Specific induction of RGS16 (regulator of G-protein signalling 16) mRNA by protein kinase C in CEM leukaemia cells is mediated via tumour necrosis factor α in a calcium-sensitive manner

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The RGS (regulator of G-protein signalling) proteins are GTPase-activating proteins for activated $G\alpha$ subunits. We investigated the effects of protein kinase C (PKC) on RGS proteins in various T cell lines by treating them with PMA. mRNA levels of both RGS16 and tumour necrosis factor α (TNF α) were found to be up-regulated in CEM leukaemia cells in a PKC-dependent manner. Mezerein, a non-phorbol-ester activator of PKC, also elevated RGS16 and TNF α mRNA levels, while the specific PKC inhibitor Go6983 abrogated their expression. In view of the slower kinetics of PMA-induced RGS16 expression and the tight correlation between TNF α and RGS16 mRNA induction among the cell lines studied, we suggest that activation of PKC up-regulates RGS16 via TNF α . Indeed, addition of recombinant

TNF α to CEM cells rapidly stimulated RGS16 mRNA expression independently of PKC. Furthermore, mobilization of calcium by A23187 and thapsigargin blocked the TNF α -mediated induction of RGS16, which was reversed by EGTA and by the immunosuppressants FK506 and cyclosporin A, suggesting that the calcineurin/NF-AT (nuclear factor of activated T cells) pathway may repress the up-regulation process. Our results demonstrate for the first time that activation of PKC induces RGS16 expression via TNF α in a calcium-sensitive manner, thereby implicating RGS16 in the regulation of T cell responses to inflammation.

Key words: cytokine function, gene expression, T cells.

INTRODUCTION

The RGS (regulator of G-protein signalling) proteins belong to a recently discovered family of proteins that enhance the GTP hydrolysis rate of activated $G\alpha$ subunits, resulting in a decrease in the duration and intensity of signalling by G-protein-coupled receptors (GPCRs) [1,2]. The first RGS protein (SST2) was discovered in the yeast *Saccharomyces cerevisiae* by Chan and Otte in 1982 [3] in a screen for mutant haploids hypersensitive to pheromone-induced cell cycle arrest. Subsequently an SST2-related gene was identified in the nematode *Caenorhabditis elegans* using a genetic screen for mutants that exhibit certain altered neuronal activities [4]. The earliest mammalian RGS protein discovered, BL34/1R20 (RGS1), was identified because its mRNA was elevated in chronic lymphocytic leukaemia and its expression could be induced specifically in B lymphocytes by mitogenic stimuli [5]. The mRNA of another mammalian RGS protein, GOS8 (RGS2), was up-regulated by cycloheximide treatment in blood mononuclear cells [6]. These results clearly indicate that transcriptional regulation of RGS plays a key role in controlling RGS expression, and hence its activity.

We have previously cloned mouse RGS16 from a mouse pituitary library [7,8]. RGS16 binds to $G_{q\alpha}$, $G_{i\alpha}$ and $G_{o\alpha}$ subunits [9] and can inhibit $G_{q\alpha}$ - and $G_{i\alpha}$ -mediated signalling pathways [10,11]. Palmitoylation of the N-terminal cysteine residues of RGS16 is required for its effect on $G_{i\alpha}$ - and $G_{q\alpha}$ -mediated signalling [12], although membrane localization of RGS16 is probably mediated through an amphipathic α -helical

N-terminal domain [13]. Interestingly, although RGS16 is expressed only in the brain, pituitary, liver and retina, as shown by RNase protection assays and Northern hybridization [7], RGS16 mRNA can be up-regulated in the heart under conditions of sepsis [14] and in interleukin 2-stimulated T cells [11]. These studies suggest that RGS16 may regulate immune responses during inflammation.

The phorbol ester PMA is commonly used to stimulate cells to proliferate and differentiate. It exerts a broad range of effects, notably stimulating protein kinase C (PKC) activity, B and T cell activation, megakaryocyte differentiation and tumour growth. Induction of RGS expression by phorbol ester has been shown for several RGS family members, including RGS1 [5], RGS2 [15] and RGS7 [16], although there is no evidence that PMA also regulates RGS16. In view of the fact that RGS16 may be implicated in inflammatory responses, we therefore investigated its induction by PMA in T cell lines. Furthermore, delineation of the induction pathway downstream of phorbol ester, especially the identification of the PKC isoforms and signalling proteins responsible for RGS16 expression, will offer an insight into the molecular mechanisms that mediate its expression. Our present study clearly demonstrates that activation of PKC can up-regulate RGS16 mRNA levels in CEM leukaemia cells. This effect is likely to be mediated through the expression of tumour necrosis factor α (TNF α), which is dependent on the non-classical PKC isoforms and the extracellular-signal-regulated kinase (ERK) protein of the mitogen-activated protein kinase (MAPK) family. However, calcium mobilization can inhibit the

Abbreviations used: RGS, regulator of G-protein signalling; GPCR, G-protein-coupled receptor; PKC, protein kinase C; TNF α , tumour necrosis factor α ; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; NF-AT, nuclear factor of activated T cells; OAG, 1-oleoyl-2-acetate-sn-glycerol.

