Protein Kinase C Isoforms Involved in the Transcriptional Activation of Cyclin D1 by Transforming Ha-Ras*

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Transformation of the cyclin D1 promoter has remained unclear. Whether PKCs are involved in signal transmission from Ras to the cyclin D1 promoter by oncogenic Ras appears to be mediated by several pathways leading to the activation of multiple transcription factors which interact with distinct elements of the cyclin D1 promoter. The present investigations revealed that cyclin D1 induction by transforming Ha-Ras is MEK-and Rac-dependent and requires the PKC isoforms ε, λ, and ζ, but not cPKC-α. This conclusion is based on observations indicating that cyclin D1 induction by transforming Ha-Ras was depressed in a dose-dependent manner by PD98059, a selective inhibitor of the mitogen-activated kinase MEK-1, demonstrating that Ha-Ras employs extracellular signal-regulated kinases (ERKs) for signal transmission to the cyclin D1 promoter. Evidence is presented that PKC isoforms ε and ζ, but not λ are required for the Ras-mediated activation of ERKs. Expression of kinase-defective, dominant negative (DN) mutants of nPKC-ε or aPKC-ζ inhibit ERK activation by constitutively active Raf-1. Phosphorylation within the TEY motif and subsequent activation of ERKs by constitutively active MEK-1 was significantly inhibited by DN aPKC-ζ, indicating that aPKC-ζ functions downstream of MEK-1 in the pathway leading to cyclin D1 induction. In contrast, TEY phosphorylation induced by constitutively active MEK-1 was not effected by nPKC-ε, suggesting another position for this kinase within the cascade investigated. Transformation by oncogenic Ras requires activation of several Ras effector pathways which may be PKC-dependent and converge on the cyclin D1 promoter. Therefore, we investigated a role for PKC isoforms in the Ras-Raf-mediated transcriptional regulation of cyclin D1. We have been able to reveal that cyclin D1 induction by oncogenic Ha-Ras is Rac-dependent and requires the PKC isoforms ε, λ, and ζ, but not cPKC-α. Evidence is presented that aPKC-λ acts upstream of Rac, between Ras and Rac, whereas the PKC isoforms ε and ζ act downstream of Rac and are required for the activation of ERKs.

Transformation by oncogenic Ras requires activation of several Ras effectors which, in turn, stimulate different signaling pathways. These Ras effectors include Raf, Ras-GDS, PI 3-kinase, and Rac-1 (1). Activation of Rac-1 by oncogenic Ras is mediated by PI 3-kinaseε-dependent (2, 3) and PI 3-kinase-independent mechanisms (2). These Ras effector pathways may converge on the cyclin D1 promoter (1, 4). Evidence for a positive regulation of the cyclin D1 promoter by Ras has been presented (4–7).

The Ras-Raf-ERK cascade has been described as the dominant pathway by which Ras transmits signals to the cyclin D1 promoter (4, 8–10), although in nontransformed cells the situation may be different (1). One effect of the stimulation of ERK1 by Ras seems to be the activation of Ets-2, since expression of plasmids encoding DN Ets (Ets-LacZ) antagonized ERK-dependent activation of the cyclin D1 promoter (4) and mutation of an Ets-binding site (term EtsB (6)) strongly reduced basal and also Ras-induced activation of cyclin D1.

Another Ras effector, Rac-1, has also been shown to induce cyclin D1 transcription in a p65 PAK-dependent manner (11, 12). Rac-1 also activates c-Jun NH2-terminal kinase, which in turn activates the JUN members of the AP-1 transcription factor family. AP-1 transcription factors activate the cyclin D1 promoter through a critical AP-1 site (4, 13).

In addition to the AP-1 site, a CRE site (13, 14) is involved in induction of cyclin D1 transcription triggered by Ras (6), pp60cra (5), or SV40 small T antigen (13). The CRE-binding protein is activated by several kinases including ERK 1/2 and a p38-dependent kinase cascade triggered by Rac-1 (15).

Thus, transcriptional activation of the cyclin D1 promoter by oncogenic Ras appears to be mediated by several pathways leading to the activation of multiple transcription factors which interact with distinct elements of the cyclin D1 promoter. Whether PKCs are involved in signal transmission from Ras to the cyclin D1 promoter has remained unclear.

