FEBS Letters 393 (1996) 48–52 FEBS 17464

Translocation of diacylglycerol kinase α to the nuclear matrix of rat thymocytes and peripheral T-lymphocytes

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Received 21 June 1996; revised version received 18 July 1996

Abstract The cytosolic α-diacylglycerol kinase (DGK) was translocated to and tightly associated with the nuclear matrix when rat thymocytes and peripheral T-lymphocytes were stimulated with concanavalin A or anti-T-cell receptor antibody. This translocation occurred rather slowly and was completed in 3–4 h after cell stimulation. We also detected significant accumulation of nuclear phosphatidic acid interpreted as being formed by the translocated enzyme. The enzyme translocation is not directly linked to phosphoinositide turnover and protein phosphorylation, since phorbol myristate acetate and calcium ionophore did not affect the cellular DGKα and since we detected no covalent modification of the enzyme molecule. Although the mechanisms underlying the enzyme translocation remain unknown, our results indicate that DGKα participates in nuclear phospholipid metabolism occurring at the intermediate stage of lymphocyte activation.

Key words: Diacylglycerol kinase, α-isozyme; T-lymphocyte, rat; Translocation; Nuclear matrix

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates DG to generate phosphatidic acid, thereby altering the balance of the two functional lipids. DG regulates many cellular functions by activating protein kinase Cs [1]. Phosphatidic acid is also known to elicit many biological responses when added to a variety of cells [2]. DGK thus can be potentially involved in the regulation of a wide range of cellular functions. The cDNA cloning of a number of mammalian DGKs [3–11] has revealed the presence of a novel and multimembered gene family, but the precise physiological significance of each DGK isozyme remains unclear. The DGK activity has been shown to exhibit a varied subcellular distribution and was described as being associated with the cytoskeleton [12] and the nuclear matrix [13] in addition to other major subcellular fractions [14]. However, it is not known which DGK isozyme so far cloned accounts for the DGK activity variably distributed within the cells. In the present work we focussed on DGKα, which is predominantly expressed in thymus and peripheral T-cells in addition to oligodendroglial cells [3–5].

It is well established that T-cell activation occurs as a two-step process. The activation of the T-cell receptor (TcR)/CD3 complex initially results in tyrosine phosphorylation of a number of proteins followed immediately by phosphoinositide turnover [15,16]. This initial signal transduction is followed at the second stage by the expression at the cell surface of high-affinity interleukin (IL)-2 receptors [17]. Recently, the role of DGKα in IL-2-dependent cell proliferation has been elucidated by the finding that DGKα could account for the most of the DGK activity, which was rapidly increased after IL-2 stimulation of T-cell lines [18]. However, the role of DGKα in phosphoinositide turnover and in other biochemical events preceding the expression of IL-2 receptors remains unknown. For example, exogenous short-chain DG repeatedly administered to human T-lymphocytes was phosphorylated by cellular DGK only to a negligible extent [19]. Van der Bend et al. [20] proposed a topological sequestration of cellular DGK molecules based on the finding that DG artificially generated by treating Jurkat and other cells with bacterial phospholipase C could not serve as substrate for the cellular DGK. These observations indicate that the metabolic role of DGKα abundantly contained in T-lymphocytes is still largely unknown and that the cellular DGKs are operating under strict control mechanisms. Here we describe a targeting of DGKα to the nuclear matrix upon T-cell activation and suggest the participation of this DGK isozyme in the nuclear phospholipid metabolism occurring in stimulated lymphocytes.

2. Materials and methods

2.1. Materials

Rabbit anti-DGKα antibody was raised against the N-terminal portion (amino acids 2–357) of rat DGKα and affinity-purified through an antigen column [5]. This antibody was previously shown to be specific to rat [5] and human [7] DGKα. Anti-rat protein kinase Cα and anti-rat TcR antibodies were obtained from Gibco BRL and Serotech, Oxford, UK, respectively. Other materials were obtained from Sigma (St. Louis, MO) unless otherwise specified.

