Dictyostelium discoideum has a single diacylglycerol kinase gene with similarity to mammalian θ isoforms

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Diacylglycerol kinases (DGKs) phosphorylate the neutral lipid diacylglycerol (DG) to produce phosphatidic acid (PA). In mammalian systems DGKs are a complex family of at least nine isoforms that are thought to participate in down-regulation of DG-based signalling pathways and perhaps activation of PA-stimulated signalling events. We report here that the simple protozoan amoeba *Dictyostelium discoideum* appears to contain a single gene encoding a DGK enzyme. This gene, *dgkA*, encodes a deduced protein that contains three C1-type cysteine-rich repeats, a DGK catalytic domain most closely related to the θ subtype of mammalian DGKs and a C-terminal segment containing a proline/glutamine-rich region and a large asparagine-repeat region. This gene corresponds to a previously reported

myosin II heavy chain kinase designated myosin heavy chain-protein kinase C (MHC-PKC), but our analysis clearly demonstrates that this protein does not, as suggested by earlier data, contain a protein kinase catalytic domain. A FLAG-tagged version of DgkA expressed in *Dictyostelium* displayed robust DGK activity. Earlier studies indicating that disruption of this locus alters myosin II assembly levels in *Dictyostelium* raise the intriguing possibility that DG and/or PA metabolism may play a role in controlling myosin II assembly in this system.

Key words: diacylglycerol kinase (DGK), myosin II, myosin II heavy chain kinase (MHCK), phosphatidic acid, protein kinase C (PKC).

INTRODUCTION

Eukaryotic diacylglycerol kinases (DGKs) are a widespread family of enzymes, with conserved representatives present in higher plants and throughout the Animal Kingdom. Based upon domain architecture, mammalian DGKs can be grouped into five classes, with some of these classes recognizable in *Drosophila* and *Caenorhabditis elegans* as well. The different classes display an array of accessory/signalling domains outside of the conserved catalytic domain, including strongly conserved cysteine-rich repeats, Pleckstrin homology (PH) domains, EF-hand motifs, Ras-associating domains, proline-rich domains and ankyrin repeats [1–3].

Earlier studies in *Dictyostelium discoideum* suggested a link between DGK signalling and myosin II assembly control [4,5]. In this system myosin II assembly and localization to discrete subcellular structures is regulated via phosphorylation of three threonine residues in the tail region of the myosin II heavy chain (MHC) [6–8]. Two distinct types of myosin II heavy chain kinase (MHCK) have been reported to participate in this process. One group is comprised of the structurally related kinases MHCK A [9,10], MHCK B [11] and MHCK C [12,13]. These enzymes all contain a novel protein kinase catalytic domain linked to a C-terminal WD-repeat domain, and represent founding members of a protein kinase family known as alpha kinases [14]. In addition, an MHCK with an apparent molecular mass of 84000 Da as determined by SDS/PAGE was purified from developmental-phase *Dictyostelium* cells [15]. Antibodies gener-

ated against this protein were used to screen a cDNA expression library, and the isolated cDNA clone was described as encoding a protein kinase C (PKC)-like protein [16]. This protein kinase was denoted myosin heavy chain-protein kinase C (MHC-PKC), reflecting its apparent PKC character and the fact that it was purified as an MHCK activity (GenBank accession nos, protein, A46136; nucleotide, M93393). In retrospective analysis of this cDNA, it was reported that the central domain of MHC-PKC was in fact a near-complete DGK-type catalytic domain [4].

We have performed further analysis of this locus, including rapid amplification of 3' cDNA ends (3'-RACE), genomic and reverse transcriptase PCR (RT-PCR), evaluation of the near-complete *Dictyostelium* genome database and Northern blotting. This analysis reveals the presence of a single gene encoding a protein that contains very strong similarity to mammalian DGKs, and a clear absence of a recognizable protein kinase catalytic domain. Previous Southern blot analysis and the near-complete *Dictyostelium* genome project indicate that this is the only DGK present in this organism. We propose that this locus be designated *dgkA*.

