Purification, cDNA-cloning and expression of human diacylglycerol kinase

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Diacylglycerol (DG) kinase attenuates the level of the second messenger DG in signal transduction, and therefore possibly modulates protein kinase C (PKC). DG kinase was purified to homogeneity from human white blood cells, showing an M_r of 86 kDa as determined by SDS-PAGE and gel filtration. Two amino acid sequences of tryptic peptides from DG kinase were determined and degenerate oligonucleotides were prepared and used in the polymerase chain reaction. An amplified DNA fragment was subsequently used to clone the full-length human DG kinase cDNA. This sequence is the human homolog of a porcine DG kinase cDNA sequence reported recently [1]. The sequence contains a double EF-hand structure typical for Ca^{2+} binding proteins. DG kinase further contains a double cysteine repeat that is present in all PKC isoforms, where it constitutes the phorbol ester (and most likely diacylglycerol) binding site. Therefore we speculate that the double cysteine repeat in DG kinase is involved in DG binding. DG kinase is transcribed as a single mRNA of 3.2 kb, that is highly expressed in T-lymphocytes. The human DG kinase cDNA when transfected in mammalian cells (COS-7) results in a 6–7-fold increase of DG kinase activity.

Diacylglycerol kinase; Human white blood cell; EF-hand; Cysteine repeat

1. INTRODUCTION

The enzyme diacylglycerol kinase (DG kinase) is thought to be mainly involved in the regeneration of phosphatidylinositol (PI) from diacylglycerol (DG) in the PI-cycle during cell signal transduction [2,3]. In this process, DG kinase can be viewed as a modulator that competes with PKC for the second messenger DG [4]. Transformation of 3T3 cells by the *ras* oncogene results in constitutively increased levels of DG and membrane-bound PKC activity, accompanied by a decrease in membrane-bound DG kinase activity, suggesting a possible role for DG kinase in *ras* cell transformation [5,6]. The demonstration that the product of DG kinase activity, phosphatidic acid (PA), is itself mitogenic provides a second way in which the enzyme may be involved in signal transduction [7,8].

Understanding the possible role of DG kinase in transmembrane signalling requires a thorough study of its molecular and enzymatic properties. At present, most studies have been performed with cell lysates and partially purified DG kinase preparations. Recent reports indicate the presence of various types of DG kinases, that differ in celluiar localization, substrate specificity and molecular weight [9–13].

Here we report the purification and characterization of a 86 kDa DG kinase from human normal white blood cells (WBC). Based on partial amino acid sequences of the purified DG kinase, primers were designed that have allowed the cloning of a human DG kinase cDNA by use of the polymerase chain reaction (PCR), resulting in the complete amino acid sequence of the enzyme. While we completed the cDNA sequence of human DG kinase, the primary structure of porcine DG kinase was reported [1]. The DG kinase sequence reported here is the human equivalent of the porcine enzyme.

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Abbreviations: DOC, deoxycholate; DG, 1,2-diacyl-*sn*-glycerol; diolein, 1,2-dioleoyl-*sn*-glycerol; PA, 3-*sn*-phosphatidic acid; PC, 3-*sn*-phosphatidylcholine; PI, 3-*sn*-phosphatidylinositol; PS, 3-*sn*-phosphatidylserine; PCR, polymerase chain reaction; PKC, protein kinase C; WBC, white blood cells

The DNA sequence will be submitted to EMBL data library

2. MATERIALS AND METHODS

2.1. *Materials*

All materials were from either Sigma, Merck or BDH unless stated otherwise. 2-Monooleoyl-*rac*-glycerol and 1-oleoyl-lyso-3-*sn*-phosphatidic acid were from Serdary (Ontario, Canada); 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol and 1,2-*O*-dioctadecyl-*sn*-glycerol from Bachem (Bubenheim, Switzerland); adenosine 5′-[γ-^{32}P]-triphosphate (3000 Ci/mmol) from Amersham; acrylamide from Serva (Heidelberg, FRG); dithiothreitol and adenosine 5′-triphosphate from Boehringer; R59022 from Janssen (Belgium). 1-Palmitoyl-2-oleoyl- and 1-*O*-hexadecyl-2-oleoyl-*sn*-glycerol were prepared in our laboratory from their respective PC homologues by reaction with phospholipase C [14] and purified by HPLC [15].

