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The Role of Protein Kinase C Signaling in Activated DRA Transcription

Niclas Setterblad,2* Isaac Onyango,† Ulla Pihlgren,* Lars Rask,† and Göran Andersson3*

Expression of human MHC HLA-DRA class II gene can be up-regulated in B cells by Ig cross-linking as well as by phorbol esters such as 12-O-tetradecanoyl phorbol 13-acetate (TPA). Induced DRA expression involves activation of restricted protein kinase C (PKC) isoforms, resulting in activated activator protein-1-dependent transcription. In this report expression profiles and activation of PKC were analyzed in human Raji B lymphoblastoid cells. Transient transfection analysis with target plasmids containing either DRA promoter (wild-type or mutated) or TPA response elements demonstrated that pretreatment with the selective PKC inhibitor GF 109203X repressed TPA-mediated activation. Western analysis performed on cellular fractions of resting cells and of TPA-activated cells revealed abundant expression of classical PKC-α (cPKC-α), cPKC-βII, and atypical PKC-ζ isoforms and identified a sustained translocation of cPKC-α and cPKC-βII from the cytosolic compartment to membranes. As expected, the distribution of atypical PKC-ζ was unaffected by TPA treatment and displayed an even distribution between cytosol and membranes. This finding was confirmed by immunofluorescence microscopy. The TPA-mediated translocation of cPKC-α and cPKC-βII was not influenced by pretreatment with GF 109203X. Finally, functional activation and translocation of PKC were investigated with a selective in vitro kinase assay. Together, these results show that activated HLA-DRA expression in response to TPA treatment is strictly dependent on PKC activation acting on the X2 box of the DRA promoter and that selective inhibition of PKC enzymatic activity does not influence subcellular localization of expressed PKC isoenzymes. Thus, the translocation event per se occurs independently of PKC activation in these cells.

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1 Abbreviations used in this paper: PI-3 kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; AP-1, activator protein-1; DAG, 1,2-diacylglycerol; TPA, 12-O-tetradecanoyl phorbol 13-acetate; cPKC, classical protein kinase C; nPKC, novel protein kinase C; aPKC, atypical protein kinase C; CAT, chloramphenicol acetyltransferase; TRE, TPA response element.

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types express defined sets of PKC isoforms that are differentially localized in subcellular compartments (15, 20).

Class II expression in B cells can be up-regulated by several means, including Ig cross-linking and PKC activation. Artificial phorbol ester treatment mimics these responses, and the induced class II expression involves activation of restricted PKC isoforms that result in activated AP-1-dependent transcription (6, 21, 22). Recently, we have shown that activated HLA-DRA transcription is dependent on PKC signaling in Raji B cells (23). To better understand the molecular mechanisms underlying this activation, we investigated the expression profiles and functional properties of PKC isoforms in resting and TPA-activated Raji B cells.

Materials and Methods

Cell culture and treatment

The human B lymphoblastoid cell line Raji (24) (American Type Culture Collection, Manassas, VA; CCL86) was grown at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, glutamine, 100 IU/ml penicillin, and 50 μg/ml streptomycin. When cells attained a density of 106 cells/ml, 150 ml of cells (i.e., ~150 × 109 cells) received different treatments: no treatment (control cells), 10 nM TPA (Sigma, St. Louis, MO), 10 nM TPA after a 15-min preincubation with 2 μM GF 109203X (catalogue no. 203290-S; K = 10 nM; Calbiochem, La Jolla, CA), and 10 μM TPA. Stock solutions of TPA and GF 109203X prepared in DMSO were used. After 24 h, the cells were harvested, washed twice with PBS, and used for protein fraction preparation. To obtain more time points, extracts from cells treated for 2 and 4 h were prepared for protein fractions (see below).