PKCs represent a family of structurally related serine/threonine protein kinases known to comprise 11 isoforms. The various PKC isoforms are classified into three major subgroups: (i) the classical or conventional PKC isoforms (cPKCs) which are Ca2+- and diacylglycerol-dependent, namely cPKC-α, cPKC-β1, cPKC-β2, and cPKC-γ; (ii) Ca2+-independent, but diacylglycerol-responsive PKC isoforms that have been termed novel PKCs (nPKCs) and comprise the isozymes nPKC-δ, nPKC-ε, and nPKC-θ; and (iii) the so-called atypical PKC isoforms that require neither Ca2+ nor diacylglycerol for activation and currently known to comprise aPKC-λ and aPKC-ζ (16).

The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; DN, dominant negative; ERK, extracellular regulated kinase; CA, constitutively active; MAP, mitogen-activated protein; GST, glutathione S-transferase.
PKC isozymes have been shown to be implicated in Ras-mediated induction of c-fos (17), activation of Raf-1 (17, 18), receptor tyrosine kinase, and Raf-mediated stimulation of ERK 1/2 (17, 19–22), RhoA, and Rac-1, as well as PI 3-kinase-dependent reorganization of the actin cytoskeleton (23). Furthermore, evidence is presented that atypical aPKC-ζ and -ε associate with Cdc42 in a GTP-dependent manner, but use different pathways as Rac for the reorganization of F-actin stress fibers (24).

In view of all these reports we investigated whether PKC isoenzymes are playing a functional role in the transcriptional activation of cyclin D1 by transforming Ras. In this paper evidence is presented that transcriptional activation of cyclin D1 by oncogenic Ras is MEK-1-, and Rac-1-dependent and requires aPKC-ζ, nPKC-ε, and aPKC-ε, but not cPKC-α.

The data support a tentative model for different signaling pathways in which aPKC-ζ acts upstream of Rac-1 and MEK-1, whereas PKC-ζ is required for the activation of ERKs working downstream of MEK-1. Expression of a DN mutant of nPKC-ε inhibits ERK activation by constitutively active (CA) Raf-1, but ERK TEY phosphorylation induced by CA MEK-1 was not affected by nPKC-ε, suggesting another position of nPKC-ε within the cascade investigated.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Cell culture media were obtained from Benderson (Vienna, Austria), epidermal growth factor and insulin were obtained from Sigma (Vienna, Austria). Restriction enzymes for molecular biological approaches were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and Promega (Mannheim, Germany). Polymerase chain reaction primers used for subcloning strategy were obtained from ARK Scientific (Darmstadt, Germany) and MWG Biotech (Ebersberg, Germany). TransFast transfection reagents and Dual-Luciferase reporter assay system were obtained from Promega (Mannheim, Germany). Opti-MEM I medium was obtained from Life Technologies, Inc. (Vienna, Austria). Aprotinin and leupeptin were purchased from Sigma (Vienna, Austria). [γ-32P]ATP (10 mCi/mmol) was obtained from PerkinElmer Life Sciences (Vienna, Austria). Mouse monoclonal 12CA5 anti- HA and anti-c-Myc 9E10 antibody were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit polyclonal anti-ERK1 and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Tachisorb Rabbit polyclonal anti-ERK1 and anti-ERK2 antibodies were purchased from Roche Molecular Biochemicals. Rabbit polyclonal anti-ERK1 and anti-ERK2 antibodies were purchased from Sigma (Vienna, Austria). p38 MAP kinase inhibitor failed to block Ras-stimulated cyclin D1 induction, even at a concentration 5-fold above the IC50 value described for living cells (25, 26) (data not shown).

RESULTS

Transcriptional Activation of Cyclin D1 by Ha-Ras Requires MEK—The Ras-Raf-ERK cascade has been described as the dominant pathway by which Ras transmits signals to cyclin D1. Therefore, we investigated whether the Ras-mediated induction of the cyclin D1 promoter can be blocked by a selective MEK-1 inhibitor in mouse mammary epithelial (HC11) cells. As shown in Fig. 1, PD98059 inhibited Ras-mediated transcriptional activation of the cyclin D1 reporter construct containing the full-length wild type promoter in a dose-dependent manner. The inhibition seems to be MEK-1-specific, since SB 203580, a p38 MAP kinase inhibitor failed to block Ras-stimulated cyclin D1 induction, even at a concentration 5-fold above the IC50 value described for living cells (25, 26) (data not shown).

