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Mitogen-induced expression of the primary response gene cMG1 in a rat intestinal epithelial cell-line (RIE-1)

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cMG1 is a primary response gene first identified in a rat intestinal epithelial (RIE-1) cell-line [(1990) Oncogene 5, 1081–1083]. A number of mitogens, including epidermal growth factor (EGF), angiotensin II (AII), serum and insulin rapidly induced 2- to 6-fold increases of cMG1 mRNA in RIE-1 cells, while transforming growth factor-β caused a small reduction. Cyclic AMP-elevating agents blocked the increase of cMG1 mRNA induced by EGF. The AII-stimulated increase of cMG1 mRNA was blocked by the depletion of protein kinase C, whereas the EGF-stimulated increase was not affected, indicating that protein kinase C-dependent and -independent signalling pathways stimulate cMG1 expression.

Primary response gene; Mitogen stimulation; Epidermal growth factor; Angiotensin II; Protein kinase C; RIE-1 epithelial cell

1. INTRODUCTION

Primary response genes are a class of genes whose expression is rapidly and transiently induced by the treatment of cells with agonists such as hormones, growth factors and mitogens (for review see [1]). Many of these genes have been identified by differential hybridization screening of cDNA libraries prepared using mRNA isolated from mitogen-stimulated cells [1,2]. Several of the known primary response genes encode nuclear proteins which have been demonstrated to be either transcription factors or transcriptional modulators, including the proto-oncogenes c-*fos* and c-*jun*, three members of the C_2H_2 zinc-finger family of proteins (egr-1, -2 and -3), and nur77, a member of the steroid hormone receptor super-family [1].

While several primary response genes have been identified using mitogen-stimulated fibroblasts, fewer studies have addressed the question of early genetic responses to growth factors in epithelial cells. We have derived an epithelial cell-line (termed RIE-1) from the small intestine of a rat [3], and demonstrated that these cells are mitogenically responsive to epidermal growth factor (EGF), insulin-like growth factors (IGFs) and the peptide hormone angiotensin II (AII) [4–6]. Recently, we used differential hybridization screening of a cDNA library prepared from EGF-treated RIE-1 cells to identify an EGF-inducible gene, which we have termed cMG1 [7,8]. This is a member of a new family of primary response genes, which currently contains three members: TIS11 [9,10] (also known as Nup475 [11] or TTP [12]), cMG1 [7,8] (also known as TIS11b [10]) and TIS11d [10]. The sequences of the proteins in this family show a marked conservation of a 67-amino acid region, the structure of which suggests the possible formation of two zinc-finger-like domains. Indeed, the TIS11/Nup475 protein has been found to bind zinc and to be localized to the nucleus [11]. These observations have led us and others to speculate that this family of proteins might constitute a novel group of mitogen-inducible transcription regulators [8,10,11].

The induction of TIS11 expression has been studied in some detail using 3T3 fibroblasts [10–13], PC12 pheochromocytoma cells [10], and a murine B-cell hybridoma [14]. In contrast, there is very little information about the induced expression of cMG1/TIS11b. The purpose of this work, therefore, was to measure the induction of cMG1/TIS11b mRNA by various mitogens in the RIE-1 epithelial cell line, and to define the signal transduction pathways regulating cMG1 gene expression.

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Abbreviations: AII, angiotensin II; EGF, epidermal growth factor; FGF, fibroblast growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

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2. EXPERIMENTAL

2.1. *Materials*

Reagents for cell culture were obtained from Flow Laboratories. EGF was prepared from mouse submaxillary glands [15]. Insulin, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 3-isobutyl-1-methylxanthine (IBMX), prostaglandin E1, forskolin and noradrenaline were obtained from Sigma. [α-^{32}P]dCTP, IGF-1 and platelet-derived growth factor were obtained from Amersham International, transforming growth factor-β (TGF-β) was from R&D Systems, and AII and bombesin were from Bachem. The cDNA probe for cMG1 was a 1262 bp *Eco*RI fragment spanning the coding sequence [8]. The probes for *fos* and glyceraldehyde phosphate dehydrogenase (GAPDH) were a 1 kb *Pst*I fragment of v-*fos* and a 250 bp *Hin*dIII-*Pst*I fragment of GAPDH provided by Dr. T. Rabbitts (MRC Laboratory of Molecular Biology, Cambridge) and Dr. L.C. Mahadevan (Department of Biochemistry, University of Oxford) respectively.

