The biology and biochemistry of diacylglycerol signalling

Meeting on Molecular Advances in Diacylglycerol Signalling

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The meeting on Molecular Advances in DAG Signalling took place at the Instituto Juan March de Estudios e Investigaciones in Madrid, Spain, between 15 and 17 November 2004, and was organized by M. Kazanietz, P. Parker and T. Iglesias.

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Introduction

A few bottles of Rioja and some late-evening dinners in the beautiful city of Madrid provided the setting for a workshop on Molecular Advances in Diacylglycerol Signalling. The participants were greeted by the impressive surroundings of the Juan March Institute with a concurrent exhibition of nineteenth-century French paintings on the human body. A series of excellent talks provided a tantalizing view of the current state of diacylglycerol (DAG) signalling. In this report, I provide an overview of the main concepts that arose at this meeting.

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The meeting began on a sad note, as P. Parker (London, UK) remembered Yasutomi Nishizuka, the father of protein kinase C (PKC), who unexpectedly passed away just a few weeks before the meeting, on 4 November 2004. In the late 1970s, Nishizuka and his colleagues discovered PKC as the first receptor for DAG, an orphan lipid at the time, which heralded an entirely new field of lipid signalling. Parker recalled the vibrant personality of Dr Nishizuka, whose infectious enthusiasm for science and PKC was well known.

Although PKC is arguably the most recognizable target of DAG, this meeting provided a forum to discuss other DAG receptors that have come centre stage, including chimaerins, Ras guanyl-releasing proteins (RasGRPs), Munc13, protein kinase Ds (PKDs) and DAG kinases. Clearly, cellular responses that are attributed to DAG, and phorbol esters that mimic DAG action, can no longer be attributed solely to PKCs, which highlights the complexity we face when ascribing cellular responses to this ubiquitous lipid second messenger.

Diacylglycerol and protein kinase C

In the first session of the meeting, A. Newton (San Diego, CA, USA) introduced the PKC family as the first discovered receptor of DAG. PKCs comprise a family of serine/threonine kinases that are identified by the presence of two copies of the C1 domain, which form the DAG-binding module (Fig 1). PKCs are sub-divided into conventional isozymes (PKC α , β I, β II and γ ; calcium, phospholipid and DAG-activated kinases), novel isozymes (PKC δ , ϵ , η and θ ; calcium-insensitive, phospholipid-dependent and DAG-dependent), and atypical isozymes (PKC ζ and ι/λ ; calcium-insensitive and DAG-insensitive enzymes). Newton presented a model of the life and death of PKC, and how its activation and subsequent degradation is controlled spatially and temporally. Recent advances in imaging technologies have allowed the visualization of PKC activity in living cells using fluorescence-based reporters that reveal the kinetics of PKC activation and inactivation in real time (Violin *et al*, 2003). The relative paucity of knowledge concerning the termination of PKC signalling prompted Newton to investigate the mechanisms of PKC degradation. She showed that a novel E3 ubiquitin ligase named RING finger that interacts with C kinase (RINCK) binds to and ubiquitinates PKC. This results in the accelerated degradation of PKC, such that a catalytically inactive

Fig 1 | Protein families with C1 domains. Diagram of protein families with C1 domains that are known to bind diacylglycerol (DAG). Other domains are also shown, including calcium and phospholipid-binding C2 domains in protein kinase Cs (PKCs) and Munc13, pleckstrin homology (PH) domains in protein kinase D (PKD), EF hands and ankyrin repeats in DAG kinases (DGKs), serine/threonine kinase domains in PKCs and PKDs, Ras guanine nucleotide exchange factor (GEF) domains in RasGRP, Rac GTPase-activating protein (GAP) domains in chimaerins, and Src-homology 2 (SH2) domains in chimaerins.

RINCK mutant stabilizes PKC and promotes an increase in the lifetime of the enzyme. Interestingly, degradation of PKC by this novel E3 ligase is independent of DAG or the phosphorylation state of PKC, which suggests that it is involved in amplitude control and quality control in PKC signalling. Conventional PKCs also require calcium and negatively charged phospholipids for activation, which was discussed by J. Gómez-Fernández (Murcia, Spain). He proposed a model in which the calcium-binding C2 domain of PKC α interacts with phosphatidylserine, which suggests a dual role for calcium in PKC activation.

