Mitogen-induced Rapid Phosphorylation of Serine 795 of the Retinoblastoma Gene Product in Vascular Smooth Muscle Cells Involves ERK Activation

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We examined the relationship between mitogen-activated MEK (mitogen and extracellular signal-regulated protein kinase kinase) and phosphorylation of the gene product encoded by retinoblastoma (hereafter referred to as Rb) in vascular smooth muscle cells. Brief treatment of the cells with 100 nM angiotensin II or 1 μM serotonin resulted in serine phosphorylation of Rb that was equal in magnitude to that induced by treating cells for 20 h with 10% fetal bovine serum (~3 × basal). There was no detectable rapid phosphorylation of two close cousins of Rb, p107 and p130. Phosphorylation state-specific antisera demonstrated that the rapid phosphorylation occurred on Ser795 but not on Ser249, Thr252, Thr273, Ser280, Ser387, or Ser411. Phosphorylation of Rb Ser795 peaked at 10 min, lagging behind phosphorylation of MEK and ERK (extracellular signal-regulated protein kinase). Rb Ser795 phosphorylation could be blocked by PD98059, a MEK inhibitor, and greatly attenuated by apigenin, an inhibitor of the Ras/Raf/MEK/ERK pathway. The effect also appears to be mediated by CDK4. Immunoprecipitation/immunoblot studies revealed that serotonin and angiotensin II induced complex formation between CDK4, cyclin D1, and phosphorylated ERK. These studies show a rapid, novel, and selective phosphorylation of Rb Ser795 by mitogens and demonstrate an unexpected rapid linkage between the actions of the Ras → Raf → MEK → ERK pathway and the phosphorylation state of Rb.

The links between pathways that translate extracellular signals into proliferative responses in cells are very complex. In particular, the relationships between signals that activate transcription cascades and those that release tonic inhibitions on cell cycle progression are only now being elucidated. The major players in proliferative transcription cascades are various types of mitogen-activated protein kinases (MAPKs)1 (1, 2), whereas the gene product encoded by Rb holds the cell cycle in check (3, 4). Phosphorylation cascades regulate the activities of the MAPKs and Rb, stimulating the former and inhibiting the latter (1–5). Although the pathways regulating the MAPKs have been reasonably well mapped out, those that regulate the cell cycle still require elucidation. What is known is that: 1) in its active hypophosphorylated state, Rb suppresses cell cycle progression at the G0-G1 interface; 2) when hyperphosphorylated, Rb becomes less active and is unable to hold the cell cycle in check; 3) Rb is a substrate for phosphorylation by several cyclin-dependent protein kinases (CDKs); and 4) dephosphorylation of Rb is mediated by protein phosphatase 1 (5).

Rb contains 16 Ser/Thr-Pro sequences that are potential CDK phosphorylation sites, and most of those sequences have been demonstrated to represent bona fide in vitro phosphorylation sites (5, 6). Hyperphosphorylation of Rb has three known effects: 1) it releases Rb from nuclear tethers, 2) it causes decreased mobility on SDS-PAGE analysis, and 3) it disrupts the binding of Rb to three distinct classes of proteins (3–5). Those are the oncogenic c-Abl (Abelson) kinase (7), cellular proteins that contain the LXXCX motif (8, 9), and the E2F family of transcription factors (10). Thr281 and Thr526 of Rb appear to be involved in the regulation of LXXCX binding, and Ser795 and Ser411 appear to be involved in c-Abl binding (11–13). Those sites do not appear to be involved in the regulation of E2F binding (11–13). In contrast, Ser795 appears to be intimately involved in the binding of E2F. Phosphorylation of this site by cyclin D1/CDK4 also has functional significance in that mutation of Ser795 to Ala prevents phosphorylation and inactivation of the cell cycle arrest function of Rb (14). The region surrounding Ser795 is important in E2F binding, but mutation of Ser795 alone is insufficient to abolish E2F binding (12). Although there is not universal agreement on which Rb sites are substrates for the different CDKs, recent evidence supports specificity in the interactions of various CDKs with Rb (12–15). The situation is further complicated by the existence of other E2F-binding proteins (p107 and p130) that may serve overlapping functions with Rb. Thus, there is opportunity for highly specific interactions among the various kinases and phosphorylation sites on Rb or p107 and p130.

