Calcium-dependent regulation of tumour necrosis factor- α receptor signalling by copine

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The role of copines in regulating signalling from the TNF- α (tumour necrosis factor- α) receptor was probed by the expression of a copine dominant-negative construct in HEK293 (human embryonic kidney 293) cells. The construct was found to reduce activation of the transcription factor NF- κ B (nuclear factor- κ B) by TNF- α . The introduction of calcium into HEK293 cells either through the activation of muscarinic cholinergic receptors or through the application of the ionophore A23187 was found to enhance TNF- α -dependent activation of NF- κ B. This effect of calcium was completely blocked by the copine dominant-negative construct. TNF- α was found to greatly enhance the expression of endogenous copine I, and the responsiveness of the TNF- α signalling pathway to muscarinic stimulation increased in parallel with the increased copine I expression. The copine dominant-negative construct also inhibited the TNF- α -dependent degradation of I κ B, a regulator of

NF- κ B. All of the effects of the dominant-negative construct could be reversed by overexpression of full-length copine I, suggesting that the construct acts specifically through competitive inhibition of copine. One of the identified targets of copine I is the NEDD8-conjugating enzyme UBC12 (ubiquitin C12), that promotes the degradation of I κ B through the ubiquitin ligase enzyme complex SCF ^{β TrCP}. Therefore the copine dominant-negative construct might inhibit TNF- α signalling by dysregulation or mislocalization of UBC12. Based on these results, a hypothesis is presented for possible roles of copines in regulating other signalling pathways in animals, plants and protozoa.

Key words: copine dominant negative, I κ B, nuclear factor- κ B (NF- κ B), ubiquitin C12 (UBC12).

INTRODUCTION

The copines are a ubiquitous family of calcium-dependent, membrane-binding proteins that are highly conserved in animals, plants and protists, and which interact with membranes through a pair of C2 domains in the N-terminal portion of the copine molecule [1–7]. The C-terminal portion of copine has a distant similarity to the protein-binding domain of certain integrins, named the A domain [8]. We have recently reported that the A domains of human copines I, II and IV mediate the binding of copines to target proteins [6]. Over 20 target proteins were identified, and a number of these are proteins involved in intracellular signalling pathways, such as MEK1 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase], protein phosphatase 5, the CDC42-regulated protein kinase, and UBC12 (ubiquitin C12), a NEDD8-conjugating enzyme involved in regulating the activity of the transcription factor NF- κ B (nuclear factor- κ B). Moreover, the copines are able to recruit these target proteins to phospholipid surfaces, suggesting that they may regulate their activities and localization in cells in response to changes in intracellular Ca²⁺ [6]. This has led to the hypothesis that a possible function of the copines may be to confer Ca²⁺ regulation on intracellular signalling pathways, although no specific cell signalling mechanism has been demonstrated to be influenced by copines.

In the present paper, we provide data suggesting that signalling in HEK293 (human embryonic kidney 293) cells from the TNF- α (tumour necrosis factor- α) receptor through the transcription factor NF- κ B is modulated in a Ca²⁺-dependent fashion by copine I. TNF- α is a potent cytokine that elicits critical biological responses, such as inflammation and apoptosis [9]. Here we

demonstrate that the ability of Ca²⁺, introduced into the cytoplasm of HEK293 cells either by activation of muscarinic receptors or by application of the ionophore A23187, to stimulate the TNF- α signalling pathway is completely abrogated by inhibition of copine function by a dominant-negative copine construct. The results represent the first demonstration of a physiological function of copines in regulating a specific intracellular signalling pathway, and provide a paradigm for the potential role of copines in other signalling pathways.

EXPERIMENTAL

Cell culture

HEK293 cells were cultured at 37 °C in 5 % CO₂ in minimal essential medium (Gibco) containing 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ g/ml streptomycin and 100 units/ml penicillin. Charcoal-stripped fetal bovine serum (Hyclone) was used to minimize basal stimulation of the cells. Cells were maintained in 100 mm tissue culture dishes (Falcon) and passaged every 2–3 days.

