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PROTEIN KINASE C-MEDIATED REGULATION OF THE CELL CYCLE

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1. ABSTRACT

Members of the protein kinase C (PKC) family of signal transduction molecules have been widely implicated in regulation of cell growth/cell cycle progression and differentiation. Increasing evidence from studies using *in vitro* and *in vivo* systems points to PKC as a key regulator of critical cell cycle transitions, including cell cycle entry and exit and the G_1 and G_2 checkpoints. PKC-mediated control of these transitions can be negative or positive, depending on the timing of PKC activation during the cell cycle and on the specific PKC isozymes involved. Most of the mechanistic information available relates to the involvement of this enzyme family in negative regulation of these transitions. Accumulating data indicate that a major target for PKC-mediated inhibition of cell cycle progression is the Cip/Kip cyclin-dependent kinase (cdk) inhibitor $p21^{waf1/cip1}$. Increased expression of $p21^{waf1/cip1}$ blocks cdk2 activity in G_1 phase, leading to hypophosphorylation of the retinoblastoma protein and inhibition of cell cycle progression into S phase. In G_2, $p21^{waf1/cip1}$ expression blocks cdc2/cyclin B activity, likely through an indirect mechanism involving inhibition of the cdk2/cyclin A complex, and prevents progression into M phase. PKC signaling can also activate a coordinated program of pocket protein regulation leading to cell cycle withdrawal into G_0. The molecular events underlying positive regulation of cell cycle progression by PKC signaling remain poorly understood, although there is evidence for a role of the enzyme in promoting $G_2 \rightarrow M$ progression by phosphorylating lamin B at sites involved in nuclear lamina disassembly. Understanding of the mechanisms underlying PKC-mediated control of the cell cycle is beginning to provide important insight into its role in uncontrolled cell growth and transformation.

2. INTRODUCTION

2.1. The Protein Kinase C Family: Role in Cell Growth Control

Protein kinase C (PKC) is a family of serine/threonine kinases which play central regulatory roles in a multitude of cellular processes, including proliferation and cell cycle progression, differentiation, apoptosis, cytoskeletal remodeling, modulation of ion channels, and secretion (1-4). The enzyme was discovered more than 20 years ago by Nishizuka and co-workers as a histone protein kinase in rat brain tissues that could be activated by limited proteolysis (5). Its involvement in signal transduction was established by the demonstration that diacylglycerol (DAG), which is generated by agonist-induced hydrolysis of phosphoinositides and other membrane phospholipids, is the primary physiological activator of the enzyme (6). Signals that activate a variety of cell surface receptors (e.g., G protein-coupled receptors, receptor tyrosine kinases) transiently produce DAG through phospholipase C-mediated breakdown of phosphatidylinositol (4,5)-bisphosphate; DAG then acts as a second messenger by activating PKC. Sustained activation of the enzyme, which is required for long-term cellular responses such as proliferation and differentiation, appears to involve additional pathways, such as the production of DAG through phosphatidylcholine metabolism (7, 8). These findings, together with the suggestion that lipids other than

DAG can activate PKC *in vitro* and *in vivo* (9), indicate that PKC is a downstream target for a plethora of different agonist-induced pathways. Activation of PKC, which is generally accompanied by increased association of the enzyme with cellular membranes (10) and/or cytoskeletal elements (11), initiates a cascade of events which relays the agonist-evoked signal to the nucleus where gene expression is modulated to produce specific cellular responses.

PKC became the subject of intense investigation when it was discovered that it is the major cellular receptor for tumor promoting phorbol esters, which can substitute for DAG in activating the enzyme (12). This finding linked PKC signaling to mitogenesis and tumor promotion. Unlike DAG, phorbol esters are not rapidly metabolized (13); thus, treatment of cells with these agents leads to a more prolonged activation of the enzyme. As a result, phorbol esters have been widely exploited as pharmacological tools for elucidating the cellular functions and mechanisms of action of members of the PKC family in a variety of systems. Other PKC agonists that have been useful in studying PKC signaling include membrane-permeant DAG analogues and the macrocyclic lactone bryostatin. It should be noted that phorbol esters and other PKC agonists can potently downregulate the enzyme following activation; caution should thus be exercised in the interpretation of data obtained using these reagents.

A major step toward understanding the basis for the diversity of physiological responses attributed to PKC involved the recognition that the enzyme is encoded by a multigene family; to date, at least ten isozymes have been identified (14, 15). These isozymes share the same basic structure but differ with respect to activator and cofactor requirements, substrate specificity, tissue expression, and subcellular distribution. Based on sequence homology and biochemical properties, PKC isozymes have been classified into three subfamilies: the 'conventional' or 'classical' PKC isozymes (cPKCs), alpha, beta I, beta II, and gamma, which are dependent on DAG and Ca^{2+} for activity and respond to phorbol esters; the 'novel' PKC isozymes (nPKCs), delta, epsilon, eta, and theta, which are insensitive to Ca^{2+}; and the atypical PKC isozymes (aPKCs), zeta and iota, which neither require Ca^{2+} nor respond to DAG or phorbol esters. The PKC beta gene is alternatively spliced to produce two gene products which differ only in their extreme C-terminal ends. Some isozymes, like PKC alpha and zeta, appear to be universally expressed; others such as PKC beta II, delta, and epsilon are widely distributed in most cells and tissues, while still others exhibit a tissue-restricted pattern of expression (e.g., PKC gamma and eta). These differences, together with the varied consequences of PKC activation in the same cell and the fact that PKC isozymes are conserved in higher organisms, argue that they are not merely isozymes of identical function, but rather that they have specialized roles in cell signaling and elicit specific cellular responses.

Although understanding of the specific functions of individual PKC isozymes and of the molecular pathways through which they exert their divergent effects remains limited, evidence obtained using a variety of pharmacological and molecular approaches clearly points to a role for the PKC family in regulation of cell growth (16-18). Work in diverse cell types has demonstrated that PKC can participate in either positive or negative regulation of cell proliferation. While early studies showed that activators of PKC, including phorbol esters (e.g., phorbol 12-myristate 13-acetate, PMA; also known as 12-O-tetradecanoylphorbol-13-acetate, TPA) and membrane-permeant DAG analogues, can stimulate DNA synthesis in some cell types (19-21), it was later shown that PKC activation induces cell growth inhibition and/or differentiation in a variety of systems (22-25). More recently, these opposing effects of PKC activation on cell growth have been demonstrated within the same cell type, either by stable overexpression of individual PKC isozymes or through the use of PKC agonists that differentially activate specific members of the PKC family. For example, overexpression of PKC beta I (26) or epsilon (27) in the R6 rat embryo fibroblast cell line resulted in enhanced growth or full transformation, respectively, while overexpression of PKC alpha led to a marked inhibition of cell growth (28). Overexpression of PKC epsilon in mouse NIH3T3 cells resulted in decreased doubling time and increased saturation density, while overexpression of PKC delta in these cells produced the opposite phenotype (29). Similarly, overexpression of PKC epsilon in rat colonic epithelial cells enhanced cell growth and induced neoplastic transformation, while overexpression of PKC delta suppressed cell proliferation (30, 31). In human erythroleukemia cells, PKC beta II was shown to be essential for cell growth, whereas PKC alpha was implicated in control of cytostasis and megakaryocytic differentiation (32). Taken together, these findings suggest that the varying growth regulatory effects of PKC agonists in different systems are the result of differential activation of individual members of the PKC family. Emerging evidence increasingly points to roles for PKC alpha, delta, and eta in negative growth regulation and/or differentiation, and for PKC beta and epsilon in positive control of cell growth. However, it should be emphasized that the actions of individual isozymes appear to be highly dependent on cellular background, and conflicting results have been obtained for each member of the PKC family in different biological systems. For example, overexpression of PKC beta I in fibroblasts resulted in enhanced growth (26), while increased levels of this isozyme in colon adenocarcinoma cells produced growth arrest and reduced tumorigenicity in nude mice (33). Furthermore, in contrast to its involvement in cell proliferation in erythroleukemia cells (32), PKC beta II has been implicated in differentiation of HL60 myelocytic leukemia cells (34).