Ha-Ras-mediated Transcriptional Activation of Cyclin D1 Requires the PKC Isozymes nPKC-ε, aPKC-ζ, and aPKC-ε, but Not cPKC-α—To characterize the biological function of PKC isozymes supposed in the pathway from transforming Ha-Ras to the cyclin D1 promoter, DN and CA mutants of several PKCs were transfected in the presence or absence of CA L61 Ha-Ras. As shown in Fig. 2A coexpression of Ras with DN mutants of PKC-ε, -ζ, or -ι significantly depressed the Ras-mediated induction of cyclin D1. To determine whether the cyclin D1 re-
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Fig. 1. Transcriptional activation of cyclin D1 by Ha-Ras requires MEK-1. Dose-response effects of the MEK-1 inhibitor PD98059. Ha-Ras-mediated transcriptional activation of the full-length wild type cyclin D1 promoter. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.15 μg of pSRα-L61-Ras, together with 0.225 μg of pA3(−1745) cyclin D1-Luc in the absence of presence of PD98059 concentrations as indicated. Ten ng of pSV40-Renilla plasmid was added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were preincubated with various concentrations of PD98059 for 1 h, harvested, and extracts were prepared for the cyclin D1 luciferase reporter assay as described under “Experimental Procedures.” No unspecified effects of PD098059 and the solvent control were detectable during the incubation period of the controls. Data are expressed as the mean (±S.D., n = 9) of three independent experiments done in triplicate.

Fig. 2. Transcriptional activation of cyclin D1 by oncogenic Ras in HC11 cells is blocked by DN nPKC-ε, DN aPKC-ζ, and DN AKPCK-λ, but not by DN cPKC-α. A, role of PKC isotypes on Ha-Ras induced transcriptional activation of cyclin D1. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.15 μg of pSRα-L61 Ras, 0.225 μg of DN cPKC-α K368R, DN nPKC-ε K436R, DN aPKC-ζ K275W, or DN aPKC-λ K275W, respectively. Ten ng of pSV40-Renilla-Luc and 0.225 μg of pA3(−1745) cyclin D1-Luc reporter plasmid were added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were harvested, extracts were prepared, and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean (±S.D., n = 12) of four independent experiments done in triplicate. B, induction of endogenous cyclin D1 by transforming Ha-Ras. HC11 cells were grown on 10-cm dishes and co-transfected with 0.6 μg of L61 Ras plus DN aPKC-ζ K368R, DN nPKC-ε K436R, DN cPKC-α K368R, DN aPKC-λ K275W, pEF-neo (2.2 μg each) or pEF-neo (2.8 μg). To select for transfected cells, all transfections were carried out with 0.6 μg of truncated pMACS-H2K as a selection marker. Forty-eight h post-transfection, cells were harvested and selected for H2K expression by using a MACS-selection system (Miltenyi Biotech). H2K positive cells were lysed and separated on a SDS-10% polyacrylamide gel. Cyclin D1 (monoclonal antibody, Santa Cruz Biotechnology, Heidelberg, Germany) and GFP (monoclonal antibody, Santa Cruz Biotechnology, Heidelberg, Germany) as a loading and expression control were detected by immunoblotting with the corresponding antibodies as described under "Experimental Procedures" for ERK.

12) of four independent experiments done in triplicate.

PKC Isoenzymes ε and ζ, but Not λ Are Involved in the Activation of ERKs by CA Ras, Raf, or MEK—In a previous publication (23) we demonstrated that transcriptional activation of c-fos by transforming Ha-Ras requires the concerted action of PKC-λ, ε, and ζ. Furthermore, evidence was presented in this paper that nPKC-ε and aPKC-ζ act downstream of Raf, whereas aPKC-λ acts either upstream or independently of the Ras-Raf pathway. To determine whether nPKC-ε and aPKC-ζ are required for ERK activation by the Ras-Raf-ERK pathway, HC11 cells were co-transfected with constructs en-

Porter reflects the behavior of the endogenous cyclin D1, it was tested whether expression of L61 Ha-Ras leads to an induction of endogenous cyclin D1 and if this effect is influenced by coexpression of DN mutants of PKC isoenzymes. Fig. 2B demonstrates that this was indeed the case.

