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Ca^{2+}/calmodulin is involved in growth factor-induced retinoblastoma gene product phosphorylation in human vascular endothelial cells

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In human vascular endothelial cells, both growth factor-induced DNA synthesis and retinoblastoma gene product (RB) phosphorylation are absolutely dependent on extracellular Ca^{2+}, and are potently inhibited by an active calmodulin antagonist, W-7, but not an inactive analogue, W-12. A reduction in the extracellular Ca^{2+} or an addition of W-7 as late as 8 h after growth factor stimulation still inhibits both RB phosphorylation and DNA synthesis to the full extent. However, once RB phosphorylation occurs 12–16 h after addition of the growth factors, it is not reversed by subsequent Ca^{2+} reduction or W-7. These results suggest the existence of a Ca^{2+}/calmodulin-dependent process relatively late in the mitogenic signalling cascade, at a step proximal to RB phosphorylation reaction itself.

Retinoblastoma gene product (RB); RB protein; Ca^{2+}; Calmodulin; Endothelial cell

1. INTRODUCTION

It has been well documented that Ca^{2+} is indispensable for normal cell growth and proliferation [1–3]. It is also suggested that calmodulin plays a role in Ca^{2+}-dependent cell growth regulation [4,5]. A wide variety of transformed cells, by contrast, show a markedly reduced dependence on Ca^{2+} [1,6,7] and increased levels of the cellular calmodulin content, as compared to normal cells [8,9]. Indeed, there is an inverse correlation observed between the Ca^{2+}-dependence and tumorigenic potency in vivo [10]. It is still unclear, however, how Ca^{2+}/calmodulin acts in the cell growth regulatory/signalling cascade. We have recently observed in Swiss-mouse 3T3 fibroblasts that a reduction in the extracellular calcium concentration ($[Ca]_{out}$) inhibits bombesin-induced DNA synthesis without inhibiting any of the bombesin-induced early cellular events, including initial transient Ca^{2+} mobilization, activation of protein kinase C and S6 kinase, and protooncogene expression, for up to 4 h after bombesin addition [11]. These results suggest the existence of a Ca^{2+}-dependent process relatively late in the signal transduction pathway that leads to DNA synthesis.

RB protein, the product of a tumor suppressor retinoblastoma gene, has been implicated as a regulator of normal cell growth: it becomes hyperphosphorylated prior to the onset of DNA synthesis in growth-stimulated cells [12,13], but remains in the underphosphorylated state in either terminally differentiating cells [13–15] or senescent cells [13,16]. The cellular mechanism underlying growth factor-induced RB protein phosphorylation is not yet fully understood.

In the present study, we provide evidence for the involvement of Ca^{2+}/calmodulin relatively late in the mitogenic signalling pathway that leads to RB phosphorylation.

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2. MATERIALS AND METHODS

Human umbilical vein endothelial cells were maintained in a subconfluent state as described [12]. Cells as passages 3–8 were used in the present study. Before each experiment confluent cells were deprived of growth factors by incubation in serum-free media containing 0.2% bovine serum albumin for 24 h. Cells were then incubated in fresh media containing various concentrations of $CaCl_2$ [11], with or without a combination of basic fibroblast growth factor (bFGF) (1 ng/ml, Collaborative Research Inc.), epidermal growth factor (EGF) (10 ng/ml, Wakunaga Pharmaceutical Co.) and 3% fetal bovine serum that had been extensively dialyzed against a 0.85% NaCl solution, in the presence or absence of W-7 or W-12 (Seikagaku Kogyo). For measurement of DNA synthesis, cells were pulse-labeled with [^{3}H]thymidine (2 μCi/ml) during the last 1 h of the indicated periods of incubations [11]. In parallel cultures RB protein phosphorylation was analyzed by 7.5% SDS-PAGE with 20 μg protein in each gel slot, followed by Western blot using a mouse monoclonal anti-human RB peptide antibody (Triton Diagnostics) and an alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody (Zymed Laboratory), as described by Decaprio et al. [12].