A full understanding of PKC isozyme functions in control of cell growth under normal and pathological conditions requires clarification of the specific actions of these molecules on cell cycle progression and on individual components of the cell cycle machinery. This is an emerging field and our current understanding of the molecular pathways involved is limited. However, accumulating evidence points to a role for member(s) of the PKC family in control of cell cycle progression at two sites: the $G_1 \rightarrow S$ and the $G_2 \rightarrow M$ transitions (14, 17, 18). Most of the available mechanistic information relates to the involvement of PKC isozymes in negative control of these transitions, although limited data are also available regarding the pathways underlying PKC-mediated positive regulation of $G_2 \rightarrow M$ progression. Evidence is also beginning to emerge regarding the involvement of PKC signaling in

control of cell cycle exit into G_0 and induction of differentiation (35-37). This review will focus on our current understanding of PKC-mediated control of these transitions, with specific emphasis on the cell cycle components targeted by PKC and the physiological relevance of information obtained from *in vitro* systems.

3. DISCUSSION

3.1. Effects of PKC activation on cell cycle progression

PKC activation has been shown to regulate cell cycle progression in a wide variety of cell types [e.g., vascular endothelial cells (38), vascular smooth muscle cells (39), melanoma cells (40), fibroblasts (41), keratinocytes (42), myeloid leukemia cells (43), breast carcinoma cells (44), intestinal epithelial cells (45)]. Addition of PKC agonists such as PMA or DAG analogues to asynchronously growing melanoma cells (40), intestinal epithelial cells (45), or HL60 myelocytic leukemia cells (46) results in accumulation of cells in G_0/G_1 and G_2/M phases, indicating that PKC signaling induces a biphasic block in cell cycle progression in these systems. Cells that are in G_1 phase when the stimulus is added remain in G_1, or perhaps exit the cell cycle into G_0 (36, 37), while cells which have progressed through the $G_1 \rightarrow S$ transition complete S phase and are arrested in G_2. Similar effects have been observed in synchronized cell populations (e.g., 38, 47). Notably, negative regulation of these transitions has also been observed as a result of overexpression of specific PKC isozymes, including PKC alpha (Leontieva and Black, unpublished data), delta (48, 49), and eta (50). It appears that PKC activation does not directly modulate progression through S phase (40, 45, 51, 52) or M phase (40, 47, 53, 54).

While PKC-mediated effects on cell cycle progression have been inhibitory in the majority of systems examined, there is also evidence for involvement of PKC in positive control of both the $G_1 \rightarrow S$ and the $G_2 \rightarrow M$ transitions. An interesting bidirectional regulation of $G_1 \rightarrow S$ progression, which depends on the timing of PKC activation, has been noted in vascular endothelial cells (38, 55). In this system, short-term application of PKC agonists early in G_1 potentiated $G_1 \rightarrow S$ phase progression, while activation of PKC in mid-to-late G_1, or the continued presence of the stimulus throughout G_1, prevented entry of cells into S phase. The ability of PKC signaling to promote cell cycle progression during early G_1 phase is consistent with the finding that, in some systems, PKC agonists such as phorbol esters and DAG analogues can mimic the action of certain growth factors and induce the expression of immediate early genes such as *c-fos* (56). Furthermore, it suggests a mechanism for the PKC-mediated stimulation of cell growth observed in certain cell types. It is noteworthy, however, that the stimulatory effects of PKC activation in early G_1 observed in vascular endothelial cells appear to be overcome by the negative growth regulatory effects of PKC signaling in mid-to-late G_1 phase (38); such a dominant inhibitory effect may have thus obscured any growth-stimulatory effects of PKC in many of the cell types examined. In addition to these G_1 effects, stimulatory effects of PKC signaling on $G_2 \rightarrow M$ progression have also been reported. In K562 erythroleukemia cells, for example, PKC beta II has been shown to be essential for cell proliferation and for the $G_2 \rightarrow M$ transition (32). Studies by Fields and co-workers have demonstrated that this isozyme is specifically targeted to the nuclear membrane during G_2/M (57, 58), where it participates in nuclear lamina disassembly prior to mitosis through phosphorylation of lamin B on Ser-405 (59). Furthermore, in *Saccharomyces cerevisiae*, which expresses a single form of PKC (PKC-1), depletion of the enzyme produces a G_2-like arrest following completion of DNA synthesis (60).

The cell cycle-specific effects of PKC activation appear to be dependent on sustained activation of PKC. Thus, in most systems examined, PKC agonist-mediated cell cycle arrest is transient, and reversal of the effect coincides with agonist-induced downregulation of the specific PKC isozyme(s) involved (40, 45, 61-63). The need for sustained PKC activation is further indicated by the fact that multiple doses of DAG analogues, which are rapidly metabolized by the cell, are required to mimic the cell cycle-specific effects of phorbol esters (39, 41, 47, 49, 64). It is noteworthy in this regard that, in many cell types, serum-stimulated DNA synthesis is most effectively inhibited if PKC agonists are added 4-6 h after release from G_0/G_1 arrest (25, 38, 45, 65); this timing of the stimulus likely ensures that high levels of activated PKC are still present at the point in mid-to-late G_1 at which they are specifically required. In systems such as the HL60 human myelocytic cell line, where PKC stimulation results in sustained cell cycle arrest followed by differentiation, activation of the relevant PKC isozymes is sustained over the time course of the experiment (32). It is noteworthy in regard to the observed role of PKC in negative regulation of cell cycle progression that depletion of phorbol ester-responsive PKC isozymes has been shown to result in shorter doubling times and acceleration of DNA synthesis in several systems (e.g., 39).

The available information, therefore, increasingly points to member(s) of the PKC family as important regulators of the $G_1 \rightarrow S$ and $G_2 \rightarrow M$ cell cycle transitions. The fact that the data have been accumulated from studies in a broad spectrum of different cell lines argues for the generality of the paradigms described, while addressing possible concerns regarding cell type specificity, tissue specificity, and species specificity. A large number of studies have explored the molecular mechanisms underlying PKC-mediated regulation of $G_1 \rightarrow S$ and $G_2 \rightarrow M$ progression, and a picture is beginning to emerge regarding the pathways leading to cell cycle arrest at these transitions. Furthermore, limited data are beginning to provide an understanding of the pathways involved in PKC-mediated cell cycle exit into G_0 and induction of differentiation.

3.2. Mechanisms of PKC-mediated cell cycle regulation

Progression through the mammalian cell cycle is regulated by the sequential activation and inactivation of a phylogenetically conserved family of protein kinases known as cyclin-dependent kinases, or cdks (66). Cdk activation requires the association of a regulatory subunit termed a cyclin, whose expression generally varies during the cell cycle (67). Particular cyclin/cdk complexes act at different cell cycle stages to control two key checkpoints, the $G_1 \rightarrow S$ and $G_2 \rightarrow M$ transitions (68). Progression through early G_1 appears to depend on cdk4 and cdk6; these cdks are activated by association with one of the three D-type cyclins (D1, D2, D3)

(69), which are induced as a delayed response to mitogens (70). Progression through late G_1 and into S phase requires activation of cdk2, which is sequentially regulated by cyclin E and cyclin A (71, 72). Cdk2 associates with cyclin E in late G_1 and, once the cell enters S phase, cyclin E is degraded and cdk2 combines with cyclin A; cyclin A is degraded in early M phase (73). The $G_2 \rightarrow M$ transition is regulated by cdc2 (cdk1) in association with cyclins A and B (74). The activity of cyclin/cdk complexes is controlled by multiple mechanisms, including positive and negative phosphorylation events and association with cdk inhibitory proteins (CKIs). Positive phosphorylation of cdks occurs within the activation loop of the catalytic domain, and is mediated by cdk activating kinase (CAK, or cdk7/cyclin H) (75). Negative phosphorylation events occur within the ATP binding domain of cdks (Thr-14 and Tyr-15), and dephosphorylation of these residues by members of the cdc25 family of phosphatases is necessary for full kinase activity. Negative regulation of cdk activity is also achieved by CKIs, which bind to cyclin/cdk complexes and render them inactive (76, 77). Two families of CKIs have been identified: the Cip/Kip family ($p21^{waf1/cip1}$, $p27^{kip1}$, and $p57^{kip2}$) and the INK4 family (p15, p16, p18, and p19). Initial studies, largely involving *in vitro* experiments or *in vivo* overexpression assays, suggested that the Cip/Kip CKIs are universal inhibitors of cdks. However, more recent work has indicated that, although these molecules inhibit cyclin E- and cyclin A-dependent kinases, they act as positive regulators of cyclin D-dependent kinases (78). The INK4 inhibitors, on the other hand, are specific for cdk4 and cdk6, the most divergent of the cell cycle-associated cdks.