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2.2. *Enzyme purification and amino acid sequencing of tryptic peptides*

WBC were isolated from 25–50 liters of blood, erythrocytes were lysed and WBC were sedimented for 15 min at 2000 × g. The resulting cell preparation was heterogeneous in composition, consisting for the predominant part of polymorphonuclear cells and for a smaller part of lymphocytes, residual erythrocytes and platelets (< 20%).

WBC pellets were diluted in 600 ml buffer A (250 mM sucrose, 1 mM EDTA, 0.01% NaN_3, 25 mM Tris-HCl (pH 7.5), 0.05 mM ATP and 0.5 mM dithiothreitol), supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μg/ml trypsin inhibitor, 0.1 TIU/ml aprotinin and homogenized with a polytron mixer on ice. After centrifugation at 100 000 × g for 1 h, the cytosolic fraction was stored at −70°C (under these conditions enzymatic activity was preserved for several months). DG kinase was purified from the cytosolic fractions using the column chromatography steps as outlined in Table I, which is similar to the procedure from Kanoh et al. [9], except that Mg^{2+} was omitted in all steps. Furthermore, ATP was omitted during salt gradient elution in the ATP-agarose column step. This was essential to separate DG kinase from other ATP-agarose binding proteins. Highly purified DG kinase (80 μg) was used for amino acid sequencing, as described [16].

2.3. *DG kinase assay*

Enzyme preparations (20 μl, maximally 20 μg protein) were incubated in an assay mixture (240 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM NaF, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM diolein, 2 mM PS, 1 mM deoxycholate and 2 mM [γ-^{32}P]ATP. The reaction was started by the addition of [γ-^{32}P]ATP and terminated after 3 min incubation at 30°C by mixing 200 μl assay mixture with 3.6 ml chloroform/methanol/2 M NaCl (1:2:0.6, v/v/v). The formation of PA was linear in time for at least 10 min. After vortexing, 1 ml chloroform and 1 ml 2 M NaCl were added, followed again by vortexing. The chloroform phase was analyzed by thin layer chromatography on silica gel plates (DC fertigplatten, Kieselgel 60; Merck) with chloroform/methanol/acetic acid/H_2O (50:30:2:4, v/v/v/v) as developing solvent. PA and LPA spots were detected by autoradiography, scraped off and quantitated by liquid scintillation counting. More than 90% of the ^{32}P-label was recovered in the PA spot.

2.4. *PCR and cDNA cloning*

The PCR mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.25 mM of each dNTP, 1 μg of each primer, and 2.5 units of Taq polymerase (native Taq or recombinant AmpliTaq, both from Perkin Elmer Cetus) in a total volume of 50 μl or 100 μl. All PCR reactions were carried out with thermocycling conditions as specified by Perkin Elmer Cetus, with a 50°C annealing temperature, using normally 30 cycles. Two λgt10 human cDNA libraries were screened to obtain a full-length DG kinase cDNA. A Jurkat T-cell cDNA library (Clontech) was screened with a randomly primed ^{32}P-labeled PCR DNA-fragment. Plaque purifications were carried out using standard procedures [17]. cDNA inserts from purified plaques were subsequently digested with *Eco*RI and cloned into an *Eco*RI digested pGEM-3Z(f+) vector. A DND41 T-cell leukemic cell cDNA library (kindly provided by E. Hochstenbach, Boston) was screened with DNA fragments from previously cloned cDNA.

Table I

Purification of DG kinase from cytosol of human WBC and pig brain

	Volume (ml)	Spec. act. (nmol/min per mg)	Activity yield (%)	Purification factor	Protein (mg)
Human white blood cells					
cytosol	600	6.5	100	1	2160
DE 52	290	26.0	68	4	371
$(NH_4)_2SO_4$	9	11.8	24	1.8	286
G150	38	53.4	10	8.2	26
ATP agarose	22	6913	6	1064	0.13

