The Hypertrophic Response in C2C12 Myoblasts Recruits the G₁ Cell Cycle Machinery*

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Hypertrophy occurs in postmitotic muscle as an adaptive response to various physiological and pathological stresses. Studies in vascular smooth muscle cells and primary cardiomyocytes suggest that angiotensin II-mediated hypertrophy activates signaling pathways associated with cell proliferation. Regulation of cyclin-dependent kinase (Cdk)-cyclin activities is essential to cell size control in lower eukaryotes, yet their role in the hypertrophic response in muscle is incompletely understood. We describe an in vitro model of hypertrophy in C2C12 skeletal myoblasts and demonstrate that induction of hypertrophy involves transient activation of Cdk4, subsequent phosphorylation of Rb, and release of HDAC1 from the Rb inhibitory complex. We also demonstrate that E2F-1 becomes transcriptionally active yet remains associated with Rb. We propose a model whereby partial inactivation of the Rb complex leads to derepression of a subset of E2F-1 targets necessary for cell growth without division during hypertrophy.

Hypertrophy occurs in postmitotic cardiac and skeletal muscle as a fundamental adaptive process in response to various stresses in both physiological and pathological situations. A number of studies indicate that AngII acts as a hypertrophic stimulus in vascular smooth muscle cells (1) and in primary cultures of cardiomyocytes (2). Recently, AngII has been shown to be required for optimal overload-induced skeletal muscle hypertrophy (3). These observations suggest that AngII can act as a hypertrophic stimulus both in vitro and in vivo across myogenic cell types.

The signaling events responsible for AngII-mediated hypertrophy have been extensively studied. AngII has been shown to induce several immediate-early genes, such as c-fos, c-jun, egr-1, and c-myc, primarily through the G protein-coupled angiotensin receptor subtype 1 in both myogenic and nonmyogenic cells (2, 4), indicating that mitogenic and hypertrophic stimuli appear to share certain intracellular responses.

Cell cycle entry and G₁ progression are controlled primarily by Cdk-cyclin complexes through their actions on the E2F-1-Rb complex (5). Cdk4 and Cdk6, assembled with their regulatory subunits, the D-type cyclins, are activated in response to mitogenic stimuli, heralding cell cycle entry and G₁ progression (6). Active Cdk4/6-cyclin D1 phosphorylates Rb during early G₁; this leads to the up-regulation of cyclin E, its assembly with Cdk2, and activation of the Cdk2-cyclin E complex, which in turn hyperphosphorylates Rb (7). Hyperphosphorylated Rb releases and thereby activates the transcription factor E2F-1, allowing the expression of genes necessary for DNA replication and mitosis (8).

Although regulation of cyclin-Cdk activities is essential to cell size control in lower eukaryotes (6), their role in the hypertrophic response in skeletal muscle is incompletely understood. In this study, we describe an in vitro model of muscle cell hypertrophy using C2C12 cells. We demonstrate for the first time that the hypertrophic response in these cells involves the transient activation of Cdk4, but not Cdk2, with subsequent phosphorylation of Rb, release of HDAC1 from the Rb inhibitory complex, and activation of the transcription factor, E2F-1. We propose a model by which partial inactivation of the Rb complex leads to the derepression of a subset of E2F targets necessary for cell growth during hypertrophy.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies to cyclin D1, cyclin D3, p21Waf1/Cip1, p27Kip1, and E2F-1, and polyclonal antibodies against cyclin E (Santa Cruz Biotechnology), Cdk4, and HDAC1 were obtained from Santa Cruz Biotechnology. Monoclonal antibody against Rb, which recognizes hypophosphorylated and hyperphosphorylated Rb, was obtained from Pharmingen. Polyclonal phospho-Rb (ST780) antibody was obtained from Cell Signaling Technology.

Cell Culture—Actively growing C2C12 skeletal myoblasts (ATCC) were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum (Invitrogen). The cells were made quiescent by serum withdrawal for 48 h and stimulated to proliferate by adding 20% fetal bovine serum. Myogenic differentiation was induced in subconfluent cultures by the addition of 2% horse serum. Cellular hypertrophy was stimulated with AngII (100 nM; Sigma).

