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Insulin and insulin-like growth factor I stimulate expression of the primary response gene cMG1/TIS11b by a wortmannin-sensitive pathway in RIE-1 cells

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Abstract The addition of insulin or insulin-like growth factor I (IGF-I) to RIE-1 cells increased the expression of the primary response gene cMG1; dose–response analysis suggested that this effect was mediated largely through type 1 IGF receptors. Insulin/IGF-I did not affect the expression of the cMG1-related genes TIS11 and TIS11d, whereas epidermal growth factor, angiotensin II or 12-*O*-tetradecanoyl phorbol-13-acetate stimulated the expression of all three genes. Incubation with wortmannin (WM) prevented the insulin/IGF-I-induced elevation of cMG1 mRNA, but not that induced by the other mitogens or the stimulation of mitogen-activated protein kinase by insulin. We conclude that WM-sensitive phosphatidylinositol 3-kinase may be involved in the specific stimulation of cMG1 expression by insulin/IGF-I.

Key words: Insulin; Primary response gene; Phosphatidylinositol 3-kinase; Mitogen-activated protein kinase; RIE-1 epithelial cell

1. Introduction

Growth factors and hormones stimulate rapid and transient increases in the expression of members of the class of genes termed primary response genes (for review see [1]). Several of these genes, such as the proto-oncogenes c-*fos* and c-*jun*, encode nuclear proteins that have been demonstrated to be either transcription factors or transcriptional modulators, and may thus be important in coordinating the genetic changes that result from cellular stimulation. Using differential hybridisation screening of a rat intestinal epithelial cell line (RIE-1) stimulated with epidermal growth factor (EGF), we identified a primary response gene named cMG1 [2,3]. This is one of three members of a new family of genes, which code for proteins containing a markedly conserved 67-amino acid region including two potential C_3H zinc-finger-like domains [4]. A second member of the family, named TIS11 [4], TTP [5] or Nup475 [6], has indeed been shown to bind zinc, and is also localised to the nucleus [6], which is consistent with a role as a transcriptional regulator. TIS11 mRNA is elevated from a low basal level by various agonists [4–6], while cMG1 (mouse TIS11b) is also stimulated but from a more readily detected basal level [4,7]; less is known about the third family member, TIS11d, which showed only limited stimulation by phorbol ester in 3T3 cells [4]. Insulin and insulin-like growth factor-I (IGF-I) were among the agonists which stimulated cMG1 expression in RIE-1 cells and Swiss 3T3 cells [7], while TIS11/TTP was identified as an insulin-stimulated gene in transfected NIH-3T3 cells expressing high numbers of insulin receptors [5]. These observations raised the possibility that the cMG1 gene family might be particularly relevant to the action of insulin/IGF-I. We have therefore investigated the specificity and mechanism of activation of this gene family by insulin/IGF-I in RIE-1 cells.

Signalling responses to many polypeptide agonists are initiated by the intrinsic protein-tyrosine kinase activity of their receptors [8–10]. Ligand-stimulated auto-phosphorylation of the receptors for platelet-derived growth factor and EGF, on tyrosine residues within specific target sequences, induces interactions with signalling molecules which are then activated by conformational change and/or phosphorylation [8,9]. A similar targeting mechanism occurs at the insulin and type 1 IGF receptors, except that the signalling molecules bind to phosphotyrosyl targets on an adaptor molecule [8,10]. The major adaptor is IRS-1, which is the principal substrate for the receptor tyrosine kinases [10], although other adaptors allow certain responses to occur after the targeted deletion of the IRS-1 gene [11,12]. Among the best characterised of the signalling pathways thus recruited to IRS-1 is the so-called mitogen-activated protein kinase (MAP kinase) pathway. The MAP kinase ERK-2 is activated by receptor tyrosine kinases via a series of components termed Grb2, Sos, $p21^{ras}$, Raf-1 and MAP kinase kinase, and a number of targets for this pathway are known [9,10,13]. Another signalling molecule that is activated when bound to IRS-1 is phosphatidylinositol 3-kinase (PI3K) [10,14,15], and insulin and IGF-I induce a marked increase in cellular phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3), the principal molecular species generated on activation of this enzyme [16,17]. Receptor-activated PI3K is inhibited by the fungal metabolite wortmannin (WM), with a substantial degree of specificity compared to various other lipid kinases [18]. The use of WM, an unrelated inhibitor LY294002, inhibitory antibodies and mutated components have implicated PI3K (and, presumably, its products) in the stimulation of cellular processes such as glucose transport, membrane ruffling, c-*fos* expression and DNA synthesis [15,19–21], although the intermediate regulatory steps are less well understood than the MAP kinase pathway. We have used WM to investigate the possible involvement of PI3K in the stimulation of cMG1 expression.