Copine expression plasmids

cDNA encoding full-length human copine I was amplified from the expression plasmid pGEX-copine I [1] by PCR and ligated into the *Hind*III and *Sal*I sites of the mammalian GFP (green fluorescent protein) fusion vectors pEGFP-C and pEGFP-N (Clontech). The resulting plasmids, pEGFP-C-copine I (labelled GFP-COP in the Figures) and pEGFP-N-copine I (COP-GFP) were used to express copine I with GFP fused to the N-terminus or the

Abbreviations used: GFP, green fluorescent protein; HEK, human embryonic kidney; I κ B, inhibitor of NF- κ B; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK (extracellular-signal-regulated kinase) kinase; NF- κ B, nuclear factor- κ B; TNF- α , tumour necrosis factor- α .

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C-terminus respectively, under the control of the CMV (cytomegalovirus) promoter. cDNA encoding the copine I A domain (residues 260–542) was similarly amplified from pGEX-copine I and ligated into the *EcoRI* and *SalI* sites of pEGFP-C. The resulting plasmid, pEGFP-C-copine IA (GFP-COP A) was used to express the A domain with GFP fused to the N-terminus. For control experiments pEGFP-C (GFP) was used without an insert to express GFP.

Luciferase reporter assay for NF- κ B activity

HEK293 cells, cultured as described above, were added to 24-well plates (2 cm²/well; 10⁵ cells/well) covered with poly(D-lysine). After 24 h the cells were co-transfected with the pNF κ B-Luc Cis-Reporter plasmid (Stratagene; 219 078), the pRL-TK (*Renilla* luciferase) plasmid (Promega) and different amounts of copine and control plasmids. pNF κ B-Luc encodes firefly luciferase as a reporter gene driven by a basic TATA box promoter element and tandem repeats of the κ B sequence motif as an enhancer. pRL-TK expresses *Renilla* luciferase driven by the HSV-TK (herpes simplex virus thymidine kinase) promoter, and was used as an internal standard for transfection efficiency. Although normalization of the firefly luciferase signal to the *Renilla* luciferase signal corrected for differences in transfection efficiency and other systematic factors that might influence the absolute measurement of the luminescence signal, we still found some variation in the signal obtained under identical stimulus conditions when comparing experiments using different cell cultures. This variation is shown in the Figures in the Results section, and is probably due to slight differences in the cell cultures due to time of passage, minor differences in culture medium, and other environmental conditions that may influence the TNF signalling pathway. However, these variations did not have a significant impact on the conclusions we were able to draw from individual experiments, all of which were performed with consistent internal controls.

Plasmid DNAs were prepared using the Endo Free kit (Qiagen) and quantified by measuring absorbances. Transfection was carried out by incubating the cells for 24 h in complete medium containing (per well) 0.75 μ l of FuGENE 6 transfection reagent (Roche), 30 ng of pNF κ B-Luc, 20 ng of pRL-TK and variable amounts of copine and control vectors. Transfection efficiency, evaluated by fluorescence microscopy of the GFP-tagged proteins, was > 90 %. For stimulation by TNF- α , cells were washed twice with 0.5 ml of serum-free medium and then incubated with 0.5 ml of serum-free medium containing 10 ng/ml recombinant mouse TNF- α (Sigma; T-7539) for various times. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). To this end, cells were washed twice with 0.5 ml of PBS (Gibco) and lysed with 100 μ l/well lysis solution for 15 min on a rocker at room temperature. Firefly and *Renilla* luciferase activities were measured sequentially immediately after lysis at room temperature in 20 μ l samples of cell lysates using a Turner 20/20 luminometer at 40 % sensitivity for 12 s. High-activity samples that exceeded the linear range of the luminometer were diluted appropriately. The data presented in the Figures are the means and sample S.D.s of triplicate determinations.

Analytical methods

SDS/12 % -PAGE gels were prepared according to Laemmli [10]. Immunoblotting was carried out on 0.2 μ m nitrocellulose membranes (Bio-Rad) using rabbit polyclonal anti-copine I antibodies prepared as described in [3] or commercial goat polyclonal anti-I κ B α antibodies (sc-371; Santa Cruz Biotechnology) (where I κ B is an inhibitor of NF- κ B). Detection was carried out by chemiluminescence using appropriate peroxidase-labelled secondary

antibodies and a commercial substrate kit (Pierce Supersignal). Protein was measured using a commercial reagent (Bio-Rad Protein Assay) based on the dye-binding method of Bradford [11] using BSA (Bio-Rad; Protein Assay Standard II) as a standard. Carbachol, atropine and Ca²⁺ ionophore A23187 were from Sigma.

