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E2F1, B-MYB AND SELECTIVE MEMBERS OF CYCLIN/CDK SUBUNITS ARE TARGETS FOR PROTEIN KINASE C-MEDIATED BIMODAL GROWTH REGULATION IN VASCULAR ENDOTHELIAL CELLS

Wei Zhou, Noriko Takuwa, Mamoru Kumada and Yoh Takuwa§

Departments of Cardiovascular Biology and Physiology,
Faculty of Medicine, University of Tokyo, Tokyo 113 , Japan

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SUMMARY : In human umbilical vein endothelial cells, the protein kinase C (PKC) stimulation during the early G1 phase leads to potentiations in growth factor-stimulated DNA synthesis, the activation of cdc2 and cdk2 cyclin-dependent kinases, and the mRNA expression of cdc2, cyclins A, D1 and E, but not cdk2 or cdk4. Conversely, the PKC stimulation in the late G1 phase completely inhibits DNA synthesis, the activation of cyclin-dependent kinases, and the mRNA expression of the same set of molecules except cyclin D1. Further, we found that the PKC stimulation bimodally regulates the message levels of E2F1 and B-*myb*, which are transcription factors implicated in the control of the mammalian cell cycle progression. These results indicate that the PKC signal transduction pathway, depending on the timing of activation in the G1 phase, either positively or negatively regulates the message level of growth-regulating genes that are crucial for the G1 to S phase progression.

Protein kinase C (PKC) has been nominated as one of the major regulators of cell growth and proliferation. Thus, activators of the PKC such as phorbol esters and cell permeable diacylglycerols, as well as a variety of natural ligands that activate the phospholipase C-PKC pathway, stimulate DNA synthesis in several types of cells [1-11]. It has also been reported, however, that under certain circumstances the PKC activation rather inhibits DNA synthesis induced by growth factors or other mitogens [12-14]. The growth regulation by the PKC activators in both stimulatory and inhibitory directions are abolished in PKC-downregulated cells [2, 5, 7, 11, 13, 14], suggesting that downregulation-sensitive isoforms of the PKC are involved in these phenomena. The cellular condition and the underlying molecular mechanism that determine the direction of the PKC-mediated growth regulation have not been well understood.

We have recently found in human umbilical vein endothelial cells that the PKC mediates a

§ All correspondence should be addressed to Yoh Takuwa, M. D., Department of Cardiovascular Biology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113,Japan. Fax:81-3-5800-6845.

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temporally well defined bimodal growth regulation [15]. Thus, the PKC activation during the early G1 phase in growth factor-stimulated endothelial cells leads to a potentiation of DNA synthesis without affecting the length of the G1 phase, whereas the PKC activation in the late G1 phase completely blocks the entry into the S phase. We have also found that the PKC activation either in the early G1 or the late G1 phase results in a potentiation or complete inhibition, respectively, of activations of cell cycle regulatory cyclin-dependent kinases cdc2 and cdk2, and of phosphorylation of one of their cellular substrates, RB protein, the product of a tumor suppressor retinoblastoma gene. The effects of the PKC activation on these cellular events are all markedly attenuated in PKC-downregulated endothelial cells.

To understand in more depth the molecular mechanism by which the PKC exerts the bimodal growth regulation on endothelial cells, we examined whether the PKC activation in the early G1 or the late G1 phase has any effect on the expression of various cyclins and cdks, the regulatory and the catalytic subunits of the cyclin-dependent kinases. In addition, we studied the effect of the PKC activation on the expression of transcription factors E2F1 and B-*myb*. It is widely known that E2F transactivates several genes including dihydrofolate reductase, thymidine kinase and DNA polymerase α, the expression of which is critical for the entry into the S phase [16]. Moreover, it has recently been demonstrated that microinjection of E2F1 cDNA induces quiescent cells to enter S phase [17]. Human B-Myb protein is a DNA binding protein with properties similar to a homologous protein c-Myb, a transcription factor that plays an essential role for G1/S transition in hemopoietic cells [18-19]. It has been reported that constitutive expression of B-*myb* cDNA in fibroblasts is accompanied by increased expression of cdc2 and cyclin D1 and a transformed phenotype [20]. The results in the present study demonstrates for the first time that E2F1 and B-*myb* as well as selective members of cyclins and cdks are targets for PKC-mediated bidirectional regulation in human vascular endothelial cells.