DNA and Protein Synthesis Assays—Quiescent cells were stimulated with AngII or 20% dialyzed fetal bovine serum for 24 h. The cells were pulse-labeled for 2 h with 2 μCi/ml [³H]thymidine to measure DNA synthesis or 1 μCi/ml [³H]leucine (PerkinElmer Life Sciences) to measure protein synthesis and then suspended in cold 10% trichloroacetic acid for 30 min at 4 °C. The trichloroacetic acid-precipitable material was washed with cold 5% trichloroacetic acid, cold 70% ethanol, and deionized water and then solubilized in 0.1 M NaOH. [³H]Thymidine and [³H]leucine incorporation was measured by liquid scintillation.

Flow Cytometry—All of the cultures were analyzed after 24 h of treatment, as described previously (9). Briefly, 10⁵ cells were stained with propidium iodide to exclude dead cells from evaluation and Hoechst 33342 (Molecular Probes) to measure DNA content in living cells. Flow cytometry was performed using a Becton Dickinson VANTAGE SE with dual argon ion lasers at 488- and 363-nm light output. Propidium iodide and Hoechst signals were acquired using 630/22- and 457/10-mm band pass filters, respectively. All of the analyses of DNA...
Hypertrophy and Cell Cycle Regulation in C2C12 Cells

AngII Stimulates Hypertrophy and G_{1} Arrest in C2C12 Myoblasts—AngII has been shown to induce hypertrophy in cardiac myocytes and vascular smooth muscle cells (1, 2); however, its effect on skeletal muscle growth has not been demonstrated. To determine whether AngII stimulates hypertrophy in C2C12 myoblasts, we treated quiescent cells with AngII (100 nm) in serum-free medium. Protein synthesis, as measured by [3H]leucine incorporation, increased 2.6-fold with AngII stimulation (Fig. 1C). This was similar to results observed for both quiescent myoblasts and differentiating myocytes. Taken together, these findings suggest that AngII stimulates hypertrophy by increasing protein synthesis in the absence of cell division.

Cdk4, but Not Cdk2, Is Active in C2C12 Myoblasts Undergoing Hypertrophy—Because cell cycle re-entry and G_{1}/S transit in proliferative cells commences with assembly and activation of Cdk4/6-cyclin D and subsequent activation of Cdk2-cyclin E, we studied the effect of AngII stimulation on G_{1} cyclin expression and Cdk activity in C2C12 cells. AngII had an up-regulatory effect on cyclin D1, whereas cyclin D3 was down-regulated in AngII-stimulated cells, compared with quiescent cells (Fig. 2A). In differentiating C2C12 cells, cyclin D1 expression was repressed, whereas cyclin D3 levels were elevated, compared with proliferating cells (Fig. 2A). These findings are consistent with previous observations that cyclin D1 is a mitogen sensor and the limiting factor in the assembly of active Cdk4 complexes necessary for G_{1}/S transit (16), whereas cyclin D3 contributes to the irreversible withdrawal of postmitotic cells from the cell cycle and is critical in the maintenance of a differentiated phenotype (17).

Although D-type cyclins control Cdk4/6 activity in early G_{1}, cyclin E regulates Cdk2 activity near the G_{1}/S transition (18). Cyclin E was repressed in differentiating cells compared with proliferating cells; however, we observed up-regulation of cyclin E expression in AngII-stimulated cells (Fig. 2A). Because Cdk activities are controlled by association with regulatory cyclins and not at the level of expression, we were not surprised to find equivalent levels of Cdk2 or Cdk4 protein in AngII-stimulated cells compared with quiescent, proliferating, and differentiating cells (Fig. 2A).

G_{1} cyclin expression was induced in AngII-stimulated cells, suggesting cell cycle re-entry. Because these cells arrested in G_{1}, however, we hypothesized that only Cdk4-cyclin D1 would be active during the hypertrophic response, because Cdk2 activity is associated with the G_{1}/S transition. To test this, we measured Cdk activities in C2C12 cells following AngII stimulation. Cdk4 and Cdk2 were immunoprecipitated from cell lysate using anti-Cdk4 or anti-Cdk2 antibody, and kinase activity was determined using GST-Rb and histone H1 as substrate, respectively. Cdk4 and Cdk2 activities were increased in proliferating myocytes and not in AngII-stimulated cells after 24 h (Fig. 2B). To determine whether kinase activities might be transient, we assayed Cdk4 and Cdk2 activities at 8, 12, 16, 20, and 24 h after stimulation with AngII. Although Cdk2 activity was not detected at any of these earlier time points (data not shown), a burst of Cdk4 activity was observed at 12 h (Fig. 2C). These results demonstrate an early increase in Cdk4 activity following AngII stimulation. The lack of Cdk2 activity in AngII-treated cells is consistent with their failure to progress through the G_{1}/S transition.

