Novel Role for Cyclin-dependent Kinase 2 in Neuregulin-induced Acetylcholine Receptor ε Subunit Expression in Differentiated Myotubes*

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Cyclin-dependent kinases (CDKs)† are a family of evolutionarily conserved serine/threonine kinases. Of the 11 CDKs identified thus far (1), six (CDK1, CDK2, CDK3, CDK4, CDK6, and CDK7) have known roles in the regulation of the cell cycle (2, 3). CDK2 acts as a checkpoint for the G_1/S transition in the cell cycle. Despite a down-regulation of CDK2 activity in postmitotic cells, many cell types, including muscle cells, maintain abundant levels of CDK2 protein. This led us to hypothesize that CDK2 may have a function in postmitotic cells. We show here for the first time that CDK2 can be activated by neuregulin (NRG) in differentiated C2C12 myotubes. In addition, this activity is required for expression of the acetylcholine receptor (AChR) ε subunit. The switch from the fetal AChRγ subunit to the adult-type AChRε is required for synapse maturation and the neuromuscular junction. Inhibition of CDK2 activity with either the specific CDK2 inhibitor peptide Tat-LFG or by RNA interference abolished neuregulin-induced AChR expression. Neuregulin-induced activation of CDK2 also depended on the ErbB receptor, MAPK, and PI3K, all of which have previously been shown to be required for AChRε expression. Neuregulin regulated CDK2 activity through coordinating phosphorylation of CDK2 on Thr-160, accumulation of CDK2 in the nucleus, and down-regulation of the CDK2 inhibitory protein p27 in the nucleus. In addition, we also observed a novel mechanism of regulation of CDK2 activity by a low molecular weight variant of cyclin E in response to NRG. These findings establish CDK2 as an intermediate molecule that integrates NRG-activated signals from both the MAPK and PI3K pathways to AChRε expression and reveal an undiscovered physiological role for CDK2 in postmitotic cells.

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† The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; AChR, acetylcholine receptor; NRG, neuregulin; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; LMW, low molecular weight; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
stream component in NRG signaling that integrates both the MAPK and PI3K pathways. In the present study, we showed that NRG activates CDK2 in differentiated C2C12 myotubes and that CDK2 is required for NRG-induced AChR gene expression. We also demonstrated novel regulation of CDK2 by a cyclin E variant. To our knowledge, this is the first evidence of a physiologic role for a cell cycle-regulating CDK in postmitotic, terminally differentiated cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant neuregulin (rNRG-j1, catalog number 396-HB) was from R & D Systems (Minneapolis, MN). The CDK2 inhibitory peptide Tat-LFG was synthesized by Synpep Corporation (Dublin, CA). AG1478, PD98059, and LY294002 were from Calbiochem.

**Cell Culture**—Murine C2C12 cells (American Type Culture Collection, Manassas, VA) were maintained as undifferentiated myoblasts in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 15 mM Hepes, 10% fetal bovine serum, and 100 units/ml penicillin and 10 units/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. Differentiation into myotubes was performed by replacing the medium with Dulbecco’s modified Eagle’s medium containing 15 mM Hepes, 2% equine serum, and penicillin + streptomycin after cells reached confluence (27). The differentiation medium was changed daily. Fully differentiated myotubes were observed by day 3–4 in differentiation medium. On day 3, the myotubes were washed with phosphate-buffered saline and cultured in serum-free Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin for 24 h prior to use.

**Reverse Transcription**—Total RNA was isolated from C2C12 cells using TRIzol (Invitrogen) according to the suggested protocol, with the addition of two extra acid phenol:chloroform extractions. RNA quantity and quality were assessed on an Agilent Bioanalyzer 2100 using the RNA 6000 Nano Lab Chip Kit (Agilent, Foster City, CA). Five μg of total RNA was used in a 20-μl reverse transcriptase reaction (SuperScript™ First-Strand synthesis system for reverse transcription PCR, Invitrogen) according to the manufacturer’s protocol. Parallel reactions without reverse transcriptase were performed to ensure that there was no amplification from genomic DNA.