2.5. *Other procedures*

SDS-PAGE on 10% gels was performed according to Laemmli [18]. Protein was determined according to Peterson [19]. DNA Sequencing was performed with a T7 DNA sequencing kit (Pharmacia, Sweden) using alkali denatured double stranded DNA. Sequence data were analysed using GCG software [20]. Deduced amino acid sequences were used to search for homology in Genbank, using 'FASTA' [20]. Total RNA and poly(A)$^+$ RNA were purified with standard techniques [17]. RNA was separated on 1% agarose formaldehyde gels, transferred to nylon membrane (Hybond-N, Amersham) and hybridized with ^{32}P-labeled randomly primed probes (according to Amersham). Total RNA from HeLa cells, A-431 cells, and GLC-2 cells as well as a human actin DNA probe were a generous gift from M. Gebbink in our institute. COS-7 cell transfections were performed as described [21], cell lysates were prepared by scraping cells in the same medium as used for WBC, followed by 10 s sonication on ice.

3. RESULTS AND DISCUSSION

3.1. *Purification and characterization of DG kinase from human normal white blood cells*

The purification of DG kinase was undertaken because an initial observation suggested the presence of DG kinase activity in highly purified PKC preparations (R.v.d.B. unpublished results), obtained after affinity column chromatochraphy on immobilized PS [22]. To investigate the possibility whether PKC could itself phosphorylate the physiological activator, DG, the separation of the two activities was the original aim.

Upon homogenization of WBC, 90% of the DG kinase activity was recovered in the cytosol fraction, which displayed a 2-fold higher specific activity than the microsomal fraction. As shown in Table I, purification resulted in preparations that were 1000-fold increased in specific activity compared with cytosol. Upon $(NH_4)_2SO_4$ precipitation, no increase in specific activity was observed. However, omittance of this step resulted in less pure preparations as judged on SDS-PAGE (data not shown). In the final step of the purification, DG kinase activity eluted from an ATP agarose column at about 400 mM NaCl (Fig. 1) and co-purified with a band of approximately 86 kDa as detected on silver stained SDS-polyacrylamide gels (Fig. 1). These fractions were devoid of PKC activity (measured as descibed in [22]). In a similar manner, DG kinase was also purified from pig brain cytosol (results not shown). In this case, final preparations also displayed an 86 kDa band on SDS-PAGE (Fig. 1). After ATP agarose chromatography the enzyme was very unstable, 90% being lost within a few hours at 0°C. Stability could not be improved by addition of known stabilizing agents, such as: bovine serum albumin, ATP [9] or PS liposomes [23]. Upon gel filtration of human WBC cytosol, DG kinase activity eluted at a molecular mass of approximately 87 kDa (results not shown). This

