Functional association of cytokine-induced SH2 protein and protein kinase C in activated T cells

Shangwu Chen¹, Per O. Anderson¹,², Li Li¹,², Hans-Olov Sjögren², Ping Wang¹ and Su-Ling Li²,³

¹Immunology Group, Institute of Cell and Molecular Sciences, St Barts & The Royal London School of Medicine, Queen Mary University of London, London EC1A 7BE, UK
²Tumor Immunology, Lund University, 22362 Lund, Sweden
³Department of Biological Science, Brunel University, Uxbridge UB8 3PH, UK

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Abstract

TCR signaling is mediated by intracellular signaling molecules and nuclear transcription factors, which are tightly regulated by interaction with regulatory proteins such as Grb2 and SLAP. We reported recently that TCR stimulation induces the expression of cytokine-induced SH2 protein (CIS). The expression of CIS promotes TCR-mediated activation. We have now found specific interactions between CIS and activated protein kinase C (PKC) α, β and θ in TCR-stimulated T cells. CIS was shown by in vitro kinase assay to associate with activated PKC. In CIS-expressing T cells isolated from CIS-transgenic mice, the amount of activated PKC associated with CIS was found to increase following TCR stimulation. By immunohistochemical analysis, CIS was also found to co-localize with PKCθ at the plasma membrane of activated T cells. In addition to the interaction and intracellular co-localization of the CIS and PKC, an increase in the activation of AP-1 and NF-κB was noted in CIS-expressing T cells, after stimulation by either anti-CD3/CD28 or phorbol myristate acetate + ionomycin. These results suggest that CIS regulates PKC activation, and that this may be important for the activation of both the AP-1 and NF-κB pathways in TCR signaling.

Introduction

Stimulation of the TCR by MHC-peptide complexes initiates a set of responses required for the immune system to function properly, including cell proliferation, cytokine production and apoptosis. The engagement of TCR by antigen leads to the recruitment and activation of cytosolic protein tyrosine kinases, such as Lck, ZAP-70 and SYK, which associate with and activate phospholipase (PLC) γ (1–3). When PLCγ is translocated to the membrane, it cleaves phosphatidylinositol (4,5)-bisphosphate, producing diacylglycerol (DAG) and inositol (1,4,5)-triphosphate. DAG activates protein kinase C (PKC), whereas inositol (1,4,5)-triphosphate mobilizes Ca²⁺ from the intracellular stores (4,5). Elevation of the Ca²⁺ level promotes early events in T cell activation by stimulating the activity of calcineurin, a Ca²⁺/calmodulin-dependent serine/threonine protein phosphatase (6,7). The activated calcineurin in turn activates NF-AT (6). NF-AT consists of a nuclear protein (AP-1) and a cytoplasmic protein (NF-ATc). NF-ATc enters the nucleus in response to elevated Ca²⁺ and binds to AP-1. This duplex, in turn, binds to the NF-AT recognition sites (8,9). Cyclosporin A, an immunosuppressant, antagonizes calcineurin, and blocks both activation and nuclear entry of NF-ATc (7,10).

DAG recognizes a cysteine-rich region of PKC and activates members of the PKC family (11,12). More than 10 members of the PKC family have been discovered. These have been divided into subgroups according to their primary structure and their binding of Ca²⁺ or phorbol ester (11,13). Although both conventional PKC (cPKC), such as α, βI, βII and γ, and novel PKC (nPKC), such as δ, ε, θ and η, are activated by phorbol myristate acetate (PMA), only cPKC bind Ca²⁺. Atypical PKC (aPKC), such as ζ and τ (λ), as well as PKC-μ/PKD are not activated by either phorbol esters or Ca²⁺, although they do bind DAG (12,13). The requirement for PKC in T cell activation has been demonstrated in both studies of dominant-negative and/or constitutively active mutants of PKC (14,15), and in studies of T cell activation using pharmaco-
logical agonists and antagonists of PKC (16). By the use of constitutively active mutants of PKCα, ε and θ, it has been demonstrated, in a mouse T cell line, that PKCα preferentially induces AP-1 transcriptional activity (15). Most of the PKC are present not only in T cells, but also other types of cell, although PKCθ is expressed primarily in hematopoietic cells (17), and during immune responses is localized to the region of contact between the TCR and the antigen-presenting cell (APC) (18).