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expression of RGS16, probably by activating the calcineurin/NF-AT (nuclear factor of activated T cells) pathway. This is the first study that shows a link between PKC and TNF α in the induction of an RGS protein, and demonstrates the participation of calcium in this process.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Calbiochem (San Diego, U.S.A.): PMA, mezerein, 1-oleoyl-2-acetate-*sn*-glycerol (OAG), FK506, cyclosporin A, Ro31-8220, Go6976, Go6983, A23187 free acid, thapsigargin and wortmannin. Recombinant TNF α was purchased from Clontech (Palo Alto, CA, U.S.A.). The rest of the reagents were purchased from Sigma.

Cell lines and RNA extraction

All cell lines were obtained from A.T.C.C. and were cultured in RPMI medium supplemented with 10% (v/v) fetal calf serum and 1% (w/v) glutamine. Cells were incubated with fresh medium for 1 h before treatment with the various reagents and chemicals. The number of CEM cells used in the experiment was approx. 6×10^6 for each sample. RNA was extracted using the guanidine thiocyanate/phenol method (RNazol; Biotex Laboratories Inc., Houston, TX, U.S.A.) according to the manufacturer's instructions. Each RNA sample was resuspended in 6 μ l of Tes buffer (10 mM Tris, 1 mM EDTA and 0.1% SDS) and heated at 60 °C for 5 min. The dissolved RNA was used immediately or stored at -70 °C until required.

Northern hybridization

Size fractionation of RNA was performed on 1.2% (w/v) agarose gels containing 16% formaldehyde, as described by Sambrook et al. [17], in Mops buffer (20 mM Mops, 5 mM sodium acetate and 1 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide (2 μ g/ml in water) for the 18 S and 28 S tRNAs, and electrophoretically transferred on to a nylon membrane (Hybond-N; Amersham) in TAE buffer (10 mM Tris base, 5 mM sodium acetate and 0.5 mM EDTA; pH adjusted to 7.8 with acetic acid). The transferred RNA was then cross-linked on to the nylon membrane using a Stratagene UV cross-linker. Probes were generated by random-primer labelling (Boehringer-Mannheim) using full-length human RGS16 and TNF α cDNAs as templates. Procedures for Northern blot analysis were as described previously [7]. To re-probe blots, they were boiled in 0.1% SSC/0.5% SDS for 30 min, before hybridization with the second probe. All experiments were performed at least three times; blots were scanned using a Bio-Rad imaging densitometer and results were analysed using the program Multi-Analyst (Bio-Rad).

RESULTS

PMA induces the production of RGS16 and TNF α mRNAs in CEM leukaemia cells

The human T cell lines Molt-3, Sup-T1 and CEM were treated with PMA (0.1 μ M) for up to 3 h, followed by RNA extraction and electrophoresis on formaldehyde/agarose gels. Full-length human RGS16 cDNA was radiolabelled and used as a probe for Northern blotting. We observed that the CEM leukaemia cell line, but not Molt-3 or Sup-T1 cells, showed a strong up-regulation of RGS16 mRNA upon PMA treatment (Figure 1A,

top panel). This induction was specific for RGS16, as neither RGS1 nor RGS10 mRNA levels were elevated (results not shown). The increase in RGS16 mRNA levels was evident at 2 h and was sustained for up to 8 h of PMA treatment (Figure 1B, top panel). However, after prolonged PMA treatment (overnight or approx. 16 h), RGS16 mRNA returned to basal levels, probably due to PMA desensitization. The level of RGS16 protein expression was correlated with its mRNA expression, with a time lag of approx. 2 h (result not shown).

