Intracellular targeting of protein kinase C isoenzymes: functional implications
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Introduction
Protein kinase C (PKC) is a family of phospholipid-dependent serine-threonine kinases that regulate cell growth and differentiation (reviewed in [1,2]). PKC is also the major intracellular receptor for tumour-promoting phorbol esters [1-3]. Molecular cloning and biochemical separation of the individual gene products has led to a classification system for the PKC isoenzymes based on domain homology and biochemical properties. Conventional PKCs (α, βI, βII, γ) are Ca2+-dependent phorbol ester receptor/kinases, whereas the novel PKCs (δ, ε, η, θ) are Ca2+-independent phorbol ester receptor/kinases [1-3]. The atypical PKCs (ζ and λ) are neither Ca2+-dependent nor phorbol ester receptors [1-3]. Most cells and tissues express several PKCs, suggesting that the isoenzymes do not have overlapping functions. The activation of PKC η by cholesterol sulphate and PKC ζ by phospha tidylinositol trisphosphate in vitro suggests the possibility that activation of individual PKCs by different second messenger pathways in vitro may result in isoenzyme-specific responses [4,5]. The fact that several agonists including platelet-derived growth factor [6,7], bombesin [7], endothelin-1 [8], phenylephrine [8] and oleic acid [9] can selectively activate novel PKCs δ and/or ε in vitro supports the hypothesis that isoenzyme-specific responses may be mediated in part by different signal transduction pathways.

In principle, the phosphorylation of different substrate proteins by individual isoenzymes should lead to distinct cellular responses. At present, little is known about isoenzyme substrate specificity in vivo. In vitro, phosphorylation of 'real' physiological substrates by different PKCs is similar [10]. The fact that isoenzyme-specific substrate interactions are difficult to demonstrate in assays in vitro suggests that isoenzyme specificity may be dictated by interactions with other proteins and/or other factors. The purpose of this article is to focus on work that suggests that targeting PKC to different locations within the cell may be a determinant of isoenzyme-specific function in vivo.

Differential localization
Targeting PKCs to discrete subcellular compartments would restrict access to potential substrates. In this way, protein targeting coupled with isoenzyme-selective activation signals could determine isoenzyme-specific phosphorylations and consequently isoenzyme-specific responses. Differential localization of endogenous PKC isoenzymes has been documented for several cell lines [11-13]. In the U937 promonocytic leukaemia cell model, phorbol esters induce non-adherent promonocytes to differentiate to adherent macrophage-like cells [14]. Because differentiation is a multi-step process, individual PKC isoenzymes may have different roles in regulating this response: U937 cells express PKCs βI(II), βII(II), ε and ζ [15] (PKCβ isoenzymes are designated according to the human cDNA nomenclature used in the work cited; the appropriate Roman numeral designation for rat cDNA is included in parentheses next to the arabic numeral designation for the human cDNA). Indirect immunofluorescence with affinity-purified PKC-specific antibodies indicates unique subcellular localization and translocation patterns for all four isoenzymes [13]. The distribution of PKCs in resting U937 cells is shown schematically in Figure 1. PKC βI(II) is associated with intracellular vesicles containing β2-integrin adhesion molecules and is also in the cytoplasm. Acute treatment with phorbol 12-myristate 13-acetate (PMA) causes redistribution of cytoplasmic and vesicle-bound PKC βI(II) to the plasma membrane [13]. PKC βII(II) is associated with the microtubule cytoskeleton (CSK) in resting cells. Additional studies suggest that PKC βII(II) binds to microtubule-associated proteins (MAPs) and not directly to tubulin [13]. PKCs βI(II) and βII(II) are splice variants and differ by only 50 amino acids at the C-terminus [16]. The distinct localization patterns displayed by these isoenzymes suggest that unique C-terminal sequences may target the enzymes to different subcellular locations [13]. PKC ε co-localizes with a novel kinesin light-chain

Abbreviations used: CSK, cytoskeleton; MAP, microtubule-associated protein; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylinerine.

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Figure 1
Subcellular distribution of PKC isoenzymes in ICRF U937 cells

In resting cells, PKC \( \beta_{11} \) is associated with intracellular vesicles containing \( \beta_1 \)-integrins, the cytochrome \( b_{558} \) component of NADPH oxidase and several proteases (see inset). PKC \( \beta_{22} \) is associated with MAPs and PKC \( \epsilon \) co-localizes with a kinesin light chain (KLC) variant, 35F, along microtubules. PKCs \( \beta_{11}, \epsilon \), and \( \zeta \) also reside in the cytoplasm. MTOC, microtubule organizing centre

Differential localization of individual PKCs suggests that PKCs are targeted to specific subcellular compartments by binding to cellular proteins. Furthermore, because MAPs and the kinesin light-chain variant 35F are also PKC substrates [17,19], targeting PKCs to specific locations may direct isoenzyme-selective responses.

