Integration of Rac-dependent Regulation of Cyclin D1 Transcription through a Nuclear Factor-κB-dependent Pathway*

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The small GTP-binding protein Rac1, a member of the Ras superfamily, plays a fundamental role in cytoskeletal reorganization, cellular transformation, the induction of DNA synthesis, and superoxide production. Cyclin D1 abundance is rate-limiting in normal G1 phase progression, and the abundance of cyclin D1 is induced by activating mutations of both Ras and Rac1. Nuclear factor-κB (NF-κB) proteins consist of cytoplasmic hetero- or homodimeric Rel-related proteins complexed to a member of the IκB family of inhibitor proteins. In the current studies, activating mutants of Rac1 (Rac-Leu-61, Rac-Val-12) induced cyclin D1 expression and the cyclin D1 promoter in NIH 3T3 cells. Induction of cyclin D1 by Rac1 required both an NF-κB and an ATF-2 binding site. Inhibiting NF-κB by overexpression of an NF-κB transdominant inhibitor (nonphosphorylatable IκBα) reduced cyclin D1 promoter activation by the Rac1 mutants, placing NF-κB in a pathway of Rac1 activation of cyclin D1. Specific amino acid mutations in the amino-terminal effector domain of Rac-Leu-61 had comparable effects on NF-κB transcriptional activity and activation of the cyclin D1 promoter. The NF-κB factors Rel A (p65) and NF-κB1 (p50) induced the cyclin D1 promoter, requiring both the NF-κB binding site and the ATF-2 site. Stable overexpression of Rac-Val-12 increased binding of Rel A and NF-κB1 to the cyclin D1 promoter NF-κB site. Activation of Rac1 in NIH 3T3 cells induces both NF-κB binding and activity and enhances expression of cyclin D1 through an NF-κB and ATF-2 site in the proximal promoter, suggesting a critical role for NF-κB in cell cycle regulation through cyclin D1 and Rac1.

Rac proteins are members of the Ras family of small GTPases, which regulate a diverse spectrum of biological effects including cytoskeletal reorganization, cellular proliferation, transformation, and transcriptional activity (1). Rac interacts with specific effector proteins through domains that coordinate activation of multiple signaling cascades (1). Biochemical activities of Rac/Rho chimerae identified the amino-terminal effector site, encompassing amino acids 30–40, which, in conjunction with the carboxyl-terminal effector site (amino acids 143–175), is needed for the induction of actin polymerization (2). Rac1 regulates downstream signaling by mitogen-activated protein kinase (MAPK)1 pathways, including the p38 pathway (3) and the Jun N-terminal kinase (JNK) pathway (4), as well as through nuclear factor κB (NF-κB) activity (5). The ability of Rac1 to activate an array of intracellular signaling pathways has confounded the elucidation of the mechanisms by which Rac1 regulates cell cycle regulatory targets. The cell cycle regulatory cyclin D1 gene is directly activated by Rac1 (6). The abundance of the cyclin D1 gene product is rate-limiting in G1 phase progression. The abundance of the cyclin D1 gene product is rate-limiting in G1 phase progression, at least in part, because of the role of this regulatory subunit in forming cyclin-dependent kinase holoenzymes, which phosphorylate and inactivate the retinoblastoma tumor suppressor (7). Rac1 induction of cyclin D1 occurred through a pathway that is distinct from JNK, extracellular signal-regulated kinase (ERK), or p38 MAPK (6), and the identity of this pathway remains to be determined.

Like Rac1, oncogenic Ras also induces NF-κB activity (8) and cyclin D1 (9), both of which are required for the induction of foci formation by Ras (8, 10). NF-κB belongs to the Rel family of transcription factors (11), which include NF-κB1 (p50), Rel A (p65), c-Rel, and Rel-B, all of which dimerize and bind DNA to induce gene transcription. In most cell types, NF-κB is sequestered in the cytoplasm in an inactive complex with IκBo or IκBβ (12). Diverse signals induce the phosphorylation and degradation of IκBα, resulting in the nuclear translocation of NF-κB and thereby enhancing DNA sequence-specific gene transcription (13). In the current studies we demonstrate that cyclin D1 is a direct transcriptional target of Rac1 through sequences that are activated by NF-κB. Both the wild type NF-κB response element and the cyclin D1 promoter are inhibited by a constitutive repressor of NF-κB activity. Rel A and NF-κB1 activate cyclin D1, and induction by NF-κB1 requires the Rac1-responsive sequences. The finding that Rac1 induction of cyclin