Mitogenic stimulation of T cells is frequently associated with enhanced expression of various cytokines, including TNF α [18]. We therefore examined a possible simultaneous induction of TNF α and its association with RGS16 expression. After the blot was stripped and re-probed with radiolabelled human TNF α cDNA, levels of TNF α mRNA were observed to be strongly up-regulated after 1 h of PMA treatment, peaking at 4 h (middle panels of Figures 1A and 1B). The increased expression of TNF α therefore occurred noticeably earlier than that of RGS16 (Figure 1B). It is noteworthy that there was a tight correlation between RGS16 and TNF α mRNA expression among the cell lines studied (Figure 1A). For example, PMA treatment of Molt-3 or Sup-T1 cells did not induce RGS16 mRNA expression, and the levels of TNF α mRNA in these cells were also not elevated.

Activation of PKC and ERK is essential for induction of RGS16 and TNF α mRNAs by PMA

PMA is a potent activator of PKC enzymes, which form a large family of serine/threonine kinases that can be divided into three subfamilies: classical, novel and atypical. To investigate whether activation of PKC is essential for RGS16 expression, a non-phorbol-ester activator of PKC, mezerein [19], together with a cell-permeable diacylglycerol analogue that activates calcium-dependent PKC, OAG, were used to treat CEM cells. Northern hybridization analysis of treated cells indicated that mezerein, but not OAG, could mimic the effect of PMA in stimulating the expression of RGS16 mRNA (Figure 1C), suggesting that the activation of the non-classical isoforms of PKC may be essential for the induction. Staurosporine, a non-specific serine/threonine kinase inhibitor that blocks the activity of various isoforms of cAMP-dependent protein kinase and PKC also abolished the induction of expression of RGS16 mRNA by PMA (Figure 1B).

To further elucidate the PKC subfamily responsible for the induction of RGS16 mRNA, the PKC inhibitors Ro31-8220, Go6976 and Go6983 were used. The activity of the calcium- and diacylglycerol-dependent classical PKC enzymes (α , β and γ) and of the novel isoform (PKC ϵ) can be blocked by the PKC inhibitor Ro31-8220 at nanomolar concentrations ($IC_{50} < 30$ nM) [20]. However, Ro31-8220 only partially abolished the effect of PMA at an inhibitor concentration of 1 μ M (Figure 1C). Go6976, another specific PKC inhibitor that inhibits the classical PKC isoforms and also PKC μ [21], failed to prevent the induction of RGS16 mRNA by PMA. Only the PKC inhibitor Go6983, which inhibits the classical PKC isoforms together with PKC δ and PKC ζ ($IC_{50} < 60$ nM), but not PKC μ [22], abrogated the effect of PMA in CEM cells (Figure 1C).

A possible involvement of MAPK was examined through the use of SB203580 and PD98059, specific inhibitors of p38 and ERK respectively. When incubated with PMA in CEM cells, the compound PD98059, but not SB203580, inhibited RGS16 mRNA expression (Figure 1C), suggesting a requirement for ERK. In human embryonic kidney cells, the p38-specific inhibitor SB203580 fully inhibited p38 kinase activity in a control experiment (result not shown), indicating that the concentration used was sufficient. A similar pattern of reduced TNF α mRNA

Figure 1 PMA induces up-regulation of RGS16 and TNF α in CEM leukaemia cells

(A) CEM, Molt-3 and Sup-T1 cells were incubated with PMA (0.1 μ M) for 0, 1 and 3 h. Total RNA was extracted as described in the Materials and methods section. The top panel shows hRGS16 (2.2 kb) mRNA levels probed by 32 P-labelled full-length human RGS16 cDNA. The middle panel shows hTNF α (1.7 kb) mRNA levels probed by 32 P-labelled human TNF α cDNA on the same blot after stripping. The bottom panel, showing ethidium bromide-stained 28 S and 18 S rRNAs in the formaldehyde/agarose gel, confirms similar loading of RNA into each lane. (B) CEM cells were incubated with PMA (0.1 μ M) at 37 $^{\circ}$ C. After the indicated time periods, 2 ml of cell suspension was removed and total RNA was extracted. The blot was hybridized with 32 P-labelled full-length human RGS16 cDNA (top panel), and then with 32 P-labelled human TNF α cDNA (second panel) after stripping. Similar amounts of RNA were loaded into each lane, as shown by the ethidium bromide-stained 28 S and 18 S rRNAs. The graph shows the levels (fold above basal) of hRGS16 (■) and hTNF α (▲) mRNAs over 16 h of stimulation with PMA. (C) CEM leukaemia cells were treated with PMA (0.1 μ M), OAG (100 μ M) or mezerein (10 μ M) for 4 h, or pretreated with staurosporine (1 μ M), Ro31-8220 (1 μ M), Go6976 (1 μ M), Go6983 (1 μ M), SB203580 (10 μ M) or PD98059 (50 μ M) for 15 min before the addition of PMA (0.1 μ M) for 4 h. The top panel shows hRGS16 mRNA levels, while the middle panel shows hTNF α mRNA levels of the same blot after stripping.

expression was observed in CEM cells when the blot was stripped and re-probed for TNF α (Figure 1C, middle panel). Apparently the conditions required for TNF α expression were identical with those required for induction of RGS16.