2.2. *Cell maintenance and treatments*

Stock cultures of RIE-1 and Swiss 3T3 cells were maintained and passaged as described previously [3,4,16]. Cells for experiments were grown on 9 cm dishes for 7–10 days, by which time they were confluent and quiescent. Approximately 10^7 cells (1 dish of RIE-1 cells, or 4 dishes of 3T3 cells) were used per treatment. In most of the experiments described, the mitogens were added directly to the cells in the serum-depleted medium; however, similar results were obtained when this medium had been replaced with fresh serum-free medium immediately before the addition of the mitogens. The cells were incubated with the mitogens for the times indicated at 37°C, and were then lysed on the dish by the addition of 0.6 ml (per dish) of denaturing solution (solution D of [17]). The cell lysates were scraped into centrifuge tubes, and the total RNA was isolated as described [17].

2.3. *Analysis of RNA*

Aliquots (10 μg) of the RNA samples were analysed by Northern blotting as described previously [18]. DNA probes were labelled with ^{32}P to specific activities of approximately 10^9 cpm/μg DNA using [α-^{32}P]dCTP as described previously [19]. Filters were prehybridized and hybridized as described [19], washed at 65°C in several changes of 0.2 × SSC/0.1% (w/v) SDS (1 × SSC consists of 150 mM NaCl, 15 mM Na citrate), and exposed to X-ray film for between 6 and 18 h at −70°C with intensifier screens. When blots were analysed sequentially with cDNA probes for different primary response genes and GAPDH, each probe was removed by the immersion of the filter in water at >90°C; the removal of the probe was checked by an overnight exposure of the filter to X-ray film as described above, before the addition of the next labelled probe. The autoradiographs were scanned using a Chromoscan densitometer (Joyce-Loebl), and the values were corrected for any differences in loading that were revealed by scans of the GAPDH autoradiographs.

3. RESULTS AND DISCUSSION

3.1. *Time-course of the induction of cMG1*

The time-course of the induction of cMG1 mRNA in RIE-1 cells treated with EGF is shown in Fig. 1. cMG1 expression rose rapidly after the addition of EGF, reaching a maximum after 60 min and subsequently returning towards basal levels by 180 min. Experiments using TPA and AII indicated that maximal stimulation of cMG1 mRNA levels by these agents also occurred after approximately 60 min (data not shown). This is a somewhat slower and less transient response than that reported for the induction of the related TIS11 mRNA in several cell types [10,13,14]. Similarly, different members of other primary response gene families (e.g. c-*fos*, *fra*-1 and *fra*-2 [20]) also show different time-courses of induced expression, but the functional significance of these differences remains to be established.

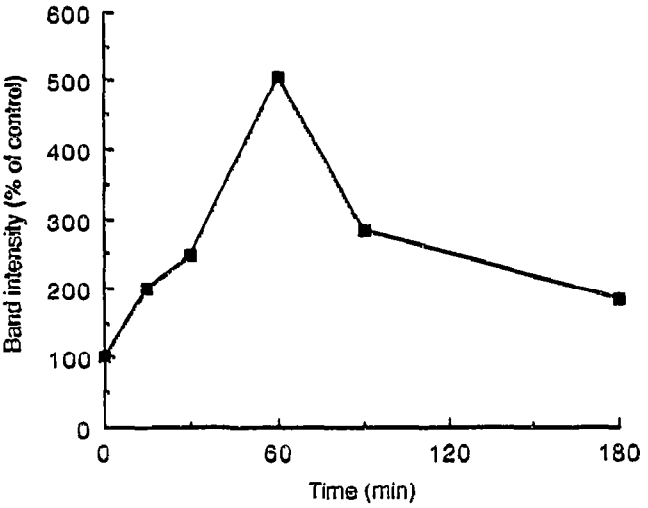

Fig. 1. Time-course of EGF-induced cMG1 expression in RIE-1 cells. Confluent, quiescent cultures of RIE-1 cells were incubated with EGF (100 ng/ml) at 37°C for the time indicated. RNA was prepared from the cells as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The blot was probed sequentially for cMG1 and GAPDH, and the autoradiographs were scanned using a Chromoscan densitometer. The results shown are the expression of cMG1 relative to that in control cells, after correction for minor differences in the loading of RNA that were revealed by the scans of the GAPDH autoradiograph. Similar results were obtained in 3 other experiments.