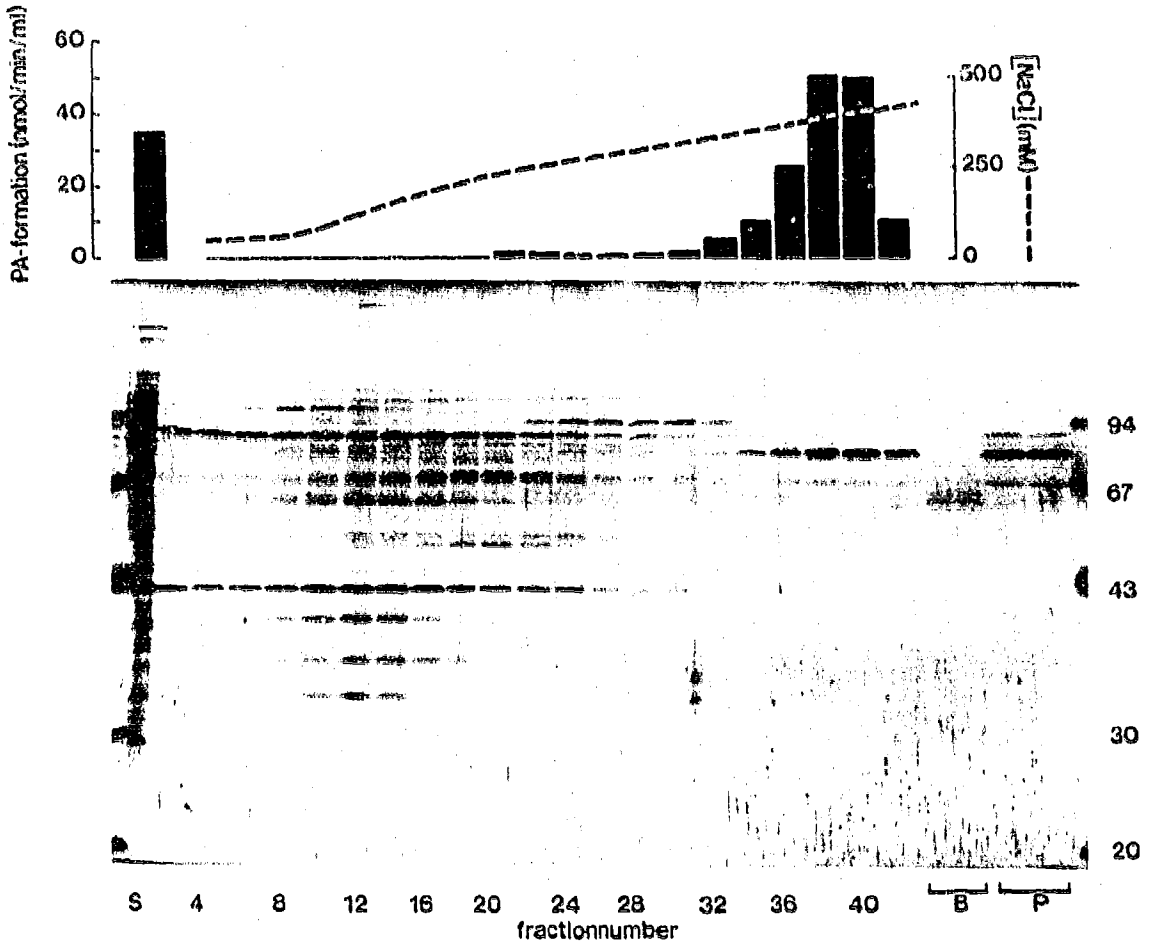

Fig. 1. SDS polyacrylamide gel analysis (silver stain) of ATP agarose column fractions of human WBC DG kinase. 50 μl of each column fraction was loaded per slot. The upper part of the figure shows the DG kinase activity and the NaCl concentration (measured by conductivity) in each fraction. B, buffer only; P, purified DG kinase from pig brain cytosol (similarly purified as human WBC DG kinase); S, starting material loaded on the ATP agarose column.

indicates that the native enzyme of human WBC consists of a single polypeptide.

It is likely that myeloid cells are the main source of our purified WBC enzyme as WBC preparations consisted for the predominant part of polymorphonuclear (myeloid) cells. This argues against the suggestion [24] that, among the different types of peripheral blood cells, the presence of DG kinase (the structure of which we report) would be restricted to lymphocytes. In addition, the 86 kDa DG kinase could also be isolated from blast cells from a patient suffering from acute myeloid leukemia (results not shown).

The WBC DG kinase shows optimal activity in the presence of PS and deoxycholate (DOC) as cofactors and lower activity in the presence of PC (results not shown). The observation that DG kinase itself is stimulated by PS, and therefore presumably possesses binding site(s) for this phospholipid could explain the observed retention of DG kinase activity on immobilized PS during purification of PKC (see above). In the presence of 2 mM PS and 1 mM DOC, a K_m for 1,2-*sn*-diolein of 0.45 mM was measured, similar to the 1 mM value reported for a bovine brain DG kinase [25], but significantly higher than reported for a 80 kDa pig brain enzyme (60 μM) [9]. The K_m for ATP with and without 1 mM DOC is 1.6 mM and 0.5 mM respectively, similar to other values of DG kinase preparations, that were determined only in the presence of DOC [9,25].

DG analogs containing an unsaturated fatty acid at the *sn*-2 position gave optimal enzyme activity (Table II), irrespective of the presence of DOC (results not shown). The low but detectable activity observed with 1,3-*sn*-diolein can be explained by a 5–10% contamination of the substrate with the 1,2-*sn* isomer, as measured by HPLC. Of the monoacylglycerols tested, only 2-monooleoyl-*rac*-glycerol was phosphorylated by our DG kinase preparation, although with low efficiency (Table II). Since DG kinase is an enzyme of the PI-cycle, its natural substrate could be 1-stearoyl-2-arachidonoyl-*sn*-glycerol, thought to be the main DG