Important targets of cdk4 and cdk2 include members of the retinoblastoma family of pocket proteins, which comprises the retinoblastoma protein (pRb), p107, and p130 (79). These growth suppressor proteins play key roles in control of cell cycle progression, cell cycle entry and exit, cell differentiation, and apoptosis (79, 80). Critical targets for the pocket protein family are the E2F transcription factors, which are required for cell cycle progression; by forming complexes with E2Fs, pocket proteins suppress the transcription of genes necessary for the $G_1 \rightarrow S$ transition. The interaction of pocket proteins with E2Fs is regulated by their phosphorylation state and their level of expression (81), both of which are controlled in response to positive and negative growth signals. In their underphosphorylated (active) state, pocket proteins bind to E2Fs and maintain cells in G_0/G_1; hyperphosphorylation of these molecules by cyclin/cdk complexes in mid-G_1 disrupts their interaction with E2Fs and allows S phase progression. Furthermore, levels of p130 and p107 change dramatically with cell growth and the cell cycle. p130 is abundantly expressed in quiescent cells, as phosphoforms 1 and 2, and is markedly downregulated following its cdk-mediated phosphorylation to form 3 in mid-G_1. Conversely, during cell cycle exit, levels of p130 forms 1 and 2 markedly increase, coinciding with the accumulation of p130/E2F complexes. p107, on the other hand, is not expressed in quiescent cells and accumulates as cells progress through the cell cycle. Levels of pRb are generally constant in quiescent and cycling cells, although a slight increase in expression has been observed during cell cycle progression in some cell types (79). It has been proposed that pocket proteins are coordinately regulated to act as competence factors to allow or impede certain cell growth and/or differentiation transitions (79).

Progress through the cell cycle is influenced by exogenous regulatory signals provided by growth factors and differentiation agents; thus, the biochemical mechanisms underlying cell cycle progression integrate information from the cellular microenvironment and regulate cell proliferation accordingly. The emerging perception is, therefore, that signal transducers (e.g., PKC isozymes), cell cycle regulators, and transcription factors act in concert to govern cell proliferation in response to environmental stimuli. In the following sections, current understanding of the cell cycle-specific targets of PKC-mediated regulation are discussed. Based on analysis of the available information as a whole, a model is proposed in which negative regulation of major cell cycle transitions by PKC occurs via common mechanisms, with induction of the Cip/Kip CKI $p21^{waf1/cip1}$ playing a pivotal role in initiating negative growth-regulatory cascades at various cell cycle stages. In addition, although the data are limited, current knowledge of the molecular pathways underlying positive control of cell cycle progression by PKC is described.

3.2.1. PKC and regulation of the $G_1 \rightarrow S$ transition

An increasing number of studies in a broad spectrum of cell lines have linked PKC signaling to regulation of the phosphorylation state/function of pRb. This link was first demonstrated by Zhou et al. (38) in vascular endothelial cells. Activation of PKC produced cell cycle-dependent, bidirectional effects on pRb phosphorylation that correlated with the bimodal growth regulatory effects of PKC agonists in these cells. Thus, PKC activation in early G_1 promoted pRb phosphorylation, concomitant with potentiation of growth factor-induced stimulation of DNA synthesis. In contrast, activation of PKC in late G_1 maintained pRb in a hypophosphorylated state and inhibited $G_1 \rightarrow S$ phase progression. The inhibitory effect of PKC activation on growth factor-induced pRb phosphorylation was also seen in vascular smooth muscle cells (52), intestinal epithelial cells (45), HL60 myelocytic leukemia cells (82), and LNCaP prostate epithelial cells (83), as well as in NIH3T3 cells stably overexpressing PKC eta (50) and vascular smooth muscle cells overexpressing PKC delta (84).

Recent studies in intestinal epithelial cells have extended these findings by demonstrating that PKC signaling can regulate the phosphorylation state and expression levels of the pRb-related proteins p107 and p130 (37). PKC activation in asynchronously growing IEC-18 rat intestinal epithelial cells resulted in hypophosphorylation and decreased expression of pRb and p107, as well as marked accumulation of phosphoforms 1 and 2 of p130; as discussed below (section 3.2.3), these changes have been shown to reflect withdrawal from the cell cycle into G_0. Thus, PKC signaling can regulate the function of all three members of the pocket protein family. Although the data are limited, effects of PKC signaling on the expression and/or activity of members of the E2F family have also been reported (55, 65, 85). Phorbol ester-induced activation of PKC resulted in bimodal regulation of E2F1 message levels in human umbilical vein endothelial cells, which depended on the timing of PKC activation in G_1 phase (55). Decreased E2F1 mRNA levels, accompanied by the

accumulation of E2F5/p130 complexes, were also observed in phorbol ester-treated U937 human myelomonocytic leukemia cells (85). Nakaigawa et al. (65) further demonstrated PKC-mediated effects on E2F transcriptional activity; in this study, PKC alpha overexpression in 3Y1 fibroblasts inhibited E2F activity while PKC delta or epsilon overexpression enhanced it. Interestingly, overexpression of any one of these isozymes resulted in enhancement of phorbol ester (PMA)-induced inhibition of E2F activity in late G_1 phase, further supporting the notion that the growth inhibitory effects of PKC signaling are dominant over PKC-mediated growth stimulatory pathways (see above).

The mechanisms underlying PKC agonist-induced control of pocket protein phosphorylation have been examined in a variety of systems. Perhaps not surprisingly, these studies generally point to modulation of G_1 cdk activity as a key component of PKC-mediated pocket protein regulation. Immune complex kinase assays in a variety of cell types have clearly demonstrated PKC-induced effects on the activity of cdk2 (37, 38, 48-50, 52, 86, 87). While these effects have generally been inhibitory, PKC-mediated bimodal regulation of cdk2 activity was seen during the $G_0 \rightarrow S$ transition in vascular endothelial cells, consistent with the bimodal effects of PKC activation on cell cycle progression in these cells (see above) (38). Since cdk2 can associate with cyclin E or with cyclin A, several studies have differentiated the effects of PKC agonists on the activity of these individual complexes. PKC-induced inhibitory effects on cyclin E/cdk2 complex activity have been observed in vascular endothelial cells (48, 49), NIH3T3 cells overexpressing PKC eta (50), and intestinal epithelial cells (37). Modulation of cyclin A-associated activity by PKC signaling has only been examined in vascular endothelial cells and intestinal epithelial cells (37, 48); in both cases, PKC had an inhibitory effect on cyclin A/cdk2 histone kinase activity.