Preparation of soluble and particulate protein fractions

Soluble and particulate protein fractions were prepared as previously described (30). Briefly, cells were harvested and washed in PBS, then lysed in a buffer (20 μM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM EGTA, 10 mM 2-ME, 50 μg/ml leupeptin, 50 μg/ml aprotonin, and 2 mM PMSF) followed by centrifugation at 100,000 × g for 30 min. The supernatant (i.e., soluble fraction) was collected, and the pellet was resuspended in the same buffer containing 1% Triton X-100. The radioactivity incorporated into the X2 box was determined by a single-well plate reader (Wallac 1420). Following phosphorylation, the membrane-bound X2 product was subjected to SDS-PAGE, transferred to a PVDF membrane, and probed with anti-PKC-α, anti-cPKC-βII, or anti-cPKC-ζ Abs for 30 min. Western blotting

Two hundred micrograms of proteins were loaded onto single-well 10% SDS-PAGE (31) prepared and run on Mighty Small II equipment ( Hoefer, San Francisco, CA). Proteins were transferred onto nitrocellulose filters (Hybond-C Extra, Amersham, Arlington Heights, IL) by semidy electroblotting for 30 min at 15 V and 400 mA. Filters were incubated for 20 min at room temperature in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBS-T) containing 5% (w/v) fat-free dried milk. The nitrocellulose membranes were incubated for 45 min with specific Abs against PKC isoforms (see above; Santa Cruz Biotechnology), all at a dilution of 0.1 μg/ml in TBS-T. This step was followed by a 1:5000 dilution of horseradish peroxidase-labeled anti-rabbit IgG was added for 30 min at room temperature. The membranes were then washed three times in TBS-T and once in TBS, and the blots were developed using enhanced chemiluminescence Western blotting reagents (Amersham). To check for reproducibility, the blots were repeated on multilwell gels, where each extract was run individually and incubated with a single Ab.

Immunofluorescence microscopy

Raji cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and were either not stimulated (control cells) or were treated with 10 nM TPA. After 24 h, the cells were harvested, washed twice with PBS, and spun down on microscope slides with a cytospin (700 rpm, 5 min). Cells were fixed with methanol and incubated with anti-cPKC-α, anti-cPKC-βII, or anti-cPKC-ζ antibodies for 30 min. The functionality of the induced PKC activity following TPA treatment was assessed using a selective PKC inhibitor (GF 109203X). Using the Western blot assay, specific repression of AP-1-dependent transcriptional activation by a selective PKC inhibitor

The functionality of the induced PKC activity following TPA treatment was studied by transient transfection analyses using a wild-type DRA promoter construct and a DRA promoter construct with point mutations in the X2 box. After a recovery period, cells were left untreated or were stimulated for 24 h with 10 nM TPA or 10 nM TPA and GF 109203X. Using the DRA wild-type promoter construct, 10 nM TPA treatment for 24 h resulted in a 1.7-fold induction of relative CAT enzymatic activity (Fig. 1, column 2) compared with the CAT activity in nonstimulated cells (Fig. 1, column 1).
Selective translocations of PKC isoforms following TPA treatment revealed by Western blotting

The aim of this experiment was to identify which isoforms are expressed in these B cells and, further, which isoforms are translocated in response to the different TPA treatments. In resting cells, three main isoforms, cPKC-α, cPKC-βII, and aPKC-ζ, were detected in both the soluble and the particulate fraction (Fig. 2A, lanes 1, 2, 8, 9, 10, and 16). The cPKC-δ and cPKC-ε were both weakly detectable and exclusively in the soluble fraction and the particulate fraction, respectively, following 10 nM TPA treatment for 24 h (Fig. 2B, lanes 3 and 13). The cPKC-α and aPKC-ζ were present as proteins with apparent molecular masses of 74 kDa in both fractions. In contrast, cPKC-βII which has the same apparent molecular mass as cPKC-α in the soluble fraction, appears as a doublet in the particulate fraction, with apparent molecular masses of 72 and 77 kDa, respectively (Fig. 2, A and B, lanes 10, and Fig. 2E, lane 1).