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Abbreviations: AII, angiotensin II; EGF, epidermal growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; MAP kinase, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PI-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; WM, wortmannin.

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2. Experimental

2.1. Materials

Reagents for cell culture were obtained from ICN-Flow and Imperial Laboratories. Insulin, EGF, AII, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 3-isobutyl-1-methylxanthine (IBMX) and forskolin were obtained from sources described previously [7,22], IGF-I was from Bachem, and WM (Sigma) was donated by Dr P.T. Hawkins. The cDNA probe for cMG1 was a 1262-bp *Eco*RI fragment spanning the coding sequence [3]. The probe for TIS11 (TTP) was a 1.8-kb *Hin*dIII/*Xba*I fragment [5] provided by Dr P. Blackshear (Howard Hughes Medical Institute, Duke University), and the probe for TIS11d was a 1.2-kb *Sal*I fragment [4] provided by Dr H. Herschman (Laboratory of Biomedical and Environmental Sciences, UCLA).

2.2. Methods

Stock cultures of RIE-1 cells were maintained and passaged as described previously [7,22]. Cells were grown on 9-cm dishes for 10–15 days in DMEM containing 5% newborn calf serum, by which time they were confluent and quiescent. If required, cellular protein kinase C (PKC) was depleted by incubation overnight with 300 nM TPA; this treatment reduces PKC in this cell line to a level undetectable by Western blotting [22]. In most of the experiments described, the mitogens were added directly to the cells in the depleted growth medium. In other experiments, the cells were rinsed and equilibrated in fresh serum-free DMEM containing BSA (1 mg/ml) for 30 min before adding the mitogens, in order to remove IGF-binding proteins. The two protocols gave similar results for all treatments except IGF-I, which had no effect unless the medium was changed as described. After incubation of the cells with the required treatments at 37°C, the medium was aspirated and the cells were lysed on the dish by the addition of 0.6 ml (per dish) of denaturing solution (solution D of [23]). The cell lysates were scraped into centrifuge tubes, and total RNA was isolated [23] and stored at −20°C.

Northern blotting, the labelling of cDNA probes using [α-^{32}P]dCTP (ICN), hybridisation to Hybond N filters, and washing of the filters, were all performed as described previously [7]. Filters were generally exposed to X-ray film for up to 24 h at −70°C with intensifier screens, except that a longer exposure (up to 7 days) was required for TIS11, which appears to be stimulated to a lower maximum level in these cells than in, for example, Swiss 3T3 cells (A.N.C., unpublished data). Autoradiographs were scanned using a Chromoscan 3 densitometer (Joyce-Loebl), and analysis of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression was used to account for minor differences in loading.

The analysis of the MAP kinase ERK-2 by Western blotting was performed essentially as described previously [22], using monoclonal or affinity-purified polyclonal anti-ERK-2 antibodies (Transduction Laboratories) followed by detection using ECL (Amersham); a low ratio of *bis*-acrylamide/acrylamide (1:60) was used in the gels, which enhanced the separation of the different phosphorylated forms of ERK-2.

3. Results and discussion

When insulin was added to confluent, quiescent RIE-1 cells, it stimulated an elevation of cMG1 mRNA, that was 2–3-fold above basal after 1 h and decreased rather slowly thereafter (Fig. 1a). In contrast, the levels of TIS11 and TIS11d mRNAs in the same samples were not affected by the incubation with insulin (Fig. 1a). However, these two genes could be activated in RIE-1 cells, since each of the other mitogens tested (EGF, AII and TPA) stimulated the expression of each of the three members of the family (Fig. 1). IGF-I also had no effect on TIS11 or TIS11d mRNA levels, and was 10–100-fold more potent than insulin in stimulating cMG1 expression (Fig. 1c), suggesting that the effects of both agonists were mediated largely through type 1 IGF receptors (although a contribution from insulin receptors cannot be excluded). Although there are differences between the times of maximal induction of the three genes by various agonists in these and other cells ([4] and