In a study of the regulation of mitogen activating protein kinases (MAPK) by PKC it was shown that, although PKCα, α and ε all activate extracellular signal-regulated kinase (ERK), PKCθ specifically cooperates with calcineurin, generating signals which converge on Rac, leading to a strong activation of c-Jun NH2-terminal kinase (JNK) (19). These studies demonstrate the importance of PKC in T cell activation, as well as their distinct biological functions.

Recently, we reported that cytokine-induced SH2 protein (CIS) is an immediate-early gene that appears following TCR stimulation (20). CIS, a member of the suppressors of cytokine signalling (SOCS) family, has been shown to be a negative regulator of cytokine signalling by inhibiting the IL-3- and erythropoietin (EPO)-mediated signal transducer and activator of transcription (STAT) S-signaling pathway (21–24). The members of the SOCS protein family have been identified on the basis of their structural similarities, all exhibiting an N-terminal region of variable length, a highly variable amino acid sequence, a central SH2 domain and a striking region of C-terminal homology designated as the SOCS box (21,25–27). Four of the members (CIS, JAB/ SOCS1/SSI, SOCS2 and SOCS3) were found to negatively regulate cytokine signal transduction by interacting with either the phosphorylated cytoplasmic domains of the cytokine receptors (21), the activated Jak kinases (26) or the STAT proteins (22). The forced expression of CIS in one myeloid cell line (M1) was found to partially suppress IL-3-induced proliferation (21) and to negatively modulate the activation of STAT5 (22). Recently, the suppression of STAT5 signalling in the liver, the mammary glands and the T cells of CIS-transgenic mice has also been reported (23). We have demonstrated that CIS can be induced by TCR stimulation in T cells (20). Through our studies of T cell activation in CIS-transgenic mice, we have also found that CD4 T cells, which express the transgene in the T cell lineage, exhibit enhanced proliferative responses upon TCR stimulation and prolonged survival of the activated T cells. The finding that this enhanced response of the CIS-expressing CD4 T cells is correlated with a marked increase in the activation of MAPK following TCR stimulation suggests that CIS regulates MAPK activation after TCR activation.

In the present study, efforts to discover the mechanisms by which CIS affects TCR-mediated T cell activation revealed a specific interaction of CIS with activated PKCα, βII and θ. The co-localization of CIS and PKCθ to the plasma membrane of the activated T cells was observed by immunohistochemical analysis. In addition to the association of CIS with activated PKC in the TCR-stimulated T cells, an increased activation of the transcription factors AP-1 and NF-κB was also noted.

**Methods**

**Mice**

C57Bl/6 mice were purchased from the Tounbjerg Breeding Centre (Ejby, Denmark). CIS-transgenic mice were established as previously described (20). Female mice were used at 4–10 weeks of age and were maintained on standard laboratory diet.

**Transfections**

CIS cDNA was subcloned into the pCEP4 expression vector under the control of the cytomegalovirus promoter. Then 10 μg of the resulting plasmid was mixed with 107 Jurkat T cells, and pulsed at 250 V and 950 μF in a 4-mm gap cuvette using a Bio-Rad (Hercules, CA) Gene Pulser. Stable transfectants were selected for in the presence of 400 μg/ml hygromycin, expanded in culture and used in the subsequent immunoprecipitation experiments.

**Electrophoretic mobility shift assay**

CD4 T cells were isolated from the spleen cells of both normal and CIS-transgenic mice by anti-CD4 coated magnetic beads (Dynabeads L3T4; Dynal, Lake Success, NY). After each separation, the efficiency of CD4 cell purification was analysed by flow cytometry and was routinely found to be >95%. Purified CD4 T cells were stimulated by anti-CD3 and anti-CD28 or 50 nM PMA + 50 nM ionomycin for 15 min. Nuclear proteins were then isolated as previously described (28). Double-stranded oligonucleotides derived from the consensus sequences of NF-κB or the AP-1 binding element (Promega, Southampton, UK) were labelled with 32P. Binding assays, using 10 μg of nuclear proteins, were performed as described previously (28). DNA–protein complexes were separated on a 4% polyacrylamide gel.