EXPERIMENTAL

Sequence analysis and cloning

The nucleotide sequence of the *dgkA* gene was assembled from independent sequence passes using the SeqMan module of the DNASTar software package. Initially 32 sequences obtained

Abbreviations used: DG, 1,2-diacylglycerol; DGK, diacylglycerol kinase; MHC, myosin II heavy chain; MHCK, myosin II heavy chain kinase; MHC-PKC, myosin heavy chain-protein kinase C; PA, phosphatidic acid; PH, Pleckstrin homology; PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; 3'-RACE, rapid amplification of 3' cDNA ends; RT-PCR, reverse transcriptase PCR.

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The sequence of DgkA is available at the GenBank Nucleotide Sequence Database under accession no. AY152858.

from the *Dictyostelium* genome project (available at <http://dictybase.org>) were used to generate a contig that still contained ambiguities and several segments with only one or two sequence passes. To resolve these ambiguities, PCR products were synthesized from *Dictyostelium* genomic DNA (prepared according to the miniprep method of Nellen et al. [17]) and sequenced. Additional clones were generated using RT-PCR and 3'-RACE (see below) so that a total of 23 additional sequence passes were pooled together with the genome project data. This resulted in a minimum coverage of four sequence passes in every portion of the gene, with up to 8–10 passes in some regions, such as the location of the stop codon and the highly repetitive poly-asparagine stretch at the C-terminus of the coding region.

Initial Clustal alignments were performed to identify the conserved core segments of the catalytic domains for the final alignment used for Figure 2 (see below). For DgkA, this segment corresponds to residues 334–576. The DNASTar MegAlign software package was used to perform Clustal W alignments and to produce the phylogenetic tree. Genbank accession numbers for the human DGKs are: θ , NP_001338; ϵ , NP_003638; ζ , NP_003637; β , XP_003156; γ , XP_003156; α , NP_001336; δ , NP_003639.

A full-length copy of the *dgkA* gene was constructed from the products described above, including RT-PCR and 3'-RACE products, to generate a spliced cDNA copy of the gene. PCR was used to introduce a *Bam*HI site next to codon 2 of the *dgkA* coding region, allowing the gene to be subcloned into the vector pTX-FLAG [25] with the N-terminal FLAG epitope coding region positioned immediately upstream of codon 2 of *dgkA*. All segments subject to PCR were sequenced subsequently to confirm the absence of any PCR-generated sequence errors.

Immunoprecipitation and DGK activity assays

Immunoprecipitations were performed as follows. Cells (5×10^7) were collected from Petri dish cultures in HL5 medium, washed with 50 mM Tes, pH 7, then suspended in buffer containing 50 mM Tes, pH 7, 5 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol and a protease inhibitor cocktail mix [18]. Cells were lysed by the addition of Triton X-100 (1% final concentration) in a volume of 400 μ l. Lysate was centrifuged briefly to remove particulate material, and a 10 μ l packed volume of anti-FLAG IgG-conjugated beads (antibody M2; Sigma) was added to the cleared supernatant. Samples were incubated at 4 °C with agitation for 60 min. Beads were collected by centrifugation, washed twice with 50 mM Tes, pH 7, 100 mM NaCl, 1 mM dithiothreitol and 1 mM EDTA. Beads were then resuspended in 20 μ l of buffer containing FLAG peptide to elute bound protein (10 mM Tes, pH 7, 0.1 mM EDTA, 1 mM dithiothreitol and 200 μ g/ml FLAG peptide; Sigma). Eluted material was stored at –80 °C until used for DGK activity assays.

DGK activity was assayed as described previously [19]. Briefly, diacylglycerol C8 (1,2 dioctanoyl glycerol; Sigma) was used as the lipid substrate in a 50 μ l reaction volume containing 4 mM sonicated 1,2-diacylglycerol (DG), 20 mM (γ - 32 P]ATP, 10 μ Ci), 5 mM MgCl₂, 10 mM Tris, pH 7.4, and 5 μ l of immunopurified FLAG-DgkA as a source of the enzyme. The phosphorylation assay was carried out for 10 min at room temperature. At the end of the assay lipids were extracted by the subsequent addition of 200 μ l of chloroform/methanol (2:1, v/v), 50 μ l of chloroform and 50 μ l of 0.1 M HCl. Organic layers were recovered, dried under a stream of nitrogen, taken up in 20 μ l of chloroform/methanol (2:1) and applied to Whatman LK6D silica gel 60 A plates along with dioctanoyl phosphatidic acid (PA) as a stan-

dard. Plates were developed with a solvent system of chloroform/methanol/4 M NH₄OH (9:7:2, by vol.). Dried plates were subjected to autoradiography and the band corresponding to [32 P]PA was identified by co-migration with non-radioactive standard visualized by iodine staining.