The molecular mechanisms that couple the cell cycle machin-

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; Ang II, angiotensin II; CDK, cyclin-dependent protein kinase; ERK, extracellular signal-regulated protein kinase; 5-HT, 5-hydroxytryptamine, serotonin; MEK, mitogen- and extracellular signal-regulated kinases kinase; P38, phosphatidylinositol-3’ kinase; Rb, retinoblastoma gene product; VSMC, vascular smooth muscle cells; FBS, fetal bovine serum.
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Materials—Drugs and reagents were obtained from the following sources. Angiotensin II, 5-HT, epidermal growth factor, and phorbol 12-myristate, 13-acetate, and agaro-conjugated anti-phospho-serine antibodies were from Sigma. PD98059 and apigenin were from Calbiochem. Rapamycin and Wortmannin were from Biomol (Plymouth Meeting, PA). Cell culture media, fetal bovine serum (FBS), and antibiotics were obtained from Invitrogen and culture flasks from Costar (Cambridge, MA). The phospho-ERK, phospho-Rb, phospho-MEK, phospho-p70 S6 kinase, and phospho-Akt antibodies and anti-Rb (C- and N-terminal) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-p107, anti-p130, anti-Rb, anti-ERF-1, and anti-CDK6 antibodies and anti-CDK2- and anti-CDK4-agro-conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CDK4, anti-cyclin D1 antibodies, and protein A-agarose were from Upstate Biotechnology (Lake Placid, NY).

Cell Culture—Rat aortic VSMC were obtained and maintained as previously described (23). Cells were used at passages 4–7. Treatment of the animal subjects conformed to guidelines of the American Veterinary Medical Association.

Phosphoprotein Immunoblots—For most experiments, protein phosphorylation of (ERK, Rb, MEK, Akt, and p70 S6 kinase) was assessed using phosphorylation state-specific antibodies (Cell Signaling Technology). The protocol was identical to that previously described by us (24, 25), except that the dilutions of the various antibodies followed the manufacturer’s recommendations and the blots were developed using Vistra ECF reagent (Amersham Biosciences).

Immunoprecipitation—Quiescent VSMC cells grown in 100-mm dishes were treated with vehicle, 1 μM 5-HT, or 100 nM Ang II for 10 min and lysed in 500 μl of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% v/v Nonidet P-40), 0.5% w/v sodium deoxycholate, 1 mM NaF, 1 mM sodium pyrophosphate, 100 mM NaVO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Preclarified by incubation with protein A-agarose beads, aliquots of the lysates were incubated overnight with mouse monoclonal anti-phosphoserine IgG conjugated to agarose beads, rabbit polyclonal anti-CDK4 IgG, mouse monoclonal anti-cyclin D1 antibody, or rabbit polyclonal anti-Rb IgG.

Immunoprecipitates were captured by addition of protein A-agarose.

The agarose beads were collected by centrifugation, washed twice in ice-cold radioimmunoprecipitation assay buffer, boiled in Laemmli sample buffer, and subjected to SDS-PAGE and subsequent immunoblot analysis. The same Western blots were stripped and reprobed with the antibody used for immunoprecipitation to assure that equal amounts of protein were loaded in each lane.