Recombinant TNF α up-regulates RGS16 mRNA with faster kinetics than PMA and acts independently of PKC and ERK

The addition of recombinant TNF α (10 ng/ml) to CEM cells resulted in a rapid rise in RGS16 mRNA levels at 1 h, with a peak at 2 h (Figure 2A). The kinetics of RGS16 up-regulation in TNF α -treated cells were therefore much more rapid than those following PMA stimulation (Figure 1B). To check whether TNF α induction of RGS16 is downstream of PKC and ERK, inhibitors that were used in the earlier study were again co-incubated with TNF α , and their effects on RGS16 mRNA production examined. None of the PKC or MAPK inhibitors had any effect on the up-regulation of RGS16 mRNA by TNF α (Figure 2B), clearly indicating that TNF α regulation of RGS16 mRNA levels is independent of PKC and of the MAPKs p38 and ERK.

Mobilization of calcium abrogates the induction of RGS16 by PMA and TNF α

In T cells, the expression of many genes can be enhanced greatly in the presence of elevated levels of intracellular calcium [18]. Calcium levels in the cell can be increased by treatment either

with ionophores, which are small hydrophobic molecules that increase the membrane permeability to calcium, or with inhibitors of the endoplasmic reticulum Ca $^{2+}$ -ATPase which mobilizes the intracellular store of calcium. CEM cells were pretreated with A23187 (1 μ M) or thapsigargin (0.2 μ M) for 15 min before incubation with PMA for 4 h in RPMI medium (Sigma R-6504), which contains approx. 1 mM calcium salts. As shown in Figure 3(A) (top panel), the calcium ionophore A23187 fully inhibited the effect of PMA on RGS16 expression, whereas thapsigargin, a sesquiterpene lactone that inhibits endoplasmic reticulum Ca $^{2+}$ -ATPase, had no discernible effect. When the blot was re-probed for TNF α mRNA expression (Figure 3A, middle panel), the level of TNF α mRNA in CEM cells pretreated with A23187 was unchanged, but there was a 3-fold increase in cells pretreated with thapsigargin. Apparently, the mobilization of intracellular calcium stores by thapsigargin had enhanced TNF α mRNA expression by PMA. Interestingly, this enhanced expression of TNF α mRNA did not result in a corresponding increase in RGS16 mRNA levels (Figure 3A). These results suggest that calcium mobilization has a direct effect on the induction of RGS16 mRNA by TNF α .

When a similar experiment was performed with recombinant TNF α , it became clear that both A23187 and thapsigargin were directly interfering with the transcription pathway of RGS16 induced by TNF α in CEM cells. Co-incubation of A23187 or thapsigargin with TNF α reduced the levels of RGS16 mRNA to a large extent (Figure 3B). A control experiment performed

Figure 2 Recombinant $\text{TNF}\alpha$ directly up-regulates RGS16 mRNA levels independently of PKC and MAPK

(A) CEM cells were incubated with $\text{TNF}\alpha$ (10 ng/ml) essentially as described in the legend to Figure 1(B). After RNA extraction, electrophoresis and blotting on to nylon membranes, the membrane was hybridized with ^{32}P -labelled human RGS16 cDNA to probe for RGS16 mRNA levels. The graph shows fold induction (above basal) of hRGS16 mRNA over 16 h of stimulation with $\text{TNF}\alpha$. (B) PKC and MAPK inhibitors were used to pretreat CEM cells for 15 min before the addition of $\text{TNF}\alpha$ (10 ng/ml) for 2 h. The Northern blot was probed with ^{32}P -labelled human RGS16 cDNA.

without any stimulant indicated that these compounds had no stimulatory effect on their own (results not shown). To validate the inhibitory role of calcium in RGS16 induction, treatment with EGTA, a calcium ion chelator, should in theory reverse the effects of the calcium ionophore and the Ca^{2+} -ATPase inhibitor. This was indeed the case when 1 mM EGTA was used to pretreat the cells for 30 min before the addition of thapsigargin or A23187 with $\text{TNF}\alpha$ for 2 h (Figure 4A). As EGTA is not a chelator of intracellular calcium, the recovery of RGS16 mRNA expression in the presence of EGTA suggests that the influx of extracellular calcium is essential for the inhibitory effect. This is possible, as thapsigargin at high doses is able to enhance the permeability to calcium of the plasma membrane [23].