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The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal kinase; NF-κB, nuclear factor-κB; ERK, extracellular signal-regulated kinase; ATF, activating transcription factor; bp, base pair(s); EMISA, electrophoretic mobility gel shift assay.
D1 involves NF-xB suggests an important role for NF-xB in Rac1-induced transformation and mitogenesis.

MATERIALS AND METHODS

Western Blot Analysis—The abundance of cyclin D1 protein was determined by Western blot analysis as described previously (9), using monoclonal antibody DC56 (Neomarkers, Fremont, CA) followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The cyclin D1 protein was visualized by the enhanced chemiluminescence system (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Plasmid and Cell Culture—The expression vectors for the activating Rac1 mutant (pcDNA3-RacLeu-61) and pCGN-RacAsn-17) and the effector domain mutants were described previously (6). The activating Rac1 mutant RacVal-12 was expressed from the pcDNA3 plasmid (RacVal-12/pcDNA3), and RacVal-12/V5His, the pCGT plasmid (RacVal-12/V5His, and RacVal-12/V5His-His), or the pEXV3 plasmid (RacAsn-17) (14), pCGN-RelA (p65) and pCGN-NF-xB (p50) expression vector (15), CMV-Luc (super-repressor) (CMV-Luc/Bsr) were described previously (16). Luciferase reporters for the gene promoters of human cyclin D1, human cyclin A (17, 18), the fos gene (c-fosLUC), JUNB (junB-2) were prepared by the method of Li et al. (19) from NIH 3T3 cells stably transfecting with the pCGN-RacLeu-61 and pcDNA3-RacLeu-61 constructs, along with derived mutants, were pooled from 50 individual colonies selected in hygromycin (pCGN vectors) or G418 (pDNA vectors). Expression of the mutant proteins was confirmed by Western blotting (6). Statistical analyses were performed using the Wilcoxon signed rank test.

Electrophoretic Mobility Gel Shift Assays (EMSA)—Nuclear extracts were prepared by the method of Li et al. (19) from NIH 3T3 cells overexpressing RacLeu-61 (pCGN-RacLeu-61) and from control cells transfected with empty pGCV vector (6). The consensus NF-xB site from murine Ig x enhancer (NF-xBex, 5'-GCG TAC GAA GGG ACT TTC CGA G 3'), a mutant NF-xB (NF-xBmut, 5'-GCA GAA Gaa ACT TTC CGA G 3') site, which abolishes NF-xB binding (15) and the NF-xB site at 39 to 50 bp in the cyclin D1 promoter (CDNFT-Luc, 5'-TAC AGG GAA GTT TTG TTG AAG-3'), were synthesized as complementary oligodeoxyribonucleotide strands for EMSA (17). For supershift analysis, 2 μg of antibody to NF-xB, RelA, Rel-B, or c-Rel (Santa Cruz Biotechnology) was added to the pre-incubation mixture.

