Diacylglycerol kinase is phosphorylated *in vivo* upon stimulation of the epidermal growth factor receptor and serine/threonine kinases, including protein kinase C-ε

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In signal transduction, diacylglycerol (DG) kinase attenuates levels of the second messenger DG by converting it to phosphatidic acid. A previously cloned full-length human 86 kDa DG kinase cDNA was expressed as fusion protein in *Escherichia coli*, to aid in the generation of DG-kinase-specific monoclonal antibodies suitable for immunoprecipitation experiments. To investigate whether phosphorylation of DG kinase is a possible mechanism for its regulation, COS-7 cells were transiently transfected with the DG kinase cDNA and phosphorylation of the expressed DG kinase was induced by various stimuli. Activation of both cyclic AMP-dependent protein kinase and protein kinase C (PKC) resulted in phosphorylation of DG kinase on serine residues *in vivo*, and both kinases induced this phosphorylation within the same tryptic phosphopeptide, suggesting that they may exert similar control over DG kinase. No phosphorylation was observed upon ionomycin treatment, intended to activate Ca^{2+}/calmodulin-dependent kinases. Co-transfections of DG kinase with either PKC-α or PKC-ε cDNA revealed that both protein kinases, when stimulated, are able to phosphorylate DG kinase. For PKC-ε, DG kinase is the first *in vivo* substrate identified. Stimulation with epidermal growth factor (EGF) of COS-7 cells transfected with both DG kinase and EGF-receptor cDNA results mainly in phosphorylation of DG kinase on tyrosine. Since the EGF receptor has an intrinsic tyrosine kinase activity, this finding implies that DG kinase may be a direct substrate for the activated EGF receptor.

INTRODUCTION

Many hormones and neurotransmitters initiate their action by phospholipase C-mediated hydrolysis of phosphoinositides, resulting in the generation of at least two second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate [1]. In the phosphoinositide cycle, DG is phosphorylated subsequently to phosphatidic acid (PA) through the action of DG kinase. This enzyme thus attenuates the levels of DG, the activator of protein kinase C (PKC), and at the same time generates PA, presumed to be another, as yet poorly defined, second messenger [2–4]. DG kinase activity can be measured not only upon stimulation of G-protein-coupled receptors, but also upon stimulation of receptor protein kinases, as concluded from observations that stimulation of cells or isolated plasma membranes by platelet-derived growth factor [5] or epidermal growth factor (EGF) [6,7] results in transient PA formation from DG.

Different forms of mammalian DG kinases have been reported [8–14], the best characterized of which is a 86 kDa cytosolic DG kinase that we and others have cloned [14,15]. It is not known whether the different types of DG kinase play distinct roles in signal transduction, nor whether DG kinase activity is regulated during cell stimulation, although several reports point in this direction. For instance, phorbol ester and DG have been reported to induce translocation of DG kinase activity from cytosol to particulate fractions [16,17], whereas translocation to the cytoskeleton has been observed in EGF-stimulated A431 cells [18]. Furthermore, *in vitro* studies have shown that purified porcine 86 kDa DG kinase is phosphorylated by PKC and by cyclic AMP-dependent protein kinase (PKA), resulting in association of the enzyme with phosphatidylserine-containing membranes [19], possibly in a Ca^{2+}-dependent manner [20]. *In vivo*, however, no agonist-induced phosphorylation of DG kinase has as yet been reported that could possibly control activity in the stimulated cell.

Studies on the regulation of DG kinase *in vivo* have been hampered thus far by the low abundance of the enzyme and the lack of antibodies suitable for its immunoprecipitation. Here we describe a panel of monoclonal antibodies (MoAbs) specific for the human 86 kDa DG kinase and their use in studies with COS-7 cells transiently expressing the enzyme to high levels. We show that in living cells DG kinase can be phosphorylated not only by PKA and by two types of PKC, but also by the EGF receptor. These results position DG kinase in multiple signal transduction pathways, and suggest the occurrence of a direct coupling of the enzyme to the activated EGF receptor.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP, [^{32}P]P_i and [^{35}S]methionine were from Amersham. Acrylamide was from Serva (Heidelberg, Germany), and dithiothreitol and ATP were from Boehringer. Anti-(EGF receptor) MoAb-108 was a gift from Dr. J. Schlessinger (New York University Medical Center). All other chemicals were of analytical grade.