**Real-time PCR and Data Analysis**—Primers were designed using MacVector™ 7.0 software, and the specificity of primers was checked against GenBank™ using Blastn software. Primers were synthesized by Operon (Alameda, CA). Primer sequences are as follows: AChrE (GenBank™ accession number NM_009603) forward, 5′-AACAGCAGCGGATTTCTGGGG-3′; reverse, 5′-GCGGAGCGGCCCTTCAATAGG-3′; CDK2 (GenBank™ accession number AB017801) forward, 5′-H11032-ACAGCAGCGGATTTCTGGGG-3′; reverse, 5′-H11032-GCGGAGCGGCCCTTCAATAGG-3′; anti-cyclin E, forward, 5′-H9252-UGGCAGUACUGGGUACACCtt-3′; CDK2 antisense, 5′-H9262-UGGCAGUACUGGGUACACCtt-3′. “Scramble” siRNA with the sequences: sense, 5′-H11032-GCGGAGCGGCCCTTCAATAGG-3′; CDK2 antisense, 5′-H9262-UGGCAGUACUGGGUACACCtt-3′ had no homology to any known mRNA sequence. To our knowledge, this is the first evidence of CDK2 expression. We also demonstrated novel regulation of CDK2 by a cyclin E variant. To our knowledge, this is the first evidence of a physiologic role for a cell cycle-regulating CDK in postmitotic, terminally differentiated cells.

**RESULTS**

CDK2 is activated by NRG in differentiated C2C12 myotubes—As a first step in testing the possibility that CDK2 is involved in AChre induction, we confirmed CDK2 expression signal substrate, catalog number 34095 (Pierce). For cyclins A and E, we were unable to find both good monoclonal and good polyclonal antibodies; therefore, we chose to use CDK2 in the co-immunoprecipitation assay, horseradish peroxidase was conjugated directly to the primary antibody. For anti-cyclin E, the antibody was purified with Nb™ spin purification kits (catalog number 45200, Pierce), desalted with protein desalting spin columns (catalog number 88962, Pierce), and then conjugated with an EZ-Link™ Plus activated peroxidase kit (catalog number 31489, Pierce). For anti-cyclin A, carrier bovine serum albumin was first removed with the SwellGel Blue albumin removal kit (catalog number 88945, Pierce), and then the antibody was subjected to the same process as anti-cyclin E.

Anti-phospho-CDK1-Tyr15-Phos (catalog number 2561), anti-MAPK (9102), anti-phospho-MAPK (9106), anti-Akt (9272), anti-phospho-Akt (9271), anti-ErbB2 (2242), anti-phospho-ErbB2 (2241), anti-c-Jun (9162), and anti-phospho-threonine (9381) antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-p27 (catalog number 610241), anti-cyclin D3 monoclonal (610279), anti-Rb (14051A), anti-PCKs (610107), and anti-CDK2 monoclonal antibodies (610145) were from BD Biosciences. Anti-CDK5 (J-3, sc-6247), anti-cyclin D3 polyclonal (sc-182), and anti-actin (sc-1616) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CDK1 antibody (catalog number ab18), anti-cyclin A (ab2948), and anti-cyclin E (ab7595) were from Abcam (Cambridge, MA).

**CDK2 and Cyclin Immunoprecipitation Assays**—C2C12 cells were harvested as described above, except the concentration of Nonidet P-40 was 0.1%. Lysates (800 μg of total protein) were incubated with 5 μg of anti-CDK2 (M2, sc-163, Santa Cruz Biotechnology), 5 μg of anti-cyclin E, or 5 μg of anti-cyclin D3 antibodies and 30 μl of protein A-agarose beads (Santa Cruz Biotechnology) for 6 h at 4 °C. For Western blot analysis, the beads were boiled with loading buffer, and then samples were separated on 12% SDS-polyacrylamide gels.

For kinase assays, the beads were washed twice each with lysis buffer and kinase reaction buffer and then incubated in 40 μl of kinase reaction buffer containing Rb for 30 min at 30 °C. The reaction was stopped by boiling in gel loading buffer. Total Rb and phospho-Rb levels were analyzed by Western blot (anti-phospho-threonine antibody (catalog number 9301) from Cell Signaling) followed by hybridization of 25 μm Tria, ph 7.5, 5 μM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₂VO₄, 10 μM MgCl₂, 200 μM ATP, and 150 ng of Rb-C fusion protein (catalog number 6022, Cell Signaling).