Table II

Substrate specificity of DG kinase purified from human WBC

Substrate	Relative DG kinase activity (%)
1-stearoyl-2-arachidonyl-*sn*-glycerol	100[a]
1,2-dioleoyl-*sn*-glycerol	109.0 (± 12.0)[b]
1,2-dipalmitoyl-*sn*-glycerol	15.0 (± 7.6)[b]
1-palmitoyl-2-oleoyl-*sn*-glycerol	96.5 (± 3.5)
1-*O*-hexadecyl-2-oleoyl-*sn*-glycerol	85.4 (± 6.2)
1,3-dioleoyl-*sn*-glycerol	10.6 (± 1.5)
1-monooleoyl-*rac*-glycerol	1.1 (± 0.2)
2-monooleoyl-*rac*-glycerol	10.7 (± 1.3)
1,2-*O*-dioctadecyl-*sn*-glycerol	< 0.1
1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol	< 0.1

DG analogs (1 mM) were tested in the presence of 2 mM PS and 1 mM DOC. The absolute values observed varied between different sets of experiments, due to enzyme instability. Therefore, mean relative values (± range of duplicates) are presented.

[a]Arbitarily set at 100%; mean absolute value (± sd) was 8.7 (± 3.4) nmol/min per ml from 3 independent experiments

[b]Data from two sets of experiments are combined (± sd); within each experiment duplicates differed by less than 15%

analog generated from phosphoinositides [26,27]. Indeed, for membrane-bound DG kinase in Swiss 3T3 fibroblasts and baboon brain, this particular DG analog has been shown to be a preferred substrate, rather than 2-oleoyl DG analogs [10,13]. However, in the cytosol of Swiss 3T3 cells DG kinase activity did not display this DG preference [10], suggesting the existence of different types of DG kinases. In WBC, we did not find such a particular DG preference for either membrane-bound DG kinase (results not shown), or for the purified cytosolic enzyme (Table II), also in the absence of DOC (not shown). With these preparations, similar activities are measured with DG substrates expected to be formed from PI- as well as PC-breakdown [28]. As receptor-mediated PC breakdown is now accepted to be a ubiquitous phenomenon (for review see [29]), the broad DG specificity of human DG kinase could also imply a role for the enzyme in this pathway. Analogs containing an ether-linked residue at the *sn*-1 position (1-*O*-hexadecyl-2-oleoyl-*rac*-glycerol) were also substrates for human DG kinase, but those containing 2 ether-linked residues (1,2-*O*-dioctadecyl-*sn*-glycerol and 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol) were not (Table II); these compounds likewise can be generated by agonist-induced hydrolysis of ether-linked PC [30].

Among the compounds which have been described to inhibit DG kinase [31,32], the compound R59022 [32] is indeed a potent inhibitor of the human WBC enzyme, but 1-monooleoylglycerol and 1,2-dioctanoylethylene glycol are not (data not shown). Furthermore, the enzyme is also inhibited by *N'*-ethylmaleimide and *N,N'*-*O*-phenylenedimaleimide (data not shown). This finding, together with the observation that the enzyme is stabilized by dithiothreitol, suggests that one or more free sulfhydryl groups in the enzyme play an essential role in its catalytic action.

3.2. *PCR cDNA cloning and sequencing of human DG kinase*

Starting from about 1000 buffy coats (which is the equivalent of approximately 400 liters of blood), 80 μg of DG kinase was obtained in highly purified form. Following electroblotting of the enzyme, tryptic peptides were prepared, and after purification by HPLC, two of them were sequenced: peptide A, EIDYDGSGSVSQAEXVR (no definitive assignment but a likely Trp at position 15), and peptide B, $^{W}/_{F}{}^{G}/_{H}{}^{G}/_{I}{}^{G}/_{M}$YEGQNLAK (two residues were detected in each of the first four cycles during gas phase sequencing). The degenerate oligonucleotide sequence of peptide A was divided in three regions, and the highly degenerate middle 16 nucleotides were amplified with PCR using degenerate primers based on the sequence from the extremities of peptide A. First strand cDNA from Jurkat cells was used as a template. The expected 51 bp fragment was amplified, extracted from gel, used for PCR reamplification, and then cloned. The unique sequence of the 16 bp middle region could thus be determined for peptide A and indeed fully matched the amino acid sequence. The 16 nucleotide sequence together with 6 bases at the 5′ end and 5 bases at the 3′ end yielded a 29 bp primer that was only 8-fold degenerate. This sequence, forward and reverse, was synthesized with a 5′ adaptor GATCGAATTC, and used in combination with B primers (deduced from peptide B) on Jurkat cell first strand cDNA. The combination of the 8-fold degenerate A forward primer with a reverse 4096-fold degenerate B primer yielded a single 0.95 kb DNA fragment.