The functional specificity of PKC signalling is also achieved by appropriate cellular localization, a concept pioneered by D. Mochly-Rosen (Stanford, CA, USA), who presented studies on peptide inhibitors and activators derived from PKC or receptors for activated C kinase (RACKs). As the name implies, RACKs are molecular chaperones that bind to and position newly activated PKCs to discrete cellular locations. Peptides that are based on the RACK–PKC interaction function as PKC isoform-specific translocation activators or inhibitors. This approach was used to probe the role of PKCs in a model of reperfusion injury after myocardial infarction in pigs. Mochly-Rosen showed that administration of a PKC δ inhibitory RACK peptide markedly improved cardiac function subsequent to the infarct, through a mechanism that involves protection of myocardial death by both apoptosis and necrosis. Intriguingly, a similar normalization of cardiac function was also observed using a specific PKC ϵ activator (pseudo- ϵ RACK peptide) before or early on during the ischaemic event. This suggests that the two PKC isoforms have an opposing role and that a balance of PKC δ and PKC ϵ activity

is crucial for recovery from ischaemia and reperfusion damage in an animal model of acute myocardial infarction. The use of such peptides in clinical trials for patients with reperfusion damage after acute infarcts was also discussed.

The issue of spatial localization of PKC was also broached by Parker, who discussed the mechanisms by which PKCs control the hepatocyte growth factor (HGF)/scatter factor receptor c-Met. Signalling through c-Met promotes cell motility. Parker showed that internalization of activated c-Met and subsequent movement through the endosomal pathway is controlled by PKC α , such that in PKC α -deficient cells, the receptor is retained in an endosomal rather than a perinuclear compartment. In turn, PKC ϵ controls signal relay from internalized c-Met to the extracellular-signal-regulated kinase (ERK) pathway in the endosomal compartment, which leads to appropriate ERK signalling. An additional level of spatial control is exerted by PKC ϵ , which controls the processing of integrin- β 1 compartments. Thus, PKC α and PKC ϵ control both receptor and integrin traffic and signal relay to ERK to control c-Met signalling responses such as migration (Kermorgant *et al*, 2004; Fig 2).

PKC knockouts were also discussed by M. Leitges (Hannover, Germany), who revealed that PKC δ /PKC ϵ double-knockout mice are embryonic lethal with several developmental vascular abnormalities. This is due to impaired endothelial cell function and smooth-muscle cell differentiation. Exploration of the molecular mechanisms of this vascular phenotype revealed attenuated cadherin levels, particularly VE- and N-cadherins, which probably accounts for the observed endothelial cell dysfunction and the inability to differentiate vascular smooth muscle cells. This is the first demonstration of a role for PKC δ and PKC ϵ in developmental vasculogenesis, and reaffirms the idea that related PKCs probably have overlapping functions.

PKCs, PKDs and chimaerins in cancer

It is not surprising that DAG activation of PKC has an important role in cancer as PKCs are targets of phorbol esters, which are potent tumour-promoting compounds. However, deciphering the mechanisms by which PKCs control the numerous phenotypes that are displayed by cancer cells remains a challenge. A variety of approaches is being taken. A. Fields (Jacksonville, FL, USA) presented a model in which both conventional PKC β II (DAG-responsive) and atypical PKC ι / λ (DAG-unresponsive) are critical for cancer-cell transformation and invasion (Murray *et al*, 2004). In particular, a remarkable upregulation of PKC ι / λ protein levels was detected in many human colon and lung tumours; in an *in vivo* model of chemical-induced colon carcinogenesis, expression of activated PKC ι / λ promoted hypersensitivity of mice to carcinogen, whereas an inactive allele blocked tumour formation. *In vitro*, PKC ι / λ also promoted growth in soft agar and invasion of colon cancer cells in a manner that was dependent on the small GTPase Rac. Fields therefore proposed that a Ras–PKC ι / λ –Rac pathway is required for oncogene- and carcinogen-induced tumour progression, particularly in colon and non-small-cell lung carcinomas, and that induction of this pathway correlates with poor patient survival. The potential for using PKC ι / λ expression levels as a prognostic for these aggressive carcinomas was also discussed.

The novel PKC δ isotype is unusual in that in addition to DAG, it is regulated by extensive tyrosine phosphorylation and by caspases, both of which are mechanisms not typically observed with other PKCs. The role of PKC δ in modulating cancer-cell apoptosis was discussed by C. Brodie (Ramat-Gan, Israel) and M. Reyland (Denver,

Fig 2 | Several mechanisms for transducing the DAG signal. A cartoon depicting the known effectors of DAG that contain C1 domains and the various cellular responses attributed to each protein family.