MATERIALS AND METHODS

Human umbilical vein endothelial cells at passages 3-8 were maintained in a subconfluent state, and before each experiment confluent cells were deprived of growth factors for 24 h [15]. Cell were then incubated in fresh media containing fetal calf serum (3 %), basic fibroblast growth factor (3 ng/ml, R & D Systems) and epidermal growth factor (3 ng/ml, Wakunaga Pharmaceutical Co.), for indicated periods of time. In some cultures phorbol-12, 13-dibutyrate (PDBu) was introduced as indicated. For measurement of [^{3}H]thymidine incorporation into DNA, cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi/ml) (Du Pont-New England Nuclear) during the last 1 h of incubations and the radioactivity incorporated into acid precipitable material was counted [21]. Histone H1 kinase activity associated with either anti-cdc2 or anti-cdk2 immunoprecipitate was measured *in vitro* as described previously [15, 21], using specific rabbit polyclonal antibodies raised against the carboxyl terminal sequence of either human cdc2 or human cdk2. Samples were analyzed on 12.5 % SDS polyacrylamide gel electrophoresis, followed by autoradiography. The radioactivity in the spot corresponding to histone H1 was determined by Fuji BAS 2000 Bio-Image Analyzer. Northern blot analysis was performed as described in detail elsewhere [22]. The following cDNA fragments were obtained with the polymerase chain reaction-amplification method and used as probes for hybridization: human cdc2 (nucleotide 721-935 when A of the initiation codon "ATG" is numbered as 1), human cdk2 (466-1083), human cdk4 (343-846), human cyclin A (76-550),

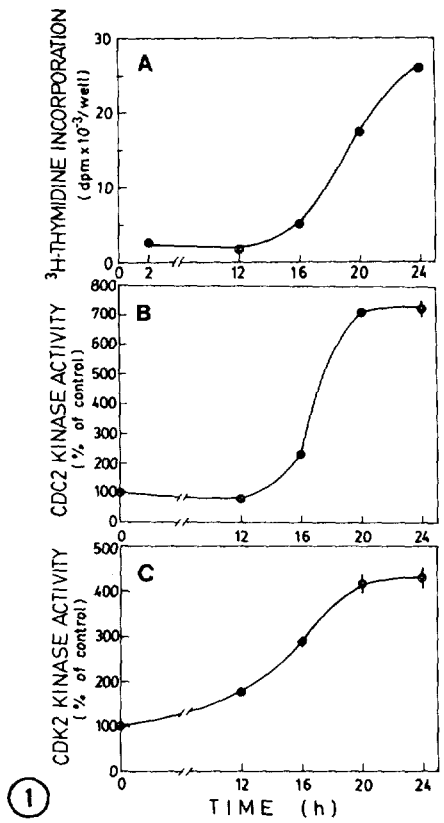
human cyclin E (677-1052), human B-*myb* (595-1145) and human E2F1 (667-1282). Mouse cyclin D1 cDNA was kindly donated by Dr. H. Matsushime (University of Tokyo). Westernblot analysis of PKC isozymes was performed as described in detail previously [23].

RESULTS AND DISCUSSION

Shown in Fig. 1 are the time courses of [^{3}H]thymidine incorporation into DNA (A), the cdc2 kinase activity (B) and the cdk2 kinase activity (C) in growth factor-stimulated human umbilical vein endothelial cells. The rate of DNA synthesis starts to increase after a lag period (= G1 phase) of 12-15 h, and continues to increase until 24 h after the growth factor stimulation. The cdc2 kinase activity starts to increase by 16 h after growth stimulation, which corresponds to G1/S boundary, and reaches the maximal plateau level at 20 h during the observation period. Although cdc2 has been implicated as a M phase kinase in vertebrates [24], we and others have found its activation to start at G1/S boundary in other types of mammalian cells as well [21, 23, 25]. The activity of cdk2 kinase, another cyclin-dependent kinase that has been shown to be indispensable for G1 to S phase progression in higher eukaryotic cells [26, 27], starts to rise by 12 h after growth stimulation, a time point prior to the onset of S phase, and continues to increase to reach the maximal plateau value at 20 h.