Abbreviations used: DG, 1,2-diacylglycerol; DMEM, Dulbecco's modified essential medium; EGF, epidermal growth factor; FCS, fetal calf serum; GST, glutathione S-transferase; HAT, hypoxanthine/aminopterin/thymidine; IBMX, 3-isobutyl-1-methylxanthine; MoAb, monoclonal antibody; PA, phosphatidic acid; PI, phosphatidylinositol; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Construction and purification of glutathione S-transferase (GST)–DG kinase fusion protein

A 2072 bp *Bam*HI–*Eco*RI restriction fragment of human DG kinase cDNA (lacking the 5′-end which contained the first 128 amino acids) was subcloned into the bacterial expression vector pRP261. This vector is an extended polylinker derivative of the pGEX-3X plasmid (Amrad) and was kindly provided by Kees Vink (The Netherlands Cancer Institute). *Escherichia coli* transfectants expressed a 90 kDa GST–DG kinase fusion protein upon induction with isopropyl β-D-thiogalactopyranoside. The fusion protein was denatured and purified by electrophoresis on a preparative SDS/polyacrylamide column, containing a 1 cm 5% stacking gel and a 2.5 cm 6% separation gel. Fractions containing 80–90% pure fusion protein at a concentration of 100–200 μg/ml were pooled, dialysed against PBS and stored at −80 °C.

Generation of MoAbs

Balb/c mice were immunized as described [21] with 50 μg of fusion protein in complete Freund's adjuvant. They received three booster injections of similar amounts of fusion protein in incomplete Freund's adjuvant. The presence of specific antibody was tested for by immunoblotting, using extracts of DG kinase-expressing COS-7 cells. Mouse spleen cells were fused with Sp2/0 hypoxanthine/aminopterin/thymidine (HAT)-sensitive myeloma cells as described [22], with minor modifications. Hybridoma colonies were selected on HAT medium for 14 days and subsequently analysed using a double e.l.i.s.a. As antigens for e.l.i.s.a., we used either GST–DG kinase fusion protein (for the identification of positive colonies) or GST–protein tyrosine phosphatase-μ fusion protein [23] (for detection of false positives). Positive hybridoma cells were diluted and seeded in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum (FCS), HAT and 20 μM β-mercaptoethanol with 10% T24 cell conditioned medium (T24 is an interleukin-6-producing human bladder carcinoma cell line). Single hybridoma clones were selected, further subcloned and finally grown in large volumes without T24 conditioned medium. Antibody-containing tissue culture supernatant was collected, divided into aliquots and stored at −20 °C.

Expression in COS-7 cells

Human DG kinase was transiently expressed in COS-7 cells as described [14,24]. To optimize expression levels of DG kinase, a perfect Kozak sequence [25] was introduced at the translation start site and a stop codon was introduced directly in front of this Kozak sequence. The 5′ non-coding sequence of DG kinase was deleted. This expression vector, termed pmtM-DGK-wt (wild type) was used in all COS-7 cell expression studies described (pmtM is a pmt-2-derived vector that contains a polylinker cloning site [23]). When indicated, pmtM-DGK-wt was co-transfected with pMT2-PKC-ε [24], pmtM-PKC-α or pmtM-EGF-receptor, expressing full-length mouse PKC-ε, full-length bovine PKC-α and full-length human EGF receptor respectively. PKC-α cDNA [26] and EGF receptor cDNA [27] were kindly provided by Dr. P. J. Parker (ICRF, London, U.K.) and Dr. M. Waterfield (Ludwig Institute for Cancer Research, London, U.K.) respectively. Transfections were performed with 20 μg of pmtM-DGK-wt, using 3.5×10^5 cells per 9 cm plate, with or without co-transfection with an additional 5 μg of pMT2-PKC-ε, pmtM-PKC-α or pmtM-EGF-receptor. Efficiency of transfection was determined by transfection of a β-galactosidase expression vector. Positive cells were visualized by colour staining (using 5-bromo-4-chloro-3-indolyl-β-D-galactoside as a substrate) and counted [28].

Radiolabelling of cells

At 24 h after transfection, cells were serum-starved for 12 h, followed by labelling for 4 h with 400 μCi of [^{32}P]P_i in phosphate-free MEM (Flow Laboratories) for 12 h with [^{35}S]methionine (125 μCi) in methionine-free RPMI (Flow).