**Cell Fractionation**—Cytosolic and nuclear fractionation of C2C12 myotubes was performed using the CellLytic™ Nuclear™ extraction kit (Sigma) with some modifications. Cells cultured in 150-mm plates were washed with warm phosphate-buffered saline and harvested with 1 ml of hypotonic buffer and placed on ice for 15 min, followed by centrifugation at 3300 g for 5 min. The supernatant was transferred to a fresh tube (S1), and the pellet was resuspended in 400 μl of hypotonic buffer. The cell suspension was passed eight times through a 27-gauge needle, followed by centrifugation at 3300 g for 4 min at 15 °C. This supernatant (S2) was combined with the S1, and this was termed the “cytosolic fraction.” The pellet was extracted with 140 μl of extraction buffer and vortexed at 4 °C for 30 min followed by centrifugation at 21,000 g for 4 min at 10 °C. The supernatant from this step was termed the “nuclear fraction.” Both the cytosolic and nuclear fractions were subjected to ultracentrifugation at 100,000 g for 30 min to obtain pure fractions.

**RNA Interference**—Small interfering RNA (siRNA) duplexes for mouse CDK2 (GenBank™ accession number NM_016756) were designed and synthesized by Ambion (Austin, TX) using the algorithm developed by Cenix BioScience (Dresden, Germany). The sequences are: CDK2 sense, 5′-GGUGUACCCAGACUGCGCAT-3′; CDK2 antisense, 5′-UGGCAUGAUCGGUACCCACt-3′. “Scramble” siRNA with the sequences: sense, 5′-GCCUCAUGUGUAUUCG-3′; antisense, 5′-CGAUCUCAACAGCGCCG-3′ had no homology to any known mammalian sequence and was synthesized by Dharmacon (LaFayette, CO) for use as a negative control. C2C12 myoblasts were plated in 6-well plates 2 days prior to transfection and used at ~30–40% confluence. Complexes were formed with ~10–20 pmol of siRNA and TransIT TKO transfection reagent (Mirus, Houston, TX) according to the manufacturer’s protocol. Two days after transfection, the cells were fully confluent, and differentiation was induced as described above.

**RESULTS**

CDK2 Is Activated by NRG in Differentiated C2C12 Myotubes—As a first step in testing the possibility that CDK2 is involved in AChre induction, we confirmed CDK2 expression...
in C2C12 myoblasts and myotubes. Undifferentiated C2C12 myoblasts fused into multinucleated myotubes ~72–96 h after switching to differentiation medium, at which time spontaneous contractions could be observed. C2C12 myoblasts expressed abundant CDK2 protein, and CDK2 protein levels in differentiated myotubes decreased only slightly, in agreement with previous reports (11, 12) (Fig. 1A). We also characterized the expression of several other CDKs. In contrast to CDK2, CDK1 was abundantly expressed in C2C12 myoblasts, but its expression decreased to undetectable levels upon myotube formation. CDK5 levels were essentially unchanged or slightly up-regulated, in agreement with a previous report that CDK5 protein levels and kinase activity increase during muscle differentiation (34). We then compared CDK2 activity in myoblasts and myotubes by measuring the ability of immunoprecipitated CDK2 to phosphorylate Rb in vitro (35, 36). We observed that basal CDK2 activity was dramatically lowered in differentiated myotubes compared with undifferentiated myoblasts (Fig. 1B).

The sustained level of CDK2 expression in myotubes led us to speculate that it might be involved in a postmitotic function. To test this possibility, we examined the response of CDK2 activity to treatment with NRG in differentiated myotubes. It has been shown that phosphorylation of CDK2 on Thr-160 is required for its activation (37). Using an antibody specific for phospho-CDK2Thr-160, we demonstrated that NRG treatment of differentiated C2C12 myotubes caused a time- and dose-dependent increase in CDK2 phosphorylation without changing the total CDK2 protein level (Fig. 1C). CDK2 was then immunoprecipitated from differentiated myotubes that had been treated with 2 nM NRG, and its kinase activity was determined in the in vitro assay. Treatment with NRG greatly enhanced CDK2 kinase activity in differentiated myotubes (Fig. 1D). These findings demonstrated that CDK2 is activated by NRG in differentiated C2C12 myotubes and suggested that CDK2 is indeed involved in postmitotic functions.