Expression of a DN mutant of cPKC-α which had previously been shown to suppress Ras-mediated phosphorylation of MARCKS protein (27) did not affect transcriptional activation of the cyclin D1 by Ras (Fig. 2, A and B). The results obtained using isotype-specific DN PKC mutants indicate that L61 Ha-Ras-mediated activation of cyclin D1 requires the PKC isotypes ε, λ, and ζ, but not α.

We next investigated whether CA mutants of PKC-ε, λ, and ζ induced cyclin D1 in the absence of L61 Ha-Ras. This was indeed the case (data not shown). Expression of CA cPKC-α, however, was ineffective with regard to cyclin D1 induction. The expression levels of all PKC mutants in transiently transfected HC11 cells were determined as described previously (17) and found to be equal. Transfection with all vectors encoding PKC mutants led to a significant overexpression of the mutants compared with the corresponding wild type (data not shown).

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Fig. 3 shows that expression of DN mutants of nPKC-ε or atypical aPKC-ζ caused a marked inhibition of ERK-1 activation by L61 Ha-Ras (Fig. 3A) or Raf-BXB (Fig. 3B). No effect of the PKC mutants on the ERK-1 protein concentration could be observed (Fig. 3, A and B). The two PKC isozymes, however, act on different targets within the Ras > Raf > ERK cascade. Whereas DN nPKC-ε did not affect MEK-1-mediated activation of ERK 1/2, DN aPKC-ζ strongly inhibited ERK-1 phosphorylation within the TEY motif by CA MEK-1 (Fig. 4A) and depressed the kinase activity of ERK-1 (Fig. 4B). Identical results were obtained for ERK-2 with these two PKC isoforms. This assumption is further strengthened by the fact that aPKC-ζ does not physically interact with MEK-1 in pull-down experiments, as well as in HC11 cells transfected with aPKC-ζ (data not shown).

Transcriptional Activation of Cyclin D1 by Ha-Ras Requires the Small GTPase Rac-1—In a previous study (23) we demonstrated that the reorganization of the actin cytoskeleton by L61 Ha-Ras is mediated by a pathway containingos aPKC-λ, Rac-1, and aPKC-ζ in this sequential order. In view of these data it
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A, CA V12-Rac-1 activates transcription of cyclin D1 in the absence of oncogenic Ras. Transcriptional activation of cyclin D1 by oncogenic Rac is depressed by coexpression of DN N17 Rac-1 in HC11 cells. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.15 μg of pSRa-L61 Ha-Ras, 0.225 μg of CA V12 Rac-1, or 0.225 μg of DN N17-Rac-1 alone, or in combination, respectively. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Ten ng of pSV40-β-galactosidase and 0.225 μg of pA3(-1745) cyclin D1-Luc reporter plasmid were included to all combinations. Forty-eight h post-transfection, cells were harvested, extracts were prepared, and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean ± S.D., n = 12 of four independent experiments done in triplicate. B, Ras-mediated cyclin D1 expression is not influenced by coexpression of DN N17-RhoA, CA V14-RhoA is not a transcriptional activator of cyclin D1 in the presence of oncogenic Ras. Forty-eight h post-transfection, cells were harvested, extracts were prepared and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean ± S.D., n = 12 of four independent experiments done in triplicate.

Table I demonstrates that CA V12-Rac activated transcription of cyclin D1, although compared with L61 Ha-Ras, proved to be a relatively weak inducer of cyclin D1. Expression of DN N17-Rac, however, completely abrogated the transcriptional activation of cyclin D1 by L61 Ha-Ras. Thus, in HC11 cells, Rac-1 is essential for the induction of cyclin D1 by oncogenic Ras. This is in contrast to RhoA, where DN N17-Rho did not inhibit cyclin D1 induction by Ras and CA V14 Rho (Table I, row 9). CA V14 Rho alone did not cause a significant transcriptional activation of the cyclin D1 luciferase reporter (Table I, row 10).

A typical aPKC-λ acts upstream of Rac-1, whereas PKC isotypes e and ζ function downstream of Rac-1—As shown in Table I, Rac-1 is essential for the transcriptional activation of cyclin D1 by L61 Ras. However, although signal transduction through Rac-1 is necessary for Ras-mediated cyclin D1 induction in HC11 cells, it cannot be concluded that signaling through Rac-1 is sufficient for cyclin D1 induction by Ras.

