelial cell growth. Recently, we have reported overexpression of HB-EGF mRNA and its polypeptide in human hepatocellular carcinoma tissue, implicating an involvement of HB-EGF in hepatocarcinogenesis [17]. However, the regulatory mechanism of HB-EGF production has not been investigated in human hepatoma cells.

Mahlavu cells express a very low or undetectable level of HB-EGF mRNA, but HB-EGF transcripts in this cell line are increased significantly by a tumor promoter, TPA. Staurosporin and H7, as well as a PKC specific inhibitor calphostin C, inhibited HB-EGF mRNA induction by TPA, strongly suggesting that PKC activation may play a central role in the induction of HB-EGF gene expression in Mahlavu cells.

Induction of HB-EGF gene expression has been reported recently in vascular endothelial cells by TNF-α or IL-1β [6], rat aortic smooth muscle cells by TPA or angiotensin II [7], and in fetal human vascular smooth muscle cells by serum, TPA or smooth muscle growth factors including PDGF, bFGF and HB-EGF [23]. HB-EGF gene expression in the above-mentioned cells is characteristic of early response genes such as nuclear transcription factors, cytoskeletal and extracellular matrix proteins, and secreted cytokines [26].

The regulatory mechanism of HB-EGF gene expression in Mahlavu cells seems to be different than in smooth muscle cells [23], since (1) the mRNA levels did not increase until after the first hour, (2) mRNA induction was more pronounced and transient, and (3) cycloheximide abrogated HB-EGF mRNA induction in Mahlavu cells. It was therefore possible that earlier molecular events preceded TPA-related induction of HB-EGF gene expression.

We have found that Mahlavu cells also secretes biologically active HB-EGF protein. Since the presence of HB-EGF mRNA is not always associated with HB-EGF protein synthesis, and HB-EGF has alternative forms includ-

ing a soluble form (mature form) and a transmembrane form (precursor form), it was also important to analyze HB-EGF protein. Mature HB-EGF protein, consisting of up to 86 amino acids [5], is processed from a 208-amino acid precursor by cleavage at the transmembrane site [5]. The transmembrane form of HB-EGF is also the receptor for diphtheria toxin, and expression of HB-EGF precursor/diphtheria toxin receptor therefore makes cells highly sensitive to this toxin [27]. Since processing of TGF-α is induced by TPA [28,29], enhanced processing of HB-EGF precursor might occur by TPA via a similar processing mechanism. In fact, treatment of a human breast carcinoma cell line synthesizing mostly transmembrane form of HB-EGF with phorbol ester resulted in loss of cell surface HB-EGF, lack of sensitivity to diphtheria toxin, and accumulation of mature HB-EGF in conditioned medium [30]. On this basis, our results show that mature HB-EGF elevated in the conditioned medium after HB-EGF gene activation by TPA treatment may be responsible, in part, for the acceleration of the processing enzyme activities by TPA. Furthermore, this processing modulated by TPA may be an alternative regulatory mechanism for the HB-EGF molecule secretion, acting in autocrine, paracrine, and juxtacrine manners.

In conclusion, we have shown that HB-EGF gene expression may be regulated via a PKC pathway, resulting in secretion of active HB-EGF into the culture medium of hepatoma-derived Mahlavu cells.

Acknowledgements

We thank Dr. D. Damm and Dr. J.A. Abraham at Socios Nava, Mountain View, CA, for providing us with the human HB-EGF cDNA probe and the neutralizing anti-human HB-EGF polyclonal antibody.

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