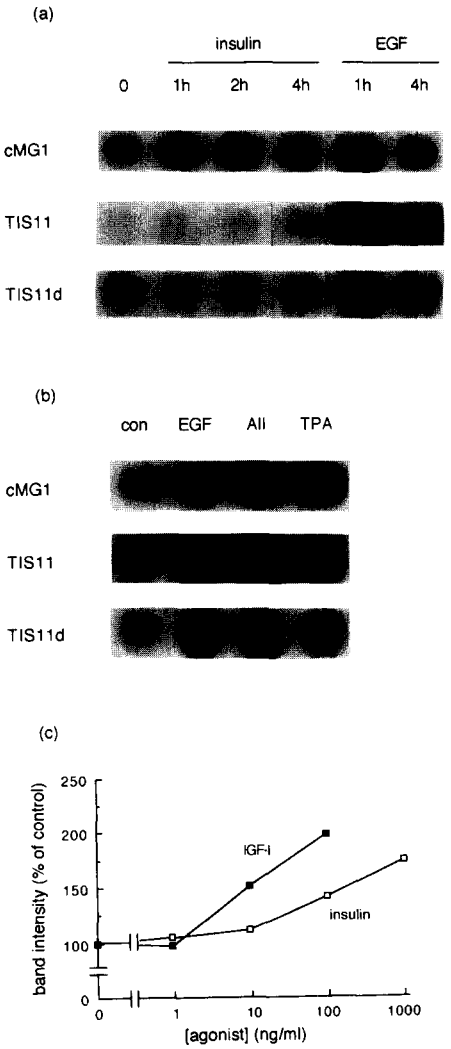

Fig. 1. Stimulation of cMG1, TIS11 and TIS11d in RIE-1 cells. Confluent, quiescent cells were incubated at 37°C for: (a) 1–4 h with insulin (1 μg/ml) or EGF (40 μg/ml); (b) 1 h with EGF (40 μg/ml), AII (100 nM) or TPA (30 nM); (c) 1 h with insulin or IGF-I at the concentrations shown; control cells received an equivalent volume of serum-free medium. RNA was prepared as described in the text, aliquots (10 μg) were analysed by Northern blotting, and the blots were probed sequentially for cMG1, TIS11 and TIS11d. Similar results were obtained in at least two other experiments with each agonist. In (c), the expression of cMG1, corrected for minor differences in loading revealed by reprobing the blots for GAPDH, is shown relative to that in control cells; mean values from 2 experiments.

A.N.C., unpublished data), this did not explain the observed lack of effect of insulin/IGF-I on TIS11 or TIS11d in RIE-1 cells: in three separate experiments no elevation of these mRNAs was observed in incubations ranging from 15 to 240 min with insulin (Fig. 1a and data not shown). It therefore appears that the receptors for insulin/IGF-I are coupled to the inducible expression of cMG1 but not of TIS11 or TIS11d in these cells. Comparison with published work indicates that the stimulation of TIS11 by insulin may only occur in cells expressing high numbers of insulin receptors, such as NIH-3T3 cells over-expressing transfected insulin receptors or 3T3-L1 cells after conversion to adipocytes [5]. Similarly, c-*fos* activation by insulin is well documented in these cell types [20,24] and in

principal insulin-responsive tissues in vivo [25]. However, cells such as 3T3 fibroblasts and RIE-1 cells mount substantial responses to insulin/IGF-I without significant changes in the level of c-*fos* mRNA compared with the effects of other mitogens ([24] and A.N.C., unpublished data). Thus, it appears that cMG1 may be unusually sensitive to activation by insulin/IGF-I.

In previous work, we showed that the addition of cyclic AMP-elevating agents such as forskolin plus IBMX markedly reduced the stimulation of cMG1 expression induced by EGF, and that the depletion of cellular PKC by pretreatment with TPA blocked the effect of AII on cMG1 expression but not that of EGF [7]. We have now found that the elevation of cyclic AMP also inhibited the stimulation of cMG1 mRNA by insulin, whereas the depletion of PKC had little effect (Table 1). Thus, the stimulation of cMG1 expression by insulin shows a similar inhibition profile to that induced by EGF, raising the possibility that the two receptor tyrosine kinases activate cMG1 expression by similar signalling pathways.

The activation of PI3K has been implicated in the regulation of various intracellular events by insulin, including the induction of c-*fos* (see Introduction). We therefore investigated the possible involvement of PI3K in the elevation of cMG1 mRNA by insulin/IGF-I in RIE-1 cells. The addition of WM markedly inhibited the stimulation of cMG1 mRNA by insulin/IGF-I (Fig. 2). Complete inhibition was obtained at a dose of WM (100 nM) which has been shown to give maximal inhibition of PI3K in other cell types without affecting other lipid kinases [18], and the specificity of WM action in the current study was indicated by two features: first, WM did not block the stimulation of cMG1 or TIS11 mRNA induced by TPA, EGF or AII (Fig. 2), indicating that it did not have a general effect on signalling pathways stimulating gene expression; secondly, it did not affect other aspects of insulin-induced signalling, such as the phosphorylation of ERK-2 (see below). Thus, PI3K may be involved in the signalling pathway by which insulin/IGF-I stimulate cMG1 expression, but it is not required for the activation of the three members of the cMG1 family by other agonists including EGF. Previous studies have shown that the EGF receptor lacks the high-affinity target sequence for PI3K recruitment [15]; the effects of EGF on PI-3,4,5-P_3 levels, which may be mediated by association of the EGF receptor with the related erb-B3 protein [26], are less marked than those of agonists such as insulin [17].