Immunoprecipitation of DG kinase

DG kinase was quantitatively immunoprecipitated (80%–90% of total DG kinase) by the following optimized procedure. One 9 cm dish of cells [$1–5 \times 10^6$ cells/plate] was washed with PBS and lysed at 4 °C in 400 μl of Nonidet P40 lysis buffer, containing 150 mM NaCl, 1% Nonidet P40, 50 mM Tris, pH 8.0, 0.5 mM EDTA, 10 μg/ml phenylmethanesulphonyl fluoride, 10 μg/ml trypsin inhibitor, and, to inhibit phosphatase activity, 100 μM Na_3VO_4, 10 mM Na_3PO_4, and 1 mM NaF. All subsequent incubations were at 4 °C. Cell lysates were collected and insoluble debris was removed by centrifugation at 12000 *g* for 2 min. The resulting supernatant was precleared by incubation for 1 h with 3 μl of normal rabbit serum, followed by 30 min of incubation with 75 μl of 10% (v/v) formalin-fixed *Staphylococcus aureus* (Staph A), followed by 5 min of centrifugation at 12000 *g*. The supernatant was again incubated for 30 min with 50 μl of Staph A to remove any residual normal rabbit serum, followed by centrifugation. Specific immunoprecipitation of DG kinase was performed for 1–2 h or overnight with 100 μl of a mixture of three different hybridoma culture supernatants, i.e. αDGK #5, 6 and 8 (see also Results). Immune complexes were removed by incubation with 85 μl of Staph A for 1 h. Immunoprecipitates were washed three times in NET buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P40, 150 mM NaCl and 5 mM EDTA), transferred to a clean tube and washed once more. Immunoprecipitated proteins were analysed by SDS/PAGE.

In vitro transcription and translation

Human DG kinase cDNA was subcloned into pGEM-3Zf(−) in the restriction sites *Pst*I and *Eco*RI, using the same fragment as was subcloned into pmtM-DGK-wt. This vector, pGEM-DGK, was linearized with restriction enzymes and transcribed *in vitro* by standard procedures [28]. Correct lengths for transcribed RNA products were verified by electrophoresis on formaldehyde agarose gels. *In vitro* transcribed RNA was translated in the presence of [^{35}S]methionine in a nuclease-treated rabbit reticulocyte lysate (Promega).

Phosphoamino acid analysis and tryptic phosphopeptide mapping

Phosphoamino acid analysis was performed as described [29]. Briefly, phosphate-labelled immunoprecipitates were separated on SDS/PAGE. The gel was dried without fixation and exposed to XAR-5 film (Kodak). Protein bands of interest were excised, reswollen and grinded as described [29]. Protein was eluted, precipitated with trichloroacetic acid, washed with 100% ethanol and dried at room temperature. Acid hydrolysis was performed for 1 h at 110 °C in 6M HCl. Samples were subsequently lyophilized and resuspended in two-dimensional running buffer