The 0.95 kb fragment was labeled by random priming and used as a probe to screen 10^6 plaques from a Jurkat cell λgt10 cDNA library. The majority of the strongly hybridising plaques contained a 2.1 kb insert. Two

clones with inserts of 2.1 kb and 1.0 kb were subcloned and used for sequence analysis. Both sequences deduced from peptides A and B, were present within the larger insert. Since both clones start at exactly the same position, because various 2.1 kb clones were isolated, and also because the longest open reading frame did not have 5′ upstream in frame stop codons, we suspected that our human DG kinase cDNA had an internal *Eco*RI site at the 5′-end of both clones. Since no additional fragments had been detected after *Eco*RI digestion of the phage DNAs from Jurkat λgt10 clones, inserts used to construct the gt10 library probably had not been properly methylated. A second λgt10 cDNA library (from human DND41 leukemic T-cells) was screened with a 1.0 kb 5′ cDNA fragment. Inserts from 20 isolated clones were directly amplified by PCR with either λgt10 primer (5′ or 3′ end) in combination with an internal DG kinase primer in reverse and complementary orientation. Two PCR products from independent phage clones were further analysed. Both PCR fragments were 0.9 kb long and indeed contained an internal *Eco*RI site. The fragments were digested with *Eco*RI, cloned and sequenced. Both clones were identical in sequence. Direct sequencing of the two original PCR-fragments derived from phage DNA (not *Eco*RI digested) using primer AGAGCCACTGCCATCAT (position 609) confirmed that these clones contained the sequence adjacent to the internal *Eco*RI site (position 449). A 5′ clone was ligated at the *Eco*RI site with a 2.1 kb clone to obtain a full-length human DG kinase cDNA.

3.3. *Human DG kinase cDNA and amino acid sequence comparison with other proteins*

The cDNA sequence of human DG kinase contains a single open reading frame of 2205 bp encoding a protein of 735 amino acids. The leader sequence contains one in frame stop codon followed downstream by an in frame start codon. Although this start codon (GACAGATGG) does not have the conserved features of a Kozak sequence (CCACCATGG) [33] it is the only apparent start for translation. The 3′ untranslated region contains the canonical polyadenylation signal AATAAA.

The deduced amino acid sequence of 735 residues corresponds with a calculated M_r of 82 671 Da, which is close to the 86 kDa actually observed for the purified DG-kinase on SDS-PAGE. The sequence of both peptide A and B were found in the human DG kinase sequence at position 166 and 473, respectively (Fig. 2). In retrospect it is clear that peptide B was contaminated with another tryptic peptide of DG kinase, FHIM, which was identified at amino acid position 537, and explains the double amino acid sequence obtained at the first four cycles of degradation. As expected, peptide A and B were both preceded by a basic amino acid, necessary for tryptic cleavage.

Fig. 2. Comparison of the human and porcine DG kinases. The sequences of human and porcine DG kinases have been aligned, and amino acid substitutions for porcine DG kinase are indicated. At position 88 for porcine DG kinase a single amino acid deletion is observed (indicated by 'x'). EF-hand structures (shaded), cysteine repeats (underlined; cysteines residues double underlined), and ATP binding sites (asterisks) are indicated.