RNA methods

Total RNA was extracted from vegetatively growing cells and at 4 h intervals during development using Trizol (Life Technologies) as described in [20]. For RT-PCR, cDNA synthesis was performed using 10 μ g of total Ax3 RNA in a reaction mixture containing 5 μ g of oligo dT₁₈, 40 units of RNasin, dNTPs and 200 units of Moloney murine leukaemia virus reverse transcriptase (Promega) for 1 h at 37 °C. PCR reactions were performed using either *Taq* DNA polymerase (Life Technologies) or Expand high-fidelity polymerase (Roche Diagnostics). Products were gel-purified using the Qiaquick gel extraction kit (Qiagen), cloned into pCR 2.1 TA (Invitrogen) and sequenced.

The 3'-RACE was performed using a 3'-RACE kit (Life Technologies) according to the manufacturer's protocol. The initial reverse transcriptase reaction was performed with 4.1 μ g of total Ax2 RNA prepared 12 h after initiation of development. Amplification was performed in two independent reactions with oligo(dT) as the downstream primer and the *dgkA*-specific primers DAGK1604 (5'-CGATAAACCCGAAGATCGTAGA-GTCATCG-3') or DAGK1837 (5'-AGAGTCGATAGATCC-ATTGAGGGTATTATC-3') as upstream primers in each amplification. Both of these primers bind within the conserved catalytic domain segment of *dgkA*. These PCR reactions yielded products that were approx. 1.3 and 1.1 kb. Products were cloned into the vector pCR4-TOPO (Invitrogen) and subjected to DNA sequencing.

Northern blotting was performed as described in [20] using RNA isolated from Ax2 cells. As a probe, a 1.3 kb cloned DNA fragment corresponding to the DGK catalytic domain (residues 1115–2358 of *dgkA*, produced as a PCR product) was labelled with [α - 32 P]dCTP using a Rediprime II kit (Amersham Biosciences). The final wash stringency was $0.2 \times \text{SSC}/0.1\%$ SDS (where $1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) at 42 °C.

RESULTS

Characterization of the *dgkA* gene

The earlier report that the MHC-PKC gene sequence contained a near-complete DGK catalytic domain [4], and the very weak similarity of the reported MHC-PKC catalytic domain to established PKC catalytic domains [16], prompted us to perform further evaluation of this locus as represented in the near-complete *Dictyostelium* genome project database. We used available genome project raw sequence runs and additional sequences generated in-house from genomic PCR products to determine the sequence of the *dgkA* gene. The genomic locus appeared to encode a single open reading frame interrupted by a single intron. RT-PCR analysis confirmed that the predicted intron was in fact spliced out in mRNA. The 3' end of the gene was mapped by 3'-RACE, using two different primers that bind within the DGK catalytic domain. The start ATG codon was deduced based upon the presence of an in-phase stop codon further upstream, and upon the presence of an extended AT-rich region further upstream with no recognizable splice junction motifs and no apparent coding segments. The resulting spliced sequence displays a single continuous open reading frame of 887

[illegible]

1 335 657 887

C1A C1B C1C CAT P N

(A) Nucleotide and deduced amino acid sequence of *dgkA*. Lower-case nucleotide residues indicate non-coding sections, and upper-case represents sections deduced to correspond to protein-coding regions of the gene. Nucleotide residue numbers are indicated on the left and amino acid residue numbers on the right. Invariant amino acid residues of the cysteine repeat domain are highlighted black for repeat C1A (residues 8–57), grey for repeat C1B (residues 141–190) and black for repeat C1C (residues 209–259). Amino acid residues corresponding to the conserved DGK catalytic domain are underlined. The asterisk beneath residue 2937 indicates the last nucleotide present in the *dgkA* transcript before the poly(A)⁺ tail, as identified from 3′-RACE product sequencing. **(B)** Schematic diagram of the domain organization of DgkA. The N-terminal segment of the protein contains three C1-type cysteine repeats (C1A, C1B and C1C). This segment is followed by a highly conserved DGK catalytic domain (CAT), a short proline-rich region (P) and large poly-asparagine stretch (N).