**CDK2 Activity Is Required for NRG-induced Expression of AChR**—We next performed experiments to determine whether CDK2 is involved in NRG-induced AChR expression. We first used a CDK2 inhibitor to determine whether blocking CDK2 activity prevents induction of AChR. Tat-LFG is a short peptide that specifically inhibits cyclin A-, B-, and E-associated CDK activities (i.e., CDK1 and CDK2) (38). The peptide serves as a docking site for cyclin/CDK complexes and blocks the phosphorylation of substrates by cyclin A/CDK2 or cyclin E/CDK2. We found that Tat-LFG effectively prevented CDK2 activation by NRG in differentiated C2C12 myotubes (Fig. 2A). We then used real-time PCR to measure AChR mRNA expression in response to NRG in the presence of Tat-LFG. NRG stimulated AChR mRNA expression, and pretreatment of C2C12 myotubes with 60 μM Tat-LFG completely blocked the NRG-induced increase in AChR mRNA (Fig. 2B). Treatment with the control peptide Tat had no effect on AChR induction. Similar results were obtained with another slightly less potent inhibitory peptide, Tat-LDL (not shown). The absence of CDK1 in differentiated myotubes (Fig. 1A) makes it unlikely that the effect of Tat-LFG on AChR expression is because of inhibition of cyclin A/CDK1 or cyclin E/CDK1 complexes. These results demonstrated that CDK2 activity is required for NRG-induced AChR expression.

To further confirm that CDK2 is involved in AChR induction, we used RNA interference to specifically knock down expression of CDK2 protein. C2C12 myoblasts were transfected with a siRNA duplex specific for CDK2 or with a control (scramble) siRNA that has no homology to any known mammalian sequence. Following the transfection, the differentiation of myoblasts into myotubes proceeded normally. Western blotting and image analysis showed that transfection with CDK2 siRNA decreased CDK2 protein levels by 80% (Fig. 2C). NRG-induced AChR expression was unaffected in cells transfected with scramble (control) siRNA and was almost completely blocked in cells transfected with CDK2 siRNA (Fig. 2D). This inhibitory effect on AChR expression was specific for CDK2, as CDK5 protein levels were unaffected by CDK2 siRNA treatment. These results confirm that CDK2 activity is required for AChR induction by NRG.

**NRG-induced CDK2 Activity Is Downstream of ErbB, MAPK, and PI3K**—We next sought to determine the location of CDK2 in the NRG signaling pathway. NRG-ErbB signaling activates both the MAPK and PI3K signaling pathways (39–42). It has been demonstrated that NRG-induced phosphorylation of ErbB receptors is necessary for subsequent MAPK and PI3K activation (30, 43, 44). Additionally, both the MAPK and PI3K path-
ways are reported to be necessary for NRG-induced AChR expression (29). We found that blockade of either the MAPK pathway with the MEK inhibitor PD98059 (50 \text{\textmu}M) or blockade of the PI3K pathway with LY294002 (40 \text{\textmu}M) abolished NRG-induced AChR expression (Fig. 3 A). NRG-induced CDK2 kinase activity (Fig. 3, B and C) was similarly abolished by inhibition of ErbB receptors (with AG1478, Ref. 45) or by inhibition of the MAPK or PI3K pathways. The specificities of these drugs are well established, and we confirmed that the PI3K pathway remained activated when the MAPK pathway was blocked and vice versa (not shown). This demonstrates that CDK2 activation by NRG is dependent on both the MAPK and PI3K pathways. Furthermore, whereas inhibition of CDK2 prevented NRG-induced AChR expression (29), we found that blockade of either the MAPK pathway with the MEK inhibitor PD98059 (50 \text{\textmu}M) or blockade of the PI3K pathway with LY294002 (40 \text{\textmu}M) abolished NRG-induced AChR expression (Fig. 3 A). NRG-induced CDK2 kinase activity (Fig. 3, B and C) was similarly abolished by inhibition of ErbB receptors (with AG1478, Ref. 45) or by inhibition of the MAPK or PI3K pathways. The specificities of these drugs are well established, and we confirmed that the PI3K pathway remained activated when the MAPK pathway was blocked and vice versa (not shown). This demonstrates that CDK2 activation by NRG is dependent on both the MAPK and PI3K pathways. Furthermore, whereas inhibition of CDK2 pre-
vented NRG-induced AChRe induction, neither treatment with Tat-LFG (Fig. 4A) nor CDK2 knockdown by siRNA (Fig. 4B) affected NRG-induced phosphorylation of ErbB2, MAPK, or Akt. Taken together, these data indicate that CDK2 is downstream of ErbB2, MAPK, and PI3K in the NRG signaling pathway.