The effects of PKC signaling on cdk4/cdk6 activity have not been as extensively studied and, collectively, the data do not as yet provide a clear picture of PKC-induced modulation of these kinases (37, 48, 52). Sasaguri et al. (52) reported that phorbol ester-induced activation of PKC did not affect the pRb kinase activity associated with cyclin D (likely cyclins D1 and/or D2) in vascular smooth muscle cells. In contrast, Ashton et al. (48) reported that overexpression of PKC delta significantly delayed the induction of cyclin D1-associated kinase activity following serum stimulation of rat microvascular endothelial cells. Furthermore, although cyclin D-associated activity was not directly measured in their study, Zezula et al. (49) proposed that phorbol ester treatment inhibits cyclin D1 activity in human venous endothelial cells based on (a) the presence of increased levels of $p21^{waf1/cip1}$ in cyclin D1/cdk4 complexes, and (b) a significant delay in bFGF-stimulated phosphorylation of pRb. A link between PKC signaling and regulation of cyclin D1 expression or subcellular distribution has also been noted in some studies. For example, reduced levels of this cyclin were detected in vascular smooth muscle cells overexpressing PKC delta (84) and in R6 fibroblasts overexpressing PKC epsilon (88). Furthermore, phorbol ester treatment resulted in transient suppression, followed by a marked induction, of cyclin D1 in melanoma cells (86) and cyclin D/D1 in intestinal epithelial cells (37). Interestingly, delayed nuclear translocation of cyclin D1 was observed in rat microvascular endothelial cells overexpressing PKC delta (48). However, it should be noted that several studies reported no change in cyclin D1 expression in response to activation or overexpression of PKC isozyme(s) (50, 52, 55, 89). An inability of PKC signaling to suppress cyclin D-associated kinase activity would indicate that other mechanisms are involved in the reported PKC-induced inhibition of growth factor-stimulated expression of cyclin E in mid-to-late G_1 phase (e.g., 49, 52, 55, 84), an event which is regulated by E2F (90-92). An exhaustive study of the modulation of D-type cyclin-associated activity by PKC signaling is required to clarify these issues.

As discussed above, cdk activity is regulated by cyclin accumulation and binding, by positive and negative phosphorylation events, and by the accumulation of cdk inhibitory proteins. Several studies have proposed a role for altered cyclin E/A expression in mediating PKC-induced suppression of cdk2 activity (17, 18). However, careful analysis of the data obtained in a variety of systems indicates that reduced expression of these cyclins is likely a consequence of PKC-induced synchronization of cells at particular phases of the cell cycle, rather than a cause of PKC-mediated cdk inhibition. Consistent with this notion, PKC-induced inhibition of cdk2 activity has been observed under conditions in which cyclin E (37, 48, 50, 86, 93) and cyclin A (37, 48) are not limiting. Furthermore, reduced expression of cyclin E has only been reported in studies in which PKC-mediated effects were examined during growth factor-stimulated $G_0 \rightarrow S$ phase progression (i.e., during progression from quiescence; 49, 52, 55, 84). Since serum-stimulated cyclin E expression occurs in mid-to-late G_1, this effect may be the result of PKC-induced cell cycle arrest prior to induction of this protein (e.g., as a consequence of inhibition of cyclin D-associated kinase activity). Similarly, reduced expression of cyclin A may be a downstream consequence of the inhibition of cdk2/cyclin E complex activity and blockade of entry into S phase (37, 52, 54, 55, 64, 86). Taken together, the data strongly indicate that PKC-induced inhibitory effects on cdk2 activity are primarily mediated by mechanisms other than the availability of partner cyclins.

Analysis of the effects of PKC signaling on G_1 regulatory molecules has consistently shown no effect on G_1 cdk expression: PKC does not appear to regulate the levels of cdk4 (37, 38, 52, 55, 86), cdk2 (37, 38, 52, 55, 86, 87), or cdk3 (52, 86). Inhibitory effects of PKC signaling on the activating phosphorylation of cdk2 at Thr160 have been reported in a limited number of studies (86, 87, 94). Coppock et al. (86) showed that addition of phorbol ester to melanoma cells in G_1 inhibits the mobility shift of cdk2 associated with its phosphorylation and conversion to the activated form, while Asiedu et al. (93, 94) proposed that PKC signaling induces dephosphorylation of cdk2 at Thr160. In one study (87), PKC-induced inhibition of serum-stimulated cdk2 phosphorylation at Thr160 was accompanied by suppression of CAK activity which correlated with decreased message levels for both cdk7 and cyclin H.

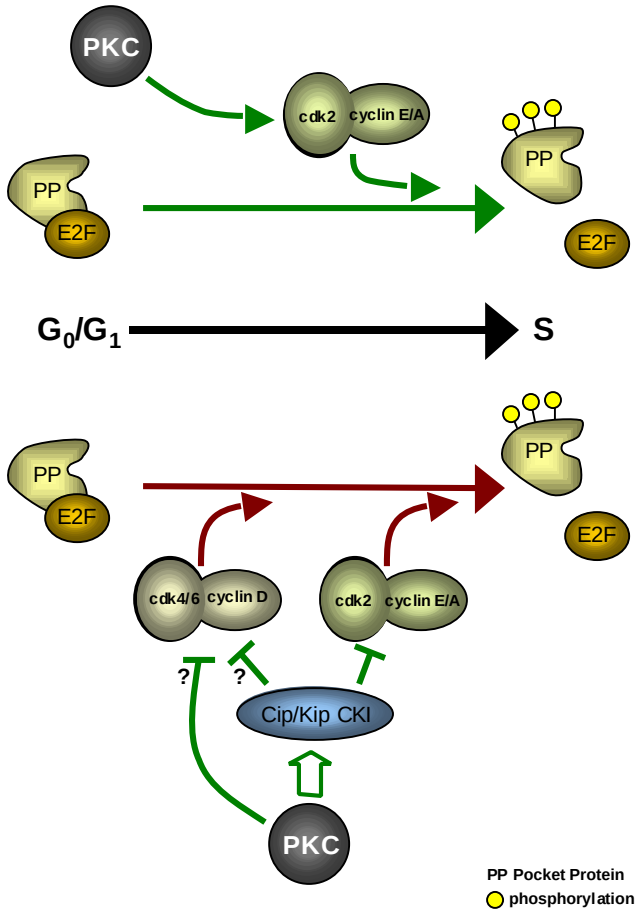

Figure 1. Model of the molecular mechanisms underlying PKC-mediated regulation of $G_1 \rightarrow S$ progression. The upper portion of the figure (above the black arrow indicating $G_0/G_1 \rightarrow S$ progression) shows the consequences of PKC activation in early G_1, while the lower portion (below the arrow) depicts events resulting from PKC activation in mid-to-late G_1 phase. Molecular events that regulate the $G_0/G_1 \rightarrow S$ transition are indicated by arrows (stimulatory events) and bars (inhibitory events); arrows/bars are colored green or red to denote events promoted or inhibited by PKC signaling, respectively. See text for details.

As mentioned above, another mechanism for inactivation of cyclin/cdk complexes involves the binding of members of the Cip/Kip and INK4 families of CKIs. In this regard, PKC signaling has been shown to induce the expression of Cip/Kip CKIs in a wide variety of cell types. PKC-mediated increases in $p21^{waf1/cip1}$ levels have been observed in melanoma cells (86), various leukemic cell lines (35, 94-98), breast cancer cells (44), non-transformed intestinal epithelial cells (37, 45), colon carcinoma cells overexpressing PKC alpha (37, 45, 99), endothelial cells overexpressing PKC delta (49), NIH3T3 cells overexpressing PKC eta (50), keratinocytes (100), and ovarian carcinoma cells (95). Induction of $p21^{waf1/cip1}$ by PKC signaling was rapid and robust, but transient, in most systems examined (e.g., 37, 45, 49, 94, 100). In addition to inducing $p21^{waf1/cip1}$ expression, PKC activation has also been shown to increase the levels of $p27^{kip1}$ in several cell types, including melanoma cells (86), vascular smooth muscle cells (84), intestinal epithelial cells (37, 45), leukemic cells (93), NIH3T3 cells overexpressing PKC eta (50), and vascular endothelial cells overexpressing PKC delta (48). It is noteworthy that, in contrast to the rapid and transient induction of $p21^{waf1/cip1}$ observed in several systems, induction of $p27^{kip1}$ has been reported to be delayed but sustained (37, 93). PKC-induced increases in levels of Cip/Kip CKIs have been detected in cyclin D1/cdk complexes (37, 49), cyclin E/cdk2 complexes (37, 48, 50, 86, 93), and cyclin A/cdk2 complexes (37), indicating that these molecule(s) could potentially be involved in PKC-mediated suppression of the activity of all three of these cyclin/cdk complexes [although, in light of more recent studies (78, see above), the significance of increased levels of Cip/Kip CKIs in cyclin D complexes is unclear]. Interestingly, there have been no reports demonstrating that PKC signaling regulates the expression of INK4 family CKIs (e.g., 37). Thus, PKC-mediated effects on CKI regulation appear to be limited to members of the Cip/Kip family.