As shown in Fig. 2A, the PKC activator PDBu, which by itself does not stimulate DNA synthesis in quiescent endothelial cells, modulates the growth factor-induced DNA synthesis in both stimulatory and inhibitory directions, depending on the timing of its addition during the cell cycle. Thus, the addition of PDBu (10^{-7} M) during the first 1 h of the growth factor stimulation (i. e. during the early G1 phase) leads to an approximately 2-fold potentiation in DNA synthesis. In contrast, when PDBu is added 6 h after the growth factor stimulation (i. e. in the late G1 phase), it completely inhibits the initiation of DNA synthesis. Under the same experimental conditions, PDBu either positively or negatively regulates the cdc2 and the cdk2 kinase activities. Thus, as shown in Figs. 2B and 2C, the PDBu treatment during the early G1 phase results in up to 2-fold potentiations in the cdc2 and the cdk2 kinase activities measured at 21 h. In sharp contrast, when PDBu is added in the late G1 phase, it strongly inhibits both of the kinase activities nearly to the basal unstimulated level. Since PDBu does not affect either the cdc2 or the cdk2 kinase activity when added directly to *in vitro* kinase assay tubes, it is likely that PDBu modulates the activation process of these kinases.

A cyclin-dependent kinase in the catalytically active form is a multiprotein complex including a catalytic subunit cdk and an activator subunit cyclin. It has been demonstrated that cyclin A binds to both cdk2 and cdc2, whereas cyclin E binds to cdk2 but not cdc2, and D type cyclins bind to cdk2 and cdk4 [28]. The molecular mechanisms for regulation of the gene expression of each cdk or cyclin have not been well understood, although recent studies suggest the involvement of E2F1 and B-*myb* in the transcriptional regulation of certain cdks and cyclins [20, 29]. In an attempt to clarify the molecular mechanisms for the PKC-mediated bidirectional regulation of DNA synthesis, we first examined the time-dependent changes after



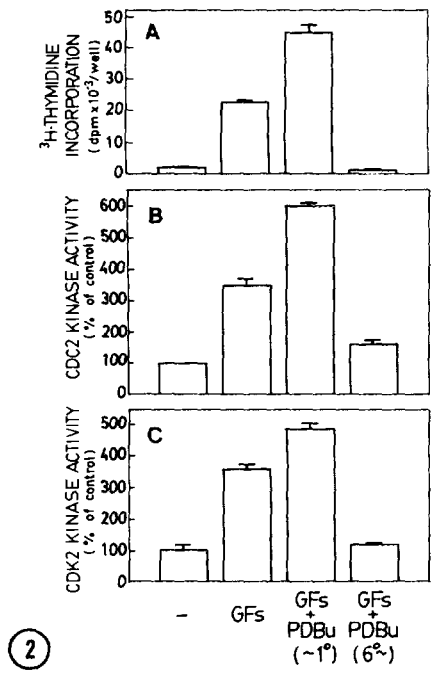


Figure 1. Time courses of [3H] thymidine incorporation into DNA (A), the cdc2 kinase activity (B) and the cdk2 kinase activity (C) in growth factor-stimulated human umbilical vein endothelial cells. Data represent the mean ± S. E. of three determinations.

Figure 2. The effects of PDBu on DNA synthesis (A), the cdc2 kinase activity (B) and the cdk2 kinase activity (C) in growth factor-stimulated endothelial cells. PDBu (10^{-7}) was introduced to the cultures during the first 1 h of the growth factor stimulation (~1°) and then removed in the continued presence of the growth factors (GFs), or was added 6 h after the addition of the growth factors (6°~). Cells were incubated for a total period of 22 h. Data represent the mean ± S. E. of three determinations.