RESULTS

Inhibition of NF- κ B activation by a copine dominant-negative construct

The isolated A domain of copine I has been demonstrated to block in a dose-dependent manner the ability of intact copine to recruit target proteins to immobilized phospholipids *in vitro* [6]. It presumably accomplishes this by binding to the copine-binding site on the target protein and preventing occupation of the site by intact copine. We therefore constructed a plasmid that would express the isolated A domain of copine I in cells to assess whether inhibition of this target protein recruitment activity of copine in a cell may have physiological effects. The A domain was fused to DNA encoding GFP so that transfection efficiencies and expression levels could be evaluated. We chose to examine signalling from the TNF- α receptor because of the presence of a κ B promoter site in the gene for copine I (see Discussion), and the observation that UBC12, a regulator of the degradation of I κ B, has been identified as a copine target [6].

As shown in Figure 1, expression of the copine A domain in HEK293 cells had a dose-dependent inhibitory effect on signalling from the TNF- α receptor to a reporter construct comprising multiple copies of the κ B promoter fused to the gene for luciferase. Expression of GFP alone had no effect on TNF- α signalling (Figure 1). The inhibition by the A domain appeared to be due specifically to inhibition of the function of endogenous copine, since overexpression of full-length copine from a second plasmid was found to overcome the inhibitory effect of the A domain (Figure 2).

Effects of the copine A domain on calcium activation of NF- κ B signalling

There are several reports of interactions between calcium fluxes and TNF- α signalling [12–15], so it is possible that the inhibition caused by the A domain of copine could involve interference with a calcium-dependent component of the TNF- α signalling pathway. To examine this possibility, we introduced calcium into the HEK293 cells either by stimulation of muscarinic receptors, which causes the release of calcium from intracellular stores, or the use of a calcium ionophore, A23187. In both cases, the signalling from the receptor was enhanced (Figure 3). Stimulation of the signalling pathway by carbachol was completely blocked by the addition of atropine (results not shown), indicating that the effect was via activation of the muscarinic cholinergic receptor. The introduction of calcium by these methods in the absence of TNF- α did not activate the signalling pathway in either case (results not shown). Therefore calcium appears to act only in a synergistic fashion with TNF- α to enhance signalling through this pathway.

The copine A domain inhibited the effect of calcium on this pathway (Figure 3). The inhibition was dose-dependent, and the highest A domain concentrations completely blocked the effect of calcium, while only partially inhibiting TNF- α signalling. The inhibition of the effect of calcium appeared to be specifically through an action on copine, since overexpression of intact copine partially restored the stimulatory effect of calcium (Figure 4).

Figure 1 Effect of the copine A domain on the activation of NF- κ B in TNF- α -stimulated HEK293 cells

The activity of NF- κ B was measured using a firefly luciferase reporter gene containing tandem repeats of the κ B enhancer sequence. Cells were co-transfected with the reporter gene (see the Experimental section) and 150 ng of plasmids for the expression of a GFP-copine I A domain fusion protein (GFP-COP A) or for the expression of GFP alone (GFP). At 24 h after transfection, cells were stimulated for 5 h with TNF- α in serum-free medium (TNF). To evaluate the efficiency of transfection, cells were also co-transfected with a plasmid for the expression of *Renilla* luciferase. Shown are the ratios of the activities of firefly and *Renilla* luciferases ('Relative luciferase activity') measured in cell lysates from cells treated as indicated. The data represent means and sample S.D.s of triplicate determinations. **(A)** Effects of TNF- α , the control GFP vector and the copine A domain expression vector. **(B)** Dose dependence of the inhibition of NF- κ B activation by increasing amounts of the GFP-COP A plasmid.

These results suggest that the ability of calcium to activate this receptor signalling pathway is critically dependent on its action on copine.

TNF- α promotes the expression of copine

Another indication of a possible link between copines and TNF- α signalling is provided by the presence of an imperfect consensus sequence for the binding of NF- κ B in the copine I gene (see Discussion). We therefore examined whether treatment of HEK293 cells with TNF- α would increase the expression level of endogenous copine I. Indeed, as shown in Figure 5, only a trace of copine I could be detected in untreated cells, while treatment with TNF- α for up to 5 h dramatically enhanced the expression of copine I.