2.2. Cell culture

Thymocytes and T-cell-enriched peripheral lymphocytes were prepared from 3-week-old male Sprague-Dawley rats [21]. Greater than 99% viable cells (assessed by trypan blue exclusion test) were obtained using Lympholyte-Rat (Cedarlane Lab.) according to the manufacturer's instructions. The cells were incubated in RPMI1640 medium (1×10^7 cells/ml) supplemented with 0.1% (w/v) bovine serum albumin at 37°C under 5% CO_2 in air. Thymocytes were usually cultured with concanavalin A (ConA, 20 μg/ml) for up to 4 h under the conditions specified in the figure legends. Throughout the present experiments, we did not detect significant changes of cell viability when the thymocytes were cultured for up to 4 h under different conditions. In the case of stimulation of peripheral T-cells, the cells (1×10^7 cells/ml) were cultured with the anti-TcR monoclonal antibody (clone R73, diluted 50-fold), which was adsorbed onto a 24-well culture plate precoated with goat anti-mouse IgG (1 μg/ml, Jackson ImmunoResearch Lab.).

2.3. Subcellular fractionation and immunoblotting

Separation of cellular components based on Triton X-100 solubility was performed by resuspending the cells (1×10^7 cells/ml) for 15 min on ice in 100 μl of extraction buffer (1% Triton X-100, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and pepstatin, and 10 mM Pipes, pH 6.8), followed by a $10000\times g$ spin for 1 min at 4°C. The aliquots of the supernatant and pellet were treated with SDS sample buffer [22] and

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PII S0014-5793(96)00857-5

were subjected to SDS-polyacrylamide gel electrophoresis (9% acrylamide). The resolved proteins were transferred onto nitrocellulose membranes (Scleicher and Schuell) as previously described [7,9]. The membrane was probed with the anti-DGKα antibody, and the immunoreactive bands were visualized using an ECL system (Amersham Corp.) as described previously [7,9]. In some experiments thymocytes were homogenized by passing through a 30 gauge needle 10 times in homogenization buffer (5 mM HEPES, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and pepstatin, pH 7.5). The homogenate was centrifuged at $100000\times g$ for 1 h to obtain cytosolic and total particulate fractions.

Purification of nuclei from thymocytes was carried out as described [23]. In brief, a crude nuclear fraction was obtained from cell homogenates in the homogenization buffer by a $500\times g$ spin for 7 min, and the nuclei were further purified by discontinuous sucrose gradient centrifugation [23]. In some experiments, the purified nuclei in the homogenization buffer were further treated with 0.75 M NaCl or DNase I, and the nuclear matrix fraction was recovered as the pellet of a $10000\times g$ centrifugation. In this case, H1 histone (Trevigen Inc. Gaithersberg, MD) was used as the marker of non-matrix nuclear proteins. In the case of the determination of phosphatidic acid associated with the isolated nuclei, 1 ml of the thymocyte suspension (1×10^7 cells) was labeled with 300 μCi of $^{32}P_i$ for 10 h in complete RPMI containing bovine serum albumin. The labeled cells were then washed twice with phosphate-buffered saline (pH 7.2), and viable cells were collected using Lympholyte-Rat. After culturing the labeled cells with ConA (20 μg/ml) for 4 h, the cells were homogenized in the homogenization buffer supplemented with phosphatase inhibitors (5 mM each of sodium fluoride and iodoacetamide). The nuclei were purified as described above, and lipid extraction and thin-layer chromatography were performed as previously described [7,9].

2.4. Indirect immunofluorescence

Thymocytes and peripheral T-lymphocytes were adhered to cover slips precoated with polylysine by incubation for 5 min at room temperature. After being cultured with various reagents, the cells were immunostained by anti-DGKα antibody (5 μg/ml) and fluorescence-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Lab.) according to previously described procedures [24]. The fluorescence image was analyzed using a Bio-Rad MRC500 confocal microscope.