Figure 3 Effects of A23187, thapsigargin, dibutyryl cAMP and wortmannin on RGS16 expression

(A) A23187 (1 μM), thapsigargin (0.2 μM), dibutyryl cAMP (500 μM) or wortmannin (0.5 μM) was used to pretreat CEM cells for 15 min before the addition of PMA (0.1 μM) for 4 h. Northern hybridization was performed with ^{32}P -labelled human RGS16 cDNA. (B) As for (A), except that $\text{TNF}\alpha$ (10 ng/ml for 2 h) was used in place of PMA.

Second messengers other than calcium which may help to delineate the pathway downstream of $\text{TNF}\alpha$ were similarly screened for any inhibitory or stimulatory effects with PMA or $\text{TNF}\alpha$. cAMP is among the most abundant second messengers, and acts on cAMP-dependent protein kinases and also the recently discovered Rap1 guanine nucleotide exchange factor Epac [24]. The non-hydrolysable analogue of cAMP, dibutyryl cAMP (0.5 mM), did not have any direct stimulatory effect, but enhanced the stimulation of RGS16 by $\text{TNF}\alpha$ (Figure 3B). Wortmannin, an inhibitor of phosphoinositide 3-kinase, did not exhibit any significant effect (Figures 3A and 3B).

FK506 and cyclosporin A relieve the calcium-mediated inhibition of RGS16 induction

Elevated levels of intracellular calcium together with a mitogen, e.g. PMA, can promote the proliferation and activation of T cells [18]. The calcium-activated calcineurin, a serine/threonine protein phosphatase, is frequently involved in this process [25]. Activated calcineurin dephosphorylates NF-AT, a transcription

Figure 4 Calcium mobilization inhibits RGS16 expression

(A) Calcium was chelated by 1 mM EGTA (pH 8) for 30 min before the addition of thapsigargin (0.2 μ M) or A23187 (1 μ M), together with TNF α (10 ng/ml), for 2 h. Controls were also carried out to assess the effects of EGTA alone or with TNF α (first four lanes). (B) CEM cells were pretreated with FK506 (200 ng/ml) or cyclosporin A (2 μ g/ml) for 15 min before the addition of TNF α (10 ng/ml), TNF α + thapsigargin (0.2 μ M) or TNF α + A23187 (1 μ M) for 2 h.

factor that translocates to the nucleus and activates a large number of genes essential for T cell proliferation and activation. The immunosuppressants FK506 and cyclosporin A inhibit this pathway by binding to FK506-binding protein and cyclophilin respectively, thereby preventing the dissociation of these proteins from calcineurin and inhibiting its activation [25]. Pretreatment of cells with FK506 (200 ng/ml) or cyclosporin A (2 μ g/ml) alone or together in the presence of TNF α did not have any discernible effect (Figure 4B). However, FK506 and cyclosporin A relieved the calcium-mediated inhibition of RGS16 mRNA expression caused by A23187 and thapsigargin (Figure 4B). In addition, the expression of RGS16 mRNA was also enhanced compared with TNF α -stimulated controls.

DISCUSSION

We show in the present study that activation of PKC up-regulates levels of RGS16 and TNF α mRNAs in CEM cells. Induction of RGS16 mRNA appears to be an event subsequent to TNF α expression, as induction of RGS16 mRNA by recombinant TNF α occurs at a much higher rate than with PMA, and is also independent of PKC and ERK activation. Mobilization of calcium by A23187 and thapsigargin blocks the

Figure 5 Schematic representation of the induction of RGS16 mRNA by PMA and TNF α in CEM cells

Abbreviation used: TNFR, TNF receptor.

induction process, but this can be reversed by the immunosuppressants cyclosporin A and FK506. Thus our results show that RGS16 mRNA expression can be modulated by signals via the cytokine receptor in T cells (Figure 5).