Figure 2 Antagonism of the effect of the copine A domain on NF- κ B activation by full-length copine in TNF- α -stimulated HEK293 cells

Activation of NF- κ B was measured using a κ B-firefly luciferase reporter gene in cells stimulated with TNF- α for 5 h, as described in the legend to Figure 1. Cells were transfected with the GFP-COP A plasmid as described in Figure 1 supplemented with plasmids (100 ng DNA/well) for the expression of full-length copine I tagged with GFP, or of GFP alone. Two different full-length copine I constructs were used: an N-terminally tagged construct (GFP-COP) and a C-terminally tagged construct (COP-GFP). Note that the full-length copine constructs blocked the inhibitory effect of the copine A domain, while GFP alone did not.

Since the effects of the dominant-negative copine construct suggested that copine was essential for calcium regulation of the TNF- α receptor signalling pathway, we sought to determine if the enhanced expression of copine I led to changes in the calcium sensitivity of the signalling pathway. We examined the effects of carbachol treatment on the activation of NF- κ B at various times after initiation of treatment with TNF- α , and found that the responsiveness to calcium increased in a time-dependent fashion (Figure 6) that paralleled the increases in levels of endogenous copine I expression (Figure 5).

Role of copine in the metabolism of I κ B

As mentioned above, one of the identified targets of copine is the NEDD8-conjugating enzyme UBC12 [6], which is involved in the degradation of I κ B, a critical regulator of NF- κ B activity. Therefore we sought to determine the effects of the copine A domain construct on the levels of I κ B in HEK293 cells. As shown in Figure 7, activation of the TNF- α receptor led to a loss of I κ B, and this loss was reduced by the presence of the copine A domain. This effect of the A domain was reversed by overexpression of full-length copine I (Figure 7).

DISCUSSION

Probing the biological roles of copines

The copines are a family of Ca²⁺-dependent, phospholipid-binding proteins expressed by a wide range of organisms, from plants to humans [1]. Although their biological role is not definitively known, it has been postulated that copines may be involved in exocytosis [1], neuronal long-term potentiation [16], phagocytosis [3] and phosphorylation of intracellular proteins [17]. In green plants, mutation of a copine gene leads to alterations

Figure 3 The copine A domain inhibits stimulation of TNF- α -dependent NF- κ B activation by carbachol or calcium ionophore A23187

Intracellular activation of NF- κ B in HEK293 cells was measured using a κ B-firefly luciferase reporter gene in cells stimulated with TNF- α for 5 h, as described in the legend to Figure 1. **(A)** Carbachol was added at a concentration of 100 μ M after 4 h of incubation with TNF- α (solid bars). Open bars represent controls without carbachol. Increasing amounts of the copine A domain plasmid (GFP-COP A) led to a dose-dependent inhibition of NF- κ B activation. Note in particular that, at the highest doses of GFP-COP A, stimulation by carbachol was eliminated. The inset shows the effect of GFP-COP A on the percentage increase in TNF- α -dependent NF- κ B activation caused by carbachol. **(B)** Calcium ionophore A23187 was added at a concentration of 10 μ M after 4 h of incubation with TNF- α (solid bars). Open bars represent controls without A23187. Increasing amounts of GFP-COP A led to a dose-dependent inhibition of NF- κ B activation. Note in particular that, at the highest dose of GFP-COP A, stimulation by A23187 was eliminated. The inset shows the effect of GFP-COP A on the percentage increase in TNF- α -dependent NF- κ B activation caused by A23187.

in plant size, stress responses and apoptosis [4,5]. The fact that copines contain two Ca^{2+} -dependent, phospholipid-binding domains, or C2 domains, similar to those of protein kinase C, synaptotagmin and other proteins involved in the transduction of Ca^{2+} signals [18], suggests the involvement of copines in Ca^{2+} -mediated intracellular processes. To advance our understanding of the biological role of copines, we have recently focused on a defining feature of this family of proteins: the C-terminal portion of the molecule—or A domain—which shows a distant relationship to the protein-binding domain of certain integrins [1]. We have demonstrated, for example, that the copine A domain binds a host of intracellular target proteins, many of which are associated with mechanisms of signal transduction [6]. Moreover, the isolated A domain of copine I blocks, in a dose-dependent manner, the ability

Figure 4 Partial restoration of the carbachol sensitivity of TNF- α signalling by overexpression of full-length copine I

Intracellular activation of NF- κ B in HEK293 cells was measured using a κ B-firefly luciferase reporter gene in cells stimulated with TNF- α for 5 h as described in the legend to Figure 1. Filled bars, carbachol was added at a concentration of 100 μ M for the final 1 h of TNF- α treatment; open bars, no carbachol was added. Cells were transfected with plasmids expressing GFP alone (150 ng), GFP-COP A (150 ng) and/or GFP-COP (100 ng), as indicated.