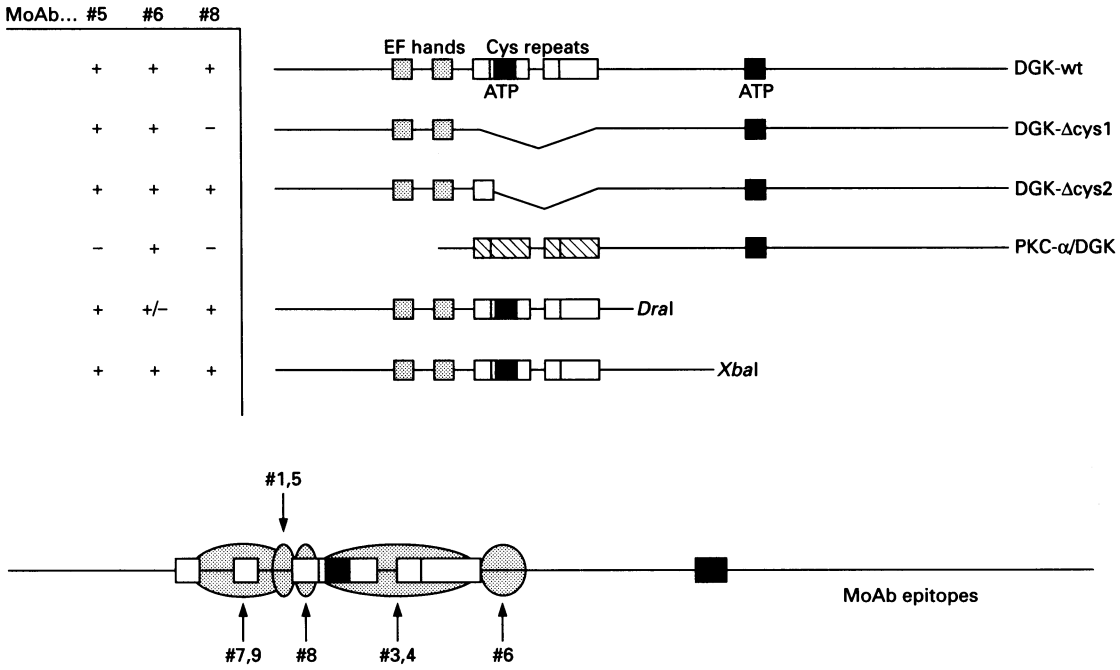

Figure 1 Characterization of MoAbs

A schematic representation of the constructs used to map the epitopes recognized by the MoAbs is given (right half of figure). The corresponding immunoreactivity of three selected MoAbs is depicted in the left part of the figure. The four constructs shown at the top of the figure were transfected in COS-7 cells and total cell lysates were analysed by immunoblotting. DGK-wt, full-length human DG kinase; DGK-Δcys1, deletion spanning amino acid residues 206–319, containing both cysteine repeats; DGK-Δcys2, deletion spanning amino acid residues 219–319, including the major portion of both cysteine repeats; PKC-α/DGK: construct in which the N-terminal portion of DG kinase, upstream from amino acid residue 320, has been exchanged with the N-terminal portion of PKC-α (amino acid residues 1–151). The constructs designated *DraI* and *XbaI* refer to restriction-enzyme-digested expression vectors used for *in vitro* transcription and translation, where the transcription was terminated by cleaving the parent vector in the DG kinase coding sequence at the indicated restriction site. The [^{35}S]methionine-labelled *in vitro*-translated products were analysed by immunoprecipitation with the αDGK MoAbs. The combined results of immunoblotting and immunoprecipitation experiments are summarized in the bottom part of the figure, where the tentative locations of the epitopes recognized by the MoAbs are indicated.

for t.l.c. [29], and labelled phosphoamino acids together with unlabelled markers were electrophoretically separated in two dimensions with a Hunter Thin Layer Electrophoresis system (HTLE 700; CBS Scientific).

Phosphopeptide mapping was performed as described [29,30]. Briefly, phosphorylated DG kinase was immunoprecipitated and separated on SDS/PAGE. Proteins were immunoblotted to Hybond-N nylon membranes (Amersham). Membranes were exposed to XAR-5 film and bands of interest were excised. Trypsin digestions were directly performed on the blotted proteins. Resulting (phospho)peptides were thoroughly eluted and separated on two-dimensional t.l.c., using electrophoresis in the first dimension with a '4.72 buffer' [29], and ascending liquid chromatography in the second dimension, with a buffer containing 1-butyric acid instead of isobutyric acid, as described [29]. T.l.c. plates were air-dried and exposed to XAR-5 film with an intensifying screen.

Other procedures

DG kinase assays were performed as described [14]. Protein was determined with the Bradford dye reagent (Bio-Rad protein assay) using bovine serum albumin as a standard. Immunoblotting was performed with standard techniques [21]. Binding of the specific antibodies was visualized using secondary antibodies conjugated with alkaline phosphatase. All molecular DNA cloning procedures were performed with standard techniques [28] and polymerase chain reactions (PCR) were performed as described [14].

RESULTS

Antibodies against DG kinase: characterization and epitope mapping

A panel of MoAbs was generated to study the regulation and function of the 86 kDa DG kinase in signal transduction. Furthermore, multiple forms of DG kinase have been described [8–14], and these MoAbs could thus be used to discriminate between the different molecular forms of DG kinase. To facilitate the production of MoAbs, *E. coli* fusion proteins of GST with 86 kDa DG kinase were used to generate eight MoAbs against DG kinase (αDGK MoAbs). A tentative antigenic map (Figure 1) was constructed based on immunoreactivity of the individual MoAbs towards wild-type and mutant proteins in immunoblots and by immunoprecipitation of DG kinase polypeptides translated *in vitro* with various truncations at the C-terminus. For further studies we selected three αDGK MoAbs, numbers 5, 6, and 8 (IgG2a, IgG1 and IgG1 isotypes respectively), since these antibodies recognized different epitopes on DG kinase (approximate amino acid positions 198–206, 320–358 and 206–219 respectively), were efficient in immunoprecipitating DG kinase and could also be used for immunoblotting. They were used as a cocktail, which increased the sensitivity of detection. Immunoblots with the αDGK MoAbs showed a single major protein, the 86 kDa DG kinase, in total cell lysates from A431 cells, Jurkat cells, human foreskin fibroblasts and COS-7 cells (results not shown). However, no 86 kDa DG kinase could be detected in HL-60 cells, in agreement with results obtained by others [31].