As discussed further below (see section 3.2.4), emerging data increasingly point to Cip/Kip CKIs, and to $p21^{waf1/cip1}$ in particular, as key mediators of PKC-induced inhibition of cdk activity and induction of G_1 arrest. Thus, PKC activation in mid-to-late G_1 appears to initiate the

following program of events (See Figure 1): increased expression of member(s) of the Cip/Kip family of CKIs, inactivation of cdk2/cyclin complexes (and possibly cdk4/6/cyclin D complexes, perhaps by other mechanisms such as downregulation of cyclin D), suppression of the phosphorylation/inactivation of members of the pocket protein family, and inhibition of cell cycle progression into S phase. While there is also evidence that PKC activation in early G_1 potentiates cdk activity/Rb phosphorylation and promotes DNA synthesis (Figure 1), to date the mechanisms underlying these effects have not been examined in detail.

3.2.2. PKC and regulation of the G_2®M transition

PKC signaling has been implicated in both negative and positive control of the $G_2 \rightarrow M$ transition, although understanding of the underlying molecular mechanisms involved remains limited. Progression through the $G_2 \rightarrow M$ transition requires the activity of a cdk-cyclin complex (maturation promoting factor) consisting of cdc2 (cdk1) kinase and a B-type cyclin (74). Activation of this complex is controlled by the accumulation of cyclin B, as well as by dephosphorylation and phosphorylation of critical residues on cdc2 (101, 102). The cdc2 kinase is maintained in an inactive state by phosphorylation on Thr-14 and Tyr-15 by inhibitory kinases such as Wee1 (103). Dephosphorylation at these sites by the dual specificity phosphatase cdc25C (104) and phosphorylation on Thr-160 by CAK activates the kinase and allows progression into M phase (105).

Several studies using cell populations synchronized at the $G_1 \rightarrow S$ border and/or near the $G_2 \rightarrow M$ transition indicate that PKC signaling can arrest cell cycle progression in G_2 phase (47, 51, 53, 54, 106). With the exception of CHO cells overexpressing PKC delta, which arrest in telophase in response to phorbol ester treatment (107), cells already in mitosis do not appear to be influenced by PKC activity (40, 47, 53, 54). Interestingly, PKC agonists can block cells from entering mitosis when added near the end of G_2, pointing to rapid activation of inhibitory mechanisms (e.g., 47, 53, 54). In general, PKC agonist-mediated cell cycle arrest in G_2 is transient, likely as a result of downregulation of requisite PKC isozymes (45, 47, 53, 54).

In all systems examined, negative regulation of $G_2 \rightarrow M$ progression by PKC signaling has been linked to inhibition of the activation of the cdc2/cyclin B complex (47, 53, 54, 106). As observed with the G_1 cyclins and cdks, PKC activation does not generally appear to affect the levels of cyclin B or cdc2 (e.g., 53, 54). Two main mechanisms appear to be associated with PKC-mediated suppression of cdc2/cyclin B activity: (1) inhibition of Tyr-15 dephosphorylation of cdc2 (47, 53, 54), likely as a result of the observed PKC-mediated downregulation of the phosphatase cdc25 (47, 51, 53), and (2) induction of the Cip/Kip CKI $p21^{waf1/cip1}$ (54, 106, 108), which has recently been implicated in negative regulation of the $G_2 \rightarrow M$ transition (109). As discussed further below (section 3.2.4), inhibition of tyrosine dephosphorylation of cdc2 may in fact be linked to the accumulation of $p21^{waf1/cip1}$, via $p21^{waf1/cip1}$-mediated inhibition of cdk2/cyclin A activity and subsequent downregulation of cdc25 (109, 110). This model for PKC-mediated negative regulation of the $G_2 \rightarrow M$ transition is presented diagrammatically in Figure 2. Interestingly, one study has reported that phorbol ester-induced $G_2 \rightarrow M$ arrest is associated with the appearance of a novel, faster migrating form of $p21^{waf1/cip1}$, which appears to lack a portion of the carboxy terminus of the molecule (108). However, the significance of this altered form of $p21^{waf1/cip1}$ in PKC-mediated G_2/M effects remains to be determined.

Studies by Fields and colleagues have demonstrated that PKC activity can also play a critical role in positive regulation of the $G_2 \rightarrow M$ transition (Figure 2). Using the PKC inhibitor chelerythrine, these investigators showed that PKC (specifically the beta II isozyme) is required for entry of human erythroleukemia cells into mitosis (58). During G_2, PKC beta II is selectively activated at the nuclear periphery, where it directly phosphorylates the nuclear envelope polypeptide lamin B at key sites involved in mitotic nuclear lamina disassembly (59). Selective nuclear activation of PKC beta II is conferred by molecular determinants within the carboxy terminus of the molecule (111), and is likely mediated by phosphatidylglycerol (112). Chelerythrine-mediated G_2 arrest does not appear to involve inhibition of cdc2/cyclin B kinase activity, indicating that PKC beta II and the cdc2/cyclin B complex act in distinct pathways to regulate $G_2 \rightarrow M$ progression in leukemic cells (58). It is possible, therefore, that specific members of the PKC family differentially regulate the $G_2 \rightarrow M$ transition; while some members arrest cells in G_2 by inhibiting cdc2 activity, others like PKC beta II promote entry into mitosis by potentiating certain mitotic events such as nuclear lamina disassembly.

3.2.3. Control of the G_1®G_0 transition by PKC signaling

PKC signaling has been shown to induce cell differentiation in a variety of systems including several leukemic cell lines [e.g., HL60 myelocytic leukemia cells (24), U937 monocytic leukemia cells (36), K562 erythroleukemia cells (57)], mouse myeloid progenitor cells overexpressing PKC alpha or delta (113), keratinocytes (114, 115), and NIH3T3 cells overexpressing PKC eta (50, 116). It is well recognized that cell and tissue differentiation require an irreversible cell cycle exit that is dominant despite the presence of optimal growth conditions (80). Thus, prior to induction of tissue-specific gene expression, differentiation-inducing signals activate mechanisms of cell cycle arrest that lead to cell cycle withdrawal into G_0. It has been proposed that differentiation signals are recognized by the restriction point machinery, leading to inactivation of cyclin/cdk holoenzymes, likely by induction of CKIs in combination with other mechanisms (117). Recent studies have demonstrated that cell cycle exit involves a coordinated program of cell cycle regulatory events including transient induction of $p21^{waf1/cip1}$, hypophosphorylation of the pocket proteins pRb and p107, inactivation of E2F-dependent transcription, disappearance of p107, accumulation of p130

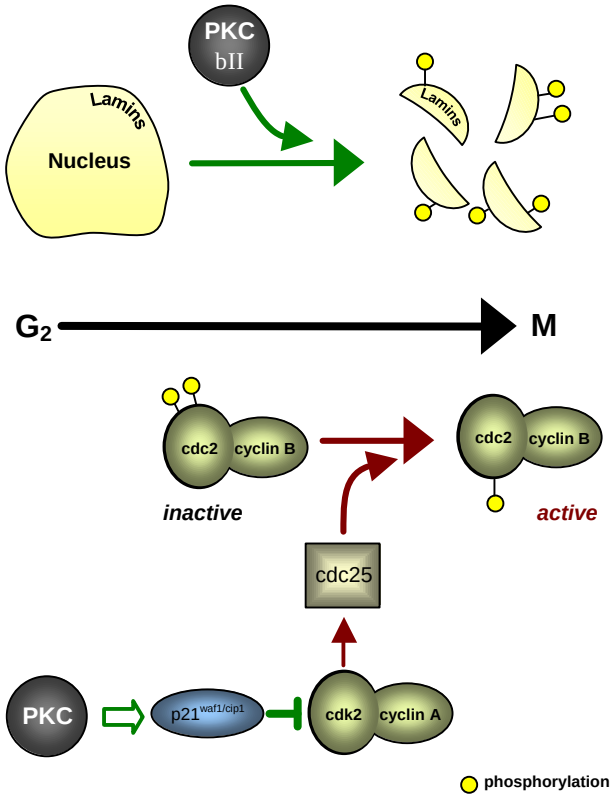

Figure 2. Model for PKC-mediated regulation of the $G_2 \rightarrow M$ transition. Stimulatory effects of PKC signaling on this transition are shown above the black arrow indicating $G_2 \rightarrow M$ progression, while inhibitory effects are shown in the lower portion of the figure. As in figure 1, molecular events involved in the $G_2 \rightarrow M$ transition are indicated by arrows (stimulatory) and bars (inhibitory), which are colored green or red to denote events promoted or inhibited by PKC signaling, respectively. PKC can stimulate $G_2 \rightarrow M$ progression by promoting nuclear lamina disassembly. Alternatively, the enzyme can inhibit this transition by blocking cdc25-mediated dephosphorylation of cdc2 on Thr-14 and Tyr-15 (indicated by two small yellow circles on inactive cdc2/cyclin B). Active cdc2/cyclin B phosphorylated by CAK on Thr-160 is shown with one yellow circle on cdc2; effects of PKC signaling on phosphorylation at this site have not been reported.