The MAP kinases ERK-1 and ERK-2 are activated by various agonists including insulin [9,10,13], and have been shown to be involved in the stimulation of primary response genes

Table 1
Effect of cyclic AMP elevation and PKC depletion on insulin-stimulated cMG1 expression in RIE-1 cells

Pretreatment	cMG1 expression (% of control)	
	control	insulin
Control	100	167
Forskolin/IBMX	117	109
Control	100	166
PKC depletion	100	152

Confluent, quiescent cells were pre-incubated for 10 min at 37°C with forskolin (10 μM) plus IBMX (50 μM); some cells were pretreated overnight with TPA (300 nM) to down-regulate PKC; control cells were given 0.1% (v/v) DMSO. Insulin (1 μg/ml) or vehicle was then added, and the incubation was continued at 37°C for 60 min. RNA was prepared as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The results shown are the expression of cMG1 mRNA relative to that in control cells, and are mean values from three experiments for cyclic AMP and two experiments for PKC depletion.

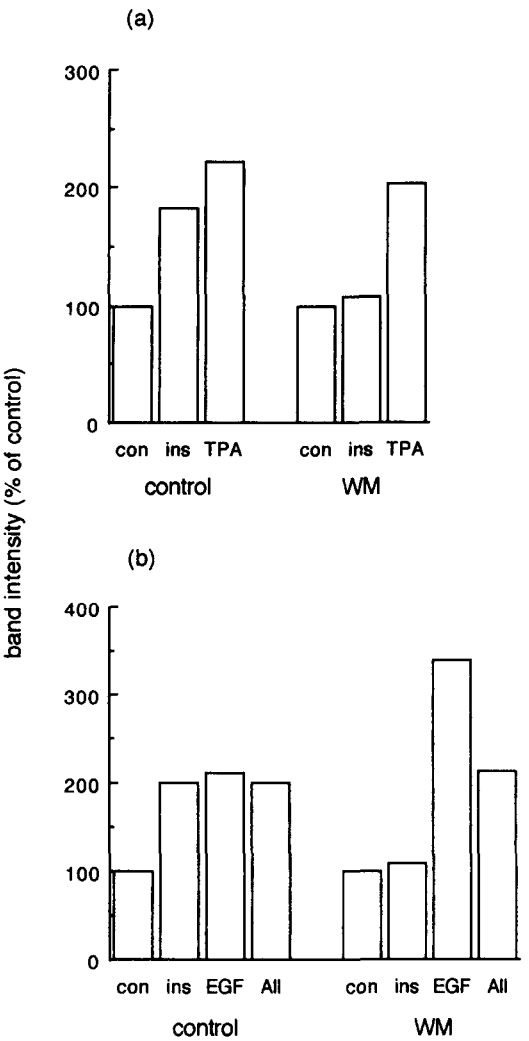

Fig. 2. Inhibition by WM of insulin-stimulated cMG1 expression in RIE-1 cells. Confluent, quiescent cells were pre-incubated for 10 min at 37°C with WM (100 nM) or vehicle (0.1% (v/v) DMSO), before incubation for 1 h at 37°C with (a) insulin (1 μg/ml) or TPA (10 nM), or (b) insulin (1 μg/ml), EGF (40 ng/ml) or AII (100 nM); control cells received an equal volume addition of serum-free medium. RNA was prepared as described in the text, and aliquots (10 μg) were analysed by Northern blotting. The blots were probed simultaneously for cMG1 and GAPDH, and the expression of cMG1 is shown relative to that in the appropriate control cells, after correction for minor differences in the loading of RNA revealed by the scans of the GAPDH autoradiograph; the addition of WM did not affect the level of GAPDH expression, but increased the basal expression of cMG1 by 68 ± 24% (mean ± S.E.M.; $n = 7$) compared with that in DMSO-treated cells. Similar results were obtained in two other experiments with insulin, such that the stimulation of cMG1 by insulin was reduced by WM from 93 ± 24% to 1 ± 9% ($n = 4$), and in two additional experiments using IGF-I. Reprobing the blots for TIS11 mRNA showed that WM did not block the stimulation of TIS11 expression induced by TPA, EGF or AII.