Western blotting of the fractions prepared from cells harvested after TPA treatments (10 nM; 10 min, 60 min, and 24 h) was performed following the same procedure. After 10 min, the distributions of the different isoforms were unchanged compared with that in the control cells (data not shown). Detectable differences were seen after 60 min of TPA treatment: the amounts of cPKC-α and -βII decreased in the soluble fraction, with a concomitant increase in the particulate fraction (not shown). The cPKC-βII band that increased the most was the 77-kDa band. The translocation was even more evident after 24 h of TPA treatment in the soluble fraction, which was almost void of cPKC-α and cPKC-βII (Fig. 2B, lanes 1 and 2). This translocation pattern from the cytosol to the membrane fraction was also seen in the extracts prepared from cells treated with 10 μM TPA for 24 h as well as in the extracts from cells treated with GF 109203X and with 10 nM TPA for 24 h (Fig. 2, C and D). After treatment with 10 μM TPA, neither cPKC-α nor cPKC-βII was detectable in the soluble fraction (Fig. 2C, lanes 1 and 2). Further, only low amounts of both these isoforms were detected in the particulate fraction (Fig. 2C, lanes 3 and 4). This difference between 10 nM vs 10 μM TPA treatment could reflect the fact that under the latter condition both cPKC-α and cPKC-βII are down-regulated. The atypical aPKC-ζ was not translocated following 10 nM TPA treatment for 24 h (Fig. 2B, lanes 8 vs 16). However, proteolytic degradation was observed in the particulate fraction (Fig. 2B, lane 16). Such proteolytic degradation was not seen after 1 h of TPA treatment (not shown).

To identify the relative molecular masses of the two cPKC-βII isoforms found in the particulate fractions, a 7.5% SDS-PAGE was run with an adequate protein size marker (see Materials and Methods). The two bands were identified to be distinct cPKC-βII isoforms with relative molecular masses of 72 and 77 kDa, respectively. Following 10 nM TPA treatment, the amounts of both 72- and 77-kDa forms were increased in the particulate fractions (Fig. 2E, lanes 1 and 2). Interestingly, after treatment with 10 μM TPA, the amount of the 72-kDa form decreased, with a concomitant increase in the 77-kDa form (Fig. 2E, lane 3). We conclude that both cPKC-α and cPKC-βII are translocated in response to TPA treatment, and that following translocation, cPKC-βII is subjected to structural modification.

Translocation of cPKC-α and cPKC-βII to the perinuclear region in response to phorbol ester administration

Immunofluorescence was performed on Raji B cells using antisera specific for the highly expressed cPKC-α and cPKC-βII (see Materials and Methods). In resting cells, cPKC-α and cPKC-βII were equally distributed between the cytosol and the membranes (Fig. 3, A and B). Following treatment of the cells for 24 h with 10 nM
TPA, the cytosol was depleted of both these PKC isoforms, with a parallel increase in staining of the perinuclear membrane (Fig. 3, C and D).

**Induced in vitro PKC activity in response to TPA treatment**

To investigate possible changes in bonafide PKC activities in response to TPA treatment, the protein extracts previously used in Western analyses (see above) were analyzed with a selective PKC assay. This assay takes advantage of the PKC-specific in vitro phosphorylation of a neurogranin peptide corresponding to amino acids 28 to 43. This peptide serves as a specific substrate for calcium- and phospholipid-dependent cPKC and calcium-independent nPKC isozymes (32). Moreover, this assay allows discrimination between activatable and active PKC by the presence or the absence of phospholipids in the reaction, respectively. The assay was performed both in the presence of phospholipids (data not shown) and with a buffer void of phospholipids to measure the active forms of PKC. The soluble fraction displayed a marked decrease in PKC activity following TPA treatment (Fig. 4, columns 1–4) paralleled by an increase in PKC activity in the particulate fraction (Fig. 4, columns 6–9). Moreover, GF 109203X inhibited all PKC activity in the particulate fraction (Fig. 4, compare columns 9 and 10), whereas GF 109203X did not influence the residual PKC activity in the soluble fraction (Fig. 4, column 5). We conclude that the enzymatic activity of phospholipid-dependent PKC isoforms was increased in the particulate fractions in response to TPA treatment, with a concomitant decrease in PKC activities in the soluble fractions.

**Discussion**

GF 109203X almost completely abrogated the TPA-induced CAT activity of the wild-type DRA promoter target plasmid and the AP-1-dependent target plasmid in response to 10 nM TPA treatment. Thus, GF 109203X efficiently inhibits TPA-mediated PKC activity in these cells. The bisindolylmaleimide GF 109203X has been shown to be a selective and potent inhibitor of PKC both in vivo and in vitro by interacting with the catalytic domain and by competing for ATP binding (34–36). In this study, the expression patterns of PKC isoforms and the functional aspects of PKC-mediated signaling were determined in human Raji B cells. The Ca\(^{2+}\)-dependent cPKC-\(\alpha\) and -\(\beta\)II as well as the atypical, Ca\(^{2+}\)- independent aPKC-\(\zeta\) could easily be detected in these cells, whereas nPKC-\(\delta\) and nPKC-\(\epsilon\) displayed weaker signals. This expression pattern is largely in agreement with the previously reported expression of PKC isoforms in other human B cells as well as in murine pre-B and B cells (37–39).