phosphoforms 1 and 2, and predominance of E2F4/p130 complexes (79, 81). It is noteworthy that form 2 of p130 only accumulates during cell cycle exit, and is maintained as a long-term binding partner and repressor of E2F4. Other events associated with cell cycle withdrawal include rapid downregulation of cyclin D (118), delayed accumulation of the CKI $p27^{kip1}$ (46), and disappearance of DNA replication licensing factors such as cdc6 (119). Recent evidence indicates that the Cip/Kip CKIs perform distinct functions in cell differentiation, with $p21^{waf1/cip1}$ playing a key role in irreversible growth arrest during early phases of the process, and $p27^{kip1}$ functioning later to induce or maintain tissue-specific gene expression (120). $p21^{waf1/cip1}$ expression may in fact play an inhibitory role during late stages of differentiation (121, 122); thus, tissue-specific gene expression appears to be associated with downregulation of the protein (e.g., 123).

Although the molecular mechanisms underlying PKC-mediated cell differentiation have not been extensively studied, data from a limited number of reports strongly suggest that certain members of the PKC family are capable of activating a conserved program of regulatory events associated with cell cycle withdrawal into G_0 and induction of tissue-specific gene expression (e.g., 35-37, 85) (Figure 3). For example, PKC activation in intestinal epithelial cells (37) results in rapid and transient induction of $p21^{waf1/cip1}$ [also see reference (36) and section 3.2.1], delayed but sustained induction of $p27^{kip1}$ (with no effect on INK4 family cdk inhibitors), rapid downregulation of cyclin D, inhibition of cyclin/cdk complex activity, decreased expression of the DNA replication licensing factor cdc6 (Clark and Black, unpublished data), and coordinated regulation of pocket protein phosphorylation state and expression levels. In this regard, PKC signaling in these cells leads to decreased expression of p107 and pRb, increased levels of p130, accumulation of the growth-suppressive forms of all three pocket proteins (i.e., hypophosphorylated pRb and p107 and forms 1 and 2 of p130), and cell cycle arrest in G_0/G_1. Studies in fibroblasts overexpressing PKC alpha, delta, or epsilon have further demonstrated that PKC-mediated cell cycle arrest is

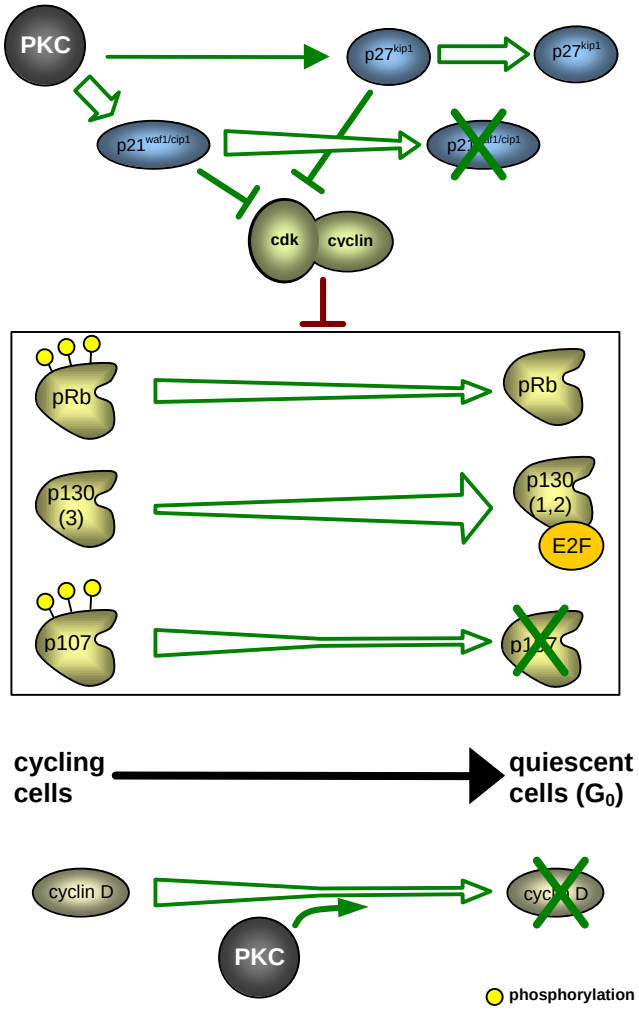

Figure 3. Model for PKC-induced cell cycle exit into G_0. Molecular events that regulate cell cycle withdrawal into G_0 are indicated by arrows (stimulatory events) and bars (inhibitory events); arrows which increase or decrease in width indicate changes in protein expression. Green arrows/bars denote pathways promoted by PKC activity while the red bar indicates events inhibited by activation of the enzyme. See text for details.

associated with decreased E2F1 activity (65); it remains to be established if PKC-mediated pathways signal the accumulation of the G_0-specific transcriptional repressor complex E2F4/p130 (124), although studies by Zhang and Chellappan (85) demonstrated the appearance of E2F5/p130 complexes during phorbol ester-induced differentiation of U937 cells.

Taken together, the limited data available strongly point to the involvement of PKC signaling cascades in cell cycle arrest and exit into G_0. Although many of the molecular mechanisms involved in PKC-mediated induction of the $G_1 \rightarrow G_0$ transition are likely to be similar to those involved in G_1 arrest, additional studies are required to further clarify the role of PKC isozymes in irreversible withdrawal from the cell cycle.

3.2.4. $p21^{waf1/cip1}$ is a key target of PKC-mediated cell cycle modulation

Comparison of the G_0/G_1 and G_2 cell cycle arrests induced by PKC activation in a variety of cell types points to the involvement of common mechanisms. In each case, PKC signaling inhibits the activity of relevant cdks (i.e., cdk2, and possibly cdk4/6, in G_1 and cdc2 in G_2). In many systems, this inhibition occurs under conditions in which cdks and partner cyclins (i.e., cyclins D and E in G_1 and cyclins B and A in G_2) are not limiting. Thus, induction of CKIs may be a key event responsible for producing PKC-induced cell cycle blockade at both the $G_1 \rightarrow S$ and $G_2 \rightarrow M$ transitions.