Analysis of the primary structure of human DG kinase reveals the presence of two EF-hand structures at amino acid positions 123 and 168, both contain the conserved sequence ZxZxZxxxZxxZ, where Z is Ser, Thr, Glx or Asx (for review see [34]). The presence of a double EF-hand structure indicates a functional Ca^{2+} binding site for DG kinase. Sequence similarity in the EF-hands extends beyond only the Z-residues, when compared with for instance calmodulin or troponin C (Fig. 3). Rather unexpectedly, we could not detect any Ca^{2+} dependence of either the purified enzyme, or of the expressed enzyme in DG kinase transfected COS-7 cells (see below); activities of the enzyme did not vary by more than 10% in the presence of 10 mM EGTA (including a 1 hour preincubation with EGTA at 4°C) when compared with varying concentrations of Ca^{2+} in the assay (irrespective of the presence of DOC). At pre-

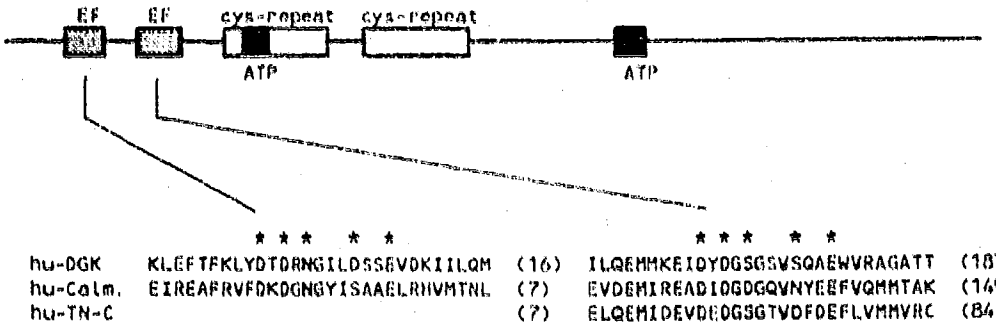

Fig. 3. Linear structure of DG kinase and comparison of the EF-hand structures with calmodulin and troponin C. The amino acid sequence of the EF-hand structures of DG kinase were aligned with the 3rd and 4th domain of human calmodulin, or the 2nd region of human troponin C [35,36]. The conserved amino acids in the EF-hand motif are indicated with asterisks. Positions of sequence identities are shaded. The number of amino acid residues that separate each pair of EF hands is shown in parentheses, and the position of the oligopeptide in the protein is indicated on the far right.

sent we do not know if DG kinase fails to bind Ca^{2+}; conceivably our assay renders the enzyme Ca^{2+} independent, or Ca^{2+} is not required for activity of DG kinase but rather fulfills another as yet unidentified function. An interesting feature is the presence of two cysteine repeats starting at positions 219 and 282. Similar double cysteine repeats are also present in the C-1 region of all PKC isotypes and in several metalloproteins (for review see [37]). For DG kinase and PKC, this homology is not restricted to the cysteine residues within each cysteine repeat, but extends to additional amino acids within these structures. Using deletion mutants and site-directed mutagenesis, the double cysteine repeat of PKC has been identified as the phorbol ester binding site [38]. Because phorbol esters and DG compete for binding to PKC [39], the double cysteine repeat of DG kinase could likewise be its DG binding site.

Finally, DG kinase contains two putative ATP binding sites (Fig. 2) one of which resides within the first cysteine repeat of DG kinase. At present it is unknown whether both constitute active ATP binding sites.

We specifically compared the sequence of human DG kinase with those of other DG metabolizing enzymes, such as *E. coli* DG kinase and *sn*-1,2-diacylglycerol cholinephosphotransferase (CPT-1) [40,41]. These proteins did not show any significant similarity with human DG kinase, suggesting that the DG binding site has not been conserved in the course of evolution. A phospholipid binding protein, that shares homology with PKC in a putative PS binding domain, referred to as p65, did not show any homology with DG kinase [42], even though DG kinase displays optimal activities in the presence of PS (results not shown).

While the sequence of our human DG kinase cDNA was being completed, a DG-kinase sequence from pig was published [1]. The pig enzyme is homologous to the human DG kinase with 51 amino acid differences and one amino acid insertion at position 88 for human DG kinase. Striking is the long stretch of 110 amino acids following the second ATP binding site, that is fully conserved between human and pig DG kinase (Fig. 2).