Figure 5 Intracellular levels of native copine I in TNF- α -stimulated cells

HEK293 cells were cultured as described in the Experimental section for luciferase measurements and stimulated with TNF- α for different periods of time. Cell lysates were subjected to SDS/PAGE (100 μ g of protein/lane), transferred to a nitrocellulose membrane and probed with antibodies against copine I.

Figure 6 Time-dependent increase in carbachol sensitivity of TNF- α signalling after exposure to TNF- α

Activation of NF- κ B in HEK293 cells was measured using a κ B-firefly luciferase reporter gene as described in the legend to Figure 1, in cells stimulated with TNF- α for the times indicated. Carbachol (100 μ M) was added where indicated for 1 h prior to cell lysis (filled bars). Open bars represent controls without the addition of carbachol.

of full-length copine to recruit target proteins to immobilized phospholipids *in vitro* [6]. Therefore the isolated copine A domain may be a useful tool to investigate copines by analysing the impact

Figure 7 Effects of the copine A domain and full-length copine on intracellular levels of $\text{I}\kappa\text{B}$ in $\text{TNF-}\alpha$ -stimulated cells

HEK293 cells were transfected and stimulated with $\text{TNF-}\alpha$ as described in the legend to Figure 1. After $\text{TNF-}\alpha$ treatment, cells were lysed and the lysates subjected to SDS/PAGE (100 μg of protein/lane), transferred to a nitrocellulose membrane and probed with anti- $\text{I}\kappa\text{B}$ antibodies. Shown are the levels of $\text{I}\kappa\text{B}$ in $\text{TNF-}\alpha$ -stimulated cells transfected with plasmids expressing the copine A domain (GFP-COP A), full-length copine (GFP-COP) or GFP alone, separately or in combination as indicated. Transfection with the copine A domain increased the levels of $\text{I}\kappa\text{B}$, reversing the effect of $\text{TNF-}\alpha$. Transfection with full-length copine did not alter the levels of $\text{I}\kappa\text{B}$, however, it blocked the effect of the copine A domain.

of overexpression of the copine A domain on different biological processes.

Copine I and $\text{TNF-}\alpha$ signalling

A possible relationship between copines and $\text{TNF-}\alpha$ signalling was suggested to us by an analysis of the sequence of the human copine I gene. Inspection of the upstream sequences shows that apparently the copine gene has no TATA box, CAAT box or initiator sequences, which is a common feature of constitutively expressed genes [19]. Unfortunately, the lack of these elements precludes the identification of the transcription start site by sequence analysis alone. We were able, however, to identify a putative promoter region based on the presence of a CG box and other elements. Sequence analysis of this region using the TRANSFAC algorithm [20] shows the presence of an imperfect κB sequence (J.L. Tomsig and C.E. Creutz, unpublished work). The consensus sequence for the κB enhancer is GGGACTTTCC, and the copine gene has a similar sequence motif (GGGATC-CTCC) at -32624 bp from the translation initiation codon. The κB sequence is a transcription enhancer that is specifically recognized by the transcription factor $\text{NF-}\kappa\text{B}$, first identified in B lymphocytes [21]. The main stimuli for $\text{NF-}\kappa\text{B}$ activation are oxidative stress and other deleterious agents, such as UV light [22], as well as $\text{TNF-}\alpha$ secreted by macrophages, monocytes, lymphocytes and other cells (see [9] for a review).

Consistent with our sequence analysis, we observed that native copine expression by HEK293 was greatly enhanced by treatment with $\text{TNF-}\alpha$ (Figure 5). We investigated the possibility that copine may influence $\text{TNF-}\alpha$ signalling, since signalling cascades often show feedback phenomena, both positive and negative. Our results suggest that $\text{TNF-}\alpha$ -dependent copine expression is a type of positive feedback mechanism, in that it sensitizes the $\text{NF-}\kappa\text{B}$ signalling pathway to positive regulation by calcium.