Signals mediated through the Ras-Rac pathway may have to combine with signals mediated by other Ras effectors which may employ PKC isotypes for signal transduction. Therefore, we investigated whether any of the three PKC isozymes required for Ras-mediated cyclin D1 induction function within the Ras-Rac pathway. Fig. 5 demonstrates that cyclin D1 induction by V12-Rac was depressed by coexpression of either DN nPKC-ε or DN aPKC-ζ, but not by DN aPKC-λ, indicating that nPKC-ε and aPKC-ζ act downstream of Rac-1, whereas aPKC-λ functions either upstream or independently of Rac-1. A model in which aPKC-λ acts upstream of Rac-1, i.e. between Ras and Rac, would be consistent with studies of Ras-mediated reorganization of the actin cytoskeleton in which aPKC-λ acts upstream and aPKC-ζ downstream of Rac-1 (23). If this model appeared conceivable that aPKC-λ mediates signals from Ras to the cyclin D1 promoter through a Ras-Rac-Rho pathway.

Fig. 4. Role of PKC isoforms in CA MEK induced ERK activity. A, activation of HA-tagged ERK1 by CA MEK-1 in the presence of a DN aPKC-ζ and DN nPKC-ε. HC11 cells growing in 6-well plates were co-transfected with the corresponding vector control pEF-neo, 0.4 μg of CA MEK-1, 0.5 μg of ERK1-HA, and 1 μg DN of nPKC-ε K436R or DN aPKC-ζ K275W, respectively. Forty-eight h post-transfection, cells were harvested and MAP kinase enzyme activities were determined as described in the legend to Fig. 3. Expression of ERK protein was confirmed by subjecting membranes to Western blotting. Representative enzyme activities out of three independent experiments are shown. Identical results were obtained with ERK-2 (data not shown). B, phosphorylation of the TEY motif by CA MEK-1 in the presence of DN aPKC-ζ. HC11 cells growing in 10-cm plates were co-transfected with the corresponding vector control pEF-neo, 1 μg of CA MEK-1, 0.8 μg of ERK1-HA, and 1.6 μg of DN aPKC-ζ K275W or aPKC-ζ A/E, respectively. Forty-eight h post-transfection, cells were harvested, HA-tagged ERK1 was immunoprecipitated with anti-HA antibody, separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with mouse monoclonal phospho-specific (Thr-202/Tyr-204) p44/p42 MAP kinase antibodies. Representative phosphorylation patterns out of three independent experiments are shown.

| A | Fold induction (± S.D.) |
|---|-----------------------|
| A | Empty vector          |
|   | L61-Ras               |
|   | N17-Ras               |
|   | L61-Ras/N17-Rac      |
|   | V12-Rac              |
| B | Empty vector          |
|   | L61-Ras               |
|   | N17-Rho               |
|   | L61-Ras/N17-Rho      |
|   | V14-Rho              |

TABLE I

Transcriptional activation of cyclin D1 by oncogenic Ha-Ras requires the small GTPase Rac, but not RhoA
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FIG. 5. In contrast to atypical aPKC-ζ, novel nPKC-ε and atypical aPKC-ζ act downstream of Rac-1 in the transcriptional activation of cyclin D1. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.2 μg of CA V12-Rac-1, 0.225 μg of DN nPKC-ε K436R, DN aPKC-ζ K275W, or DN aPKC-λ K275W, respectively. Ten ng of pSV40-Renilla-Luc and 0.225 μg of pA3(-1745) cyclin D1-Luc reporter plasmids were added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were harvested, extracts were prepared, and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean (±S.D., n = 12) of four independent experiments done in triplicate.

FIG. 6. Expression of CA V12-Rac-1 overcomes the inhibition of Ras-mediated cyclin D1 induction by DN aPKC-λ, but not the repression caused by DN nPKC-ε or DN aPKC-ζ. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.15 μg of pSRα-L61 Ha-Ras, 0.2 μg of CA V12-Rac-1, 0.225 μg of DN nPKC-ε K436R, DN aPKC-ζ K275W, or DN aPKC-λ K275W, respectively. Ten ng of pSV40-Renilla-Luc and 0.225 μg of pA3(-1745) cyclin D1-Luc reporter plasmid were added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were harvested, extracts were prepared, and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean (±S.D., n = 12) of four independent experiments done in triplicate.

is correct, the inhibitory effect of DN aPKC-λ on Ras-mediated cyclin D1 induction should be selectively overcome by CA V12-Rac1, whereas the depression exerted by DN mutants of nPKC-ε or aPKC-ζ should not be affected by CA V12-Rac. The data shown in Fig. 6 support this model as expression of V12-Rac overcomes the inhibition of Ras-mediated cyclin D1 induction by DN aPKC-λ, but does not overcome the repression caused by DN mutants of nPKC-ε or aPKC-ζ.