3. Results and discussion

In untreated thymocytes, most of the cellular DGKα could be extracted by Triton X-100, although a considerable portion (20–30%) of the enzyme was associated with the detergent-insoluble fraction (Fig. 1A). We earlier demonstrated [25] that in thymocytes and peripheral T cells DGKα is present mostly in the cytosolic fraction. In accordance with this observation, we confirmed in separate experiments that the Triton X-100-soluble DGKα represented the cytosolic enzyme and that the insoluble enzyme was associated with the total particulate fraction (not shown). In cells stimulated with ConA, the detergent-soluble DGKα gradually decreased and became almost completely detergent-insoluble at 3–4 h after the treatment (Fig. 1A). ConA ranging from 2 to 20 μg/ml resulted in similar translocation of DGKα (not shown). Little enzyme translocation was detected within the first 1 h of ConA treatment or in a control incubation without ConA for up to 4 h. In order to confirm the physiological significance of the DGK translocation in a better-defined experimental system, we prepared peripheral lymphocytes from the rat spleen and cross-linked the TcR with the anti-TcR antibody. As shown in Fig. 1B, the activation of TcR also caused a gradual translocation of DGKα from the detergent-soluble to -insoluble fractions as observed with the ConA-stimulated thymocytes. These results suggest that the association of DGKα with the Triton X-100-insoluble compartment is related to a physiological activation process occurring in T-

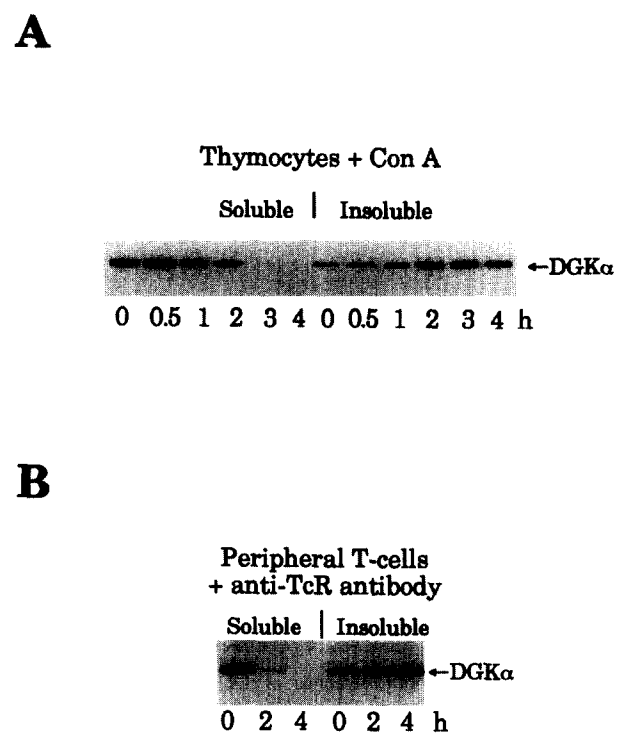

Fig. 1. Stimuli cause translocation of DGKα to the Triton X-100-insoluble fraction in rat thymocytes and peripheral T-cells. (A) Thymocytes were cultured with ConA for the time periods indicated. After stimulation, equal aliquots of Triton X-100-soluble and -insoluble fractions were subjected to immunoblotting with anti-DGKα antibody. (B) Peripheral T-cells were stimulated with anti-TcR antibody for up to 4 h as indicated. Analysis of the Triton X-100-soluble and -insoluble fractions was performed as in A.

lymphocytes. During these experiments we did not detect significant changes of cell viability. Furthermore, a prolonged treatment of thymocytes with dexamethasone (1 μM for 6 h), a well-established apoptotic reagent for thymocytes [26], did not affect the detergent solubility of DGKα (not shown). These results indicated that the enzyme translocation was not due to the secondary effects of cell apoptosis.