TNF α appears to play a crucial role in the induction of RGS16 in CEM cells. In the T cell lines studied here, RGS16 mRNA was up-regulated in CEM cells upon PMA treatment. Furthermore, a tight correlation was observed between the expression of RGS16 and TNF α mRNAs in this cell line, which is a CD4+ leukaemia cell line commonly used to study HIV infection [26]. Addition of exogenous TNF α also rapidly stimulated RGS16 mRNA expression in these cells. In contrast, Sup-T1 and Molt-3 cells failed to respond to either PMA or TNF α , with no induction of RGS16 expression. These cells also did not express TNF α when stimulated by PMA within the time course of the study. Although all three cell lines studied are lymphoblastic leukaemia T cells, they may exhibit differences in their cell signalling components. For example, both Sup-T1 and CEM cells express the chemokine receptor CXCR4 [27], which acts as a co-receptor for HIV entry into the cell, but only CEM cells can resist the virally induced cytopathic effects [26].

Recent evidence has shown that TNF α can stimulate RGS1 directly in normal B cells [28], while TNF α treatment stabilized RGS7 mRNA through the activation of p38 [29]. In that study, RGS7 was shown to be rapidly up-regulated in mouse brain after exposure to either endotoxin or TNF α , but not in mice lacking TNF receptor 1. In our study, since the kinetics of TNF α mRNA expression preceded those of RGS16 mRNA expression in PMA-treated CEM cells (Figure 1B), together with the finding that recombinant TNF α rapidly induced the up-regulation of RGS16 mRNA (Figure 2A), we suggest that PMA induces RGS16 via TNF α . This is further supported by our observations in the T cell lines and results obtained through the use of various PKC activators and inhibitors (Figure 1C), where a similar pattern of decreased RGS16 and TNF α mRNA expression was noted. The delay in the appearance of RGS16 mRNA in PMA-

stimulated CEM cells is therefore likely to be an event subsequent to TNF α expression, possibly via an autocrine feedback mechanism (see Figure 5).

Our results also show that PKC and ERK are essential for the induction of TNF α mRNA by PMA. The specific PKC isoforms necessary for induction were not identified, due to the overlapping PKC inhibitory profiles of the inhibitors and the extremely low efficiency in transfecting CEM cells. However, we noted that inhibition of the classical PKC isoforms was not sufficient to block the induction process, while inhibition of ERK activity completely blocked the up-regulation of both RGS16 and TNF α (Figure 1C). Interestingly, the non-classical PKC isoforms and ERK1/2 were also found to be important for TNF α production in PMA-stimulated mast cells [30].

TNF α is known to signal through the calcineurin/NF-AT pathway and various MAPK pathways. In an attempt to delineate the transcription pathway of RGS16, MAPK inhibitors were used, but these had no effect (Figure 2B). In contrast, the stabilization of RGS7 by TNF α requires activation of MAPK p38 and the presence of candidate MAPK phosphorylation sites [29]. Therefore, in CEM leukaemia cells, it is likely that TNF α transcriptionally regulates RGS16 via a pathway that is independent of ERK and p38.

Furthermore, we found that calcium-mobilizing agents abrogated the induction of RGS16 by both PMA and TNF α . This is unexpected, as most genes are up-regulated in T cells in the presence of phorbol ester and high intracellular calcium, which culminate in T cell activation [18]. A speculative explanation for the observed inhibition of RGS16 levels is that the lower abundance of RGS16 allows the signalling of GPCRs, which may be essential for proper T cell differentiation and propagation. Interestingly, one study suggested that sepsis attenuated the mitogen-mediated elevation in intracellular calcium, and thereby suppressed T lymphocyte proliferation [31]. This suppression acts via prostaglandin E₂ through its second messenger cAMP, which can antagonize intracellular calcium signalling in T cells. In our experiment, addition of dibutyryl cAMP along with TNF α enhanced RGS16 mRNA levels (Figure 3B), possibly due to a similar inhibitory effect of cAMP on calcium signalling in CEM cells. It must also be noted that dibutyryl cAMP was unable to stimulate RGS16 expression when used alone (results not shown), indicating that antagonism of calcium signalling or high levels of cAMP is insufficient to induce RGS16 expression.