Cyclin-dependent Kinase Assays—Cells were treated with vehicle, 1 μM 5-HT, or 100 nM Ang II for 5 min, and then protein extracts were prepared as described (26). For each condition, 5 × 10⁶ cells were scraped into lysis buffer containing 50 mM Hepes, pH 7.5, 10 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM MgCl_2, 1 mM EDTA, 25 μg/ml of leupeptin, 2 mM Na_3VO_4, 1 mg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. The lysates were vortexed, placed on ice for 10 min, and then the lysates were cleared by centrifugation. Aliquots of the lysates were incubated with rabbit antibodies raised against CDK2, CDK4, and CDK6 (Santa Cruz Biotechnology). The CDK2 and CDK4 antibodies were precoupled with protein A-agarose. Immunoprecipitates were resuspended in 10 μl of buffer containing 50 mM Hepes, pH 7.4, 10 mM MgCl_2, 5 mM MnCl_2, 1 mM diithiothreitol, 10 μl of [γ-32P]ATP, and 20 μg/ml of recombinant protein fusion between maltose-binding protein and Rb residues 701-928 (Cell Signaling Technology). The mixtures were incubated for 30 min at 30 °C in a shaking water bath, and then the reactions were terminated by the addition of equal volumes of 2× Laemmli sample buffer. The samples were separated on precast 4–20% SDS-PAGE gels (Novex, La Jolla, CA) and visualized on a Phosphorimag (Amersham Biosciences).

Statistical Analysis—Data were analyzed for repeated measures by Student’s t test for unpaired two-tailed analysis. p values <0.05 were considered significant.

RESULTS

To study phosphorylation of Rb by mitogens in VSMC, we used phosphorylation state-specific antibodies that have been recently developed and extensively characterized. Their specificity has been validated using mutant recombinant Rb proteins (27). Cells were incubated with 5-HT (1 μM) for 10 min, 10% FBS for 20 h (positive control), or vehicle and then subjected to immunoblot. Fig. 1A shows that 5-HT induced a marked increase in the level of phospho-Rb-Ser^795 that was equal in magnitude to that induced by FBS. 5-HT did not increase the levels of phospho-Rb-Ser^790, phospho-Rb-Thr^287, or phospho-Rb-Ser^370/Thr^383, all of which are phosphorylation targets of the Raf-1/MAP2K1/MAPK pathway in VSMC (28).
which have been suggested to be bona fide and functionally significant Rb phosphorylation sites (11–14, 27). In contrast, FBS increased the level of phosphorylation of Ser795/Thr252, Thr72, Ser249, and Ser807/811.

To establish that brief treatment of VSMC with mitogens could alter the phosphoserine content of Rb, VSMC were treated with 5-HT or Ang II. After lysis, VSMC extracts were incubated with agarose-conjugated anti-phosphoserine antibody, precipitated, and subjected to immunoblot with antisera specific for Rb, p107, and p130. Fig. 1B shows that treatments for 10 min with Ang II (100 nM) and 5-HT (1 μM) increased the phosphoserine content of Rb in VSMC to nearly three times the basal levels. The magnitude of the phosphorylation was similar to that induced by 10% FBS (20 h, Fig. 1B) and epidermal growth factor factor (10 ng/ml × 10 min, not shown). The increases were apparent whether C-terminal Rb or N-terminal Rb antisera were used. In contrast, treatment with Ang II and 5-HT did not alter the phosphoserine content of p107, whereas pre-treatment with FBS for 20 h markedly increased p107 phosphoserine content (not shown). We did not detect p130 in immunoprecipitates from VSMC. These data suggested that 5-HT and Ang II can rapidly induce serine phosphorylation of Rb, but not p107 or p130, in VSMC.

We next used the Ser795 phospho-specific antibody to confirm that both Ang II and 5-HT rapidly induce phosphorylation of Rb on serine 795. Fig. 1C shows that both 5-HT and Ang II increased the phosphorylation of Rb on Ser795 after 10 min of incubation. The increases in phosphorylation were similar in magnitude to those induced by treatment with 10% FBS (20 h, Fig. 1B) and 1 μM phorbol 12-myristate, 13-acetate or epidermal growth factor (10 ng/ml × 10 min, not shown).