Figure 2 Phylogenetic analysis of the catalytic domains of DgkA and human DGKs

Sequences were aligned using the Clustal W algorithm in DNASTar 5.0. Very similar results were obtained with Clustal V. Sequence ends were trimmed to the core conserved catalytic domain for all sequences, which corresponds to residues 334–576 of DgkA. Dd, *D. discoideum*; Hs, *Homo sapiens*.

amino acids, encoding a polypeptide with a predicted molecular mass of 102.7 kDa and a pI of 7.34 (Figure 1A).

Domain organization of DgkA

The deduced protein sequence of DgkA contains cysteine-rich repeats at the N-terminus, a structural feature that is common to both PKCs and DGKs [21]. In both classes of enzyme these repeats display the strongly conserved H_2C_6 motif: His-Xaa₁₂-Cys-Xaa₂-Cys-Xaa_{13/14}-Cys-Xaa₂-Cys-Xaa₄-His-Xaa₂-Cys-Xaa₇-Cys.

This repeat motif is referred to as a C1-type repeat in accord with the original PKC nomenclature for the cysteine-rich repeat domain first identified in PKCs (reviewed in [21]). DgkA displays three complete C1 repeats (highlighted in black and grey in Figure 1A), followed by a conserved DGK catalytic domain (underlined in Figure 1A). C-terminal to the apparent DGK catalytic domain, DgkA contains a proline/glutamine-rich segment, followed by a large poly-asparagine segment.

In view of the unusually repetitive character of this downstream region, this segment of the gene was sequenced with a minimum of 8–10-fold redundancy, confirming the sequence as presented in Figure 1(A). In addition, 3'-RACE reactions revealed no

splicing events anywhere in or downstream of the DGK catalytic domain. The genomic sequence and characterization of the mRNA thus confirm the domain organization indicated in Figure 1(B), with three C1-type repeats, a highly conserved DGK catalytic domain, a short proline-rich domain and a large poly-asparagine segment.

Domain analysis of DgkA

BLAST searches with the DGK-like catalytic domain segment of DgkA indicated strong conservation with known DGKs from a variety of organisms (e.g. 40% identity and 56% similarity reported by BLAST 2 sequences compared with human DGK θ). To evaluate this similarity further, we performed Clustal W alignments of the catalytic segment of DgkA, together with a set of DGK catalytic domains that included representatives of each of the five classes of human DGKs. In this analysis the DgkA catalytic domain consistently grouped most closely with the catalytic domain of the human DGK θ (Figure 2).

C1-type cysteine-rich repeats can be classified as 'typical' or 'atypical', based upon the presence in typical repeats of specific conserved residues such as proline at position 11 of the repeat and a glycine at position 23 that are thought to be critical for stabilizing the conformation of the DG-binding site (Figure 3; reviewed in [21]). Conventional PKCs display two C1 repeats in their N-terminal domain, and at least one of these always fits the consensus for the typical classification. This structural pattern is associated with the ability to bind DG and phorbol esters with high affinity (reviewed in [21]). Most DGKs also contain two C1-repeat motifs. However, with the exception of the C1A repeat of β and γ DGKs, DGK C1 repeats do not fit the typical profile. Evaluation of the three C1 repeats of DgkA revealed disagreements with the typical pattern in all three (Figure 3), indicating that these repeats fit the pattern normally present in mammalian DGKs rather than the pattern present in PKCs.