In general, the role of Ca^{2+} in $\text{TNF-}\alpha$ signalling is poorly understood. It has been reported previously that there is a potentiation of $\text{TNF-}\alpha$ signalling by Ca^{2+} influx, whether from intracellular [12,15] or extracellular [13,14] sources, and, in the case of $\text{TNF-}\alpha$ -induced apoptosis, Ca^{2+} has been reported to be the intracellular mediator of the effects of $\text{TNF-}\alpha$ [23]. In the present study we observed that both muscarinic stimulation and ionophore-mediated Ca^{2+} influx enhanced $\text{TNF-}\alpha$ signalling. The Ca^{2+} -stimulated component of $\text{TNF-}\alpha$ signalling appeared to be more sensitive to the inhibitory effect of the copine A domain than basal signalling. However, the A domain also partially inhibited

signalling from the $\text{TNF-}\alpha$ receptor when an additional calcium-mobilizing stimulus was not provided. It is possible that this effect was also due to an action on the role of calcium, which might be mobilized to a lesser extent by $\text{TNF-}\alpha$ receptor activation alone.

The exact mechanism by which copine affects $\text{NF-}\kappa\text{B}$ signalling is not immediately obvious. Since there are multiple target proteins for copines [6], including components of the MAPK signalling pathway (MEK1) and pathways involving the CDC42 regulated kinase and protein phosphatase 5, the effects may come about through more than one mechanism. However, a possible direct link between copines and $\text{NF-}\kappa\text{B}$ signalling is represented by the interaction between the NEDD8-conjugating enzyme UBC12 and the copine I A domain [6]. NEDD8 is a ubiquitin-like protein that is covalently attached to proteins targeted for degradation through the co-ordinated action of the conjugating enzyme UBC12 and other enzymes [24]. It has recently been demonstrated that attachment of NEDD8 to Cul-1, a component of the ubiquitin ligase system that ubiquitinates $\text{I}\kappa\text{B}$, is an important requirement for the degradation of $\text{I}\kappa\text{B}$ and may therefore constitute a regulatory mechanism for $\text{NF-}\kappa\text{B}$ signalling [25,26]. Our observations suggest that copines may enhance $\text{NF-}\kappa\text{B}$ signalling by promoting $\text{I}\kappa\text{B}$ degradation via an activatory effect on UBC12. Possibly, endogenous copine binds UBC12 and promotes its association with other components of the signalling pathway on a membrane surface, or regulates its activity directly in a calcium-dependent fashion.

General paradigm for the role of copines in signal regulation

The apparent role of copine I in regulating the $\text{TNF-}\alpha$ signalling pathway suggests a general model for the role of copines in regulating diverse signalling pathways. In a series of signal transduction events involving a sequence of interactions between proteins, the ability of copines to localize one or several of the component proteins on a membrane surface could accelerate the pathway. This effect of copine would typically be regulated by Ca^{2+} acting through the C2 domains of copine. However, changes in the lipid composition of membranes could also regulate copine function since, for example, copine I binds with high affinity to phosphatidic acid in the absence of calcium [3]. Inhibitory roles could also be envisioned if the binding of a target to a membrane by copine sequesters an important component of a signalling pathway away from other components. The hallmark of the involvement of a copine would be a role for Ca^{2+} in regulating the pathway, including pathways in which Ca^{2+} does not play a central role through other known Ca^{2+} -binding proteins. Because copines have multiple targets in cells, and their expression may be regulated in response to the activation of a signalling pathway, this family of proteins may generally provide mechanisms for cross-talk between signalling pathways, as demonstrated here in the case of signalling from the muscarinic receptor and the $\text{TNF-}\alpha$ receptor. The dominant-negative copine A domain construct described here may prove to be an important probe for identifying other signalling pathways that are subject to regulation by a copine.

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REFERENCES

- Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J. and Cohen, J. (1998) The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from *Paramecium* to humans. *J. Biol. Chem.* **273**, 1393–1402