Because Rb phosphorylation has previously been linked to the Ras → Raf → MEK → ERK pathway, we performed experiments to test the role of that pathway in phosphorylation of Rb Ser795. Fig. 2A shows that both Ang II and 5-HT stimulate phosphorylation of MEK (6 and 5 × basal levels, respectively) and ERK (to 5 × basal levels for both), as has been previously shown by others (28, 29). The time courses for MEK and ERK phosphorylation by each hormone were similar, peaking at 5 min and persisting for at least 60 min. The peak phosphorylation of Rb lagged slightly behind MEK and ERK (peaking at 10 min), consistent with a potential role for MEK and ERK upstream of Rb (Fig. 2B).

We next examined the ability of two inhibitors of the Ras → Raf → MEK → ERK pathway to prevent phosphorylation of MEK, ERK, and Rb. Phosphorylation of both ERK and Rb was greatly attenuated by preincubation with the MEK1 inhibitor, PD98059, (10 μM) (30) for 15 min prior to stimulation with 5-HT or Ang II. Fig. 3 shows that phosphorylation of ERK and Rb was also blocked significantly by apigenin (5 μM). At this concentration, apigenin has been shown to arrest synchronized human diploid fibroblasts at the G0-G1 interface (31) and to block Ras-mediated activation of ERK (32, 33). In contrast, apigenin and PD98059 did not significantly affect the phosphorylation of Rb. Thus, these results confirm that both apigenin and PD98059 interfered with phosphorylation of ERK and Rb Ser795 without significantly affecting the phosphorylation of MEK. Thus, both compounds appear to block MEK function, resulting in inhibition of phosphorylation of ERK and Rb.

Because there is also evidence that a second pathway involving phosphatidylinositol-3′-kinase (PI3K) and p70 S6 kinase can regulate the cell cycle by modulating Rb function, we tested the effects of inhibitors of those two targets on Ang II- and 5-HT-induced phosphorylation of Rb Ser795. Those experiments showed no effect of wortmannin or rapamycin on Ang II- and 5-HT-induced phosphorylation of Rb Ser795 (Fig. 3). At the
same time, treatment cells with wortmannin completely inhibited 5-HT- or Ang II-induced phosphorylation of Akt, the major known effector of PI3K (data not shown), supporting the inhibition of PI3K in our conditions. Similarly, rapamycin treatment blocked epidermal growth factor or phorbol 12-myristate 13-acetate-induced phosphorylation of p70 S6 kinase (data not shown), indicating that p70 S6 kinase was indeed inhibited under our experimental conditions. Those experiments establish a clear link between MEK (and not PI3K or p70 S6 kinase) and the phosphorylation of Rb Ser795.

Multiple proline-directed kinases have been demonstrated to phosphorylate Rb, including CDK2, CDK4, CDK6, and ERK (13, 34, 35). To further assess the biochemical consequences of brief 5-HT and Ang II treatments on vascular smooth muscle cells, the ability of those agents to activate possible intermediate kinases was assessed using an immunoprecipitation kinase assay in which a C-terminal Rb fusion protein (which includes CDK2, CDK4, CDK6, and ERK kinases was assessed using an immunoprecipitation kinase assay, using the C-terminal region of Rb fused to maltose-binding protein as the substrate. Data are expressed as mean ± S.E. * indicates significance against vehicle-treated cells (black bars) at p < 0.01. ** indicates significance against vehicle-treated cells (black bars) at p < 0.05. † indicates significance against 5-HT or Ang II without blockers.

CDK2 or CDK6 is involved in the phosphorylation of Rb Ser795 induced by Ang II and 5-HT in VSMC.