The presence of three C1 repeats in the DgkA protein is also noteworthy. To our knowledge the only other class of proteins known to contain three C1 repeats are the θ isoform subgroup of mammalian and nematode DGKs [1,2,22,23]. The conservation of the DgkA catalytic domain with mammalian DGK θ catalytic domains, and the conservation of the C1-repeat organization

Figure 3 C1 cysteine-rich repeats of DgkA compared with those of human DGK θ

Invariant residues of the H_2C_6 repeat are highlighted in light grey. Residues conforming to the 'extended C1' motif described previously [24] are highlighted in dark grey. In DgkA, as in the human DGK θ , only the C1 repeat closest to the catalytic domain (C1C in each case) displays the profile of the extended C1 motif. typ indicates the profile conserved in classic typical DG/phorbol ester-binding C1 domains such as present in PKCs. Bold residues on the Typ line represent residues that are characteristically invariant in typical PKC-type C1 domains. ext indicates the profile residues for the extended C1 motif. Bold residues on the Ext line represent residues that are characteristically invariant in DGK C1 domains that lie closest to the DGK catalytic domain. In each profile, upper-case letters refer to specific conserved amino acid residues, and lower-case symbols are: h, large hydrophobic (Trp, Tyr, Phe, Leu, Ile, Val, Met); a, aromatic; +, basic [21].

Figure 4 Expression and biochemical activity of FLAG-DgkA

(A) Control *Dictyostelium* cells and cells transfected with a FLAG-tagged version of *dgkA* were subjected to Western blot analysis using an anti-FLAG antibody. A 120 kDa band corresponding to FLAG-DgkA was apparent in crude cell lysates and in anti-FLAG immunoprecipitates from cells transfected with the FLAG-tagged *dgkA* gene. (B) Anti-FLAG immunoprecipitates from control cells and cells expressing FLAG-DgkA were assayed for DGK activity as described in the Experimental section. An autoradiogram shows that immunoprecipitates containing FLAG-DgkA phosphorylate DG to generate ^{32}P -labelled PA. PA indicates the position of migration of an unlabelled PA standard.

with θ isoforms may reflect conserved regulation or cellular roles between these enzymes as well. It should be noted that DgkA does display some differences from mammalian DGK θ isoforms. The mammalian isoforms contain a PH domain positioned between the C1 repeats and the catalytic domain, with the PH domain also reported to have Ras-interacting activity (reviewed in [2,3]). The relatively close placement of the DgkA C1C repeat to the catalytic domain appears to rule out the presence of a PH domain at this position, and we cannot detect any similarity to PH domains in other regions of the protein. Mammalian DGK θ also contains a proline-rich segment at the N-terminus. DgkA also displays a proline-rich segment, but it lies downstream of the catalytic domain rather than upstream. In common with the proline-rich segment of mammalian DGK θ , this downstream region contains examples of the motif PXXP, typical for proteins that can bind Src homology 3 domains.

Another diagnostic feature of DGK C1 repeats is that the C-terminal C1 repeat, located closest to the DGK catalytic domain, contain a characteristic conserved set of residues in and beyond the core H_2C_6 motif. This set of residues has been termed the 'extended' cysteine rich domain, and may be involved in the recognition/binding of substrate DG [24]. The DgkA C1C motif explicitly fits the extended C1 pattern (Figure 3, dark grey highlights), further supporting the hypothesis that this enzyme functions as a conventional DGK rather than a PKC-like activity.

Biochemical activity of DgkA

A full-length epitope-tagged version of the *dgkA* gene was constructed and the resultant cDNA was subcloned into the vector pTX-FLAG to allow constitutive expression of FLAG-tagged DgkA. Upon transfection into *Dictyostelium* cells, the expressed FLAG-DgkA protein migrates with an apparent mass of 120 kDa (Figure 4A). FLAG-DgkA was immunoprecipitated with anti-FLAG epitope beads (Sigma) and was assayed for

Figure 5 Northern blot indicating expression pattern of the *dgkA* gene

Ax2 cells were developed on buffered filter pads for the indicated number of hours prior to RNA isolation.

DGK activity using an assay described previously [19]. A control immunoprecipitation from non-transfected *Dictyostelium* cells shows no DGK activity, while the immunoprecipitate from FLAG-DgkA *Dictyostelium* cells revealed the presence of robust DGK activity (Figure 4B).

Expression and locus organization of *dgkA*

Northern-blot analysis was performed with RNA harvested from growth-phase cells and cells collected during development. A single message of approx. 3.0–3.5 kb was detected (Figure 5). This message was detected at all growth stages, but expression was significantly up-regulated in the 12–24 h time points of development.