Phosphorylation of DG kinase transfected in COS-7 cells

The 86 kDA DG kinase has been shown to be a substrate for PKC and PKA *in vitro* [19]. To investigate whether DG kinase can also be phosphorylated by these protein kinases in living cells, we used COS-7 cells transfected with DG kinase cDNA to attain transiently high levels of expression. To simplify detection of phosphorylated DG kinase, we co-transfected the cells with PKC-α, PKC-ϵ or EGF receptor cDNA. The panel of transfectants thus obtained was used to test four different stimuli for induction of phosphorylation of DG kinase: (1) phorbol 12-myristate 13-acetate (PMA), a phorbol ester that directly activates PKC; (2) 3-isobutyl-1-methylxanthine (IBMX) together with forskolin (to block cyclic AMP-phosphodiesterase and activate adenylate cyclase respectively), resulting in increased cyclic AMP levels and thus activating PKA; (3) EGF, which activates the EGF receptor, a tyrosine kinase; and (4) ionomycin, which artificially raises intracellular Ca^{2+} levels, to activate Ca^{2+}/calmodulin kinases. IBMX/forskolin and PMA both induce phosphorylation of DG kinase, whereas Ca^{2+}/ionomycin does not (Figure 2). Furthermore, PMA stimulation of cells co-transfected with cDNAs of DG kinase and PKC-ϵ (lane 4), or EGF stimulation of cells co-transfected with cDNAs of DG kinase and EGF receptor (lane 7), resulted in greatly enhanced levels of DG kinase phosphorylation in comparison with cells transfected with DG kinase cDNA alone (lanes 3 and 6 respectively). In both cases, phosphorylation was completely dose- and stimulus-dependent. In addition, PMA stimulation of cells co-transfected with cDNAs for DG kinase and PKC-α resulted in a similarly enhanced phosphorylation of DG kinase as was observed for co-transfected PKC-ϵ (results not shown).

As an internal control for the specificity of phosphorylation induced by the various stimuli, we also analysed phosphorylation of the endogenous EGF receptor. In agreement with published data, PMA induced phosphorylation of the EGF receptor, while IBMX/forskolin or Ca^{2+}/ionomycin did not [32–34], indicating that, at least for PKA stimulation, phosphorylation of DG kinase occurs in a specific manner. PMA-induced phosphorylation of the EGF receptor was consistently decreased upon co-transfection of DG kinase with PKC-ϵ. Such a decrease also occurred upon co-transfection of PKC-ϵ with other cDNAs (results not shown), and we have no explanation for this phenomenon. As expected, EGF stimulation induced a major autophosphorylation of EGF receptors in all transfectants (compare lanes 1 and 6 in Figure 2). In addition, cells co-transfected with EGF receptor cDNA showed an approx. 2-fold greater autophosphorylation upon EGF stimulation (lanes 8 and 7, Figure 2). It is obvious that EGF receptor overexpression leads to a dramatic increase in phosphorylation of DG kinase, but not to a proportional increase in autophosphorylation of the EGF receptor (compare lanes 6 and 7 in Figure 2). This may be explained as follows: first, only a small fraction of the cells become transfected (1–3%); secondly, cells were co-transfected with a relatively small amount of EGF receptor cDNA (5 μg) as compared with DG kinase cDNA (20 μg), which will lead to a 2–5-fold increase in total EGF receptor protein (endogenous and transfected) as judged by immunoblotting (G. Verheijden, unpublished work); and thirdly, it is very likely that co-transfection of DG kinase and EGF receptor cDNAs leads to overexpression of both proteins in the same cells, resulting in amplification of DG kinase phosphorylation, but not to a proportional amplification of EGF receptor autophosphorylation, for which the whole cell population should be taken into account.

Identification of phosphorylation sites in DG kinase

Phosphoamino acid analysis revealed that EGF stimulation of transfected COS-7 cells resulted in phosphorylation of DG kinase, mainly on tyrosine (Figure 3). Phosphorylation is dependent on the EGF stimulus and on the level of expression of the EGF receptor in these cells. Therefore the activated EGF receptor tyrosine kinase most likely phosphorylates DG kinase directly. Stimulation of cells with IBMX/forskolin on PMA (with or without co-transfected PKC-ϵ) resulted mainly in phosphorylation of DG kinase on serine (Figure 3).