RESULTS

Rac1-responsive Cyclin D1 Promoter Elements—In previous studies we showed that the cyclin D1 promoter was activated by transforming Rac1 mutants (6). The current studies were performed to identify the molecular mechanisms governing cyclin D1 induction by Rac1. Cyclin D1 abundance was assessed in NIH 3T3 cells overexpressing RacLeu-61. In the serum-starved state, cyclin D1 levels were increased 2-fold in the RacLeu-61 cell line compared with NIH 3T3 cells stably transfected with the empty pCDNA vector (Fig. 1A). Serum induced cyclin D1 protein levels 5-fold at 24 h in the parental cell line. Cyclin D1 protein levels were elevated 2- to 3-fold fold at each time point in the RacLeu-61 stable line compared with NIH 3T3 cells stably transfected with an empty vector (Fig. 1A). The cyclin D1 promoter luciferase reporter gene (−1745 CD1LUC) was co-transfected with the transforming Rac1 mutants (RacVal-12 and RacAsn-17) into NIH 3T3 cells (Fig. 1B). The cyclin D1 promoter luciferase reporter gene was induced 6-fold by the RacVal-12 mutants. Co-expression of a dominant negative Rac1 mutant, RacAsn-17 suppressed base-line expression of −1745 CD1LUC by 20%. To identify Rac1-responsive DNA sequences, a series of cyclin D1 promoter deletion constructions or point mutants was assessed (Fig. 1C). Induction of the cyclin D1 promoter by RacVal-12 was reduced to 2-fold by deletion to −66 bp, indicating that minimal Rac1 responsive elements were located within the proximal 66 bp. Mutation of the cyclin D1 promoter CRE/ATF site at −54 (20–23) abolished RacLeu-61 induction (Fig. 1C). Because an activating Rac31 mutant-induced NF-xB activity (5), and we had identified sequences resembling an NF-xB site in the cyclin D1 promoter at −39 to −50 bp, the effect of mutating the NF-xB site on RacLeu-61 induction of the cyclin D1 promoter was assessed. Mutation of the NF-xB site also abolished RacLeu-61 induction of the cyclin D1 promoter (Fig. 1C).

NF-xB Activity Regulation by RacLeu-61—Our studies suggested that RacLeu-61 might induce NF-xB activity in NIH 3T3 cells, consistent with previous studies in which NF-xB activity was induced by Ras in NIH 3T3 cells (8) or by Rac60 in COS cells (5). We therefore determined whether RacLeu-61 directly activated the NF-xB responsive reporter 3xBLUC in NIH 3T3 cells. The 3xBLUC reporter was induced 22-fold by RacVal-12 and 11.7-fold by RacLeu-61 (Fig. 2A). RacAsn-17 dominant negative mutant suppressed basal activity of 3xBLUC to 68 ± 12% of control (n = 6, p < 0.05). These studies suggest that basal 3xBLUC activity is sustained in part by endogenous Rac1-dependent activity. Rel A also induced the NF-xB respon-
Regulation of the Cyclin D1 Promoter by Rac1

The Effector Domains of Rac1 that Induce Cyclin D1 and NF-κB Activity—Recent studies identified a role for the Rac1 amino-terminal effector domain in activation of the ERK, JNK, p38, and NF-κB pathways (1, 6). We examined the role of the amino-terminal effector domain in Rac1/NF-κB activation of the cyclin D1 promoter (Fig. 4B). The amino-terminal effector domain mutants exhibited a similar pattern of activation of the 3xBLUC reporter and the cyclin D1 promoter but that residues 26 and 40 are less critical for induction of either reporter construction. Residue 43 is required preferentially for induction of cyclin D1.

NF-κB Activates Cyclin D1 Promoter Activity—Because induction of cyclin D1 by Rac1 required an NF-κB binding site, we examined the role of NF-κB in directly regulating cyclin D1. p65 induced the cyclin D1 promoter 50-fold (Fig. 4A) but not the c-fos, c-myc, Myb promoter, and junB promoter were not induced by p65, and the cyclin A promoter were induced only 1.8-fold (Fig. 4B).

An IκB "super-repressor" expression plasmid pCMV-IκB(Sr) in which IκBα serine residues 32 and 36 in pCMV-IκB(Sr) have been mutated to alanines, inhibited activity of the 3xBLUC reporter and the cyclin D1 promoter (Fig. 4B) but not the c-fos promoter (data not shown). Rac1 enhanced p65 induction of 3xBLUC and p65 enhanced Rac1 activation of the cyclin D1 promoter (Fig. 4C). To determine the cyclin D1 promoter DNA sequences required for induction by NF-κB, a series of cyclin D1 promoter constructions were examined in the presence of p50 (Fig. 4D) or p65 (not shown). p50 induced the cyclin D1 promoter 50-fold.