Because ongoing progress towards completion of the *Dictyostelium* genome project has become available, it is now possible to identify an assembled genome project contig (JC2a94d04) that spans the entire *dgkA* locus. This contig was evaluated with respect to positions of restriction sites. This analysis reveals restriction sites within and around the *dgkA* gene, as indicated in Figure 6. Predicted restriction-fragment sizes determined for several enzymes all match observed fragment sizes for each of the genomic restriction digests analysed by Southern-blot analysis in the original molecular characterization of the MHC-PKC cDNA [16]. The earlier Southern analysis was performed with a segment of the MHC-PKC cDNA that is now recognized as encoding the DGK-like catalytic domain. The clear identification of only one hybridizing segment of DNA in the original characterization further suggests that *Dictyostelium* contains only a single DGK gene, and that the correct structure of this locus is as reported here. In addition, extensive BLAST searches of the nearly complete *Dictyostelium* genome database did not reveal any other genes sharing significant similarity with the catalytic domain of *dgkA*.

Figure 6 Restriction map of the genomic locus of the *dgkA* gene derived from the *Dictyostelium* genome project, contig JC2a94d04

H, *HindIII*; E, *EcoRI*; Bc, *BclI*; K, *KpnI*; C, *ClaI*; Bg, *BglII*. Calculated restriction-digest patterns correspond to digests described previously for the MHC-PKC gene locus [16].

DISCUSSION

The *dgkA* gene described here appears to be the only gene in *Dictyostelium* that contains a DGK-like catalytic domain, based on genomic database analysis, Northern blotting and previous Southern blot analysis. Biochemical analysis of DgkA, expressed as a FLAG-tagged protein in *Dictyostelium*, provides direct evidence for its ability to phosphorylate DG. The strong similarity with mammalian θ DGK isoforms is intriguing, and may provide hints as to the functions of the DgkA protein. Previous studies in rat small arteries have demonstrated translocation of DGK θ to the plasma membrane in response to noradrenaline, but not angiotensin II [26], suggesting a role for this enzyme in regulating DG and PA levels during noradrenaline receptor activation. DGK θ activation, but not translocation, was furthermore found to be dependent on activation of phosphoinositide 3-kinase (PI-3K). It was also found that DGK activity could be co-immunoprecipitated with protein kinase B (Akt/PKB) in this setting, although the DGK isoform was not identified. It is noteworthy that the PI-3K and Akt/PKB pathways are critically involved in cell polarity and actomyosin organization during chemotaxis in *Dictyostelium* [27–29]. Further studies are needed to determine whether interactions between DgkA and the PI-3K pathway participate in cytoskeletal control in this system.

The relationship between DgkA and the previously reported MHC-PKC is difficult to resolve fully, but we suggest that problems with the cDNA clone originally described as encoding the MHC-PKC enzyme led to the appearance of a domain architecture resembling a PKC. The fact that DGKs were not recognized as a family at the time of the original MHC-PKC cloning probably contributed to this misinterpretation. In addition, at least two short chimaeric inserts are clearly present in the MHC-PKC cDNA entry in GenBank, which probably also contributed to misinterpretation of the protein structure. Nucleotides 1–149 of the MHC-PKC cDNA (GenBank accession no. M93933) do not display similarity to the *dgkA* locus. In BLAST searches performed against all GenBank sequences, this segment does display a stretch of 98 nucleotides that is nearly identical to the *D. discoideum* EF1-I gene, encoding elongation factor 1 α . Within the segment of MHC-PKC reported to resemble a PKC catalytic domain there also appears to be a segment of DNA that is chimaeric, in that nucleotides 1800–2280 of MHC-PKC are not present in the *dgkA* locus. BLAST searches performed with this segment of the MHC-PKC do not reveal significant similarity to GenBank entries from *Dictyostelium* or from any other source. The regions of the MHC-PKC sequence surrounding this block display strong similarity but not identity

with the *dgkA* locus, and scattered single nucleotide differences in these flanking regions create several phase-shift disagreements between the two sequences.