3. RESULTS AND DISCUSSION

Stimulation of growth factor-deprived human umbilical vein endothelial cells with a combination of growth factors (bFGF, EGF and dialyzed serum) results in an increase in DNA synthesis after a lag period (G1 phase) of approximately 15 h (Fig. 1A). The growth factor-

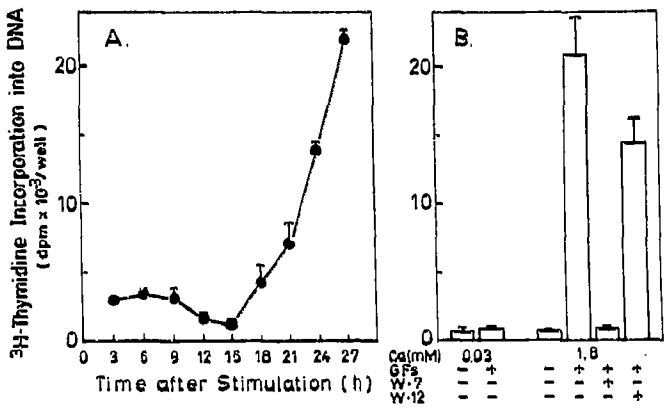

Fig. 1. (A) Time-course of DNA synthesis induced by a combination of growth factors, i.e. bFGF(1 ng/ml), EGF(10 ng/ml) and dialyzed serum (3%), at 1.8 mM extracellular calcium concentration ($[Ca]_{out}$). (B) Effects of $[Ca]_{out}$ (0.03 vs. 1.8 mM) and a calmodulin antagonist, W-7, vs. inactive analogue, W-12 (30 μM each), on DNA synthesis. Cells were incubated for 24 h. GFs, growth factors. Results are from typical sets of experiments performed in triplicate (mean ± S.E.M.).

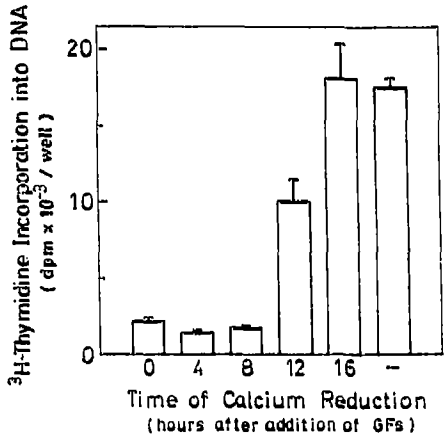

Fig. 2. A reduction in $[Ca]_{out}$ 8 h but not 16 h after addition of the growth factors potently inhibits DNA synthesis. $[Ca]_{out}$ was reduced from 1.8 to 0.03 mM at indicated time points (h) in the continued presence of the growth factors (GFs), and [^{3}H]thymidine incorporation into DNA was compared at 24 h. – denotes that $[Ca]_{out}$ was fixed at 1.8 mM throughout the 24 h experimental period. Data represent the mean ± S.E.M. of three determinations.

induced increase in DNA synthesis is absolutely dependent on $[Ca]_{out}$ (Fig. 1B); it is barely detectable at 0.03 mM calcium, increases linearly with increasing calcium concentration for up to 0.5 mM, and reaches a maximal value at approximately 1 mM calcium. Cells are fully viable under these and the following experimental conditions, as judged by phase contrast microscopy and Trypan blue exclusion. The combined effect of growth factors on DNA synthesis at 1.8 mM calcium is totally abolished by the potent calmodulin antagonist, W-7 (30 μM), but not by the inactive analogue, W-12 (30 μM), which has approximately 10-times lower affinity for calmodulin than W-7 [9] (Fig. 1B), indicating that the effect of W-7 is relatively specific and is likely based on antagonism against calmodulin.

To explore the site of action of Ca^{2+} along the mitogenic signal transduction pathway, calcium was reduced at various time points after addition of the growth factors, and the rate of DNA synthesis was compared at 24 h. As shown in Fig. 2, a reduction in $[Ca]_{out}$ as late as 8 h after growth factor stimulation inhibits DNA synthesis to a comparable extent as in cells for which $[Ca]_{out}$ was 0.03 mM from the beginning. However, a reduction in $[Ca]_{out}$ at much later time points fails to inhibit DNA synthesis measured at 24 h. These results are consistent with our previous observation [11] and suggest the existence of a Ca^{2+}-dependent process relatively late in the G1 phase of the cell cycle, i.e. after growth factor-induced early cellular events, such as receptor/tyrosine kinase activation, an initial transient Ca^{2+} mobilization and immediate–early protooncogene expression.