Levels of Cdk4-bound Cdk Inhibitors, p21^{Waf1/Cip1} and p27^{Kip1}, Are Unchanged in Hypertrophic C2C12 Myoblasts—G_{1} progression is controlled through Cdk activity, and this in turn is regulated in part by CKIs such as p21^{Waf1/Cip1} and p27^{Kip1} (19). To further examine the mechanism by which C2C12 cells recruit the cell cycle machinery but remain arrested in G_{1} during the hypertrophic response, we determined the levels of p21^{Waf1/Cip1} and p27^{Kip1} expression following AngII treatment (Fig. 3A). As expected, levels of both proteins were down-regulated in proliferating myoblasts and up-regulated in differentiating myotubes, compared with quiescent cells. Expression of both proteins was elevated in AngII-treated myoblasts compared with quiescent cells, at levels similar to those observed in differentiating myotubes. Other investigators have observed...
elevated p27Kip1 levels in AngII-stimulated, vascular smooth muscle cells, resulting in diminished Cdk2 activity and hypertrophy (15). In addition, up-regulation of p21 Waf1/Cip1 and p27Kip1 has been observed in the mesangial cell hypertrophic response to high glucose (20, 21). Our results are consistent with those reported for other cell types and extend a general role for these G1-active CKIs in the response to hypertrophic stimuli.

Although increased expression of CKIs may suggest a role for these proteins in AngII-stimulated hypertrophy in C2C12 cells, their effects as cell cycle regulators are delivered through interaction with Cdk complexes. To correlate the increased p21Waf1/Cip1 and p27Kip1 levels that we had observed in response to AngII with a downstream effect on Cdk4 activity and G1 progression, we next measured their association with the Cdk4-cyclin D complex. We observed co-immunoprecipitation of p21Waf1/Cip1 with Cdk4 complexes in differentiating cells, and this association appeared to be decreased in proliferating cells, as expected. Surprisingly, the p21Waf1/Cip1-Cdk4 interaction was not significantly disrupted in AngII-stimulated cells, compared with proliferating cells (Fig. 3B).

Our observation that Cdk4 activity is low or absent in differentiating cells and elevated in proliferating cells and at 12 h following AngII stimulation suggests that p21Waf1/Cip1 may function through several mechanisms in the same cell line. Although p21Waf1/Cip1 appears to associate with inactive Cdk4 complexes in differentiating and quiescent C2C12 cells, it also interacts with active Cdk4 in C2C12 cells responding to hypertrophic stimuli. It has been shown in other systems that despite its initial description as a Cdk inhibitor, p21Waf1/Cip1 may be found in active Cdk complexes and may facilitate the assembly of active Cdk4-cyclin D1 complexes in particular (22). Our data suggest the possibility that both activities of p21Waf1/Cip1 are present in skeletal muscle myoblasts responding to different stimuli. Although the interaction between p21Waf1/Cip1 and Cdk4 in differentiating cells may be inhibitory, p21Waf1/Cip1 also may stabilize Cdk4-cyclin D1 activity in cells undergoing hypertrophy, allowing for the brief but nonsustained Cdk4 activity observed.

In contrast, p27Kip1 co-immunoprecipitated with inactive Cdk2 in differentiating and AngII-treated cells compared with proliferating C2C12 myoblasts (data not shown). This suggests that p27Kip1 functions primarily as a Cdk2 inhibitor at the G1/S transition in hypertrophic C2C12 myocytes.