CO, USA). PKC δ is expressed at low levels in gliomas, which are also characterized by deregulated apoptosis and resistance to chemotherapeutic agents such as etoposide. Curiously, in these cells, PKC δ can have both pro- and anti-apoptotic effects, such that in etoposide-treated cells, PKC δ is pro-apoptotic. Conversely, on exposure to tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), PKC δ is protective. Brodie showed that the mechanisms that can account for these differences are differential tyrosine phosphorylation of specific residues of PKC δ and intracellular translocation. For example, TRAIL promotes translocation of PKC δ to the endoplasmic reticulum, whereas PKC δ is nuclear in etoposide-treated cells. Both ligands also stimulated the caspase-dependent cleavage of PKC δ , which releases a catalytically active fragment. Reyland also addressed the function of PKC δ in apoptosis, and showed that etoposide-induced nuclear translocation of PKC δ is required for apoptosis. Induction and phosphorylation of signal transducer and activator of transcription 1 (STAT1) by PKC δ was shown to occur in etoposide-treated cells, which indicates that the PKC δ -STAT1 signalling axis is required for apoptosis *in vitro*. The contribution of the PKC δ -STAT axis to apoptosis was also analysed *in vivo* using a branching morphogenesis model, in which apoptosis controls mammary gland development and involution. In PKC δ -deficient mice, increased alveolar development during pregnancy was accompanied by subsequent delayed involution, which correlates with the resistance of primary mammary epithelial cells to apoptosis. Thus, PKC δ is

clearly a critical molecular component of the apoptotic machinery in both development and cancer, and the mechanisms through which it regulates this phenotype are being elucidated.

So, are we to believe that PKCs are the only molecular targets of DAG in cancer? Certainly not. M. Kazanietz (Philadelphia, PA, USA) introduced the chimaerins, so-called because of their two-domain structure, which includes a high-affinity DAG-binding C1 domain and a GTPase-activating protein (GAP) domain that targets Rho family GTPases (Fig 1). The $\beta 2$ chimaerin isoform acts as a GAP for Rac, and therefore inactivates Rac signalling by promoting the GDP-bound form. The structural basis for this mechanism was recently solved (Canagarajah *et al*, 2004). Expression of $\beta 2$ chimaerin blocks Rac signalling, cell proliferation and cytoskeletal reorganization in a manner dependent on a functional GAP domain. Interestingly, reduced levels of $\beta 2$ chimaerin were found in breast cancer cell lines and in duodenal carcinomas, which prompted Kazanietz to speculate that $\beta 2$ chimaerin might function as a tumour suppressor (Fig 2). He also showed that DAG binding to $\beta 2$ chimaerin promotes its association with GTP-Rac at the membrane and has constructed hyperactive chimaerins with an exposed C1 domain and enhanced sensitivity to DAG and phorbol esters.

Another target of DAG is PKD, a serine/threonine kinase that was formerly named PKC μ , but that actually has more homology to calcium/calmodulin-dependent kinases than to PKC. Although PKD has two copies of the C1 domain that bind to DAG (Fig 1), this serves

primarily to position the enzyme at appropriate locations in cells, rather than to function as an allosteric activator. An additional level of PKD regulation is phosphorylation, which activates the kinase. Our lab (A. Toker, Boston, MA, USA) found that PKD is phosphorylated by PKC δ in cancer cells that have been exposed to oxidative stress (Storz *et al*, 2004). Cancer cells show elevated levels of intracellular reactive oxygen species (ROS) and are also exposed to exogenous ROS as the host mounts an immune response to the tumour. Paradoxically, these same cells are protected from the death-inducing effects of elevated ROS, which suggests the induction of survival pathways. We found that activation of the PKC δ /PKD signalling pathway protects cancer cells from death. Exposure of cells to oxidative stress, or the release of ROS from the mitochondria, activates PKD. This results in the activation of two key transcription factors—nuclear factor (NF)- κ B and forkhead box O3a (FOXO3a). These factors in turn induce pro-survival genes such as manganese superoxide dismutase (MnSOD) that then detoxify ROS and promote survival (Fig 2).