We next examined whether 5-HT and Ang II could induce a physical interaction between cyclin D1/CDK4 and ERK1/2. We explored this possibility using immunoprecipitation of lysates from cells pretreated with vehicle or PD98059 for 30 min and then treated with vehicle, 5-HT, or Ang II with a polyclonal CDK4 antibody, followed by Western blotting with monoclonal antibodies to cyclin D1 and to ERK1/2. Exposure of VSMC to 5-HT or Ang II for 10 min increased the amount of cyclin D1 in CDK4 immunoprecipitates by ~250% (Fig. 5A). Pretreatment of VSMC with PD98059 completely abolished the increase of cyclin D1 in CDK4 immunoprecipitates induced by 5-HT and Ang II, supporting an important role of MEK-ERK activity for formation of a complex between CDK4 and cyclin D1. Interestingly, active (phosphorylated) ERK1/2 appears to form a complex with CDK4/ cyclin D1, and the amount of phospho-ERK in CDK4 immunoprecipitates was increased by ~300% in VSMC stimulated with 5-HT or Ang II for 10 min. There was no detectable amount of phospho-ERK in CDK4 immunoprecipitates from cells pretreated with PD98059 (Fig. 5B).

Because Rb function depends, at least in part, on interactions with the E2F family of DNA-binding transcription factors, we wanted to explore the possibility that 5-HT and Ang II stimulate a dissociation of E2F from Rb. To answer this question we pretreated VSMC with vehicle or PD98059 for 30 min, treated them with vehicle, 5-HT, or Ang II, and performed immunoprecipitation of Rb from cell lysates with a polyclonal Rb antibody followed by Western blotting with a monoclonal antibody to the E2F-1 transcription regulator. Treatment of...
VSMC for 10 min with 1 μM of 5-HT or 100 nM of Ang II resulted in a ~30% decrease in the amount of E2F-1 in Rb immunoprecipitates (Fig. 6). Interestingly, pretreatment of VSMC with PD98059 caused a slight but significant ~20% increase in the amount of E2F-1 in Rb immunoprecipitates from cell lysates that did not depend on 5-HT or Ang II treatment.

**DISCUSSION**

The specific role of the Ras → Raf → MEK → ERK pathway in the regulation of the cell cycle is still controversial. Evidence from at least five studies supports a direct role for certain components of this pathway in the stimulation of the cell cycle, and this regulation usually takes place over a period of several or more hours. First, the cyclin D1 promoter and protein were shown to be increased by ERK activation in CCL39 cells; these effects occurred within 6–9 hours. Serum stimulation or transfection with constitutively activated ERK or the ERK phosphatase MKP-1 was sufficient to induce hyperphosphorylation of Rb, whereas transfection with an inducible version of constitutively activated Raf was not (34, 36). Moreover, some mitogens have previously been reported to cause hyperphosphorylation of Rb (serum, thrombin), whereas others have not (epidermal growth factor, insulin). Sustained ERK activation has also been shown to positively regulate cyclin D1 expression in IIC9 fibroblasts (38). In addition, insulin-like growth factor, insulin-like growth factor stimulated cyclin D1 synthesis and hyperphosphorylation of Rb, but these effects are not sensitive to blockade of MEK by PD98059 (50, 51). Rather, they are sensitive to chemical inhibitors of PI3K (50). Similary, phosphorylation of Rb by interleukin 2 in T-cells depends upon PI3K, but not the Ras → Raf → MEK → ERK pathway (52). In VSMC, rapamycin, an inhibitor of p70 S6 kinase, blocked phosphorylation of Rb induced by 20% serum, but this effect was apparent only after 6 hours (53). In addition, activation of PI3K alone has been shown to promote cell cycle entry in 3Y1 rat embryo fibroblasts (54). In our study, we found no evidence that PI3K or p70 S6 kinase participate in the rapid phosphorylation of Rb induced by Ang II or 5-HT. Although most studies have focused on longer term phosphorylation of Rb, others have shown that Rb can be phosphorylated within minutes by mitogens (55).