NRG Regulates CDK2 Activity through CDK Phosphorylation, CDK Nuclear Accumulation, and Nuclear Loss of p27—We then performed experiments to begin to elucidate the mechanisms of CDK2 regulation by NRG in differentiated myotubes. The NRG-induced phosphorylation of CDK2 on Thr-160 (Fig. 1C) was abolished by inhibition of either the ErbB receptors or the MAPK or PI3K signaling pathways (Fig. 5A). In addition to CDK2 phosphorylation, the MAPK pathway regulates CDK2 nuclear translocation in proliferative cells (46, 47). We therefore examined the cellular localization of CDK2 in differentiated C2C12 myotubes. In untreated myotubes, the majority of CDK2 localized to the cytoplasm (Fig. 5B, top panel), was consistent with the observed decrease in CDK2 activity following differentiation. NRG treatment caused a dose-dependent increase of CDK2 in the nucleus. Upon inhibition of ErbB or of either the MAPK or PI3K pathways, NRG-induced nuclear accumulation of CDK2 was completely blocked (Fig. 5C, top panel). Finally, in untreated differentiated myotubes, the CDK2 inhibitor p27 was equally distributed between the cytoplasmic and nuclear fractions (Fig. 5B, second panel). Upon NRG treatment, the level of cytoplasmic p27 remained unchanged, whereas nuclear p27 was greatly decreased. Inhibition of ErbB, MAPK or PI3K also blocked the NRG-induced loss of nuclear p27 (Fig. 5C, second panel). In fact, inhibition of
these pathways caused an increase in nuclear p27 protein to levels above that seen in untreated cells. These results indicate that NRG, through the activation of both the MAPK and PI3K pathways, synchronously induced CDK2 phosphorylation on Thr-160, CDK2 nuclear accumulation, and loss of p27 in the nucleus, all of which contributed to the CDK2 activation in differentiated myotubes.

**CDK2 Activity in Differentiated Myotubes**

**Is Associated with a Cyclin E Variant**—Because the activity of CDKs requires binding to a cyclin partner, we next sought to determine which cyclin(s) associate with CDK2 in differentiated myotubes. In proliferating cells, CDK2 associates with cyclin E and cyclin A (2, 48). We therefore first determined expression levels of cyclin A and cyclin E in differentiated myotubes. A cyclin A band of the expected size of ~60 kDa (49) and another of ~51 kDa were observed in Western blots from undifferentiated myotubes (Fig. 6A, top panel). The 60-kDa form remained unchanged upon myotube differentiation, whereas the 51-kDa band disappeared. In contrast, a single cyclin E band of the expected size of ~51 kDa (50) was observed in undifferentiated myotubes (Fig. 6A, second panel). Upon differentiation, the amount of protein in the 51-kDa band decreased and a second, smaller band of ~45 kDa appeared. This smaller band is consistent in size with several reported low molecular weight (LMW) forms of cyclin E (51–54). Image analysis indicated cyclin E levels in myoblasts and myotubes were virtually identical and that the smaller band contained approximately half of the cyclin E immunoreactivity. Finally, cyclin D3 levels have been reported to increase in C2C12 cells upon differentiation (16). However, we observed a decrease in cyclin D3 levels (Fig. 6A, third panel).

We then used immunoprecipitation assays to determine which cyclin(s) associated with CDK2 in differentiated myotubes and in NRG-induced CDK2 activity. We found that no cyclin A co-immunoprecipitated with CDK2 in differentiated myotubes, either before or after NRG stimulation (not shown). We also observed little, if any, co-immunoprecipitation of CDK2 and cyclin E in unstimulated differentiated myotubes (Fig. 6B, and C, middle panels). However, upon NRG stimulation, there was a dramatic increase in the amount of CDK2 and cyclin E that co-immunoprecipitated. Interestingly, immunoprecipitation with anti-CDK2 antibody showed that only the 45-kDa form of cyclin E associated with CDK2 (Fig. 6B). In contrast, equal amounts of cyclin D3 co-immunoprecipitated with CDK2 in both unstimulated and NRG-stimulated myotubes (Fig. 6B, B and D, middle panels). Consistent with these findings, no increase in cyclin D3-associated kinase activity was observed upon NRG treatment, whereas NRG induced a dramatic increase in cyclin E-associated kinase activity (Fig. 6, C and D, bottom panels). Thus, CDK2 appears to undergo a novel form of regulation by LMW cyclin E in differentiated myotubes.