**Immunoprecipitation and immunoblot analysis**

Cell lysates of stimulated CD4 T cells were prepared in 1% NP-40 lysis buffer (containing protease inhibitors (Boehringer Mannheim, Basel, Switzerland) and sodium vanadate) and immunoprecipitated with either anti-CIS (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-PKCθ antibodies (Santa Cruz Biotechnology). Immune complexes were resolved by 10% SDS–PAGE. The anti-CIS precipitates were sequentially immunoblotted with antibodies specific for CIS and PKC (α, βII and θ) respectively. The anti-PKCθ precipitates were likewise immunoblotted with antibodies to CIS and PKCθ. The Jurkat cells transfected with myc-tagged CIS were lysed in 1% NP-40 lysis buffer and precipitated with anti-myc antibody (9E10). The precipitates were blotted with anti-myc 9E10 and anti-PKCθ antibodies.

**Confocal immunofluorescence microscopy**

CD4 T cells isolated from the spleens of normal mice were stimulated with or without anti-CD3 and anti-CD28 for 6 h. After stimulation, the cells were washed, fixed and permeabilized as described previously (29). The fixed cells were then incubated for 4°C with 1 μg/ml of anti-PKCθ and with 2 μg/ml of anti-CIS antibody in PBS containing 1% BSA. After washing, cells were incubated with phycoerythrin-conjugated donkey anti-rabbit IgG antibody (1:200 dilution; Jackson, West Grove,
PA) for the detection of rabbit anti-PKCq and FITC-conjugated donkey anti-goat IgG antibody (1:250 dilution; Santa Cruz Biotechnology) for the detection of goat anti-CIS antibody. The cells were then washed, mounted on glass coverslips using a SlowFade kit (Molecular Probes, Eugene, OR) and analyzed by conventional fluorescence microscopy or by confocal microscopy (MRC 1024S; Bio-Rad).

In vitro kinase assay
PKCθ from cell lysates of anti-CD3 (5 μg/ml) and anti-CD28 (4 μg/ml) or PMA + ionomycin-stimulated CD4 T cells were purified by immunoprecipitation with anti-PKCθ (Santa Cruz Biotechnology), as previously described (30). The kinase activity of PKCθ was measured by a 20-min incubation of immunoprecipitated PKCθ with myelin basic protein (MBP) in the presence of 10 μCi [γ-32P]ATP in kinase buffer (containing 20mM HEPES (pH 7.0), 5 mM 2-mercatoethanol, 10 mM MgCl₂, 0.1 mg/ml BSA and 10 μM unlabeled ATP) at 37°C. The phosphorylated MBP was analysed by SDS–PAGE.

Results

Co-localization of PKCθ with CIS to the plasma membrane in activated T cells

The function of PKC is associated with its membrane translocation (4). More significantly, PKCθ is selectively translocated to the site of contact between the T cells and the APC (18). This selective translocation was found to correlate with a selective increase in the activity of PKCθ (15,19). None of the other cytosolic proteins co-localized with PKCθ in the cytoplasmic membrane of activated T cells (18). To our surprise, CIS translocated to the plasma membrane after TCR stimulation, as shown by immunohistochemical analysis. The co-localization of CIS with PKCθ in anti-CD3/CD28 stimulated normal T cells was examined using confocal immunofluorescence microscopy. Consistent with earlier published results, PKCθ was translocated to the cytoplasmic membrane of the activated T cells (Fig. 1E). A similar membrane localization of CIS was also revealed in the activated T cells (Fig. 1D). Upon staining of the activated T cells with both anti-PKCθ and...
CIS interacts with PKCβ, βII and θ in T cells after stimulation with anti-CD3/CD28 antibodies