3.2. *The stimulation of cMG1 expression by various mitogens*

We have investigated the ability of all the known mitogens for RIE-1 cells to stimulate cMG1 expression in these cells. As shown in Fig. 2a, EGF, AII and TPA all substantially stimulated cMG1 expression (between 3- to 6-fold over the basal level in different experiments). In general, AII and TPA gave the greatest stimulation of cMG1 expression, while EGF was slightly less effective. In addition, insulin and IGF-1 induced smaller but consistent increases (about 2- to 3-fold) in the level of cMG1 mRNA (Fig. 2b). The addition of serum consistently gave a smaller stimulation than that obtained using the individual mitogens AII, TPA and EGF (Fig. 2). In contrast to the other factors tested, TGFβ caused a small but reproducible reduction of the level of cMG1 mRNA (Fig. 2a). This is an interesting result because, as in other epithelial cell lines, TGFβ is a potent inhibitor of RIE-1 cell proliferation (R.D. Smith and K.D.B., unpublished results).

Varnum et al. [10] found only 'modest induction' of cMG1/TIS11b mRNA in 3T3 fibroblasts stimulated using EGF or fibroblast growth factor (FGF). These workers suggested that our original cloning of cMG1

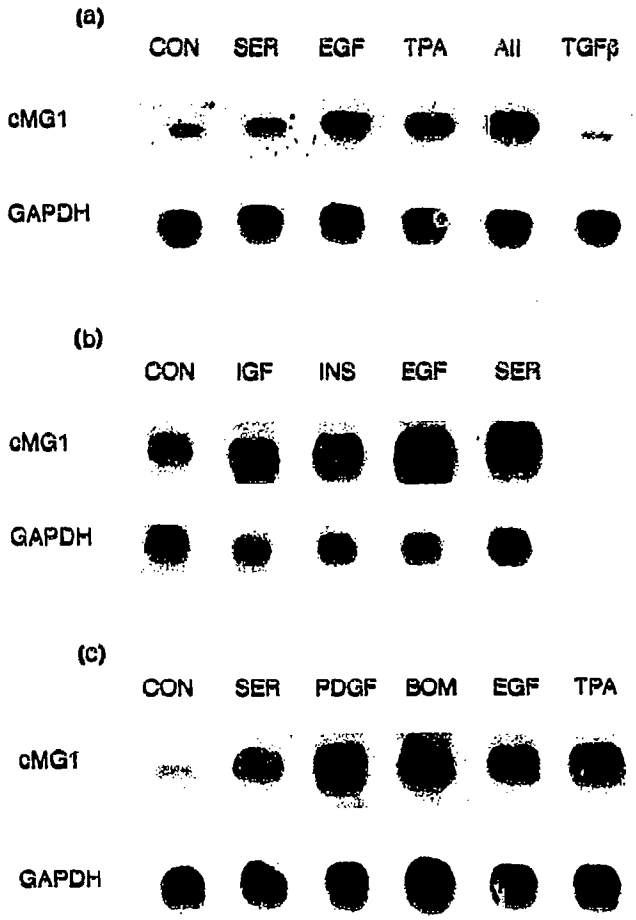

Fig. 2. Stimulation of cMG1 expression in RIE-1 cells (a,b) and Swiss 3T3 cells (c) by various mitogens. Confluent, quiescent cell cultures were incubated at 37°C for 60 min, in the presence of the following mitogens: serum (SER; 10%, v/v), EGF (100 ng/ml), TPA (30 nM), angiotensin II (AII; 100 nM), TGFβ (1 ng/ml), IGF-1 (100 ng/ml), insulin (INS; 1 μg/ml), PDGF (10 ng/ml) or bombesin (BOM; 10 nM). Control cells (CON) received an equal volume addition of serum-free medium. RNA was prepared from the cells as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The blots were probed sequentially for cMG1 and GAPDH. In (a) and (c), mitogens were added to the cells in the serum-depleted medium in which they had been grown. In (b), mitogens were added to cells whose medium had been replaced with serum-free medium immediately before the experiment (to remove IGF-binding proteins which could interfere with the response to IGF-1). Similar results were obtained in at least two other experiments with each mitogen.