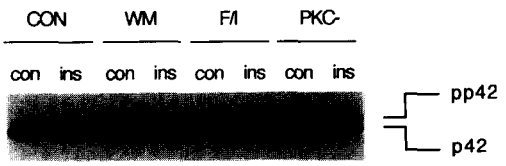

Fig. 3. Phosphorylation of MAP kinase ERK-2 in RIE-1 cells stimulated by insulin. Confluent, quiescent cells were pre-incubated for 10 min at 37°C with WM (100 nM) or forskolin (10 μM) plus IBMX (50 μM) (F/I); cells indicated PKC⁻ had been pretreated overnight with TPA to down-regulate PKC; control cells were given 0.1% (v/v) DMSO. Insulin (1 μg/ml) or vehicle was then added, and the incubation was continued at 37°C for 10 min. Cell lysis, SDS/PAGE, Western blotting and analysis of ERK-2 were performed as described in the text. The positions of the shifted bands are indicated (p42/pp42). Similar results were obtained in a second experiment of this design.

[9,13,27]. The pathway from Raf-1 to ERK-2 is activated by receptor tyrosine kinases through Grb2, Sos and $p21^{ras}$ [9,10,13], and in some cell types (but not others [28]) this activation of ERK-2 has been shown to be blocked at Raf-1 by cyclic AMP analogues or cyclic AMP-elevating agents [28,29]. In addition, recent studies using WM have suggested that insulin may also activate this pathway through PI3K [30,31]. It was therefore possible that the inhibitory effects of cyclic AMP elevation and/or WM on insulin-stimulated cMG1 expression might be explained by inhibition of the MAP kinase pathway. Insulin stimulated a partial activation of ERK-2 in RIE-1 cells, as revealed by the decreased electrophoretic mobility (Fig. 3) that is associated with its activation by phosphorylation [22,28]. However, this effect was not prevented when the cells were preincubated with WM or with IBMX plus forskolin (Fig. 3). These results indicate that the stimulation by insulin of ERK-2 in RIE-1 cells is not mediated through PI3K, that the inhibition of insulin-stimulated cMG1 expression by WM is not due to inhibition of the ERK-2 pathway, and that we cannot use inhibition by cyclic AMP to deduce a contribution of this pathway to the insulin-stimulated expression of cMG1. The latter conclusion also applies to EGF-stimulated expression of cMG1, since the addition of IBMX plus forskolin did not affect the stimulation of ERK-2 by EGF (A.N.C., unpublished data).

Taken together, our results suggest that insulin/IGF-I specifically activate cMG1 expression by a signalling pathway which involves WM-sensitive PI3K and is inhibited by cyclic AMP beyond a point of convergence with the separate signalling pathway stimulated by EGF. If there is a contribution of the MAP kinase pathway to cMG1 stimulation by insulin/IGF-I, then this is not sufficient for detectable stimulation to remain when the PI3K pathway is blocked. The signalling components downstream from PI3K remain to be determined. Recently, the rapamycin-sensitive pathway to $p70^{s6k}$ been shown to be blocked by WM and LY294002, and thus may lie downstream of PI3K [19,30]; although $p70^{s6k}$ is principally considered to be involved in the activation of translation rather than transcription, recent evidence suggests that it may also stimulate members of the CREM family of transcription modulators [32]. However, in preliminary experiments (not shown) rapamycin did not block the stimulation of cMG1 expression by insulin/IGF-I, suggesting that the $p70^{s6k}$ pathway may not be involved. Another potential signalling pathway may be deduced from reports that PI 3,4,5-P_3 stimulates PKC-z [33] and that PKC-z activates the transcription factor NFkB [34]. The PKC-z isozyme is not affected by the TPA pretreatment used to deplete conventional PKC [35], and we have indeed found that such pretreatment with TPA, although inhibiting stimulation by AII [7], does not prevent the stimulation of cMG1 expression by insulin (Table 1). However, there are no obvious NFkB consensus sequences in about 1 kb of proximal 5′-flanking sequence of the cMG1 gene (A.N.C., J.C. Pascall, K.M. Hadfield and K.D.B., manuscript in preparation), and further work will therefore be required to elucidate the mechanism of activation of cMG1 at the gene level. It will also be interesting to determine whether the inhibition by cyclic AMP of the stimulation of cMG1 expression by both insulin (Table 1) and EGF [7] results from an effect of cyclic AMP on a common signalling component, or on a negative regulatory element in the cMG1 gene.

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