Cleveland peptide mappings [35] of DG kinase, ^{32}P-phosphorylated upon stimulation of cells with IBMX/forskolin or PMA (with or without co-transfected PKC-ϵ), revealed similar

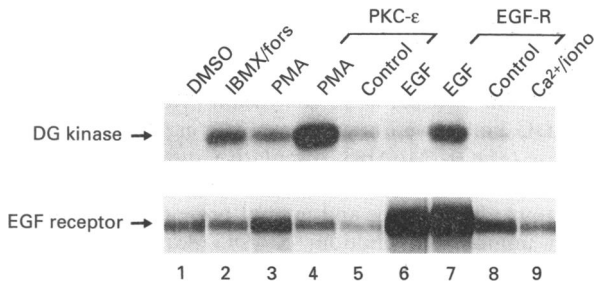

Figure 2 Phosphorylation of DG kinase in response to different activators of protein kinases

COS-7 cells were transfected with the DG kinase expression vector, without or in conjunction with an expression vector for PKC-ϵ cDNA (lanes 4 and 5) or EGF receptor (EGF-R) cDNA (lanes 7 and 8). [^{32}P]P_i-labelled cells were stimulated as indicated. DMSO, (dimethyl sulphoxide) solvent control; IBMX/fors, 0.5 mM IBMX for 10 min followed by 20 μM forskolin for 15 min; PMA, 200 nM PMA for 10 min; EGF, 100 ng/ml EGF for 5 min; Ca^{2+}/iono, 5 μg/ml ionomycin for 5 min in Hepes-buffered saline with EGTA/Ca^{2+}, resulting in approx. 200 μM free Ca^{2+} in the medium. Following stimulation of cells, detergent lysates were prepared and immunoprecipitated sequentially with αDGK MoAbs and anti-(EGF receptor) antibodies, starting with equal amounts of protein. After SDS/PAGE (9% gel), radioactive proteins were visualized by autoradiography (12 h exposure with an intensifying screen). Only regions of interest are shown.

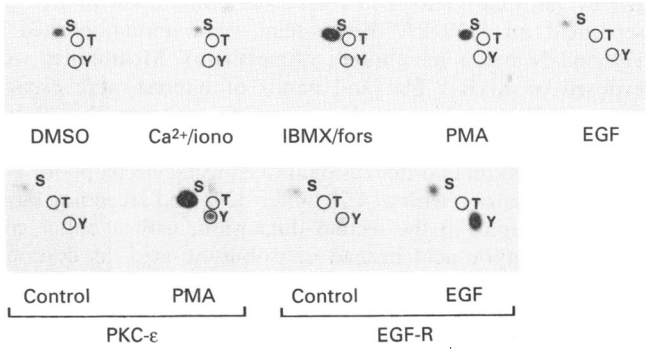

Figure 3 Analysis of phosphoamino acids of DG kinase phosphorylated in response to agonists

Phosphorylation in DG kinase cDNA-transfected COS-7 cells (co-transfected with PKC-ϵ cDNA or EGF receptor (EGF-R) cDNA, as indicated) was induced as described in the legend to Figure 2. ^{32}P-labelled DG kinase was immunoprecipitated and separated on SDS/PAGE, visualized by autoradiography, excised and partially hydrolysed in 6 M HCl as described in the Experimental section. Phosphoamino acids (quantitatively proportional to excised bands) were resolved by two dimensional t.l.c. electrophoresis. Non-radioactive phosphotyrosine, phosphothreonine and phosphoserine markers (indicated with Y, T and S respectively) were visualized by staining with ninhydrin. Autoradiograms were exposed for 10 days with an intensifying screen. DMSO, dimethyl sulphoxide; iono, ionomycin; fors, forskolin.