On analysis of the intracellular distribution of DGKα in unstimulated thymocytes by indirect immunofluorescence, a prominent signal was observed in the extranuclear space (Fig. 2A), indicating the cytoplasmic localization of most of this enzyme. We noted that the anti-DGKα antibody stained a limited population (30–45%) of total thymocytes for unknown reasons. When the cells were stimulated for 4 h with ConA, the signal in the cytoplasm had largely disappeared, resulting in uneven staining of the nuclei (Fig. 2C). A similar staining pattern was observed with the peripheral T-cells treated with anti-TcR antibody for 4 h (not shown). Based on the results of immunostaining and the Triton X-100 insolubility of the translocated DGKα, we considered that the nuclear matrix was the structure with which the cellular DGKα was associated. In order to confirm this, we purified the nuclei from thymocytes pretreated with ConA for 4 h, and examined the resistance of DGKα to the treatments with high salt extraction or with DNase I. In this case H1 histone was shown to be extracted by high concentrations of NaCl (Fig. 3A) or by DNase I treatment (not shown). DGKα translocated to the nuclei, on the other hand, remained insoluble in both treatments (Fig. 3). It was thus confirmed that DGKα

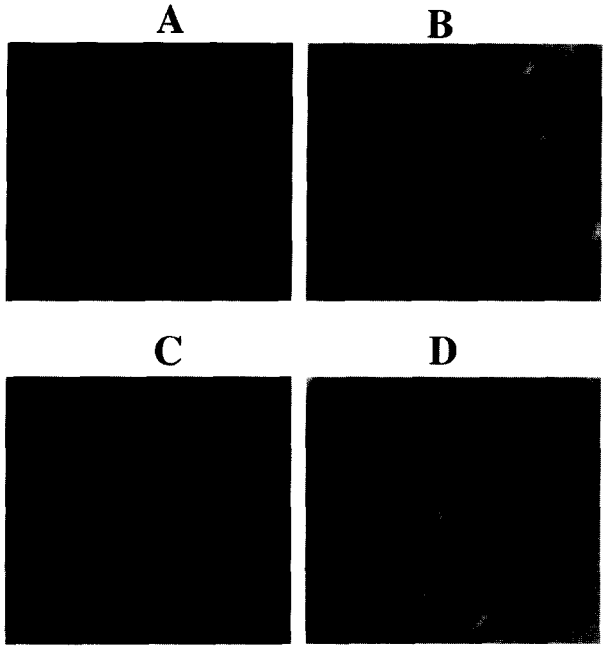

Fig. 2. Indirect immunofluorescence of DGKα in rat thymocytes. Thymocytes were incubated with (C,D) or without (A,B) ConA for 4 h. Shown are the flurorescence (A,C) and phase-contrast images (B,D) of the same view.

was tightly bound to the nuclear structure which is resistant to high salt or DNase I, i.e. the nuclear matrix [27].

We next attempted to determine whether the DGK associated with the nuclear matrix could contribute to nuclear phospholipid metabolism. We found that the nuclear phosphatidic acid was increased by approx. 70% as a result of the surface-cross-linking of thymocytes with ConA for 4 h (Fig. 4A). Since phosphatidic acid is also known to be generated by the action of phospholipase D [28], we down-regulated protein kinases C, which activate phospholipase D [28], by culturing thymocytes for 10 h in the presence of 100 nM phorbol myristate acetate (PMA). In this case we confirmed by Western blotting analysis of protein kinase Cα (not shown) that at least classical protein kinases C were down-regulated. We observed that the translocation of DGKα in ConA-stimulated thymocytes occurred similarly even after prolonged culture with PMA (not shown) with persistent accumulation of nuclear phosphatidic acid (Fig. 4B). We also confirmed that the addition of 1% ethanol did not sigificantly affect the level of nuclear phosphatidic acid in thymocytes treated with ConA for 4 h (not shown). These results support the notion that the nuclear phosphatidic acid was formed by the translocated DGKα rather than by phospholipase D.

DGK has generally been believed to be involved in phosphoinositide turnover occurring immediately after TcR activation [14,16]. However, the translocation of DGKα to the nuclear matrix exhibited a lag period, which is not consistent with the rapid kinetics of phospholipase C activation [16]. In an attempt to elucidate the mechanism of enzyme translocation, we examined the effects on the cellular DGKα of protein kinase C activators. As shown in Fig. 5, PMA, oleoylacetylglycerol (tested at 50 μM, not shown), and the calcium ionophore, A23187, had little effect on cellular DGKα, when tested alone or in combination. In contrast, protein kinase Cα in PMA-treated thymocytes was rapidly recruited to membranes (Fig. 5B). These results further confirmed that the association of DGKα with the nuclear matrix was not directly linked to protein kinase C activation and calcium mobilization known to occur in phosphoinositide turnover.