The Effector Domains of Rac1 that Induce Cyclin D1 and NF-κB Activity—Recent studies identified a role for the Rac1 amino-terminal effector domain in activation of the ERK, JNK, p38, and NF-κB pathways (1, 6). We examined the role of the amino-terminal effector domain in Rac1/NF-κB activation of the cyclin D1 promoter by comparing the effects of specific effector domain mutations on activation of the 3xBLUC and -1745 CD1LUC reporters. Mutating residue 33 or 37 in pCGT-RacVal-12 reduced induction of -1745 CD1LUC by 95% (Fig. 3B). Mutation of residues 30, 31, 33, and 45 in pCGN-RacLeu-61 suppressed induction by smaller amounts (60–70%, Fig. 3B). Mutation of residue 40 reduced induction by pCGT-RacVal-12 some 25% (Fig. 3A). The amino-terminal effector domain mutants exhibited a similar pattern of activation of the 3xBLUC reporter. Again, residues 30, 31, 33, 37, and 45 mutants, in their respective pCGN-RacVal-12 or pCGN-RacLeu-61 contexts, had diminished activity, but the activity was relatively preserved in the other mutants. These studies suggest that residues 30, 31, 33, 37, and 45 are required for full induction of the 3xBLUC reporter and the cyclin D1 promoter but that residues 26 and 40 are less critical for induction of either reporter construction. Residue 43 is required preferentially for induction of cyclin D1.

NF-κB regulation by RacLeu-61. A, the NF-κB responsive reporter 3xBLUC was co-transfected with expression vectors encoding RacVal-12, or RacLeu-61, or the dominant negative Rac1 mutant, RacAsn-17. Luciferase activity is expressed (striped bars) relative to activity cells co-transfected with equal amounts of empty expression vector cassette (solid bars). The data are shown as means ± S.E. for four (RacVal-12 and RacLeu-61) or six (RacAsn-17) independent experiments. B, induction of 3xBLUC by Rel A is shown for comparison versus its cognate empty vector.

The cyclin D1 promoter is induced by NF-κB through an NF-κB response element. A, co-transfections were conducted with pCGN-RelA (p65) and the reporter constructions as shown. B, CMV-IκB(Sr) suppressed base-line expression of 3xBLUC and -1745 CD1LUC compared with the cognate empty expression vector. C, the effect of RacVal-12 on cyclin D1 promoter activity in the presence of co-transfected Rel A. The RacVal-12 induction of the -1745 CD1LUC reporter was induced further by the addition of Rel A. D, cyclin D1 promoter deletion constructions or point mutants in a luciferase expression vector were co-transfected with pCGN-NF-κB1 (p50).

The Effector Domains of Rac1 that Induce Cyclin D1 and NF-κB Activity—Recent studies identified a role for the Rac1 amino-terminal effector domain in activation of the ERK, JNK, p38, and NF-κB pathways (1, 6). We examined the role of the amino-terminal effector domain in Rac1/NF-κB activation of the cyclin D1 promoter by comparing the effects of specific effector domain mutations on activation of the 3xBLUC and -1745 CD1LUC reporters. Mutating residue 33 or 37 in pCGT-RacVal-12 reduced induction of -1745 CD1LUC by 95% (Fig. 3B). Mutation of residues 30, 31, 33, and 45 in pCGN-RacLeu-61 suppressed induction by smaller amounts (60–70%, Fig. 3B). Mutation of residue 40 reduced induction by pCGT-RacVal-12 some 25% (Fig. 3A). The amino-terminal effector domain mutants exhibited a similar pattern of activation of the 3xBLUC reporter. Again, residues 30, 31, 33, 37, and 45 mutants, in their respective pCGN-RacVal-12 or pCGN-RacLeu-61 contexts, had diminished activity, but the activity was relatively preserved in the other mutants. These studies suggest that residues 30, 31, 33, 37, and 45 are required for full induction of the 3xBLUC reporter and the cyclin D1 promoter but that residues 26 and 40 are less critical for induction of either reporter construction. Residue 43 is required preferentially for induction of cyclin D1.

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Mutation of the ATF-2 site reduced cyclin D1 promoter induction by 75% (Fig. 4D), and mutation of the NF-κB site abolished induction by p50 (Fig. 4D).