Compelling evidence in support of a major role for $p21^{waf1/cip1}$ in mediating PKC-induced cell cycle arrest comes from recent information on the specific actions of

Table 1: Comparison of the cell cycle-specific effects of PKC activation and $p21^{waf1/cip1}$ expression

Cell cycle-specific effects	PKC activation	$p21^{waf1/cip1}$ expression
G_0/G_1 arrest	+	+
G_2 arrest	+	+
S phase effects	-	-
Mitotic arrest	-	-
Cyclin availability	+	+
Inhibition of cdk activity		
Cdk4/6/Cyclin D	?	+
Cdk2/Cyclin E	+	+
Cdk2/Cyclin A	+	+
Cdc2/Cyclin B	+	+
Hypophosphorylation of Rb	+	+
Hyperphosphorylated cdc2 (Thr-14, Tyr-15)	+	+

this molecule on $G_1 \rightarrow S$ and $G_2 \rightarrow M$ progression. Reed and co-workers (109) performed an in-depth analysis of the function(s) of $p21^{waf1/cip1}$ in regulation of critical cell cycle transitions by conditionally expressing approximately physiological levels of the protein (i.e., comparable with those seen in response to gamma-irradiation) in a variety of transformed and non-transformed cell types. The cell cycle-specific effects of $p21^{waf1/cip1}$ expression seen in this study showed remarkable parallels with those seen in response to PKC activation or overexpression in a variety of cell types (see above and Table 1). First, in addition to negative regulation of the $G_1 \rightarrow S$ transition and maintenance of cellular quiescence, $p21^{waf1/cip1}$ was also shown to play a role in negative regulation of the $G_2 \rightarrow M$ transition. As seen in response to PKC activation, $p21^{waf1/cip1}$-expressing cells showed no evidence of mitosis, indicating that $p21^{waf1/cip1}$ inhibits the initiation of mitosis rather than the mitotic state or the ability of cells to exit mitosis (also see 110). Second, G_1-arrested cells expressed cyclins D1 and E, while G_2-arrested cells expressed cyclins A and B. Thus, as seen in response to PKC agonist treatment, the relevant cyclins were not limiting in G_1- or G_2-arrested cells. Third, $p21^{waf1/cip1}$-induction resulted in inhibition of cyclin D- and E-associated kinase activity, which correlated with increased levels of $p21^{waf1/cip1}$ in cyclin D- and E- containing complexes and with hypophosphorylation of Rb. Reduced cyclin A-associated kinase activity and cyclin B/cdc2 activity (which could account for the observed G_2 arrest) were also observed, although $p21^{waf1/cip1}$ was only detected in cyclin A-containing complexes. Fourth, as seen in response to PKC agonists, cdc2 bound to cyclin B in $p21^{waf1/cip1}$-arrested cells was predominantly in the hyperphosphorylated state associated with inhibitory phosphorylation on Thr-14 and Tyr-15. This effect on cdc2 has been attributed to $p21^{waf1/cip1}$-mediated inhibition of cyclin A/cdk2 activity, based on the suggestion that cdk2 activity upregulates the cdc25 phosphatases that remove inhibitory phosphorylation from the cdc2/cyclin B complex (110). Therefore, as discussed above (section 3.2.2), in addition to mediating PKC-induced G_1 arrest, $p21^{waf1/cip1}$ induction may also play a major role in PKC-induced G_2 arrest via a mechanism involving blockade of cdk2/cyclin A activity, inhibition of cdc25 upregulation, accumulation of inhibitory phosphorylation on cdc2, and suppression of cdc2/cyclin B activity (see Figure 2).

It should be noted that the study by Reed and co-workers (109) demonstrated that $p27^{kip1}$ expression also results in a biphasic inhibition of cell cycle progression in G_1 and G_2 phases. Thus, PKC-agonist induced cell cycle-specific effects could also involve this CKI. However, the potential role of $p27^{kip1}$ in PKC-induced effects is unclear since (a) induction of the molecule occurs with delayed kinetics relative to $p21^{waf1/cip1}$ (37), (b) $p27^{kip1}$ is unable to mediate PKC-induced cell cycle arrest in the absence of $p21^{waf1/cip1}$ (35, 36), and (c) $p27^{kip1}$ has been implicated in regulating tissue-specific gene expression rather than in mediating cell cycle arrest (121). The timing of $p27^{kip1}$ induction suggests that it is a downstream event of $p21^{waf1/cip1}$ expression involved in initiation and/or maintenance of differentiation (35, 37). Regardless of any potential role of $p27^{kip1}$, the close parallels between the cell cycle-specific effects of PKC agonists and of $p21^{waf1/cip1}$ expression (Table 1) indicate that $p21^{waf1/cip1}$ induction alone could account for the majority of PKC-mediated cell cycle inhibitory events. Interestingly, increased levels of this CKI could also account for the inhibitory effects of PKC activation on CAK-mediated phosphorylation of cdk2 observed in some systems (see section 3.2.1) (86). Therefore, $p21^{waf1/cip1}$ is likely to be a major target for PKC-mediated negative growth regulatory signaling.

The molecular events underlying PKC-mediated control of Cip/Kip CKI levels are currently under investigation in several laboratories. Increasing evidence indicates that PKC is among the various signals that regulate $p21^{waf1/cip1}$ expression through p53-independent pathways. In this regard, PKC-mediated induction of this CKI has been observed in a number of p53 null cell lines, as well as in the absence of parallel increases in p53 expression (e.g., 95-97, 100). Control of $p21^{waf1/cip1}$ levels by PKC signaling appears to be complex, involving transcriptional (49, 95, 97) and post-transcriptional mechanisms (49, 95, 100); PKC can regulate the amounts of $p21^{waf1/cip1}$ at the level of mRNA transcription, stabilization and translation, as well as by enhanced stability of the protein. Notably, in systems in which PKC-mediated induction of $p21^{waf1/cip1}$ is transient, levels of $p21^{waf1/cip1}$ RNA decline substantially before decreases in protein levels become apparent [e.g., >10 h in human venous endothelial cells, (49)].

Several studies point to the role of members of the Sp1 family of transcription factors, including Sp1 and Sp3, in mediating PKC-induced activation of the $p21^{waf1/cip1}$ promoter (125, 127). Furthermore, there is increasing evidence supporting a requirement for the mitogen-activated protein kinase (MAPK) cascade in PKC agonist-mediated induction of $p21^{waf1/cip1}$ (49, 95, 126, 128; Frey, Clark, and Black, unpublished data). In this regard, phorbol ester or DAG analogue induction of $p21^{waf1/cip1}$ expression can be abrogated by the MAPK kinase 1 (MEK) inhibitor PD098059 (49, 95), or by expression of dominant-negative mutants of MEK and Ras (126). Studies in intestinal epithelial cells using a panel of

PKC agonists have further demonstrated that prolonged, rather than short-term, activation of the MAPK signaling pathway is required for PKC-mediated cell cycle arrest (Frey, Clark, and Black, unpublished data). This finding is of particular significance in light of emerging evidence indicating that, while mitogenic signaling involves transient activation of the MAPK signaling cascade, sustained activation of this pathway is required for growth arrest and differentiation (129-131).

Although the evidence for p53-independent regulation of $p21^{waf1/cip1}$ expression by PKC is compelling, the participation of p53 in PKC-induced cell cycle-specific effects has not been formally ruled out. It is interesting in this regard that PKC has been shown to phosphorylate p53 *in vitro*, with concomitant activation of sequence-specific DNA binding that is thought to be important for the growth inhibitory function of this protein (132, 133). It should be noted, however, that recent evidence indicates that p53 may not be a direct target for PKC phosphorylation *in vivo* (134-136), although nuclear translocation and accumulation of the protein may be dependent on PKC activity through an indirect mechanism (135). Evidence that erk2 can phosphorylate p53 *in vitro* and possibly *in vivo* suggests that PKC may regulate p53 activity through the MAPK pathway (137). Additional studies are clearly required to establish whether p53 plays a role in PKC-mediated negative regulation of cell cycle progression.

3.2.5. The role of individual members of the PKC family in regulation of cell cycle progression

A major challenge for the future remains understanding the specific functions of individual PKC isozyme(s) in control of cell cycle progression. However, information is beginning to emerge from studies in a broad spectrum of cell types (described throughout this review) regarding isozyme-specific functions in regulation of the major cell cycle transitions. Thus, PKC isozymes which are likely to play an important role in negative regulation of the $G_1 \rightarrow S$ transition include PKC alpha (39, 45, 55, 113, 138, 139), PKC delta (42, 45, 54, 55, 84, 113), and PKC eta (42, 50). Indirect evidence has also implicated PKC epsilon in control of $G_1 \rightarrow S$ progression (39, 42, 45, 50, 55, 140); however, the reported involvement of this isozyme in producing transformation in several systems (e.g., 27) points to a role in positive rather than negative regulation of this transition. PKC isozymes which have been linked to regulation of cell cycle exit into G_0 and induction of differentiation include PKC alpha (32, 37, 57, 99, 113, 115, 138, 141), PKC delta (37, 113), and PKC eta (50, 115, 116). Negative regulation of the G_2? M transition appears to involve PKC alpha and/or delta (45, 54, 107), while PKC beta II has been strongly implicated in positive control of this transition (58). It is clear, however, that much work remains to be done in order to elucidate the specific functions of individual members of the PKC family in control of cell cycle exit/entry and progression through the major cell cycle transitions.