The current work demonstrates that application of 5-HT or
Ang II to quiescent VSMC results in the rapid phosphorylation of Ser795 of Rb and that this phosphorylation requires activation of MEK/ERK pathway. Moreover, it is highly likely that the phosphorylation of Ser795 is mediated in large part by CDK4. These results are consistent with the findings of Connell-Crowley et al. (14), who used a microinjection assay to show that phosphorylation of Rb by a factor derived from cyclosporin D caused nuclear accumulation of Rb. These results suggest that phosphorylation of Rb is mediated by a factor derived from cyclosporin D.

Rb function depends, at least in part, on interactions with the E2F family of DNA-binding transcription factors. E2F sites are found in the promoters of many genes that are important for cell cycle progression, and Rb appears to repress transcription of these genes through its interaction with E2F (57, 58). Rb-mediated inactivation of E2F may occur by at least two different mechanisms. First, Rb can bind to the E2F transcription factor within its transactivation domain, thus blocking its ability to activate transcription. Second, Rb can be recruited to DNA by E2F to assemble a repressor complex that can actively repress transcription (58). However, because many of the studies in tissue culture cells have relied on overexpression of E2F and Rb-family proteins, the relative contributions of transactivation by free E2F or displacement of an active Rb-E2F repressor complex to the cell cycle progression through the cell cycle remains unclear. The E2F family of transcription factors binds DNA as heterodimers in conjunction with the DP family of factors (59). DP-1, the best studied member of the DP family of proteins, by itself has little transcriptional activity, but it cooperates with the E2Fs to activate transcription of the E2F target genes (59). Studies using a dominant negative mutant of DP-1 showed inhibition of progression of cells into S phase, suggesting that interaction of E2F/DP with promoters is important for cell cycle progression (60).

Our data suggest that the rapid phosphorylation of Ser795 of Rb induced by 5-HT or Ang II in VSMC could be functionally significant, because treatment of cells with mitogens resulted in an ~30% decrease in the amount of E2F-1 in Rb immunoprecipitates (Fig. 6). At the same time, inhibition of the MEK/ERK pathway by pretreatment of VSMC with PDB0059 caused a slight increase in the amount of E2F-1 in Rb immunoprecipitates, suggesting that ERK-dependent phosphorylation of Rb plays a role in E2F-Rb interactions. The relatively small level of dissociation of E2F-1 from Rb in our experiments could mean that G protein-coupled receptors need to collaborate with the other mechanisms to fully inactivate Rb. This is not surprising, because binding of Rb to E2F most likely is regulated by multiple phosphorylation sites (12, 61, 62). It has been suggested that phosphorylation by cyclin D-CDK4/6 and cyclin E-CDK2 was necessary to completely hyperphosphorylate Rb and block Rb binding to E2F (62). Moreover, regulation of Rb function by phosphorylation appears to be even more complex after recent studies have suggested that unphosphorylated Rb is inactive in G1 and that initial phosphorylation by CDK4/6 leads to a hypophosphorylated, active protein that is assembled with E2Fs in vivo (63). Thus, it does not seem likely that 5-HT- and Ang II-induced selective phosphorylation of Rb Ser795 is sufficient to cause the transition of cells through G1. The subsequent fate of E2F-1 released from Rb after 5-HT and Ang II treatment remains unclear. One possibility is that E2F-1 interacts with DP-1 to deliver it to the nucleus. It has been shown that DP-1 lacks an autonomous nuclear localization signal; therefore, its presence in nuclei depends upon an interaction with the appropriate E2F partner, which subsequently causes the efficient nuclear accumulation of DP proteins (64, 65). Taking into consideration that DP-1 in most cell types is constitutively expressed, it is likely that the phosphorylation of E2F induced by 5-HT or Ang II requires the activity of MEK-ERK pathway (Fig. 7). Moreover, this rapid phosphorylation is functionally significant, because it correlates with dissociation of E2F-1 from Rb. These findings support the possibility that mitogens can regulate cell cycle machinery within minutes in addition to their well established interactions that require hours to manifest either functionally or biochemically.

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