DAG signalling in the nervous system

Chimaerins also made an appearance in discussions that related to DAG signalling in neuronal development. The α 2 chimaerin isoform cooperates with Rac and Cdc42 to induce lamellipodia and filopodial extensions in neuroblastoma cells. C. Hall (London, UK) showed that expression of α 2 chimaerin in neurons stimulates neurite extension. However, in response to phorbol ester, activation of the GAP activity of α 2 chimaerin results in neurite retraction, which is independent of PKC activity. Two proteins that interact with phorbol-ester-activated α 2 chimaerin—Cdk5 and collapsin response mediator protein 2 (CRMP2)—promoted neurite guidance in response to semaphorin 3A. This pathway also involves α 2 chimaerin GAP activity. N. Brose (Göttingen, Germany) introduced the Munc13 family of proteins, which are essential for synaptic plasticity. They function as synaptic vesicle priming factors in a manner that is thought to involve the regulation of the target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) syntaxin. Mice that lack Munc13 show a complete lack of fusion-competent synaptic vesicles and subsequent transmitter release, and are embryonic lethal. Because Munc13s have C1 domains that bind DAG (Fig 1), Brose proposed that not all of the known effects of phorbol esters on vesicle fusion and transmitter release can be attributed to PKC activity. Indeed, Munc13 binds to DAG, and C1 domain knock-in mutations that impair this binding reveal a synaptic depression phenotype, indicating regulation of short-term synaptic plasticity by the DAG–Munc13 interaction (Fig 2).

In the PKC field, C. Larsson (Malmö, Sweden) reported further on his recently published findings on the role of the C1 domains of PKCs in controlling Golgi localization and how this relates to neurite outgrowth in neuroblastoma cells (Schultz *et al*, 2004). R. Messing (Emeryville, CA, USA) summarized his recent findings on the behavioural response to alcohol in PKC δ knockout mice. The observation that these mice display reduced ethanol-induced ataxia and anxiety-like behaviour led to an analysis of γ -aminobutyric acid A (GABA-A) receptors, which showed that PKC δ -knockout mice show attenuated responses to drugs that act at extrasynaptic GABA-A receptors. These mice also displayed reduced infarct size after focal cerebral ischaemia and reperfusion. This phenotype was associated with deficits in neutrophil function and could be transferred to wild-type

mice through transplantation with bone marrow from knockout donors. The results led Messing to propose that PKC δ could be a target for treatment of stroke reperfusion injury.

T. Iglesias (Madrid, Spain) discussed Kidins220, a neuronal PKD substrate protein. Studies have shown that PKD controls Golgi organization and function, particularly the transport of cargo from the trans-Golgi network to the plasma membrane (Yeaman *et al*, 2004). Indeed, Kidins220 transport to the membrane is blocked in cells that express kinase-inactive PKD. It had been assumed that the effect of this mutant PKD is the impaired phosphorylation of substrates that may control the transport machinery, but Iglesias proposed a different model. Autophosphorylation of a carboxy-terminal residue in PKD is required for Kidins220 transport, and interestingly, both PKD and Kidins220 have a PDZ-domain-binding motif at the carboxyl terminus. When mutated, this blocks transport, raising the possibility that a PDZ-domain-containing protein could control PKD-dependent Golgi-to-membrane transport. Although mammalian PKD knockouts have not yet been developed, C. Rubin (New York, NY, USA) presented the *Caenorhabditis elegans* phenotypes of DKF-1 and DKF-2, which are worm orthologues of PKDs that contain functional C1 domains that bind DAG and phorbol ester. Deletion of DKF-1 caused a marked loss of neuromuscular control of lower body movement and severe muscular immobility in the tail region, forcing animals into a non-productive circular movement. Conversely, overexpression of wild-type DKF-1 resulted in a smaller body size. Disruption of the DKF-2 gene elicited a 35% increase in *C. elegans* lifespan. The field anxiously awaits the development of mouse PKD knockouts to see if similar phenotypes are recapitulated in mammals.

DAG kinases and receptors in the immune system

There was much to discuss on DAG signalling in immune cells. Carrying on with the PKD theme, S. Matthews from the Cantrell laboratory (Dundee, UK) presented recently published studies in which expression of a membrane-anchored PKD in PKD-deficient T cells resulted in the partial rescue of lymphocyte development (Marklund *et al*, 2003). This model system was used in an array screen for targets of PKD, which may give clues to the molecular basis for PKD signalling in lymphocytes. Genes that were induced by both cytosolic and membrane PKD were found, but there was also a large number of genes that were specific to each PKD allele. Of particular relevance to lymphocyte development, cyclins and CDK inhibitors were induced by membrane PKD, but repressed by cytosolic PKD. Identification of substrates of PKD is also a pressing question in the field, and Matthews discussed a phospho-proteomic approach to identify such substrates, revealing that several proteins that control the actin cytoskeleton in lymphocytes are phosphorylated by PKD.