**DISCUSSION**

In this study we demonstrated that CDK2 protein is abundantly expressed and can be activated by NRG in differentiated C2C12 myotubes. NRG caused phosphorylation of CDK2 on Thr-160, CDK2 nuclear accumulation, and nuclear loss of the CDK2 inhibitory protein p27. In addition, NRG-induced CDK2 activity is regulated by a LMW variant of cyclin E. Our finding that CDK2 activity (but not protein levels) decreases during C2C12 differentiation is in agreement with previously published reports (11, 12). As a positive regulator of cellular proliferation, it is not surprising that CDK2 activity must be turned off in order for terminal differentiation to proceed. An important finding in this study is that CDK2 activity was essential for NRG-induced AChRe expression, as confirmed by both the inhibition of CDK2 with the specific inhibitory peptide Tat-LFG and knock down of CDK2 with RNA interference. AChRe expression is important not only because it alters the electrophysiological properties of the AChR channel as it switches from its fetal to its adult configuration. AChRe expression is also essential for morphological and molecular changes characteristic of the mature synapse, such as clustering of AChRs into high density patches, restriction of AChR synthesis to subsynaptic nuclei, formation of junctional folds, formation of a raised endplate, and proper distribution of synaptic components, such as rapsin, utrophin, ErbB4, and MuSK (re-
CDK2 in NRG-induced AChRe Expression

viewed in Refs. 17 and 19). Our results suggest that, in addition to cell cycle regulation, CDK2 is also involved in the AChR maturation in differentiated myotubes. Thus, there appears to be a requirement in differentiated myotubes for CDK2 activity outside its cell cycle-regulatory function. To our knowledge, this is the first evidence of a physiological role for a cell cycle-regulating CDK in postmitotic, terminally differentiated cells.

Tansey et al. (29) demonstrated that both the MAPK and PI3K signaling pathways were required for NRG-induced AChRe expression, although the involvement of PI3K has been questioned (27, 55). Tansey et al. showed that pharmacological inhibition of the MAPK had no effect on NRG-induced PI3K activity, and vice versa, although inhibition of either pathway blocked AChRe reporter expression. This suggests the existence of a downstream integrating molecule that coordinates the signals from these two pathways. We showed that CDK2 lies downstream of the ErbB2 receptor and both MAPK and PI3K in the NRG signaling pathway, and that ErbB2, MAPK, and PI3K are all required for both NRG-induced CDK2 activity and NRG-induced AChRe expression. These results position CDK2 as an ideal candidate for this integrating function.

Activation of CDK2 is complex and depends upon many factors including phosphorylation of Thr-160 (37), dephosphorylation of Thr-14 and Tyr-15 (56), disassociation of CDK2 from the inhibitory proteins p21, p27, or p57 (57–59), and assembly of CDK2 with cyclins (60). Both phosphorylation of Thr-160 by CDK-activating kinase (61) and dephosphorylation of Thr-14 and Tyr-15 by CDC25 (62) take place in the nucleus, and nuclear localization has been shown to correlate with CDK2 activation (63). Inhibition of MAPK has been shown to prevent CDK2 Thr-160 phosphorylation, nuclear localization, and kinase activity in response to mitogenic stimuli (46, 47, 64). We showed that, in differentiated C2C12 myotubes, NRG-induced nuclear accumulation and Thr-160 phosphorylation of CDK2 were dependent on both the MAPK and PI3K pathways. Furthermore, in some cells accumulation of p27 and growth arrest is dependent only upon PI3K (65, 66), whereas in other cells, MAPK is also involved (67, 68). We showed that, in differentiated C2C12 cells, inhibition of either MAPK or PI3K not only blocked the NRG-induced nuclear loss of p27, but increased nuclear p27 levels to above that seen in untreated cells. These results demonstrate that NRG-induced MAPK and PI3K signaling converge at CDK2 and regulate CDK2 activity through coordinating multiple events.

In proliferating cells, CDK2 requires cyclin E for the entry into the S phase and the initiation of DNA replication, whereas it requires cyclin A for S phase transition and further control of the replication cycle (2, 48). We found that CDK2 no longer associates with cyclin A in differentiated myotubes, in agreement with a previous study (16). Also in agreement with this study, we observed association of cyclin D3 with CDK2 in differentiated myotubes. However, cyclin D3 does not appear to be involved in NRG-induced CDK2 activity. In contrast, both cyclin E interaction with CDK2 and cyclin E-associated kinase activity increased dramatically upon NRG stimulation in differentiated myotubes. Interestingly, we observed the appearance of a LMW cyclin E upon myotube differentiation, and it is this smaller form of cyclin E that co-immunoprecipitated with CDK2.