Ras-mediated Transcriptional Activation of the Cyclin D1 Luciferase Reporter Containing Mutated AP-1 and CRE Sites within the Promoter: Role of PKC Isoforms—Previously, we had demonstrated that the induction of c-fos by Ha-Ras requires the cooperative function of the PKC isozymes ε, λ, and ζ (17).

As the cyclin D1 promoter also contains AP-1 and CRE sites (4, 6, 28–30) which interact with c-Fos containing heterodimers (4, 14), it might be suggested that our observations may merely reflect an essential role of c-Fos in the induction of cyclin D1 by Ras.

In that case, one would expect that a deletion of the AP-1 and the CRE sites depresses the responsiveness of Ras to the cyclin D1 promoter and abolishes the PKC dependence of any remaining transcriptional activation. This was not the case, since Ras could activate the mutated promoter as efficient as the wild type promoter (compare Fig. 7, lane 2, with Fig. 1, lane 2), indicating that under these conditions signal transmission from Ras to the cyclin D1 involves other sites within the promoter. Furthermore, the activation of the mutated cyclin D1 promoter predominantly involves the Ras > Raf > ERK pathway, since PD098059 concentrations above 5 μM completely abrogates the Ras effect (Fig. 7, lanes 4 and 5). In contrast to Ras, deletion of both the AP-1 and the CRE sites abrogated the responsiveness of V12-Rac (data not shown), indicating that Rac activates the cyclin D1 promoter in part by these two sites.

We next investigated for a role of PKC isotypes. Fig. 8 demonstrates that Ras-mediated transcriptional activation of a cyclin D1 in which both the AP-1 and the CRE sites had been deleted was suppressed by DN mutants of nPKC-ε and aPKC-ζ, but not by DN aPKC-λ. As this mutated cyclin D1 promoter no longer responded to Rac (data not shown), these findings are consistent with the assumption that in our system, PKC-λ is required, in a Rac-dependent manner, for signal transmission from Ras to cyclin D1.

In summary, the data presented so far suggest that Ras mediates the effects on cyclin D1 via two pathways containing MEK-1 and Rac-1. In the Rac-1-dependent pathway evidence is presented that aPKC-ζ acts upstream of Rac, between Ras and Rac, as well as upstream of MEK-1, whereas aPKC-ζ acts downstream of Rac and is required for the activation of ERKs. The position of nPKC-ε in the signaling pathway from Ha-Ras to the cyclin D1 promoter has to be further apprised.

DISCUSSION

The data presented here demonstrate that in HC11 cells, transcriptional activation of cyclin D1 by transforming L61 Ha-Ras is MEK-1- and Rac-1-dependent and requires the three PKC isozymes λ, ε and ζ. This conclusion is based on the observation that DN mutants of aPKC-ζ, nPKC-ε, and aPKC-λ inhibit cyclin D1 induction by transforming Ras, whereas CA mutants of these enzyme family were found to activate cyclin
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**Fig. 7.** Dose-response effects of the MEK-1 inhibitor PD98059 on an AP-1 and CRE double mutant. HC11 cells growing in 24-well plates were co-transfected as described in the legend to Fig. 1 and the wild type cyclin D1 promoter construct was replaced by an AP-1/CRE double mutant (0.225 μg of pA3(−1745) cyclin D1-AP1/CREmut-Luc). Ten ng of pSV40-Renilla plasmid was added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were preincubated with various concentrations of PD98059 for 1 h, harvested, and extracts were prepared for the cyclin D1 luciferase reporter assay as described under “Experimental Procedures.” No unspecific effects of PD98059 and the solvent control were detectable during the incubation period of the controls. Data are expressed as the mean (±S.D., n = 9) of three independent experiments done in triplicate.