Rb Is Hyperphosphorylated in Hypertrophic C2C12 Myoblasts—Rb is an important in vivo substrate of Cdk4-cyclin D1. As a critical regulator of the G1/S transition of the cell cycle, Rb can exist in hypophosphorylated and hyperphosphorylated

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**FIG. 1. AngII elicits a hypertrophic response in C2C12 myoblasts.** Quiescent (Q) C2C12 cells were stimulated to proliferate with 20% fetal bovine serum (P), differentiate with 2% horse serum (D), or hypertrophy with AngII for 24 h. A, protein and DNA synthesis in AngII-stimulated cells. Protein synthesis was measured as [3H]leucine incorporation (counts/min/cell number). DNA synthesis was measured as [3H]thymidine incorporation (counts/min/cell number). The data shown are the means ± S.E. (n = 3). The protein/DNA synthesis ratio increased in response to AngII. B, cell growth with AngII stimulation. The cells were analyzed by forward angle light scatter (FALS). A representative experiment is shown (n = 3). A rightward shift (*) indicated an increase in cell size with AngII treatment. C, cell cycle progression in AngII-stimulated cells. The cells were stained for DNA content with Hoechst 33342 and analyzed by flow cytometry. A representative experiment is shown (n = 3). The majority of cells treated with AngII demonstrated 2 N DNA content, compared with the normal distribution between 2 and 4 N DNA content seen in proliferating (P) cells.
lated cells, compared with differentiating or quiescent cells. B histone H1 and GST-Rb as Cdk2 and Cdk4 substrates, respectively. tated from cell lysates, and kinase activities were measured with indicated antibodies by immunoblot. A representative experiment is shown (n = 3). Cyclins D1 and E were up-regulated, cyclin D3 was down-regulated, and G1 Cdk levels were unchanged in AngII-stimulated cells, compared with differentiating or quiescent cells. B, Cdk activities with AngII stimulation, Cdk2 and Cdk4 were immunoprecipitated from cell lysates, and kinase activities were measured using histone H1 and GST-Rb as Cdk2 and Cdk4 substrates, respectively. 32P-Labeled substrates were separated by SDS-PAGE and analyzed by autoradiography. A representative experiment is shown (n = 3). Cdk2 and Cdk4 are active only in proliferating cells at this time point. Cdk4 activity over 24 h of AngII stimulation. Cdk4 activity was assayed at indicated time points (top panel). Kinase activity was quantitated as picromoles of 32P incorporated/min/mg substrate (bottom panel). The data shown are the means ± S.E. (n = 3). A transient, ~3-fold increase in Cdk4 activity was measured following AngII stimulation for 12 h.

forms (23). Although the active, hypophosphorylated form binds to and inhibits E2F transcription factors required for expression of genes involved in DNA synthesis, the inactive, hyperphosphorylated form releases E2F, allowing for its activation (24). To determine whether the transient Cdk4 activity observed in cells undergoing hypertrophy inactivates Rb, we examined Rb phosphorylation in AngII-stimulated cells. Whereas hypophosphorylated (active) Rb persisted in quiescent myoblasts and differentiated myotubes, Rb phosphorylated on serine 780, which has been shown to be preferentially phosphorylated by Cdk4-cyclin D1 (25), was detected in both proliferating cells and in hypertrophic cells stimulated with AngII (Fig. 4A). Hyperphosphorylated Rb similarly was de-

tected in C2C12 myoblasts treated with other known hypertrophic stimuli, including prostaglandin F2α, and 1,25-dihydroxyvitamin D3 (data not shown). These results suggest that Rb is inactivated by Cdk4 phosphorylation during the hypertrophic response in C2C12 cells and that this occurs regardless of the initial hypertrophic stimulus.

Rb Hyperphosphorylation by Cdk4-Cyclin D Is Associated with the Release of HDAC1—Rb plays a fundamental role in cell cycle progression through its association with the E2F
family of transcription factors (5). These in turn regulate expression of genes important for G₁/S transition and DNA synthesis (26). Rb represses transcription from promoters containing E2F binding sites by complexing with HDAC enzymes (27–29) and SWI/SNF nucleosome remodeling factors (30). The first molecular event for cell cycle re-entry caused by mitogenic stimuli in nonmyogenic, proliferating cells is activation of Cdk4/6-cyclin D1 followed by initial phosphorylation of Rb by activated Cdk complexes. Phosphorylation of Rb displaces HDAC1 from the inhibitory HDAC1-Rb-SWI/SNF complex (31). This partial disruption of the Rb inhibitory complex relieves repression of the cyclin E gene (32). Cyclin E expression and assembly with Cdk2 lead to formation of active Cdk2-cyclin E complexes, which hyperphosphorylate Rb, further disrupting the inhibitory complex (24). This leads to the complete release of E2F and transcriptional activation of a wide variety of E2F target genes necessary for DNA replication and mitosis (8). The association between Rb and HDAC1 recently has been shown to coincide with myogenic differentiation and myocyte cell cycle withdrawal (33). Because of this and our observation of Cdk4 activity (Fig. 2C) without subsequent Cdk2 activity and Rb hyperphosphorylation (Fig. 4A) in C2C12 cells undergoing hypertrophy, we wanted to determine the downstream effect of this transient Cdk4 activity on HDAC1 association with the Rb inhibitory complex. To accomplish this, we performed co-immunoprecipitation experiments of Rb with HDAC1 in AngII-treated C2C12 cells (Fig. 4B). Although Rb co-immunoprecipitated with HDAC1 in quiescent and differentiating C2C12 cells, indicating active repression by the Rb inhibitory complex, the amount of HDAC1 associated with the Rb complex in AngII-treated cells was markedly reduced. These data show for the first time that HDAC1 is released upon phosphorylation of Rb by Cdk4-cyclin D complexes in myogenic cells during the hypertrophic response.