PKC targeting proteins
Targeting PKC to specific CSK structures such as microtubules [13], focal contacts [20] and cell-cell junctions [21] suggests the existence of anchoring proteins for PKC that mediate its subcellular location. An overlay assay was developed to detect proteins that interact with PKC [22]. In this assay, cellular proteins are separated on denaturing gels and blotted to nitrocellulose. The blocked nitrocellulose membrane is incubated with purified PKC in the presence or absence of activating cofactors [18,22]. Bound PKC, as well as endogenous PKC in the sample, is detected with PKC isoenzyme-specific antibodies [18,22]. PKC interactions with binding proteins depend on the presence of phosphatidylserine (PS) and phorbol ester or \( \text{Ca}^{2+} \); i.e. \( \text{Ca}^{2+} \) can substitute for phorbol ester for the conventional \( \text{Ca}^{2+} \)-dependent PKCs, but not for the novel \( \text{Ca}^{2+} \)-independent PKCs [18,22]. Overlay assays with \( ^{14}\text{C}] \text{PS} \) indicated that all of the PKC-\( \alpha \)-binding proteins are also PS-binding proteins, but not all PS-binding proteins bind PKC [18]. These studies suggest that PS provides a bridge between the proteins, but additional protein–protein interactions confer stability on the ternary complex.

Two lines of evidence suggest that the proteins detected with the overlay assay are of biological significance: first, several of the binding proteins in REF52 cells appear to be transformation sensitive [22–24], and secondly, several of the PKC-binding proteins are also substrates [24]. In fact, the major PKC substrate, myristoylated alanine-rich C-kinase substrate (MARCKS), is also a PKC-binding protein [24]. PKC binding to MARCKS is modulated by the phosphorylation state of MARCKS [24]. The fact that the PKC-binding proteins detected with the overlay assay are also substrates underscores the utility of this approach for identifying downstream targets of PKC action. Additional PKC binding protein/substrates were identified by adapting the overlay assay for screening \( \lambda \text{gt}11 \) expression libraries [17]. Interaction cloning yielded 11 clones: 5 known sequences and 6 unique clones. The best characterized of these clones are: 35A, a rat homologue of MARCKS-related protein; 35H, a novel member of the adducin family; and 35F, a novel splice-variant of kinesin light chain [17,18]. All of the binding proteins identified by interaction cloning are CSK-associated, PS-binding proteins that are also PKC substrates ([17,18] and S. Jaken, unpublished work). Proteins with these characteristics are ideally suited to targeting PKC isoenzymes to discrete intracellular sites. Work is in progress to determine whether the PKC-binding sequences within these proteins can, in fact, target PKCs to different subcellular locations.

Differential localization studies in U937 cells
indicated that two of the four endogenous PKCs co-localize with cytoskeletal structures. PKC $\beta_{20}$ co-localized with microtubules. To determine whether the interaction was mediated directly through binding to tubulin, or indirectly through binding to MAPs, the PKC overlay assay was used to determine whether recombinant PKC $\beta_{20}$ would preferentially bind purified tubulin or a semi-purified preparation of heat-soluble MAPs. PKC $\beta_{20}$ bound to MAPs in a cofactor-dependent manner, but did not bind to tubulin [13]. Additional studies with MAPs prepared from resting and from PMA-treated U937 cells suggest that the phosphorylation state of the binding proteins may regulate PKC binding to that protein; i.e. PKC binds to dephosphorylated protein substrates with greater affinity than to the phosphorylated form of the same protein [13]. This has already been demonstrated for several established PKC-binding protein/substrates [17,24]. Thus MAPs isolated from U937 cells appear to manifest all of the properties attributed to PKC downstream targets: PS binding, CSK localization and phosphorylation by PKC [13,19]. It is possible that MAPs anchor PKC $\beta_{20}$ to microtubules and also function as downstream targets during cellular responses, because changes in the phosphorylation state of MAPs are known to influence the stability (crosslinking) of the microtubule network [25].

**Altered localization: altered phenotype**

Changes in the expression of downstream targets could have profound effects on PKC signalling and localization; i.e. a loss of downstream targets could effectively abrogate a PKC-mediated pathway and result in an altered phenotype. In normal REF52 fibroblasts, PKC $\alpha$ is concentrated in the CSK at sites of cell-substratum adhesion, known as focal contacts [20]. SV40-transformation causes a change in PKC $\alpha$ localization that correlates with the loss of two major binding proteins [23]. In other studies with temperature-sensitive SV40-transformed REF52 fibroblasts, PKC $\alpha$ is not found in focal adhesions at the permissive temperature, but accumulates in the focal contacts 4–5 h after the switch to the non-permissive temperature for functional T-antigen expression [26]. 35H, a novel form of adducin identified by interaction cloning [17], is localized in the cell borders of normal rat proximal tubule epithelial (RPTE) cells and adenovirus E1A-immortalized RPTE ([21] and S. Jaken, unpublished work). PKC $\alpha$ co-localizes with 35H in both normal and immortalized RPTE cells [21]. However, when cells are transformed with SV40, both proteins are coordinately lost from cell borders (S. Jaken, unpublished work). Similarly, the loss of PKC $\beta_{20}$ staining along microtubules in differentiated U937 cells correlates with the loss of MAP-binding proteins in the PKC overlay assay [13]. Taken together these results suggest a strong correlation between changes in both PKC and PKC-binding protein/substrate localization and alterations in phenotype.