3.4. *RNA blotting analysis*

Northern blot analysis showed a single mRNA species of 3.2 kb (Fig. 4). Expression of DG kinase mRNA was high in normal human T-cells and Jurkat

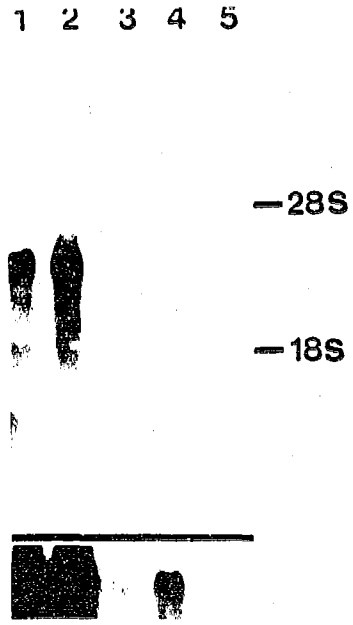

Fig. 4. Northern blot analysis of DG kinase mRNA. Total RNA (20 μg/lane) from Jurkat cells, normal human T-cells, A431 cells, HeLa cells, and GLC2 cells (lanes 1, 2, 3, 4 and 5, respectively) were separated on a 1% agarose formaldehyde gel and transferred to a nylon membrane. The filter was hybridized with a 1.0 kb DNA fragment (starting at the internal *Eco*RI site) and exposed for 4 h. DG kinase mRNA is detectable as a single 3.2 kb mRNA. The bottom panel shows an overexposure (3 days) of the 3.2 kb DG kinase region of the blot. This exposure reveals DG kinase mRNA in HeLa cells, and a faint band for A431 cells and GLC2 cells. Equal quantities of RNA had been loaded, as judged from hybridisation with a human actin cDNA probe (data not shown).

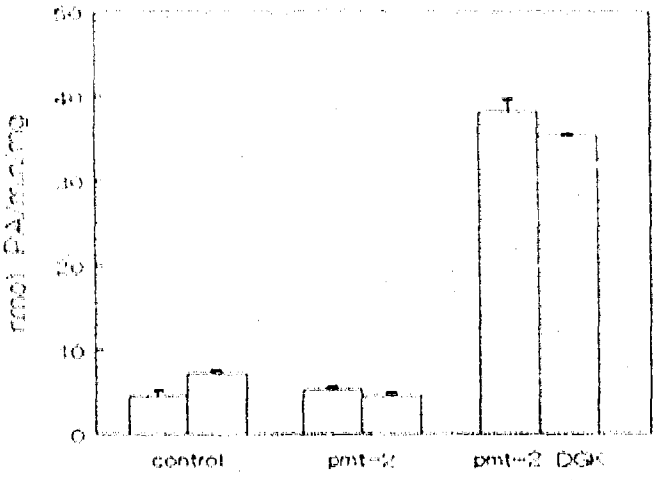

Fig. 5. DG kinase activity in transfected COS-7 cells. Total cell lysates from COS-7 cells transfected with either no plasmid, pMT-2 (vector alone), or pMT-2-DGK (vector plus DG kinase) were analysed for activity. Each bar in the figure shows a separate transfection, and the activity shown is the mean of two independent assays. The data shown are from one experiment, but could be reproduced with similar results.

cells (human leukemic T-cells). This is in agreement with immunological quantitation studies of DG kinase [24]. DG kinase mRNA was expressed at a low level in HeLa cells (human cervix carcinoma), and barely detectable in A431 cells (human epidermoid carcinoma) and GLC2 cells (small cellular lung carcinoma).

3.5. *Expression of DG kinase in COS-7 cells*

The full-length DG kinase cDNA was subcloned into the expression vector pMT-2, and transfected into COS-7 cells. After 2 days cells were harvested, lysed and assayed for DG kinase activity. As shown in Fig. 5, cells transfected with human DG kinase contained 6–7-fold higher activity compared with control cells. Absolute values of activities for total cell lysates were 35–38 nmol PA/min/mg for DG kinase transfected cells. This overexpression yields high DG kinase activities, when compared with non-transfected COS-7 cells (4–5 nmol/min/mg), pig brain cytosol (2–3 nmol/min/mg), or WBC cytosol (6–7 nmol/min/mg). These experiments are the first to clearly demonstrate high DG kinase activity in a eukaryotic system, associated with the expression of DG kinase cDNA.

3.6. *Concluding remarks*

The cloning and expression of the human DG kinase cDNA allows us to study enzymatic and regulatory properties of this enzyme, and to determine its role in signal transduction. Not only as a possible regulator of PKC, but also as an enzyme that generates PA, which may function as a second messenger in its own right.

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