We initially reasoned that some covalent modifications of the DGKα molecule might have led to its translocation to the nuclear matrix. However, we failed to detect evidence of enzyme phosphorylation or other modifications in repeated

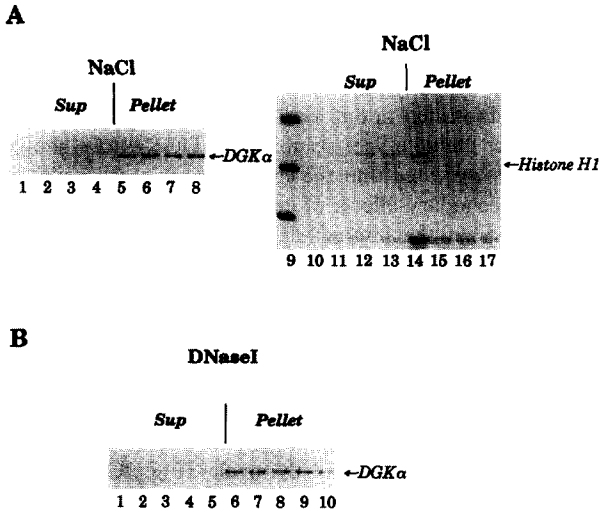

Fig. 3. DGKα translocated to thymocyte nuclei is resistant to high salt or DNase I treatment. (A) Nuclei were purified from thymocytes preincubated with ConA for 4 h. The suspension of nuclei was incubated for 30 min on ice with 0 (lanes 1,5,10,14), 0.25 (lanes 2,6,11,15), 0.5 (lanes 3,7,12,16) and 0.75 M (lanes 4,8,13,17) NaCl. The nuclei were then centrifuged at 10000×*g* for 1 min, and equal aliquots of the resultant supernatant and pellet were subjected to immunoblotting with anti-DGKα antibody (lanes 1–8). In the same experiments, H1 histone was visualized by Coomassie blue staining of the SDS gel (lanes 10–17). In this case molecular mass standards (45, 31, 21.5 and 14.5 kDa) are shown in lane 9. (B) The nuclei were treated for 1 h at 30°C with 0 (lanes 1,6), 0.025 (lanes 2,7), 0.05 (lanes 3,8), 0.1 (lanes 4,9), and 0.15% (w/v) (lanes 5,10) of DNase I.

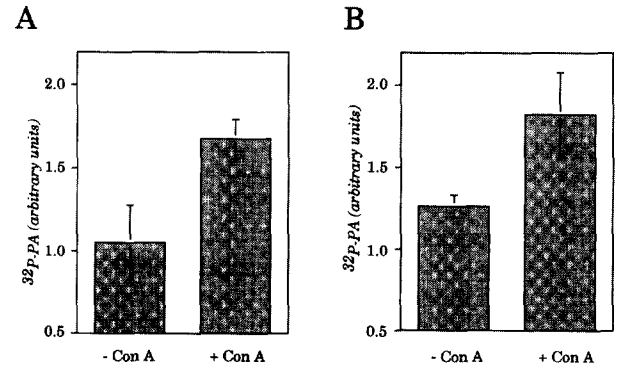

Fig. 4. Analysis of nuclear phosphatidic acid in ConA-treated thymocytes. Thymocytes were prelabeled with $^{32}P_i$ for 10 h in the absence (A) or presence of 100 nM PMA (B). Labeled cells were purified using Lympholyte-Rat, and were further incubated for 4 h with or without ConA. [^{32}P]Phosphatidic acid (PA) extracted from the isolated nuclei (500 μg of protein) was separated by thin-layer chromatography, and quantitated using a BAS2000 analyzer. The results are averages of three determinations with the range of S.D.