Cellular differentiation is a complex process involving the co-ordinate up-regulation of some genes and the down-modulation of others. Phorbol ester-resistant HL60 cell variants, deficient in PKC $\beta$, have been used to demonstrate that this isoenzyme is required for macrophage differentiation [27–29]. The fact that these cells were not growth inhibited and did not express $\beta_2$-integrin adhesion molecules on the cell surface when treated with phorbol ester suggests that PKC $\beta$ mediates an early event in the differentiation process [27–29]. PMA-resistant U937 cells (cloned by S.K.) differ from the HL60 variants in several respects. First, all the PKC isoenzymes expressed in wild-type U937$s$ are also expressed at comparable levels in the resistant cells (S. C. Kiley, R. Whelan, P. Adams and P. J. Parker, unpublished work). Secondly, the isoenzymes are activated and down-modulated by PMA to the same extent as in wild-type cells (S. C. Kiley, R. Whelan, P. Adams and P. J. Parker, unpublished work). Thirdly, despite the fact that PMA does not induce surface expression of $\beta_2$-integrins or superoxide production in these cells, expression of p47$^{phox}$ (one of the cytoplasmic components of the phagocyte NADPH oxidase) is induced (S. C. Kiley, R. Whelan, P. Adams and P. J. Parker, unpublished work). Thus PMA can stimulate some elements of the macrophage differentiation programme in the resistant cell lines. Subcellular localization studies indicated that only the microtubule-associated pattern of PKC $\beta_{20}$ in wild-type U937$s$ changed in the resistant cells [13] (S. C. Kiley, R. Whelan, P. Adams and P. J. Parker, unpublished work). The change in PKC $\beta_{20}$ localization correlates with a loss of heat-soluble MAP/PKC binding proteins in the resistant cell lines (S. C. Kiley, R. Whelan, P. Adams and P. J. Parker, unpublished work). Therefore down-modulation of PKC substrates could be the cause of PMA resistance in the U937 cell lines.

The intracellular storage vesicles depicted in Figure 1 contain several proteins that mediate differentiated functions in macrophages. $\beta_1$-Integrins, cd11b and cd11c, mediate substratum adhesion,
motility and phagocytosis [30], whereas cytochrome b_{558} is the membrane/CSK anchor with which cytoplasmic components p47, p67 and rac associate to form the NADPH oxidase complex [31–33]. These vesicles normally translocate to the cell surface within 3 min of PMA treatment, as shown in Figure 2 [30]. Immunocytotofluorescence indicates that this vesicle translocation does not occur in PMA-treated resistant U937 cells, despite the fact that the integrins are expressed intracellularly (S. C. Kiley, S. Jaken P. J. Parker, unpublished work). The integrin-containing vesicles are larger than secretory granules and cannot reach the plasma membrane without some degree of cytoskeletal reorganization. CSK-associated proteins such as MAPs participate in the reorganization of the microtubule CSK [25]. If MAPs are substrates for PKC \( \beta_{210} \), the loss of MAP expression may effectively abrogate a PKC-mediated pathway critical for subsequent steps in the differentiation programme. If this hypothesis is correct, it should be possible to reconstitute the PMA-sensitive phenotype with microtubule-disrupting drugs. In fact, this turns out to be true. Both colchicine and nocodazole pretreatments followed by 72 h treatment with PMA restore integrin cd11b/c expression (adherence) and superoxide production (functional NADPH oxidase activity) for two of the PMA-resistant cell lines (Figure 2) (S. C. Kiley, S. Jaken and P. J. Parker, unpublished work). Cytochalasin D pretreatment was ineffective in reconstituting the normal phenotype, underscoring the importance of microtubule reorganization in this step of the differentiation process (S. C. Kiley, S. Jaken and P. J. Parker, unpublished work). These studies emphasize that PKC co-localization with CSK proteins may be indicative of a functional association between them, and that coordinate changes in PKC localization and binding protein/substrate expression may result in an altered phenotype.

**Conclusion**

Although much of the work reviewed here is correlative in nature, all of these studies point to the same conclusion; i.e. PKCs do not float freely in the cytoplasm or inner leaflet of the plasma membrane, but are targeted to different subcellular locations via interactions with other proteins. In situations where the targeting protein is also a PKC substrate, coordinate changes in the expression and localization of PKC and its substrate have functional consequences that may be manifested in an altered phenotype. As targeting proteins are identified by interaction cloning [17] or by yeast two-hybrid.
approaches [34], it will be possible to evaluate these protein–protein interactions critically by mutating essential sequences in the binding domains of the respective proteins.

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14-3-3 proteins: biological function and domain structure
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Introduction
The name ‘14-3-3’ was given to a protein family because of their particular migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis [1]. There are seven major mammalian brain forms of 14-3-3, named α–η after their respective elution positions on HPLC [2,3]. Sequences from a number of species have been determined [4] and we recently showed that α and δ isoforms are phosphorylated forms of β and ς isoforms respectively [5]. The 14-3-3