growth factor stimulation in the mRNA levels of cdc2 (= cdk1), cdk2, cdk4, cyclins A, D1, and E, and transcription factors E2F1 and B-*myb* (Fig. 3). The cdk3 message was not detected in this cell type under the present experimental conditions. The mRNAs for cdc2, cyclin A, E2F1 and B-*myb* are barely detectable in quiescent cells. They are induced to become detectable between 8 and 12 h, and reach the maximal levels during the S phase between 20 and 24 h. The cyclin E mRNA shows a similar time course of expression, although quiescent cells show an easily detectable level of basal expression. The mRNAs for cdk2 and cyclin D1 are at moderate levels in quiescent cells and become maximal at 12 h after growth factor stimulation. The expression levels of the cdk2 and cyclin D1 mRNAs decrease as cells progress into the S phase. The basal expression level of cdk4, which is another candidate RB kinase [30], is quite high in quiescent cells and increases only slightly in response to the growth stimulation.

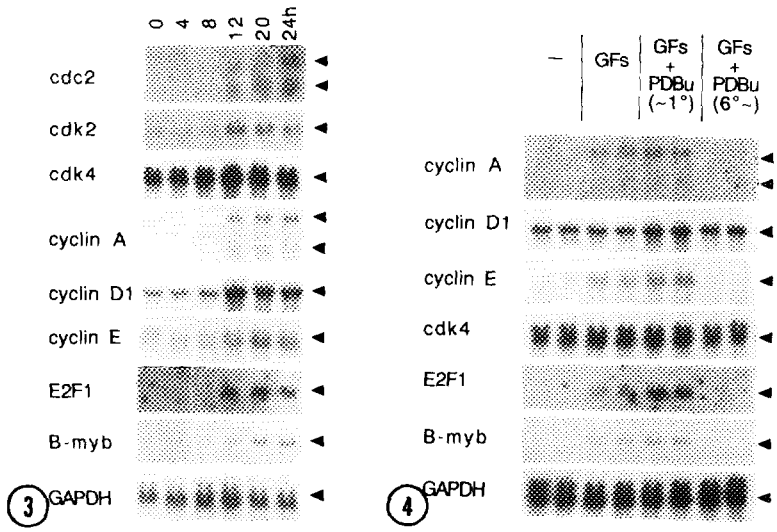


Figure 3. Northern blot analysis of time courses of changes in the message levels of cyclin/cdk subunits and transcription factors E2F1 and B-*myb* in growth factor-stimulated endothelial cells. Northern blot analysis of glyceraldehyde-3-phosphate dehydrogenese (GAPDH) is also shown as an internal control. Cells were incubated with the growth factors for the indicated periods of time (h). Arrowhead indicates the position of respective mRNA.

Figure 4. The effects of PDBu on the mRNA levels of cyclins, cdk4, E2F1 and B-*myb*. PDBu was introduced to the cultures as described in the legend to Fig. 2. Cells were incubated for 14 h (cyclin D1) or 22 h (others).

We next examined the effects of the PKC activation during either the early G1 or the late G1 phase on the mRNA levels of these growth-regulating molecules. As we previously reported, the PKC activation in the early G1 phase results in marked potentiations in the expression levels of cdc2 mRNA and protein, whereas the PKC activation in the late G1 phase leads to suppression of expression of the cdc2 mRNA and protein [15]. In sharp contrast, the cdk2 mRNA and protein levels are barely affected, if any, by the PKC activation either in the early G1 or the late G1 phase [15], despite the fact that the cdk2 kinase activity is regulated bimodally by the PKC activation just like the cdc2 kinase activity (Fig. 2C). These observations raise a possibility that the PKC regulates the cdk2 kinase activity through regulation on the expression of cdk2-associated cyclins. To test this, we treated cells in the same way as shown in Fig. 2 and then analyzed the mRNA levels of various cyclins by Northern blotting. As shown in Fig. 4, the PKC activation during the early G1 phase of the growth factor stimulation results in enhancement of the mRNA expression for cyclins A, D1 and E, all of which have been shown to associate with and activate cdk2 [28]. Conversely, the PKC activation in the late G1 phase completely abolishes the mRNA expression of cyclins A and E, but not cyclin D1. The cyclin D1 mRNA level at 22 h shows a paradoxical increase in response to the PKC activation in the late G1 phase. We have also studied the effect of the PKC activation on the mRNA level of cdk4, a cyclin-dependent kinase that is activated for the most part by D type cyclins [28]. As in the case with cdk2, the message level for cdk4 is