**Fig. 8.** PKC dependence of Ras-mediated transcriptional activation of cyclin D1 lacking functional AP-1 and CRE sites. HC11 cells growing in 24-well plates were co-transfected with the corresponding vector control pEF-neo, 0.15 μg of pSRα-L61 Ha-Ras, 0.225 μg of DN cPKC-α K368R, DN nPKC-ε K368R, DN aPKC-ζ K275W or DN aPKC-λ K275W and pA3(−1745) cyclin D1-AP1/CREmut-Luc reporter plasmids, respectively. Ten ng of pSV40-Renilla-Luc plasmid was added to all samples. The total amount of plasmid DNA was adjusted to 0.7 μg with empty pEF-neo vector. Forty-eight h post-transfection, cells were harvested, extracts were prepared, and cyclin D1 luciferase expression was determined as described under “Experimental Procedures.” Data are expressed as the mean (±S.D., n = 9) of three independent experiments done in triplicate.

D1 by a Ras-independent mechanism (data not shown).

PKC-α which is also expressed in HC11 cells, is not employed by Ras for the induction of cyclin D1. Neither DN nor CA versions of cPKC-α were found to affect cyclin D1 induction by Ras.

As suggested in our recent publication (23), aPKC-λ acts upstream of Rac in Ras-mediated reorganization of the F-actin cytoskeleton. PKC isotypes ε and ζ were found to function downstream of Raf-1 (17).

The Ras-Raf pathways have been shown to be interconnected by PAK (31). Activation of PAK by Rac is considered essential for cyclin D1 induction by Rac, whereas activation of c-Jun NH₂-terminal kinase or p38 are found to be dispensable for cyclin D1 induction by Rac (11). PAK-1 has been shown to phosphorylate and enhance the activity of MEK-1 (32). Thus, Rac may require the Rac > PAK > MEK pathway for a full activation of ERK 1/2. Overexpression of ERK-1 directly induced cyclin D1 (4). All these findings indicate that ERKs are involved in transcriptional activation of cyclin D1 by Ras, as well as by Rac.

In our system the MEK-1 specific inhibitor PD098059 significantly inhibited transcriptional activation of cyclin D1 by Ras. PD098059 abrogated transcriptional activation by Ras when both, the AP-1 and the CRE sites were deleted, demonstrating that under these conditions the Ras-Raf-ERK pathway, presumably resulting in activation of an Ets site, is predominant for signal transmission from Ras to the cyclin D1 promoter. This is in agreement with the findings reported by others (4), indicating that ERK induction of cyclin D1 was blocked by dominant negative Ets.

We previously demonstrated that PKC isozymes ε and ζ act downstream of Raf-1 in the Ras-Raf-MEK-ERK pathway activating transcription of c-fos (17). The data presented here further enlarge the assumption that PKC isotypes ε and ζ are involved in the activation of ERKs by Ras and are in agreement with data published by others (20, 33–39).

The molecular mechanism by which PKC stimulates ERK is, however, still unclear. It was demonstrated that conventional and novel PKCs (α and ε) are potent activators of c-Raf-1, whereas atypical aPKC-ζ stimulates MEK by a different mechanism (20, 38).

DN PKC-ζ strongly inhibits phosphorylation and activation of ERK 1/2 by CA MEK-1, indicating that aPKC-ζ functions downstream of MEK-1. This assumption is further strengthened by the fact that aPKC-ζ does not physically interact with MEK-1 in pull-down experiments (data not shown). The mechanism by which nPKC-ε and aPKC-ζ regulate ERK activation remains to be elucidated. A direct PKC-catalyzed phosphorylation of ERKs could be excluded. Furthermore, no evidence for an inhibition of MAP kinase phosphatases by PKC isotypes could be detected (not shown).

Another model which is presently under investigation, concerns a PKC-regulated interaction with scaffold proteins which may be essential for the selective activation of ERKs (40–43). As recently reported Ha-Ras employs the same PKC isotypes for the induction of c-fos (17). Evidence for a functional role of c-fos in the transcriptional regulation of cyclin D1 has been
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published (14, 44, 45). Thus, it may appear that the findings presented here simply reflect a requirement for c-fos in the transcriptional activation of cyclin D1, but the following considerations, however, suggest that this is not the case.