Some studies have indicated that ligands of GPCRs can directly up-regulate RGS mRNA levels [15,32]. However, we did not observe any effect with stromal-derived factor-1 α , RANTES (regulated upon activation, normal T-cell expressed and secreted), interleukin 8, bradykinin or lysophosphatidic acid (C. W. Fong, unpublished work). Even interleukin 1 β , a cytokine that triggers effects that are biologically indistinguishable from those of TNF α by activating the same set of transcription factors, failed to stimulate RGS16 expression (C. W. Fong, unpublished work). This indicates that a pathway specific to TNF α is critical for its up-regulatory effect. The regulation by calcium of RGS16 mRNA levels may also extend to other cells, for example the B cell line HS-Sultan, in which the induction by PMA of RGS16, but not RGS1, mRNA was enhanced by a calcium ionophore (C. W. Fong, unpublished work). It is possible that RGS proteins are regulated by different intracellular second messengers; for example, the expression of RGS2 is consistently shown to be dependent on cAMP or agents that stimulate the production of cAMP [11,33]. The dramatic expression and tight transcriptional control of RGS16 in CEM cells strongly suggest an important role for RGS16 in regulating T cell responses. Beadling et al. [11]

have shown that RGS16 binds G_i α and G_q α proteins in T cells and inhibits their signalling pathways. The induction of RGS16 in response to bacterial lipopolysaccharide also resulted in elevated c-fos promoter expression [14]. As RGS proteins have different specificities for the various G α proteins, and even for the GPCRs that couple to the same G α protein [34], it is possible that the expression of RGS16 protein may act to modulate the signalling of only a subset of GPCRs in CEM cells.

Our study clearly indicates that TNF α acts downstream of PKC and can directly up-regulate RGS16 mRNA levels. This further extends the number of RGS family members that are known to be induced in the presence of this pro-inflammatory cytokine. In view of the fact that RGS16 expression can be modulated by the immunosuppressants cyclosporin A and FK506 in the presence of calcium, it is likely that RGS16 plays an important role in regulating T cell responses during inflammation. We have thus provided evidence of signalling cross-talk between the cytokine receptors and GPCRs through the up-regulation of an RGS protein.

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REFERENCES

- Dohlman, H. G. and Thorner, J. (1997) RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* **272**, 3871–3874
- Berman, D. M. and Gilman, A. G. (1998) Mammalian RGS proteins: barbarians at the gate. *J. Biol. Chem.* **273**, 1269–1272
- Chan, R. K. and Otte, C. A. (1982) Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a factor and alpha factor pheromones. *Mol. Cell. Biol.* **2**, 11–20
- Koelle, M. R. and Horvitz, H. R. (1996) EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell* **84**, 115–125
- Hong, J. X., Wilson, G. L., Fox, C. H. and Kehrl, J. H. (1993) Isolation and characterization of a novel B cell activation gene. *J. Immunol.* **150**, 3895–3904
- Siderovski, D. P., Heximer, S. P. and Forsdyke, D. R. (1994) A human gene encoding a putative basic helix–loop–helix phosphoprotein whose mRNA increases rapidly in cycloheximide-treated blood mononuclear cells. *DNA Cell. Biol.* **13**, 125–147
- Chen, C., Zheng, B., Han, J. and Lin, S. C. (1997) Characterization of a novel mammalian RGS protein that binds to G α proteins and inhibits pheromone signaling in yeast. *J. Biol. Chem.* **272**, 8679–8685
- Chen, C. K., Wieland, T. and Simon, M. I. (1996) RGS-r, a retinal specific RGS protein, binds an intermediate conformation of transducin and enhances recycling. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12885–12889
- Chen, C. and Lin, S. C. (1998) The core domain of RGS16 retains G-protein binding and GAP activity in vitro, but is not functional in vivo. *FEBS Lett.* **422**, 359–362
- Zhang, Y., Neo, S. Y., Han, J., Yaw, L. P. and Lin, S. C. (1999) RGS16 attenuates galphag-dependent p38 mitogen-activated protein kinase activation by platelet-activating factor. *J. Biol. Chem.* **274**, 2851–2857
- Beadling, C., Druey, K. M., Richter, G., Kehrl, J. H. and Smith, K. A. (1999) Regulators of G protein signaling exhibit distinct patterns of gene expression and target G protein specificity in human lymphocytes. *J. Immunol.* **162**, 2677–2682
- Druey, K. M., Ugur, O., Caron, J. M., Chen, C. K., Backlund, P. S. and Jones, T. L. (1999) Amino-terminal cysteine residues of RGS16 are required for palmitoylation and modulation of Gi- and Gq-mediated signaling. *J. Biol. Chem.* **274**, 18836–18842
- Chen, C., Seow, K. T., Guo, K., Yaw, L. P. and Lin, S. C. (1999) The membrane association domain of RGS16 contains unique amphipathic features that are conserved in RGS4 and RGS5. *J. Biol. Chem.* **274**, 19799–19806
- Panetta, R., Guo, Y., Magder, S. and Greenwood, M. T. (1999) Regulators of G-protein signaling (RGS) 1 and 16 are induced in response to bacterial lipopolysaccharide and stimulate c-fos promoter expression. *Biochem. Biophys. Res. Commun.* **259**, 550–556
- Song, L., De Sarno, P. and Jope, R. S. (1999) Muscarinic receptor stimulation increases regulators of G-protein signaling 2 mRNA levels through a protein kinase C-dependent mechanism. *J. Biol. Chem.* **274**, 29689–29693