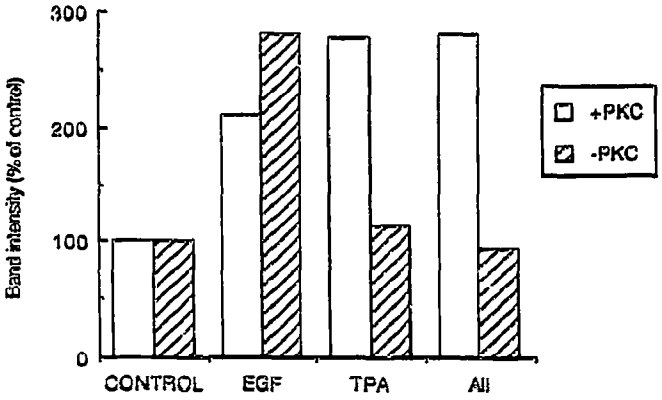

Fig. 3. Dependence of the stimulation of cMG1 expression on protein kinase C (PKC). Confluent, quiescent cultures of RIE-1 cells were pretreated for 72 h with TPA (300 nM) to down-modulate PKC; immunoreactive PKC was decreased to <5% of control levels, as assessed by Western blotting. Control cells (open columns) and PKC-depleted cells (hatched columns) were incubated at 37°C for 60 min in the presence of EGF (100 ng/ml), TPA (30 nM) or angiotensin II (AII; 100 nM). RNA was prepared from the cells as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The blot was probed sequentially for cMG1 and GAPDH, and the autoradiographs were scanned using a Chromoscan densitometer. The results shown are the expression of cMG1 relative to that in the appropriate control cells, after correction for minor differences in the loading of RNA that were revealed by the scans of the GAPDH autoradiograph. Very similar results were obtained in a second experiment of this design.

from an EGF-stimulated epithelial cell line by differential hybridization raised 'the exciting possibility that cell-type-specific differences' occur in the response of this gene to the same ligand. In order to explore this possibility, we have studied cMG1 mRNA levels in Swiss 3T3 cells stimulated by various mitogens. As in RIE-1 cells, cMG1 mRNA levels were increased up to 5-fold by serum, TPA or growth factors including EGF (Fig. 2c). Thus, the size of the induction of cMG1 mRNA in Swiss 3T3 fibroblasts is apparently not significantly different from that in the RIE-1 epithelial cells. However, in both cell lines there is a significant basal expression of cMG1/TIS11b mRNA (Fig. 2 and [10]), and this reduces the proportional stimulation observed when compared to those primary response genes which show virtually undetectable basal expression, such as TIS11 [10] or c-*fos* [7,21,22] (see also Fig. 4).

3.3. *Signalling pathways involved in the induction of cMG1 expression*

The marked increase in cMG1 mRNA levels in TPA-stimulated cells indicates that cMG1 gene expression can be induced via the activation of protein kinase C. We have therefore tested whether this control enzyme is involved in the signalling pathways by which other mitogens activate the cMG1 gene, by pretreating RIE-1 cells with TPA to down-regulate protein kinase C [23] before stimulating them with TPA, EGF or AII (Fig. 3). In the absence of pretreatment with TPA, each mitogen increased cMG1 mRNA levels. However, the responses to TPA and AII were effectively eliminated in protein kinase C-depleted cells (Fig. 3), indicating that there is an absolute requirement for protein kinase C in the stimulation of cMG1 expression by AII. Thus, although this concentration of AII transiently increases cytoplasmic [Ca^{2+}] from approximately 100 nM to 800 nM in these cells [6], this Ca^{2+} signal is apparently not sufficient for the AII-induced expression of cMG1. In contrast to the response to TPA and AII, the induction of