A number of LMW forms of cyclin E, ranging from Mr 36,000–45,000 have been described. These LMW molecules are the result of both alternative splicing of the cyclin E transcript and proteolytic processing (50, 52, 54, 69–72). Two of the splice variants, cyclin Eα (52) and cyclin Eγ (53) contain internal deletions at or near the cyclin box and are unable to bind CDK2. Other LMW forms can bind CDK2 and do so with a higher affinity than full-length cyclin E (50, 69–71). Transcripts for these splice variants are found in both normal and tumor cells, although the amount of cyclin E and the number of different forms are greater in tumor cells (51, 69, 70, 73). In addition, when epitope-tagged full-length cyclin E is expressed in normal cells, it undergoes post-translational proteolytic processing, indicating that normal cells have the capacity to form LMW cyclin E (54). We observed that the total amount of cyclin E did not change upon myotube differentiation, and that the LMW form constituted approximately half of the cyclin E in differentiated cells. This expression pattern remained constant through at least eight days in differentiation medium (not shown) and suggests that LMW cyclin E in differentiated C2C12 cells is produced at least in part by proteolytic processing. In cancer cells, LMW cyclin E forms are hyperactive and cause deregulation of the cell cycle, and their overexpression in tumors is associated with disease progression and poor clinical outcome (73–76). In differentiated C2C12 myotubes, however, LMW cyclin E appears to represent a novel mechanism of physiological regulation of CDK2.

CDK2 has received attention lately as recent studies have suggested that CDK2 may not have an essential function in cell cycle control. A number of CDKs and cyclins including CDK2 (77, 78), CDK3, CDK4, CDK6, cyclin A1, cyclin B2, cyclin E1, and cyclin D2 (reviewed in Ref. 79) appear to be fairly dispensable in embryonic development and cell cycle progression. In CDK2 knock-out mice, the only phenotypic defect observed is impaired gametogenesis. It has been postulated that the dispensability of the various CDKs may be due to redundancy in mammalian cell cycle control, and that, at least in vivo, other cyclins and CDKs might compensate for the missing genes (reviewed in Refs. 8, 79–81). Indeed, there is a good degree of overlap in the substrates that can be phosphorylated by the various cyclin/CDK complexes, and much of the substrate specificity resides in the temporal and spatial patterns of expression of these proteins (48, 82). However, the ability of CDKs to compensate for one another is poorly understood at present.

Nevertheless, primary mouse embryonic fibroblasts isolated from CDK2 knock-out mice demonstrate lower proliferation rates compared with wild-type mouse embryonic fibroblasts at later passages in culture despite identical proliferation rates at earlier passages (77, 78). In addition, late passage CDK2−/− mouse embryonic fibroblasts that are synchronized by serum starvation display delayed re-entry into the S phase compared with wild-type mouse embryonic fibroblasts. These data indicate that, although other CDKs may be able to partially compensate for the lack of CDK2, they are not able to do so as efficiently as CDK2. This suggests that cell cycle control proteins may be optimized for certain functions as a means of fine-tuning a complex process. The same reasoning may be applicable to the role of CDK2 in postmitotic cells. Although CDK2 knock-out mice do not display the severe muscle atrophy and reduced viability seen in AChRe knock-out mice, it would be interesting to see whether they possess any subtle defects in muscle function.

In postmitotic cells, aberrant activation of CDK2 is associated with the death of neurons and cardiomyocytes in hypoxia/ischemia and graft coronary arteriosclerosis (83–85). Here we report for the first time that CDK2 activation in a differentiated cell type by a physiological stimulus is an essential step for a physiological phenotype, i.e. the expression of the AChRe subunit. We also demonstrate a novel form of physiological regulation of CDK2 that appears to be specific to differentiated cells. It is therefore critical to gain a thorough understanding of what accounts for the differential functions of CDK2 in various physiological and pathological
states. In differentiated myotubes, it appears that a primary function of CDK2 may be to integrate signals from the pathways activated by NRG that are required for AChR expression and subsequent synapse maturation.

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