Although there appear to be errors in the original MHC-PKC cDNA sequence, it is intriguing that gene-targeting studies performed with that cDNA resulted in clear cytoskeletal defects. Based upon Southern blot analysis, the gene-targeting construct made with the MHC-PKC cDNA did appear to target to the *dgkA* locus and disrupt the *dgkA* gene [5]. These knockout cells were reported to accumulate a dense ring of myosin II filaments at the cell cortex and to have substantially more myosin II associated with the Triton-insoluble cytoskeleton than control cells. [5]. These cells also displayed aberrant fruiting body formation. This latter phenotype is unlikely to result solely from myosin II overassembly, as cell lines that express mutant forms of myosin II that cannot be phosphorylated on the heavy chain ($3 \times$ ALA myosin) display severe myosin II overassembly but still form relatively normal fruiting bodies [6].

Although the ambiguity regarding the cDNA clone used in the earlier studies complicates a definitive conclusion, the results of the gene-targeting studies point towards a possible role of the DGK catalytic domain functions of DgkA as an upstream regulator of myosin II assembly control. If the MHCK purification, antibody production and cDNA library screening strategy that was used to originally isolate MHC-PKC was successful, then it may be concluded that the strongly conserved DGK-type catalytic domain of DgkA must have protein kinase activity in addition to DGK activity, and be capable of directly phosphorylating myosin II. Although there is no precedence from other systems for DGKs to display protein kinase activity, biochemical analysis performed with recombinant DgkA will be necessary to test this possibility. Alternatively, if *dgkA* is not the gene encoding the originally isolated MHCK activity, it is possible that DgkA may act upstream of MHCKs or MHC phosphatases during chemoattractant responses, to activate the localized assembly and recruitment of myosin II to the cytoskeleton that is critical for polarization and efficient migration. Based upon earlier studies in mammalian systems with DGK θ [26], and studies in *Dictyostelium* [27], we suggest that DgkA may function in a regulatory pathway that includes PI-3K and Akt/PKB during this process. Further studies with a full-length *dgkA* cDNA will be necessary to clarify fully the role of this enzyme in cytoskeletal organization and myosin II function.

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REFERENCES

- 1 Kano H, Yamada K, and Sakane F. (2002) Diacylglycerol kinases: emerging downstream regulators in cell signaling systems. *J. Biochem. (Tokyo)* **131**, 629–633
- 2 van Blitterswijk W. J. and Houssa B. (2000) Properties and functions of diacylglycerol kinases. *Cell Signal.* **12**, 595–605