Engagement of the T-cell receptor (TCR) also activates RasGRP1, a guanine nucleotide exchange factor for Ras that contains a C1 domain and binds to DAG (Fig 1). J. Stone (Alberta, Canada) introduced the RasGRP family. The importance of RasGRP1 in lymphocyte signalling is highlighted by the finding that knockout mice show defects in Ras signalling and thymocyte differentiation. Older mice go on to develop autoimmunity and lymphoproliferative disorders. Stone showed that RasGRP3, which is expressed in B cells, also appears to function in a similar fashion downstream of the B-cell receptor. However, RasGRP3 is also a substrate of PKC and requires phosphorylation for full activity. This

suggests two DAG inputs to RasGRP3, one from DAG binding directly to the C1 domain, and the other from DAG activation of PKC, which phosphorylates RasGRP3.

From the PKC world, L. Bosca (Madrid, Spain) discussed the use of PKC ϵ -knockout mice to evaluate its role in the inflammatory response. These mice are much more sensitive to infection and reveal impaired macrophage activation with lowered TNF- α , prostaglandin and IL-1 β production. The underlying mechanisms for these phenotypes were shown to be attenuated NF- κ B, STAT1 and ERK activation. Therefore, PKC ϵ is an important DAG effector in the inflammatory response, and specific inhibitors of this isotype may prove useful in pathologies in which chronic inflammation is observed, such as septic shock.

Phosphatidic acid (PA), an important lipid second messenger, is produced by the phosphorylation of DAG in a reaction catalysed by DAG kinases (DGKs). Considerable effort has gone into understanding the role of DGKs in immune signalling, and was stimulated by observations that DGK ζ inhibits signalling by the TCR. G. Koretzky (Philadelphia, PA, USA) provided an update on recently published data showing that DGK ζ negatively regulates T-cell and mast-cell function (Zhong *et al*, 2003). Overexpression of DGK ζ blocks T-cell responses, such as NF- κ B induction and IL-2 production, resulting in the decreased appearance of peripheral T cells. However, the DGK ζ -knockout reveals, as expected, decreased PA production, enhanced IL-2 production and hyperproliferation. Why might this be relevant? Because a productive immune response is of obvious importance during infection, and indeed DGK ζ -deficient mice respond to viral and tumour challenge with increased IL-2 production and enhanced T-cell responses. Therefore, DGK ζ is a negative regulator of T-cell responses, which has clear implications for immunotherapy in humans (Fig 2). This concept was reinforced by I. Merida (Madrid, Spain), who showed that the non-canonical C1 domains of both DGK ζ and DGK α are required for membrane localization, such that receptor-derived signals modulate distinct regulatory domains to allow proper localization of DGK isoforms. Merida has also developed fluorescence-based reporters based on C1 domains that have revealed DAG production at the T-cell synapse. Because DGKs attenuate DAG levels, it would be expected that signalling enzymes such as RasGRP and PKCs would be impaired in T cells in which DAG kinases are activated, and this is indeed the case. In addition, M. Izquierdo (Madrid, Spain) showed that inhibition of DGK α promotes activation-induced cell death in T lymphocytes by a mechanism that seems to involve the secretion of Fas ligand by specialized organelles called lethal exosomes, which implicates this DGK in exocytosis. Finally, M. Topham (Salt Lake City, UT, USA) presented an interesting model for DGK ζ signalling based on the finding that DGK ζ binds directly to and inhibits RasGRP. This blocks Ras activation through the TCR, which explains why T cells derived from DGK ζ -deficient mice show enhanced ERK activation. Again, this confirms the idea that DGK ζ is a negative regulator of T-cell function. Interestingly, the various DGKs seem to have non-redundant functions; Topham presented the DGK ι knockout, which revealed increased levels of active Rap1, suggesting that DGK ι might affect signalling to Ras/ERK via this GTPase.

Concluding remarks

The pace of diacylglycerol research is accelerating. Given the expansion of families of proteins that contain classical and atypical C1 domains that are competent in binding DAG and phorbol esters, there is reason to be excited about future progress in this field. An open forum at the end of the meeting summarized the emerging concepts in DAG signalling, and also stimulated discussions on the tasks ahead. One important issue that was raised is the realization that PKCs are not unique in promoting DAG signal relay, and that more molecular targets of this lipid second messenger await discovery. Second, the identification and visualization of intracellular pools of DAG, and not just at the plasma membrane, will probably provide an important step forwards in our understanding of how DAG can exert such diverse biological functions. This meeting certainly provided a stimulating and interactive environment to discuss these developments.

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