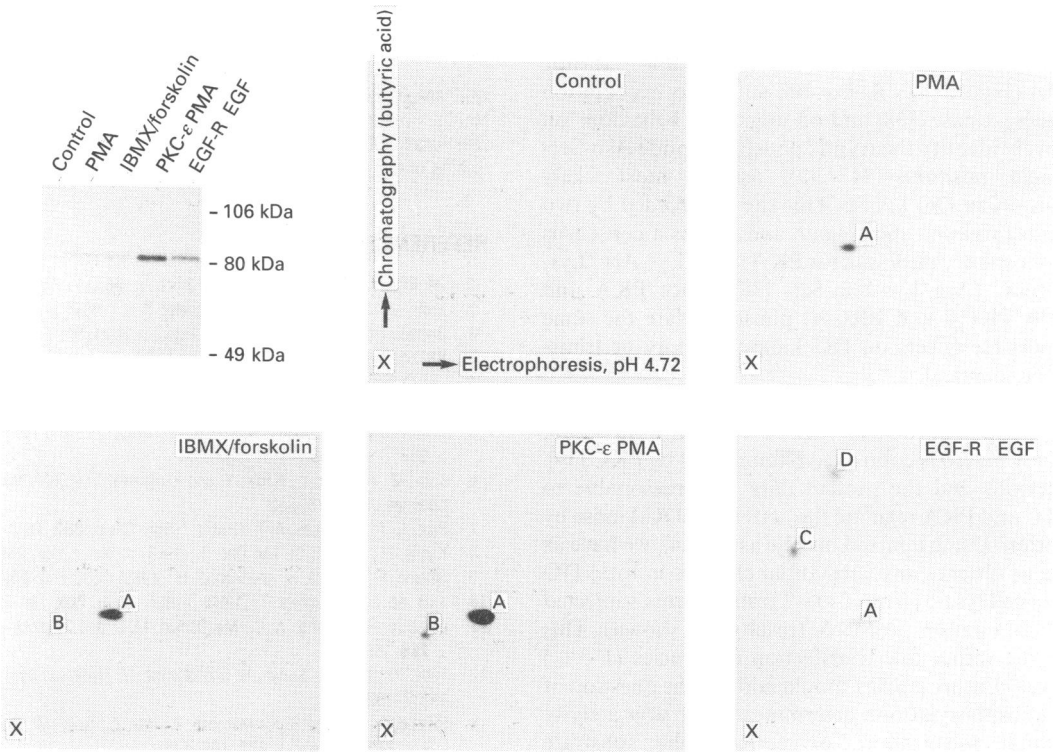

Figure 4 Activation of PKC and PKA results in phosphorylation of the same tryptic peptide in DG kinase

Phosphorylation of DG kinase overexpressed in COS-7 cells [in the absence or presence of co-transfected PKC-ε or EGF receptor (EGF-R) as indicated] was induced by the indicated stimuli, as described in the legend to Figure 2. DG kinase was immunoprecipitated, resolved by SDS/PAGE and transferred to nitrocellulose (top left panel; autoradiogram exposed for 4 h with two intensifying screens). DG kinase was excised and trypsin-treated directly on the nitrocellulose, and soluble peptides were resolved by two-dimensional t.l.c. electrophoresis in the directions indicated. The origin X is in the lower left corner of each panel. Radioactive peptides were visualized by autoradiography (7 days exposure with one intensifying screen). Note that, upon EGF stimulation, two unique ^{32}P-labelled peptides can be detected, denoted C and D.

V8 proteolysis maps (on SDS/PAGE) for both stimuli (results not shown). EGF stimulation, however, resulted in a different proteolysis map (not shown), confirming that in this case the phosphorylation site (on tyrosine) differs from that for the other two stimuli.

To further investigate whether PKA, endogenous PKC and PKC-ε phosphorylate DG kinase on the same or on different serine residues, we performed a complete tryptic digestion of ^{32}P-phosphorylated DG kinase and resolved the resulting phosphopeptides by two-dimensional t.l.c. Figure 4 shows that stimulation with IBMX/forskolin or PMA (with or without co-transfected PKC-ε) yielded the same major phosphopeptide, termed A. To separate phosphopeptide A in the second dimension we used a butyric acid-containing solvent system, since this peptide did not migrate in a 'regular chromatography buffer' [29], indicating the hydrophilic nature of this peptide. To prove that these peptides are identical, we mixed the tryptic phosphopeptides obtained by stimulation with IBMX/forskolin and PMA. This resulted in one major labelled spot (results not shown), demonstrating that, for both stimuli, the resulting phosphopeptides co-migrated and are probably identical. In addition, a minor phosphopeptide, B, is visible in IBMX/forskolin- and PMA (with PKC-ε)-stimulated cells. It is possible that this phosphopeptide B corresponds to the tyrosine-phosphorylated peptide observed in Figure 3 for DG-kinase- and PKC-ε-cotransfected cells stimulated with PMA (although no phosphotyrosine is visible in the IBMX/forskolin sample in Figure 3, it is faintly present on the original autoradiograph). The tryptic phosphopeptide map of DG kinase from EGF-stimulated cells shows phosphopeptides C and D, being distinct from phosphopeptides A and B, as expected. In addition, EGF also induced a small amount of phosphopeptide A.

DISCUSSION

DG kinase plays an important role in signal transduction, since it regulates the levels of the second messengers DG and PA [36]. In establishing this role, however, progress has been rather slow because the enzyme is laborious to purify and is unstable in a purified state. To facilitate biochemical analysis we have cloned a 86 kDa human DG kinase [14] and have now generated a panel of eight MoAbs, the first to be described for DG kinase. The epitopes recognized by these antibodies were mapped and were shown to reside at different positions, predominantly in the N-terminal part of the 86 kDa DG kinase.