We next examined the effects of $[Ca]_{out}$ and the calmodulin antagonist on growth factor-induced RB protein phosphorylation (Fig. 3). As reported for various human cell types, including endothelial cells [12], hyperphosphorylated forms of RB protein (H in Fig. 3A) show delayed mobility on SDS-PAGE and are clearly identified on Western blot as at least three distinct bands above the underphosphorylated form (U in Fig. 3A). A time-dependent increase in RB protein phosphorylation in growth factor-stimulated cells at 1.8 mM of calcium is shown in Fig. 3A. The amount of the hyperphosphorylated forms of RB protein starts to increase between 12 and 16 h after addition of the growth factors, and continues to increase for up to 24 h. By contrast, there is little change in the amount of the underphosphorylated form of RB protein throughout the observation period. As shown in Fig. 3B, at 0.03 mM of $[Ca]_{out}$, the growth factors fail to induce RB phosphorylation even after 24 h, just like DNA synthesis (Fig. 1B). A reduction in $[Ca]_{out}$ from 1.8 to 0.03 mM 6 h, but not 16 h after growth stimulation, nearly completely inhibits RB phosphorylation at 24 h (Fig. 3C), just as in the case with DNA synthesis (Fig. 2). Similarly, an addition of W-7, but not W-12, as late as 8 h after growth stimulation effectively inhibits RB phosphorylation at 24 h, however, it is without effect if applied at 12 h or later (Fig. 3D). These results strongly suggest that at least one site of action of Ca^{2+}/calmodulin is located in the late G1 phase, at a step proximal to RB phosphorylation reaction itself since, once RB becomes phosphorylated 12–16 h after addition of the growth factors (Fig. 3A), it is not dephosphorylated thereafter by either a reduction in $[Ca]_{out}$ or the calmodulin antagonist (Fig. 3C and D). Indeed, it has been reported that a putative RB kinase, $p34^{cdc2}$, which phosphorylates RB protein with an identical tryptic phosphopeptide map as that observed in proliferating cells [17,18], requires neither Ca^{2+} nor calmodulin for in vitro kinase activity

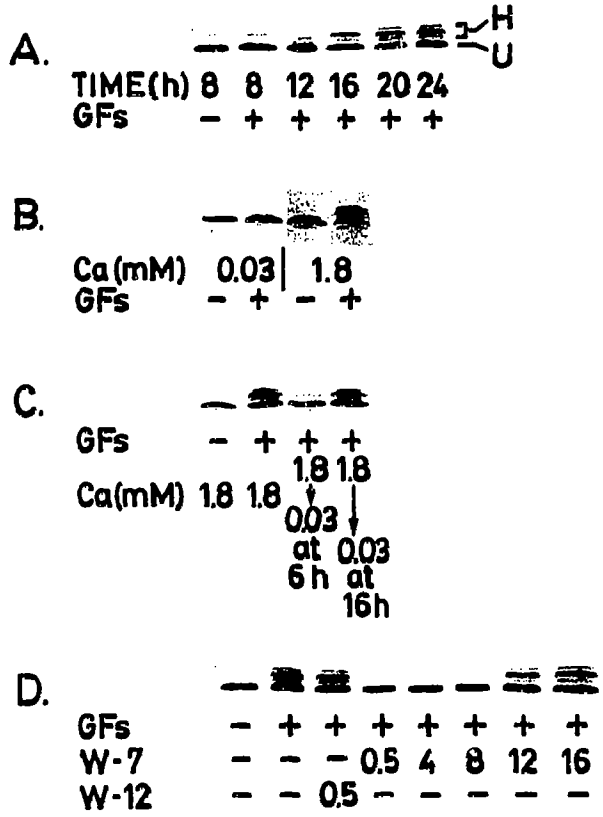

Fig. 3. Analysis of RB protein phosphorylation by Western blotting. (A) Time-course of RB phosphorylation after addition of the growth factors (GFs). Hyperphosphorylated (H) and underphosphorylated (U) forms of RB protein are indicated to the right. (B) The effect of $[Ca]_{out}$ on RB phosphorylation. Cells were incubated at either 0.03 or 1.8 mM of $[Ca]_{out}$ in the presence or absence of the growth factors for 24 h. (C) A reduction in $[Ca]_{out}$ 6 h but not 16 h after addition of the growth factors potently inhibits RB phosphorylation at 24 h. (D) Effects of W-7 and W-12 on RB phosphorylation. The antagonists (30 μM) were introduced to the cultures at indicated time points (h) after addition of the growth factors, and RB phosphorylation was analyzed at 24 h.

[17–19]. It would be of particular interest to test the possibility whether either the expression or phosphorylation state of $p34^{cdc2}$ or related kinases (and/or of their regulatory protein(s) such as cyclin) is regulated in a manner dependent on Ca^{2+} and calmodulin.

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