3.2.6. PKC-mediated control of cell growth and cell cycle progression in self-renewing tissues *in situ*

A complete understanding of the mechanisms involved in PKC-mediated regulation of cell cycle progression will require integration of the information gathered from relatively simple, well-defined systems such as cultured cell lines with that obtained from their *in situ* normal and pathological counterparts. The intestinal epithelium is an attractive system for these studies because of its well-defined crypt/villus unit, where proliferating, differentiating, functional, and senescent cells are topologically restricted to distinct compartments (lower half of crypt, upper half of crypt, villus, and villus tip, respectively). Using a combined morphological and biochemical approach, Saxon et al. (142) characterized the profile of PKC isozyme expression and activation status along the crypt/villus unit at the individual cell level. This analysis revealed that (a) levels of PKC activity markedly increase as intestinal epithelial cells migrate from the proliferative to the functional zone, (b) most members of the PKC family (PKC alpha, beta II, delta, epsilon, zeta, eta, theta, and iota) are expressed in intestinal epithelial cells, (c) the expression and activation of these isozymes are differentially regulated with respect to cell growth and differentiation in intestinal tissues, and (d) several PKC isozymes, (e.g., PKC alpha and delta), are activated precisely at the point within intestinal crypts at which cells cease dividing. These data indicated that one or more members of the PKC family are involved in negative regulation of cell growth and cell cycle progression in this system. Analysis of the developmental control of G_1 regulatory molecules along the crypt/villus axis (37) revealed that PKC activation in the mid-crypt region correlates with induction of $p21^{waf1/cip1}$ and $p27^{kip1}$, but not with alterations in the expression or subcellular distribution of INK4 CKIs. Elevated levels of $p21^{waf1/cip1}$ are maintained during early stages of differentiation, but return to baseline at later stages of the process (also see 123). In contrast, $p27^{kip1}$ induction is sustained along most of the length of the villus. PKC activation also coincided with the disappearance of p107, decreased levels of pRb, and marked accumulation of p130 (37). Biochemical analysis of isolated intestinal epithelial cells at different developmental stages demonstrated that pRb is hypophosphorylated in post-mitotic cells, while p130 accumulates as forms 1/2 of the molecule. Thus, activated PKC appears to be appropriately positioned along the crypt/villus axis to initiate a coordinated program of events associated with cell cycle exit into G_0 and induction of differentiation (79, 81). A direct link between these events and PKC signaling is supported by the fact that they could be recapitulated in the IEC-18 intestinal crypt cell line by activation of PKC with phorbol esters or DAG analogues (37, 45). As described above, treatment of IEC-18 cells with these agents resulted in induction of Cip/Kip CKIs, inhibition of cdk2 activity, coordinated changes in pocket protein phosphorylation and expression levels, disappearance of the DNA licensing factor cdc6, and cell cycle arrest in a G_0-like state. Many of these cell cycle regulatory changes have been observed in models of intestinal epithelial differentiation *in vitro* [e.g., the CaCo-2 colon adenocarcinoma cell line (143-145) and conditionally immortalized human fetal intestinal epithelial cells (120)], indicating that they are part of a normal program of intestinal maturation. Taken together, these findings support the notion that certain members of the PKC family participate in negative control of cell cycle progression

during tissue renewal *in vivo*. It is notable in regard to this proposed function of PKC that intestinal tumors exhibit a marked reduction in the expression of certain PKC isozymes (146-148), including PKC alpha which seems to be downregulated at early stages of intestinal carcinogenesis (Black, unpublished data). The disappearance of this negative regulator of cell cycle progression may confer a growth advantage to intestinal cells and thus contribute to the development of intestinal neoplasms (141, 147, 148).

Recent studies in transgenic mice have indicated that PKC activity can also be associated with positive control of intestinal cell growth. Targeted overexpression of PKC beta II in mouse colonic crypt cells resulted in hyperproliferation of the colonic epithelium and an increased susceptibility to azoxymethane-induced aberrant crypt foci (149). These findings are consistent with reports demonstrating the involvement of PKC beta I and beta II in stimulation of HD3 colon carcinoma cell growth (150, 151), and with evidence that colon tumor cells express increased levels of these isozymes relative to normal colonoctyes (148, 152, 153). Accumulating data, therefore, indicate that individual members of the PKC family may mediate positive or negative regulation of cell growth and cycle progression in the intestinal epithelium, and that the growth-regulatory consequences of PKC agonists depend on the panel of PKC isozyme(s) that are activated in these cells.

Studies in the skin model of epithelial renewal further support the idea that certain PKC isozymes are important regulators of the cell cycle. Epidermal keratinocyte differentiation is a tightly regulated, stepwise process that requires PKC activation (114, 115); recent evidence has implicated the PKC alpha (114), delta and eta (115) isozymes in triggering a terminal differentiation program in these cells. Early studies demonstrated that a single topical application of the phorbol ester PMA to hairless mouse epidermis produced profound alterations in cell population kinetics in this tissue (154). The time course of the response to PMA treatment could be divided into two periods: the first period (0-12h) was characterized by a transient block in cell cycle progression into S and M phases, while the second period (12-96h) was associated with partly synchronized cells displaying multiple waves of DNA synthesis and cell division, with a considerable reduction in cell cycle time. The end result was a hyperplastic epithelium composed of a relatively high proportion of immature cells. Later studies (155) showed that a single application of PMA to mouse skin causes a rapid and sustained loss of PKC activity lasting for 3-4 days. Interpreted in the context of current understanding of PKC-mediated regulation of the cell cycle, these findings indicate that PMA-induced PKC activation in skin results in an initial bimodal cell cycle arrest in G_1 and G_2 phases (42), which is reversed coincident with downregulation of the enzyme. These findings further point to the role of some members of the PKC family in promoting cell cycle arrest and differentiation, and demonstrate that loss of certain PKCs can result in uncontrolled growth and possibly transformation.

4. PERSPECTIVES

Accumulating evidence points to PKC as an important regulator of cell growth and cell cycle progression. Members of this family negatively or positively regulate critical cell cycle transitions, including cell cycle entry and exit and the G_1 and G_2 checkpoints. Data from studies in a broad spectrum of cell types indicate that PKC-mediated cell cycle arrest in G_0/G_1 and G_2 phases involves common mechanisms, with the Cip/Kip CKI $p21^{waf1/cip1}$ being a key player at both transitions. Although there is evidence that PKC beta II promotes the $G_2 \rightarrow M$ transition, understanding of the molecular mechanisms underlying positive regulation of cell cycle progression by PKC remains an important challenge for the future. Other major challenges include elucidating the specific functions of individual members of the PKC family in regulation of cell growth/cell cycle progression, and understanding the signaling pathways activated by these molecules to control major cell cycle transitions.

The importance of PKC in regulation of cell growth is supported by evidence of its involvement in transformation. It has been proposed that member(s) of this family can act as tumor suppressors in some systems, a potential role which is well exemplified in intestinal epithelial cells. Overexpression of PKC alpha (99; Leontieva, Uronis, and Black, unpublished data), beta I (33), or delta (31) in colon carcinoma cell lines or in non-transformed intestinal epithelial cells retards cell growth and, in the case of tumor cell lines, inhibits tumorigenicity in nude mice. Expression of PKC alpha is abrogated in the majority of colon adenocarcinomas (147, 148), and its absence from ApcMIN mouse adenomas (Black, unpublished data) indicates that it is lost early during intestinal carcinogenesis. In contrast, PKC isozymes have also been implicated in promoting carcinogenesis in many cell types. In colonic epithelial cells, for example, both PKC epsilon (30) and PKC beta II (149) have been linked to uncontrolled cell growth and transformation. A better understanding of the cell cycle targets regulated by PKC isozymes may, therefore, point to novel sites for intervention in the treatment of cancer.

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