Recently, we have reported that CIS is an immediate-early gene induced by TCR stimulation (20). After TCR stimulation, the expression of CIS can strongly increase the proliferative response and survival of T cells (20). Moreover, both CIS and PKCβ are translocated to the cell membrane of stimulated T cells. To elucidate any direct interaction between CIS and PKCβ, we first stimulated CD4 T cells isolated from the spleens of C57bl/6 for 6 h with or without anti-CD3 (5 μg/ml) and anti-CD28 (4 μg/ml). The stimulated CD4 T cells were then lysed in 1% NP-40 lysis buffer. The co-precipitated proteins were sequentially blotted with anti-CIS antibodies, an exclusive co-localization of PKCβ and CIS to the cytoplasmic membrane was demonstrated (Fig. 1F). In contrast, it was found that in unstimulated CD4 T cells only the cytoplasm was stained with anti-CIS antibody (Fig. 1B). The co-localization of CIS and PKCβ in Jurkat cells. Jurkat cells were transfected with myc-tagged CIS and anti-PKCβ, and anti-myc antibodies. (Fig. 2a). These data indicate that the expression of CIS can be induced by TCR stimulation, which is consistent with our previous findings (20). In anti-CD3/CD28-stimulated CD4 T cells, a specific interaction of PKCα, βII and θ, but not PKCζ and ε, with CIS was demonstrated (Fig. 2a). To further confirm the interaction of PKC and CIS, lysates of unstimulated and anti-CD3/CD28-stimulated CD4 T cells were precipitated with anti-PKCβ. The co-precipitation of CIS was confirmed by immunoblotting with anti-CIS antiserum. Consistent with this, anti-CIS antisera was shown to co-precipitate PKCβ from unstimulated CD4 T cells (Fig. 2b). In order to further confirm the association of CIS with PKCβ, Jurkat cells were transfected with myc-tagged CIS cDNA and the transfectants subjected to immunoprecipitation with anti-myc antibody. The blotting analysis of anti-myc and anti-PKCβ showed an interaction between the transfected CIS and endogenous PKCβ (Fig. 2c). Since it has been reported that PKCβ is preferentially expressed in the T cells, selectively regulates JNK and co-localizes with TCR (18), the association of CIS with PKCβ suggests an important role for CIS in the regulation of T cell activation.

Association of CIS with activated PKC

The activity of CIS-associated PKC was analyzed by in vitro kinase assay to further investigate its function. CD4 T cells
PKC.

that CIS preferentially associates with the activated form of PKC. Study of the CD4 T cells from CIS-transgenic mice indicated (24,31,32). CIS has thus been proposed to serve as a negative regulator, but also enhances activation of the AP-1 transcription factor by regulation of PKC activation in T cells.

CIS is one of the STAT5-regulated genes, as the STAT-dependent expression of CIS in the thymus and the discovery of two STAT5 binding sites within the CIS gene promoter indicated (24,31,32). CIS has thus been proposed to serve as a feedback modulator for STAT5, since expression of CIS is induced by STAT5 and it has a negative modulating effect on STAT5 activation. The mechanism mediating the inhibitory effect of CIS on STAT5 activation appears to be the interaction

increased PKC activation in the CIS-expressing T cells or may, alternatively, be directly associated with the CIS molecules.

We have previously reported that CIS-expressing CD4 T cells exhibited a marked increase in the activation of MAPK following TCR stimulation (20). MAPK regulates activation of the AP-1 transcription factors, which promote the expression of IL-2 and leads to the generation of a proliferative response in T cells following TCR stimulation (1–3). Once initiated, the proliferative response of T cells, whether induced by TCR stimulation or exposure to mitogens, is regulated by the transcription factor NF-κB, which is not directly controlled by MAPK (1–3). In order to clarify the role of CIS–PKC association in the AP-1 and NF-κB pathways, we analysed AP-1 and NF-κB from both anti-CD3/CD28 or PMA + ionomycin-activated CD4 T cells for their ability to bind oligonucleotides carrying a binding site specific for each transcription factor. In normal CD4 T cells, stimulation by anti-CD3/CD28 or PMA + ionomycin induced the binding of NF-κB and AP-1 to the oligonucleotides. We expected to see a greater increase in the binding of AP-1 to its consensus nucleotides in the activated CIS-expressing T cells. However, after anti-CD3/CD28 or PMA + ionomycin stimulation, we observed that the activity of both NF-κB and AP-1 was increased in the CIS-expressing T cells, compared with normal T cells (Fig. 4a and b). The increased activity of the NF-κB signalling cascade may be due to the increased PKC activation in the CIS-expressing T cells or may,