The use of soluble and particulate protein fractions prepared from resting cells and cells treated either with 10 nM TPA or 10 \(\mu\)M TPA in Western blotting analyses revealed that both cPKC-\(\alpha\) and -\(\beta\)II were translocated to the particulate fraction. Treatment with 10 \(\mu\)M TPA for 24 h led to a complete translocation from the soluble to the particulate fraction (Fig. 2B), which was confirmed by immunofluorescence analyses (Fig. 3). In contrast, the subcellular localization of aPKC-\(\zeta\), in agreement with the findings of other studies (reviewed in Ref. 15), remained unaffected by phorbol ester treatment.
When soluble and particulate fractions prepared from cells treated with 10 nM TPA for 24 h were compared, PKC-βII appeared as a 72-/77-kDa doublet in the particulate fraction (Fig. 2, B and E). Two distinct cPKC-βII bands have been reported previously in the B lymphoblastoid cell lines IM-9 and BJA-B (40). Most likely, this size difference reflects a posttranslational modification in response to activation. The larger (77-kDa) band was reported to resist down-regulation by long term phorbol ester treatment (40). This is in agreement with our finding, which shows a sustained amount of the 77-kDa band of cPKC-βII in the particulate fraction after both long term and high dose TPA treatment. Under these conditions, the 72-kDa cPKC-βII band was almost undetectable. Moreover, these two cPKC-βII forms were never detected in soluble fractions of either resting or TPA-treated cells. Thus, the translocation induced by TPA also involves a structural modification of cPKC-βII resulting in the doublet.

Additionally, treatment with the selective PKC inhibitor GF 109203X was performed before treatment with 10 nM TPA. This treatment did not influence translocation of the PKC isoforms in response to TPA treatment. However, selective PKC inhibition was revealed by two criteria. Firstly, in the PKC assay, complete inhibition of the induction mediated by 10 nM TPA was observed in the particulate fraction when the cells were pretreated with GF 109203X. Therefore, the actual translocation event appears to be independent of binding of ATP to the PKC enzymes. At the concentration of 2 μM used in the present investigation, GF 109203X is expected to inhibit all PKC isoforms expressed in these B cells with a ranked order of potency (classical > novel > atypical) (35). Also, the binding of GF 109203X is regulated by a binding constant of $K_i = 10$ nM, and during the preparation of the extracts, some of the inhibitory effect might be lost, explaining the basal PKC activities in the in vitro PKC assay. The PKC isoforms involved in activation of AP-1 in Raji B cells remain to be determined. Further analyses using immunodepletion and isoform-specific inhibition by expression of dominant negative PKCs should resolve this question.
Raji B cells have rapid phosphoinositol breakdown and PKC activation in response to B cell Ag-induced signal transduction and should represent an appropriate model for B lymphocyte signaling. However, the basal PKC activities seen in resting cells suggest that the expression of EBV proteins that leads to the transformation of B cells has preactivated PKC signaling to some extent (41). Recently, TPA-activated expression of the HLA-DRA gene was investigated in Raji B cells. AP-1 heterodimers containing c-Fos were shown to be essential for activated transcription of the HLA-DRA gene in response to PKC signaling (23). PKC-dependent up-regulation of MHC class II expression has also been reported on the cell surface of macrophages (22). Thus, activation of PKC appears to be a crucial mechanism for efficient Ag presentation by modulation of MHC class II gene expression in both macrophages and B cells (42, 43). The exact contribution of selective PKC isoforms to MHC class II expression in these cells remains to be investigated. However, it was recently reported that targeted disruption of the murine gene encoding cPKC-β and cPKC-βII resulted in immunodeficiency with impaired humoral immune responses, implicating a crucial role for this PKC isoenzyme in immunity (44). The data from our study are in accordance with these findings, suggesting that cPKC-βII may be a key component in the PKC-dependent triggering of a functional immune response, including potentiated expression of MHC class II Ags. The functional implication of the structural modification of cPKC-βII remains to be investigated.

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