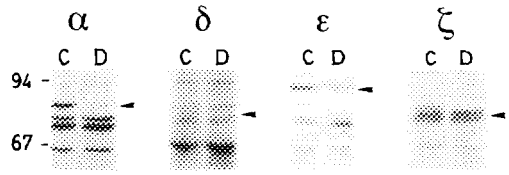


Figure 5. Western blot analysis of PKC isozymes in control endothelial cells (C) and PDBu-pretreated, PKC-downregulated endothelial cells (D). Arrowhead indicates the position of respective PKC isozyme.

minimally affected by the PKC activation either in the early G1 or the late G1 phase. We further examined whether the PKC has any regulatory role for the expression of the transcription factors E2F1 and B-*myb*. The results clearly show that the PKC bimodally regulates the message levels of both E2F1 and B-*myb*, exactly in the same way as those of cdc2, cyclins A and E.

In an attempt to identify the PKC isozyme (s) responsible for the bidirectional regulation of the cell growth, we studied the amount of PKCα, -β, -γ, -δ, -ε and -ζ isoforms in endothelial cells. Shown in Fig. 5 are Western blot analyses of PKC isozymes in quiescent human umbilical vein endothelial cells and PKC-downregulated, quiescent endothelial cells. PKCα, -δ and -ε are present in control cells and markedly decrease in PKC-downregulated cells. In sharp contrast, the protein levels of PKC-ζ does not significantly change after the procedure of PKC downregulation [15]. Under our experimental condition, specific bands corresponding to the PKCβ and -γ isoforms are not detected. The results suggest the involvement of either PKCα, -δ or -ε in PDBu-induced growth regulation of endothelial cells in both stimulatory and inhibitory directions.

The present study demonstrates that in human umbilical vein endothelial cells the PKC-mediated bimodal growth control involves the regulation of the mRNA expression for E2F1, B-*myb*, and selective members of cyclin/cdk subunits including cdc2, cyclins A and E. Since these molecules, especially E2F1, cyclins A and E, have been shown to play pivotal roles for G1/S transition and S phase progression [17, 31-33], it is likely that the PKC exerts the growth-regulatory actions at least in part through the effects on the expression of these genes. It is not known at present, however, whether the PKC exerts these effects at the level of the transcription, the degradation of mRNA, or both. The function of E2F1 may also be regulated at a posttranslational level by the PKC signaling pathway. Thus, the PKC stimulation in the late G1 phase inhibits the activation of RB kinases cdc2 and cdk2 (Fig. 2B and 2C) and keeps RB protein in the unphosphorylated state [15]. It is thought that unphosphorylated RB functions as a negative regulator for G1/S transition, at least in part by forming a complex with and negatively regulating the function of transcription factors including E2F1 [16]. In fact, the RB/E2F1 complex has been shown to repress the transcription of cdc2 [29] and other genes by binding to E2F-binding motif in the promoter regions of these genes.

The present study also demonstrates two points of interest regarding the mechanism for PKC-mediated regulation of cyclin-dependent kinases. First, the PKC stimulation regulates

the activities of cdc2 and cdk2 cyclin-dependent kinases in the same directions but through distinct molecular mechanisms. Second, cdk4/cyclin D1 complex, if it ever functions as a kinase in endothelial cells, would be relatively constitutively active in quiescent cells, and even in growth-arrested cells by the late G1 PKC activation (Figs. 3 and 4). Since RB protein is nearly exclusively in its unphosphorylated form in the quiescent cells as well as the growth-arrested cells [15], it is unlikely that cdk4/cyclin D1 is a major kinase responsible for the phosphorylation of RB protein in human umbilical endothelial cells. However, there is a possibility that cdk4 serves as an RB kinase in a complex with either cyclin D2 or cyclin D3, and that at least a portion of the cdk4 kinase activity is under the control of the PKC signaling pathway, through regulation of the expression of these particular D type cyclins. Further studies, including measurement of the cdk4 kinase activity using RB protein as a substrate (cdk4 does not phosphorylate histone H1), are required to address these possibilities.

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