We have applied the MoAbs to study phosphorylation of DG kinase as a potential control mechanism for activation of this enzyme in living cells. To facilitate detection and characterization of phosphorylated DG kinase, transfected COS-7 cells were used to express DG kinase to high levels. In these cells DG kinase can be phosphorylated by stimulating different kinases, such as the EGF receptor, PKA and at least two types of PKC, i.e. PKC-α and PKC-ε, but not by stimulation of Ca^{2+}/calmodulin-dependent kinase. It should be noted that DG kinase is the first substrate identified in living cells for PKC-ε, a new member of the PKC family with a restricted substrate preference [24,37]. Our *in vivo* results on PKA- and PKC-mediated DG kinase phosphorylation are in agreement with earlier *in vitro* observations [19]. Phosphorylation of the 86 kDa DG kinase by

PKA and PKC (isotypes α and ϵ) occurs at the same or nearby sites, as concluded from our finding that both kinases phosphorylate serine residues on the same tryptic phosphopeptide (Figure 4). Based on substrate recognition sequences for these kinases [38] and on migration behaviour on t.l.c., we tentatively identify the tryptic phosphopeptide Asn-Ser-Lys (amino acid positions 347–349) as the most likely phosphorylation site in DG kinase. This site is preceded by two basic amino acids (arginine and lysine), and is thus a consensus protein kinase phosphorylation site for PKA (Arg/Lys-Arg/Lys-Xaa-Ser) and PKC (Arg/Lys-Xaa-Ser) [38]. Since PKA and PKC (specifically PKC-ϵ and PKC-α) phosphorylate the same peptide, their possible effects on DG kinase (activity or translocation) must be identical.

We have previously presented experimental evidence suggesting that, in human fibroblasts stimulated with bradykinin, DG kinase activity *in vivo* is controlled (stimulated) by PKC [39]. From these previous and the present data it is reasonable to suggest that PKC and PKA regulate the activity of DG kinase by its phosphorylation. Using a mixed micelle assay [14], we have as yet been unable to observe any substantial changes in total DG kinase activity in cell lysates from COS-7 transfectants subjected to the different stimulation protocols (results not shown). This may be due to the rather low transfection efficiencies (1–3%) routinely observed. Future studies should address the question of whether these phosphorylations determine the *in vivo* activity and/or subcellular localization, or perhaps the substrate preference, of DG kinase.

A novelty that emerges from this study is that DG kinase may be a direct substrate of the EGF receptor *in vivo*. This notion is supported by the findings that (a) stimulation of cells with EGF results in DG kinase phosphorylation on tyrosine residues, and (b) the extent of this tyrosine phosphorylation is dependent on the level of expression of the EGF receptor. Known *in vivo* substrates of the EGF receptor, such as phospholipase-γ and GTPase-activating protein [40–42], interact with the receptor via so-called SH-2 domains. DG kinase would be the first *in vivo* EGF receptor substrate that does not have such a domain, and thus would interact in a different manner. In addition, two phosphatidylinositol (PI) kinase activities, PI 4-kinase and PI4*P* 5-kinase, have recently been described to be associated with the EGF receptor, in a region of the receptor that lacks tyrosine residues [43]. However, the molecular structures of these kinases are still unknown. We have not as yet determined a physiological function for the phosphorylation of DG kinase by the EGF receptor. However, preliminary data indicate that DG kinase and the EGF receptor can form a physical complex (D. Schaap, unpublished work). At present we are studying whether phosphorylation plays a role in the complex formation of DG kinase with the EGF receptor.

In conclusion, DG kinase can be phosphorylated *in vivo* on tyrosine and serine residues by activation of the EGF receptor and by activation of PKA and PKCs respectively. Evidence is increasing that the phosphoinositide-metabolizing enzymes are controlled in a complex manner by different protein kinases operating in distinct signalling routes. One such enzyme, phospholipase C-γ, has previously been shown to be an *in vivo* substrate for the EGF receptor [40,41], as well as for PKC and PKA [44]. Interestingly, phospholipase C-γ is phosphorylated on the same serine residue by both PKC and PKA, analogous to what we describe here for DG kinase. For phospholipase C-γ this serine phosphorylation is suggested to decrease the enzyme activity [44]. The observations described here suggest that DG kinase is regulated by the same kinases. Future studies should address the question of whether these phosphorylations determine the *in vivo* activity, subcellular localization, and/or substrate preference of DG kinase.

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