isolated from the spleen of both normal and transgenic mice were treated with anti-CD3/CD28 for 15 min. The cells were then lysed and anti-CIS antisera used to precipitate the cleared lysates. Co-precipitated PKC were assayed for their ability to phosphorylate MBP, a substrate for PKC. The kinase activity of CIS-associated PKC was confirmed (Fig. 3a). We have shown that the expression of CIS can enhance both TCR and PMA + ionomycin induced TCR signalling (20). In order to examine the activity of CIS-associated PKC, CIS-expressing CD4 T cells were isolated from the spleens of CIS-transgenic mice and stimulated for different periods of time with PMA + ionomycin. After stimulation, the cells were lysed and cleared lysates precipitated with anti-CIS antiserum. The precipitates were divided into four aliquots, one for kinase assay, and the others for immunoblotting with anti-PKCα, anti-PKCβII and anti-PKCβI antibodies respectively. As shown in Fig. 3(b and c), the activity of CIS-associated PKC was increased following stimulation. These results suggest that the association of CIS with PKC is induced by T cell activation and that CIS preferentially associates with the activated form of PKC.

Discussion

CIS, a member of the SOCS protein family, exhibits a negative feedback function in cytokine signalling (21,22,25–27). Studies have shown that CIS exerts this function on IL-3-, IL-2- and EPO-activated Jak–STAT5 signalling, by blocking the interaction of STAT5 and its docking sites on the cytoplasmic domain of the receptor chain (21). In examining gene expression in activated T cells, we found that CIS is an immediate early gene induced by TCR stimulation (20). Contrary to its inhibitory effect on STAT5 activation, the mechanism mediating the inhibitory effect of CIS on STAT5 appears to be the interaction

Increased activity of both the AP-1 and NF-κB transcription factors in CIS-expressing T cells

We have previously reported that CIS-expressing CD4 T cells exhibited a marked increase in the activation of MAPK following TCR stimulation (20). MAPK regulates activation of the AP-1 transcription factor, which promotes the expression of IL-2 and leads to the generation of a proliferative response in T cells following TCR stimulation (1–3). Once initiated, the proliferative response of T cells, whether induced by TCR stimulation or exposure to mitogens, is regulated by the transcription factor NF-κB, which is not directly controlled by MAPK (1–3). In order to clarify the role of CIS–PKC association in the AP-1 and NF-κB pathways, we analysed AP-1 and NF-κB from both anti-CD3/CD28 or PMA + ionomycin-activated CD4 T cells for their ability to bind oligonucleotides carrying a binding site specific for each transcription factor. In normal CD4 T cells, stimulation by anti-CD3/CD28 or PMA + ionomycin induced the binding of NF-κB and AP-1 to the oligonucleotides. We expected to see a greater increase in the binding of AP-1 to its consensus nucleotides in the activated CIS-expressing T cells. However, after anti-CD3/CD28 or PMA + ionomycin stimulation, we observed that the activity of both NF-κB and AP-1 was increased in the CIS-expressing T cells, compared with normal T cells (Fig. 4a and b). The increased activity of the NF-κB signalling cascade may be due to the increased PKC activation in the CIS-expressing T cells or may,
of CIS with the STAT5 binding sites in the receptors of IL-3, IL-2 and EPO (21–24). The early induction of CIS expression in TCR-stimulated T cells indicates that there is an alternative pathway to STAT5 for the regulation of CIS expression (20). The strong enhancement of the proliferative response to TCR stimulation obtained in the CIS-expressing T cells suggests that the function of CIS in T cell activation is not associated with STAT5 (20). Rather, we have shown in the present study that there is a functional interaction between CIS and PKCα, βII and θ, resulting in an up-regulation of PKC activity and increased intracellular translocation of the activated PKC. Thus, the inhibitory effect of CIS on cytokine signalling is distinct from its stimulatory effect on TCR signalling.