E2F-1 Remains Associated with Rb but Is Transcriptionally Active in Hypertrophic C2C12 Cells—Although AngII-stimulated cells arrested in G₁ (Fig. 1C) and did not demonstrate Cdk2 activity, Rb phosphorylation coincident with Cdk4 activity was observed (Figs. 4A and 2C). In addition, HDAC1 was partially released from the Rb inhibitory complex (Fig. 4B), and cyclin E expression was induced (Fig. 2A). This suggested that E2F-1, which regulates cyclin E expression (32) and is regulated by the Rb complex, may be active at a subset of target promoters during the hypertrophic response. To test this, we first examined whether E2F-1 was released from Rb upon AngII stimulation. Surprisingly, co-immunoprecipitation of E2F-1 with Rb in proliferating and AngII-stimulated cells demonstrated that E2F-1 remained associated with Rb after AngII stimulation, although perhaps to a lesser extent than in quiescent, unstimulated cells (Fig. 5A).

To determine whether E2F-1 in complex with Rb but after HDAC1 release was transcriptionally active, we performed reporter assays in AngII-stimulated C2C12 cells. We used one reporter containing four tandem repeats of the E2F-1 binding site (E2F4B-luc) to examine E2F-1 DNA binding (12) and another containing the entire E2F-1 promoter (E2F1-luc) to evaluate transcriptional activation (13), as E2F-1 positively regulates its own expression. With both reporters, we observed a modest but significant increase (~3-fold) in activity in C2C12 cells stimulated with AngII for 12 h (Fig. 5B), compared with unstimulated, quiescent cells. Although this represents only one-third of the E2F-1 activity seen in proliferating cells, it coincided with the increase in cyclin E expression (Fig. 5B), as well as the peak of Cdk4 activity (Fig. 2C), and Rb phosphorylation (Fig. 4A) also observed. These data demonstrate for the first time that during the hypertrophic response, E2F-1 is
transcriptionally active without Rb hyperphosphorylation by Cdk2 or complete release from the Rb inhibitory complex.

The G1 Cell Cycle Machinery Plays an Important Role in Skeletal Muscle Cell Hypertrophy—We have shown that AngII stimulation of C2C12 cells simulates the hypertrophic cell growth and G1 arrest observed in primary cardiomyocytes and vascular smooth muscle cells. We have used this model system to elucidate the mechanisms by which the G1 cell cycle regulatory apparatus controls the hypertrophic response in myoblasts. The early G1 Cdk4-cyclin D1 complex is transiently activated in response to AngII in C2C12 cells, whereas the later G1 Cdk2-cyclin E complex is not. Although p27Kip1 is associated with inactive Cdk2 during this process, p21Waf1/Cip1 may have two functions in these cells: inhibiting Cdk4 activity in differentiating cells and permitting a transient burst of Cdk4 activity during hypertrophy, perhaps through stability effects. Coincident with peak Cdk4 activity, Rb is hyperphosphorylated, resulting in the release of HDAC1 from the Rb inhibitory complex. Although E2F-1 remains associated with Rb, it becomes transcriptionally active, inducing the expression of at least one target gene, cyclin E.

Based on these observations, we propose a model in which hypertrophy results from the derepression of a subset of E2F-1 targets necessary for cell growth during the hypertrophic response (Fig. 6). The identification of this subset of E2F-1-regulated genes and other components of the Rb complex that also may mediate partial E2F-1 activity will further validate such a model and provide potential targets for manipulating the hypertrophy response in muscle tissues.

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