- 2 Nakayama, T., Yaoi, T., Yasui, M. and Kuwajima, G. (1998) N-copine: a novel C2-domain-containing protein with neuronal activity-regulated expression. *FEBS Lett.* **428**, 80–84
- 3 Tomsig, J. L. and Creutz, C. E. (2000) Purification and biochemical characterization of copine: a ubiquitous Ca^{2+} -dependent, phospholipid-binding protein. *Biochemistry* **39**, 16163–16175
- 4 Hua, J., Grisafi, P., Cheng, S. H. and Fink, G. R. (2001) Plant growth homeostasis is controlled by the *Arabidopsis* BON1 and BAP1 genes. *Genes Dev.* **15**, 2263–2272
- 5 Jambunathan, N., Siani, J. M. and McNellis, T. W. (2001) A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. *Plant Cell* **13**, 2225–2240
- 6 Tomsig, J. L., Snyder, S. L. and Creutz, C. E. (2003) Identification of targets for calcium signalling through the copine family of proteins. *J. Biol. Chem.* **278**, 10048–10054
- 7 Tomsig, J. L. and Creutz, C. E. (2002) Copines: a ubiquitous family of Ca^{2+} -dependent, phospholipid-binding proteins. *Cell. Mol. Life Sci.* **59**, 1467–1477
- 8 Lee, J. O., Rieu, P., Arnaout, M. A. and Liddington, R. (1995) Crystal structure of the A domain from the alpha subunit of the integrin CR3 (CD11b/CD18) *Cell* **80**, 631–638
- 9 Baud, V. and Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11**, 372–377
- 10 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (London)* **227**, 680–685
- 11 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 12 Chen, K., Chang, B. H., Younan, P., Shlykov, S. G., Sanborn, B. M. and Chan, L. (2002) Increased intracellular calcium transients by calmodulin antagonists differentially modulate tumor necrosis factor- α -induced E-selectin and ICAM-1 expression. *Atherosclerosis* **165**, 5–13
- 13 Bernal-Mizrachi, E., Wen, W., Shornick, M. and Permutt, A. (2002) Activation of nuclear factor- κ B by depolarization and Ca^{2+} influx in MIN6 insulinoma cells. *Diabetes* **51**, S484–S488
- 14 De, A., Krueger, J. M. and Simasko, S. M. (2003) Tumor necrosis α increases cytosolic calcium responses to AMPA and KCl in primary cultures of rat hippocampal neurons. *Brain Res.* **981**, 133–142
- 15 Pollock, J., McFarlane, S. M., Connell, M. C., Zehavi, U., Vandenabeele, P., MacEwan, D. J. and Scott, R. H. (2002) TNF- α receptors simultaneously activate Ca^{2+} mobilisation and stress kinases in cultured sensory neurons. *Neuropharmacology* **42**, 93–106
- 16 Nakayama, T., Yaoi, T. and Kuwajima, G. (1999) Localization and subcellular distribution of N-copine in mouse brain. *J. Neurochem.* **72**, 373–379
- 17 Caudell, E. G., Caudell, J. J., Tang, C. H., Yu, T. K., Frederick, M. J. and Grimm, E. A. (2000) Characterization of copine III as a phosphoprotein with associated kinase activity. *Biochemistry* **39**, 13034–13043
- 18 Rizzo, J. and Südhof, T. (1998) C2-domains, structure and function of a universal Ca^{2+} -binding domain. *J. Biol. Chem.* **273**, 15879–15882
- 19 Novina, C. D. and Roy, A. L. (1996) Core promoters and transcriptional control. *Trends Genet.* **12**, 351–355
- 20 Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Meinhardt, T., Prüss, M., Reuter, I. and Schacherer, F. (2000) TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res.* **28**, 316–319
- 21 Sen, R. and Baltimore, D. (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46**, 705–716
- 22 Schmidt, K. N., Amstad, P., Cerutti, P. and Bäuerle, P. A. (1995) The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF- κ B. *Chem. Biol.* **2**, 13–22
- 23 Kim, B., Kim, H., Mamura, M., Ambudkar, I. S., Choi, K. and Kim, S. (2002) Tumor necrosis factor induces apoptosis in hepatoma cells by increasing Ca^{2+} release from the endoplasmic reticulum and suppressing Bcl-2 expression. *J. Biol. Chem.* **277**, 31381–31389
- 24 Gong, L. and Yeh, E. T. H. (1999) Identification of the activation and conjugating enzymes of the NEDD8 conjugation pathway. *J. Biol. Chem.* **274**, 12036–12042
- 25 Read, M. A., Brownell, J. E., Gladysheva, T., Hottlet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R., Chau, V. and Palombella, V. J. (2000) Nedd8 modification of Cul-1 activates SCF ^{β -TrCP}-dependent ubiquitination of I κ B α . *Mol. Cell. Biol.* **20**, 2326–2333
- 26 Amir, R. E., Iwai, K. and Ciechanover, A. (2002) The NEDD8 pathway is essential for SCF ^{β -TrCP}-mediated ubiquitination and processing of the NF- κ B precursor p105. *J. Biol. Chem.* **277**, 23253–23259

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