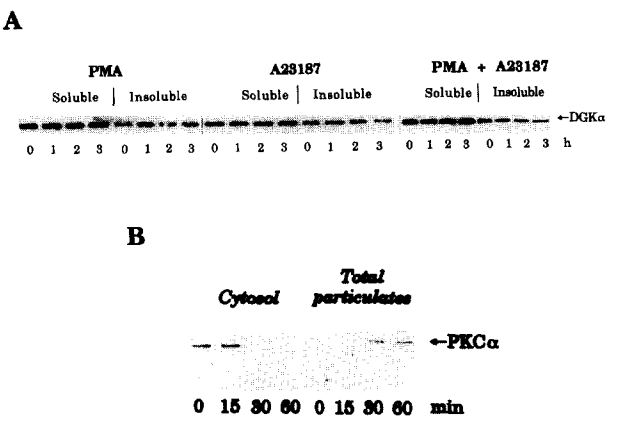

Fig. 5. PMA and A23187 treatments have no effects on cellular DGKα. Thymocytes were incubated with 100 nM each of PMA and A23187, tested alone or in combination, for the time periods indicated. DGKα in the Triton X-100-soluble and -insoluble fractions was visualized by immunoblots as in Fig. 1. (B) Cytosol and total particulate fractions were obtained from cells incubated with 100 nM PMA for the periods indicated. Equal aliquots of both fractions were subjected to immunoblotting analysis using anti-protein kinase Cα antibody. Protein kinase Cα associated with the particulate fraction was Triton X-100-soluble (not shown).

analyses (not shown). These include (1) immunoblotting of DGKα using anti-phosphotyrosine and -phosphoserine antibodies, (2) isoelectrofocusing of the cell extracts in urea-containing gels followed by immunoblotting with anti-DGKα antibody, and (3) side-by-side comparison of the mobility of the enzyme proteins upon Western blotting. We therefore concluded that protein kinases known to be activated immediately after the TcR occupancy [15,16], were not directly involved in the DGKα translocation.

The present work suggests a novel function of DGKα in nuclear phospholipid metabolism, although the mechanism of its translocation to the nucleus could not be elucidated. We previously demonstrated that DGKα is a high-affinity calcium-binding protein [29], however, calcium binding is not involved in the enzyme translocation in view of its time course (Fig. 1) and the effects of calcium ionophore (Fig. 5). Increased production of nuclear DG upon cell stimulation has been detected in many cells [30,31], and the importance of the nuclear localization of protein kinases C has been addressed [32]. It is thus likely that DGKα is the isozyme participating in the metabolic processing of nuclear DG. The present work has shown that at least a part of the DGK activity previously found associated with the nuclear matrix [13] can be accounted for by DGKα. However, it is not known whether other DGKs with a related structure would also be translocated similarly upon cell stimulation. There have been several reports describing the translocation of crude DGK activities to membranes under varied conditions ([33], reviewed in [34]). It thus appears that DGK translocation is caused by different mechanisms depending on the types of cells and DGK isozymes involved. Despite its presence in the cytosol of resting lymphocytes [25], DGKα was shown to be mainly located in the nuclei of IL-2-dependent lymphocyte cell line [18]. Although its intranuclear localization was not studied, nuclear DGKα was shown to be translocated to the perinuclear region upon IL-2 stimulation of these cells [18]. These findings suggest that the binding of DGKα with the nuclear matrix may be a reversible phenomenon. DGK is generally believed to be involved in the resynthesis of phosphatidylinositols consumed by the action of phospholipase Cs [14]. However, the time course of DGKα translocation and its apparent independency of phosphoinositide turnover suggest that DGKs other than the α-isozyme, in particular arachidonoyl DG-specific DGK [11,35], may be involved in the phosphoinositide turnover. Further work on the molecular machinery operating for the translocation of DGKα and on the control of other DGKs is needed to define the function of the enzyme family.

Acknowledgements: We thank Dr. H. Tobioka (Department of Pathology, Sapporo Medical University) for his help in the confocal microscopy. This work was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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