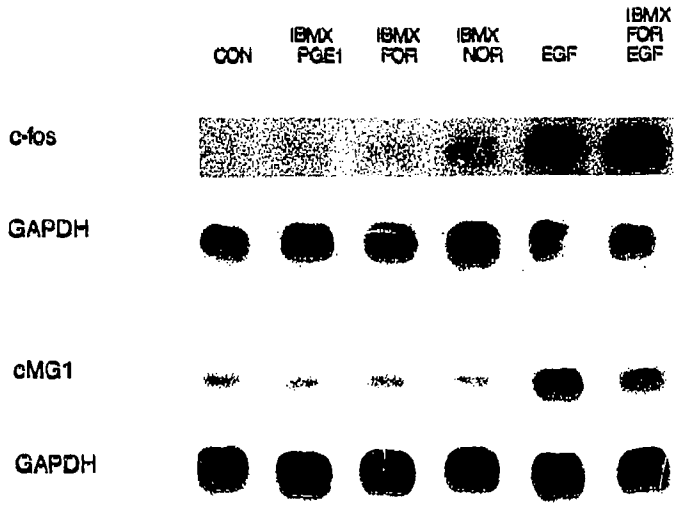

Fig. 4. Expression of c-*fos* and cMG1 in response to cyclic AMP-elevating agents. Confluent, quiescent cultures of RIE-1 cells were incubated at 37°C for 30 min or 60 min, in the presence of EGF (30 ng/ml), IBMX (100 μM), prostaglandin E1 (PGE1; 1 μM), forskolin (FOR; 10 μM) or noradrenaline (NOR; 10 μM) as indicated. RNA was prepared as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The blots were probed sequentially for cMG1, c-*fos* and GAPDH, and the results are shown for c-*fos* at 30 min and cMG1 at 60 min. The potentiation by IBMX/FOR of the EGF-stimulated c-*fos* response was also detected in the 60-min samples, and the reduction by IBMX/FOR of the EGF-stimulated cMG1 response was also detected in the 30-min samples (data not shown).

cMG1 by EGF is not inhibited in TPA-pretreated cells (Fig. 3), indicating that a protein kinase C-independent signalling pathway is utilised by the EGF receptor in inducing cMG1 expression. Similarly, the induction of TIS11 by EGF or FGF in fibroblasts [13] or by interleukin-6 in a B-cell hybridoma [14] can occur through protein kinase C-independent pathways.

Among the agonists that have been found to stimulate the expression of certain primary response genes are those which activate the cyclic AMP pathway. For example, TIS11 expression in 3T3 fibroblasts is induced by treatment with forskolin [11]. In RIE-1 cells, forskolin, prostaglandin E1 and noradrenaline, each used in the presence of the phosphodiesterase inhibitor IBMX, stimulate 10- to 50-fold accumulations of cyclic AMP (R.D. Smith and K.D.B., unpublished data). However, these cyclic AMP-elevating agents had no significant effect on the level of cMG1 mRNA in unstimulated RIE-1 cells, and forskolin and IBMX caused a reduction in the stimulation of cMG1 expression by EGF (Fig. 4). This was not a non-specific inhibitory effect on gene expression, since the cyclic AMP-elevating agents induced a small stimulation of c-*fos* mRNA in the same experiment, and forskolin and IBMX potentiated the induction of c-*fos* mRNA by EGF (Fig. 4). Similarly, the elevation of cyclic AMP has previously been reported to potentiate the induction of c-*fos* mRNA by bombesin in Swiss 3T3 cells [24]. Thus, the elevation of cyclic AMP has opposite effects on the expression of c-*fos* and cMG1 in RIE-1 cells.

Taking all of these results together, it is clear that a similar range of agonists (and thus, presumably, signalling pathways) regulate cMG1 mRNA expression in both RIE-1 and Swiss 3T3 cells. It is also apparent that there are both similarities and differences between the regulation of cMG1 expression and that reported for the cMG1-related gene, TIS11. It will therefore be of considerable interest to use reporter gene constructs to dissect and compare the promoter elements present in the cMG1 and TIS11 genes.

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