- 3 Topham, M. K. and Prescott, S. M. (1999) Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* **274**, 11447–11450
- 4 Thanos, C. D. and Bowie, J. U. (1996) Developmentally expressed myosin heavy-chain kinase possesses a diacylglycerol kinase domain. *Protein Sci.* **5**, 782–785
- 5 Abu-Elneel, K., Karchi, M. and Ravid, S. (1996) *Dictyostelium* myosin II is regulated during chemotaxis by a novel protein kinase C. *J. Biol. Chem.* **271**, 977–984
- 6 Egelhoff, T. T., Lee, R. J. and Spudich, J. A. (1993) *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization *in vivo*. *Cell* **75**, 363–371
- 7 Vaillancourt, J. P., Lyons, C. and Côté, G. P. (1988) Identification of two phosphorylated threonines in the tail region of *Dictyostelium* myosin II. *J. Biol. Chem.* **263**, 10082–10087
- 8 Sabry, J. H., Moores, S. L., Ryan, S., Zang, J. H. and Spudich, J. A. (1997) Myosin heavy chain phosphorylation sites regulate myosin localization during cytokinesis in live cells. *Mol. Biol. Cell* **8**, 2605–2615
- 9 Côté, G. P., Luo, X., Murphy, M. B. and Egelhoff, T. T. (1997) Mapping of the novel protein kinase catalytic domain of *Dictyostelium* myosin II heavy chain kinase A. *J. Biol. Chem.* **272**, 6846–6849
- 10 Futey, L. M., Medley, Q. G., Côté, G. P. and Egelhoff, T. T. (1995) Structural analysis of myosin heavy chain kinase A from *Dictyostelium*. Evidence for a highly divergent protein kinase domain, an amino-terminal coiled-coil domain, and a domain homologous to the β -subunit of heterotrimeric G proteins. *J. Biol. Chem.* **270**, 523–529
- 11 Clancy, C. E., Mendoza, M. G., Naismith, T. V., Kolman, M. F. and Egelhoff, T. T. (1997) Identification of a protein kinase from *Dictyostelium* with homology to the novel catalytic domain of myosin heavy chain kinase A. *J. Biol. Chem.* **272**, 11812–11815
- 12 Luo, X., Crawley, S. W., Steimle, P. A., Egelhoff, T. T. and Côté, G. P. (2001) Specific phosphorylation of threonine by the dictyostelium myosin II heavy chain kinase family. *J. Biol. Chem.* **276**, 17836–17843
- 13 Liang, W., Licate, L., Warrick, H., Spudich, J. and Egelhoff, T. (2002) Differential localization in cells of myosin II heavy chain kinases during cytokinesis and polarized migration. *BMC Cell Biol.* **3**, 19
- 14 Ryazanov, A. G., Pavur, K. S. and Dorovkov, M. V. (1999) Alpha-kinases: a new class of protein kinases with a novel catalytic domain. *Curr. Biol.* **9**, R43–R45
- 15 Ravid, S. and Spudich, J. A. (1989) Myosin heavy chain kinase from developed *Dictyostelium* cells. Purification and characterization. *J. Biol. Chem.* **264**, 15144–15150
- 16 Ravid, S. and Spudich, J. A. (1992) Membrane-bound *Dictyostelium* myosin heavy chain kinase: a developmentally regulated substrate-specific member of the protein kinase C family. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5877–5881
- 17 Nellen, W., Datta, S., Reymond, C. D., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1987) in *Dictyostelium discoideum*: Molecular Approaches to Cell Biology (Spudich, J. A., ed.), pp. 67–100, Academic Press, London
- 18 Steimle, P. A., Naismith, T., Licate, L. and Egelhoff, T. T. (2001) WD repeat domains target *Dictyostelium* myosin heavy chain kinases by binding directly to myosin filaments. *J. Biol. Chem.* **276**, 6853–6860
- 19 Flores, I., Casaseca, T., Martinez, A., Kanoh, H. and Merida, I. (1996) Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. *J. Biol. Chem.* **271**, 10334–10340
- 20 Goldberg, J. M., Bosgraaf, L., Van Haastert, P. J. and Smith, J. L. (2002) Identification of four candidate cGMP targets in *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 6749–6754
- 21 Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M. and Nishizuka, Y. (1997) Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* **6**, 477–480
- 22 van Blitterswijk, W. J. and Houssa, B. (1999) Diacylglycerol kinases in signal transduction. *Chem. Phys. Lipids* **98**, 95–108
- 23 Houssa, B., Schaap, D., van der, W. J., Goto, K., Kondo, H., Yamakawa, A., Shibata, M., Takenawa, T. and van Blitterswijk, W. J. (1997) Cloning of a novel human diacylglycerol kinase (DGK θ) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. *J. Biol. Chem.* **272**, 10422–10428
- 24 Houssa, B. and van Blitterswijk, W. J. (1998) Specificity of cysteine-rich domains in diacylglycerol kinases and protein kinases C. *Biochem. J.* **331**, 677–679
- 25 Levi, S., Polyakov, M. and Egelhoff, T. T. (2000) Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231–238
- 26 Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V. and Ohanian, J. (2001) Diacylglycerol kinase θ is translocated and phosphoinositide 3-kinase-dependently activated by noradrenaline but not angiotensin II in intact small arteries. *Biochem. J.* **353**, 129–137
- 27 Chung, C. Y., Potikyan, G. and Firtel, R. A. (2001) Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKs. *Mol. Cell* **7**, 937–947
- 28 Firtel, R. A. and Chung, C. Y. (2000) The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients. *Bioessays* **22**, 603–615
- 29 Chung, C. Y. and Firtel, R. A. (1999) PAKs, a putative PAK family member, is required for cytokinesis and the regulation of the cytoskeleton in *Dictyostelium discoideum* cells during chemotaxis. *J. Cell Biol.* **147**, 559–576