The cyclin D1 promoter contains two sites that could mediate a direct induction by c-Fos: a classical AP-1 site (at approximately −950) and a CRE/ATF site at approximately −60 (4, 28). Both sites have been shown to bind c-Fos containing heterodimers (4, 14).

These findings were confirmed by us in HC11 cells where stimulation of the Ras pathway by epidermal growth factor was shown to result in binding of c-Fos/c-Jun heterodimers to both the AP-1 and the CRE sites (not shown). The observation that deletion of both the AP-1 and the CRE sites neither depressed cyclin D1 induction by Ras nor abolished the PKC dependence of Ras-mediated transcriptional activation, suggests that transcriptional induction of c-fos is not sufficient for cyclin D1 induction by oncogenic Ha-Ras. Candidates for additional sites are Ets-binding domains and E2F sites. Multiple putative Ets-binding sites can be detected within the cyclin D1 promoter. Ets sites essential for Ras-mediated cyclin D1 induction have been located at position −778 (6) and within the proximal promoter region (4). Mutation of a E2F site located in a truncated cyclin D1 promoter lacking the sequence upstream of position −163 strongly reduced the responsiveness to transcriptional activation by Neu (46).

In agreement with a previous publication (11), it was also found that transforming Ha-Ras employs the Ras-homology protein Rac-1 for transcriptional activation of cyclin D1. DN N17-Rac-1 blocked Ras-mediated induction of cyclin D1 and CA V12-Rac activated transcription of cyclin D1 in the absence of transforming Ras. Induction of cyclin D1 by V12-Rac is depressed by DN mutants of nPKC-ε and aPKC-ζ, but not, however, by DN aPKC-λ, indicating that PKC isotypes ε and ζ act downstream of Rac. The inhibitory effect of DN aPKC-λ, however, was overcame by V12-Rac, whereas V12-Rac had no effect on the suppression of Ras-mediated cyclin D1 induction by DN PKC isoforms ε and ζ. Therefore, it can be concluded that aPKC-λ acts upstream of Rac-1, whereas PKC isozymes ε and ζ function downstream of Rac-1.

The ability of Rac-1 to bind and activate p21-activated kinase p65 PAK was shown to correlate with its ability for transcriptional activation of the cyclin D1 promoter (11). It has also been reported that signals from Ras and Rac converge on PAK and evidence for a signaling pathway Rac → Rac → PAK has been presented (47), suggesting that Rac activates transcription of cyclin D1 predominantly via Rac.

If this is correct, Ras and Rac should activate cyclin D1 transcription through the same promoter elements. This, however, was not found to be the case. Since Ras could activate the mutated promoter as efficient as the wild type promoter we have suggested that under these conditions signal transmission from Ras to the cyclin D1 promoter involves other sites.

In HC11 cells, Rac-1 partially activates transcription of cyclin D1 through AP-1 and CRE sites. In accordance with findings by others (31), our data suggest that Rac-1 additionally is involved in the pathway from Ras to the cyclin D1 promoter via the Ras → Raf → ERK cascade, presumably by activating p65 PAK-1 (32). The recent suggestion that PAK-3 could stimulate Raf-1 activity by directly phosphorylating Ser-338 through a Ras-phosphatidylinositol 3-kinase/Cdc42-dependent pathway has attracted much attention (48). However, in HC11 cells, this pathway seems to be of minor importance, as the PI 3-kinase inhibitors wortmannin and LY 294002 did not interfere with cyclin D1 induction by Ras (not shown).

In addition to these sites, binding elements for several other transcription factors have been identified within the cyclin D1 promoter, comprising sites for Sp1, TCF/LEF-1, NF-κB, E2F, Ets, and STAT5 (6, 7, 28–30, 49). The biological function of PKC isoforms in pathways corresponding with these additionally described factors remains to be elucidated.

The observations described in this paper allow us to draw novel and exiting models for the regulation of cyclin D1 by PKCs, however, they also raise many questions that need to be addressed. For example, how can we explain that aPKC-ζ is involved in signal transmission from Ras to cyclin D1 without direct interaction of MEK-1 and/or phosphorylation events necessary for ERK activation. Genetic models, including conditional PKC knock-out mice, will give us more information concerning the biological functions of the PKC isoforms found in these pathways. Experiments in this direction are under way. Together with findings from others (50), explaining the absolute dependence on cyclin D1 from Neu- and Ras-mediated malignant transformation in mammary epithelial cells, inter-

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