The involvement of PKC isoforms in T cell activation was demonstrated by the activation of PKC by DAG after TCR stimulation (12,13). PKC has been found to be recruited to the membrane/cytoskeletal compartments of activated T cells (18,33). The activation and subcellular localization of PKC are important for promoting the TCR-mediated proliferation (19,34). In particular, the PKC isotype θ was found to be selectively translocated to the site of cell contact between the APC and the T cell (18). This was accompanied by an increased activity of PKCθ (18). In this study, PKCθ activity was enhanced in CIS-expressing T cells upon TCR stimulation. These results indicate the importance of CIS in the regulation of PKCθ in activated T cells. Increased activity of JNK in CIS-expressing CD4 T cells (20) supports the recent findings of the involvement of PKC in the regulation of JNK activation (19). The association of an increased activation of PKC and NF-κB in CIS-expressing T cells suggests that CIS-associated PKC are involved in the regulation of TCR-mediated NF-κB activation. Indeed, the requirement of PKCθ for the activation of NF-κB in T cell activation was reported recently (35,36). In a study of reporter genes in cells transfected with different PKC isoforms, the TCR-mediated activation of both AP-1 and NF-κB was induced only in the PKCθ transfectants (35). Even more interesting is the report that in PKCθ knockout mice, the activation of MAP kinases (JNK, ERK and p38) is not altered, but the activation of both AP-1 and NF-κB is abolished in mature T cells (36). Intact activity of JNK in T cells of PKCθ knockout mice may suggest that JNK can be regulated by other PKC isoforms than PKCθ or is co-regulated by PKCθ and other PKC (36). Since it has been shown that PKCθ is required for the NF-κB activity in mature T cells (36), the strong increase of NF-κB activity in CIS-expressing CD4 T cells suggests the importance of CIS–PKCθ association in the regulation of NF-κB activation in T cells.

PKCθ has also been found to be functionally associated with Vav in T cells (34). By in vitro transfection of PKCθ either with or without Vav, Vav has been shown to promote the translocation of PKCθ from the cytosol to the membrane as well as to the cytoskeleton (34). The co-localization of CIS and PKCθ at the plasma membrane of activated T cells suggests that TCR activation and/or co-stimulatory signals may induce CIS to become involved in the formation of the T cell synapse. CIS associates with multi-isofoms of PKC, although only PKCθ has been found to localize to the point of cellular contact between APC and T cells or to be involved in the regulation of JNK activity (19,34). However, other PKC isoforms have also been shown to be involved in TCR signalling (16,37,38).

Involvement of CIS in cytokine signalling is distinct from its stimulatory effect on TCR signalling. Our results, and those of previous studies concerning the role of CIS in cytokine signalling, point to CIS having important biological functions in different signalling pathways, although the specific function is dependent upon the particular pathway. The association of CIS with PKC and the increase in NF-κB activity in CIS-transgenic T cells after TCR stimulation may suggest the function of CIS in the regulation of T cell activation to be distinct from its inhibitory effect on cytokine signalling. Immune homeostasis requires the limitation of clonal expansion of activated T cells. It was recently reported that TCR-mediated feedback inhibition of T cell expansion, results from an inhibitory effect of STAT5 on IL-2 signalling (41). Therefore, the reciprocal regulation of TCR and IL-2 receptor signalling by CIS may be essential for the control of both immune responses and homeostasis.

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Abbreviations
APC antigen-presenting cell
CIS cytokine induced–SH2 protein (s)
DAG diacylglycerol
EPO erythropoietin
ERK extracellular signal-regulated kinase
JNK c-Jun NH2-terminal kinase
MAPK mitogen-activated protein kinase
MBP myelin basic protein
PKC protein kinase C
PLC phospholipase C
PMA phorbol myristate acetate
SOCS suppressors of cytokine signaling
STAT signal transducer and activator of transcription

References
CIS associates with PKC in activated T cells