- 16 Khawaja, X. Z., Liang, J. J., Saugstad, J. A., Jones, P. G., Harnish, S., Conn, P. J. and Cockett, M. I. (1999) Immunohistochemical distribution of RGS7 protein and cellular selectivity in colocalizing with Galphaq proteins in the adult rat brain. *J. Neurochem.* **72**, 174–184
- 17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In *Molecular Cloning: A Laboratory Manual*, 2nd edn, vol. 1, Section 7.43, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 18 Favero, J. and Lafont, V. (1998) Effector pathways regulating T cell activation. *Biochem. Pharmacol.* **56**, 1539–1547
- 19 Johnson, M. S., Simpson, J., MacEwan, D. J., Ison, A., Clegg, R. A., Connor, K. and Mitchell, R. (1995) Phorbol ester and diacylglycerol activation of native protein kinase C species from various tissues. *Mol. Cell. Biochem.* **146**, 127–137
- 20 Wilkinson, S. E., Parker, P. J. and Nixon, J. S. (1993) Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.* **294**, 335–337
- 21 Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* **268**, 9194–9197
- 22 Gschwendt, M., Dieterich, S., Rennecke, J., Kittstein, W., Mueller, H. J. and Johannes, F. J. (1996) Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett.* **392**, 77–80
- 23 Llopis, J., Kass, G. E., Gahn, A. and Orrenius, S. (1992) Evidence for two pathways of receptor-mediated Ca^{2+} entry in hepatocytes. *Biochem. J.* **284**, 243–247
- 24 de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature (London)* **396**, 474–477
- 25 Guerini, D. (1997) Calcineurin: not just a simple protein phosphatase. *Biochem. Biophys. Res. Commun.* **235**, 271–275
- 26 Casareale, D., Stevenson, M., Sakai, K. and Volsky, D. J. (1987) A human T-cell line resistant to cytopathic effects of the human immunodeficiency virus (HIV). *Virology* **156**, 40–49
- 27 Hesselgesser, J., Liang, M., Hoxie, J., Greenberg, M., Brass, L. F., Orsini, M. J., Taub, D. and Horuk, R. (1998) Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. *J. Immunol.* **160**, 877–883
- 28 Moratz, C., Kang, V. H., Druey, K. M., Shi, C. S., Scheschonka, A., Murphy, P. M., Kozasa, T. and Kehrl, J. H. (2000) Regulator of G protein signaling 1 (RGS1) markedly impairs G α signaling responses of B lymphocytes. *J. Immunol.* **164**, 1829–1838
- 29 Benzing, T., Brandes, R., Sellin, L., Schermer, B., Lecker, S., Walz, G. and Kim, E. (1999) Upregulation of RGS7 may contribute to tumor necrosis factor-induced changes in central nervous function. *Nat. Med. (NY)* **5**, 913–918
- 30 Csonga, R., Prieschl, E. E., Jaksche, D., Novotny, V. and Baumruker, T. (1998) Common and distinct signaling pathways mediate the induction of TNF-alpha and IL-5 in IgE plus antigen-stimulated mast cells. *J. Immunol.* **160**, 273–283
- 31 Sayeed, M. M. (1996) Alterations in calcium signaling and cellular responses in septic injury. *New Horiz.* **4**, 72–86
- 32 Burchett, S. A., Bannon, M. J. and Granneman, J. G. (1999) RGS mRNA expression in rat striatum: modulation by dopamine receptors and effects of repeated amphetamine administration. *J. Neurochem.* **72**, 1529–1533
- 33 Pepperl, D. J., Shah-Basu, S., VanLeeuwen, D., Granneman, J. G. and MacKenzie, R. G. (1998) Regulation of RGS mRNAs by cAMP in PC12 cells. *Biochem. Biophys. Res. Commun.* **243**, 52–55
- 34 Xu, X., Zeng, W., Popov, S., Berman, D. M., Davignon, I., Yu, K., Yowe, D., Offermanns, S., Muallem, S. and Wilkie, T. M. (1999) RGS proteins determine signaling specificity of Gq-coupled receptors. *J. Biol. Chem.* **274**, 3549–3556

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