Transforming Rac1 Mutants Increase p50/p65 Binding to the Cyclin D1 NF-κB Site—To determine whether Rac<sup>Leu-61</sup> altered NF-κB binding activity in NIH 3T3 cells, EMSA were performed using either the cyclin D1 NF-κB site or a consensus NF-κB site. Nuclear extracts were obtained after 24 h of serum deprivation and after restoration of serum for 4 h (Fig. 5A) from cells stably overexpressing Rac<sup>Leu-61</sup> (6) or the empty expression vector cassette (pCDNA3). The cyclin D1 NF-κB site bound a complex (Fig. 5A, lane 1, bands A and B), which was competed by specific competitor (lane 2) and was supershifted with antibodies to p65 (lane 4) and p50 (lane 5) but not control IgG (lane 3), Rel-B (lane 6), or c-Rel (lane 7). Under the same serum-starved conditions, equal amounts of nuclear extracts from the Rac<sup>Leu-61</sup> stable cell line conveyed increased binding to the same site (compare Fig. 5A, lanes 1 and 8). The cyclin D1 NF-κB site bound both p65 and p50 (lanes 11, 12). The addition of serum for 4 h further induced the binding to the cyclin D1 promoter NF-κB site (compare Fig. 5A, lanes 8 and 15). The proteins binding to the cyclin D1 NF-κB site in the Rac<sup>Leu-61</sup> cell line included p65 (lane 11) and p50 (lane 12). These studies suggested that band A consisted predominantly of p50 and the lower band B of p50/p65. The complexes binding to the consensus NF-κB probe are indicated as a’, b’, and c’ (Fig. 5B, lane 1). Complexes a’ and c’ were supershifted with p65 antibody (Fig. 5B, lanes 3, 10, and 16) and complex b’ was shifted with the p50 antibody (lanes 4, 11, and 17). The relative abundance of these complexes was increased in the Rac<sup>Leu-61</sup> stable cell line extracts (Fig. 5B, lane 1 versus 8) and was increased further with 4 h of serum treatment (Fig. 5B, lane 8 versus 15).

**DISCUSSION**

Cyclin D1 encodes a rate-limiting component of the cell cycle regulatory holoenzyme required for G<sub>1</sub> phase progression, cellular mitogenesis, and Ras-induced transformation (10, 25). Recent studies have identified a role for Rac1 in promoting G<sub>1</sub> phase progression, mitogenesis, and Ras transformation (1, 26) and have shown that cyclin D1 is activated by transforming mutants of Ras, Rac1, and the Dbl-related proteins (6, 9, 27). Rac1 was previously shown to induce cyclin D1 expression through a pathway distinct from the JNK or ERK pathway (6). In the current studies, Rac1 induction of cyclin D1 was inhibited by an NF-κB <i>trans</i>-dominant inhibitor; Rac1 activated the cyclin D1 promoter through DNA sequences that were also activated by NF-κB; Rac1 induced NF-κB binding to the cyclin D1 NF-κB site and Rac1 effector domain mutations correlated in their effect on NF-κB and cyclin D1 promoter activity. Thus, we demonstrate a critical role for NF-κB in Rac1-induction of cyclin D1 and also demonstrate that the cyclin D1 gene is a direct transcriptional target of NF-κB.

In the current studies, the ATF-2 site contributed to cyclin D1 induction by Rac1 and p50. The cyclin D1 ATF-2 site contributes to several distinct signaling pathways that induce the cyclin D1 gene in distinct cell types. The ATF-2 site contributed to induction of cyclin D1 by SV40 small t antigen (21), and was required for induction of cyclin D1 by pp60<sup>c-src</sup> in MCF7 breast epithelial cells (20), by serum in fibroblasts (22), and by ATF-2 in chondrocytes (23). The abundance of cyclin D1 is rate-limiting in serum-induced cellular proliferation in fibroblasts (22), in chondrocyte proliferation (23), and in Ras-induced transformation in NIH 3T3 cells (10), implicating the cyclin D1 ATF-2 site as a critical target in these proliferative and transforming pathways. The cyclin D1 ATF-2 site binds predominantly c-Fos/FosB proteins in fibroblasts (22). NF-κB, both its p50 and p65 components, enhances activation by c-Fos (28). The ATF-2/c-Jun proteins form synergistic functional interactions with NF-κB (13, 28); in conjunction with the finding that the NF-κB <i>trans</i>-dominant inhibitor blocked Rac1 induction of cyclin D1, these studies underscore the importance of combinatorial interactions between NF-κB and other transcription factors. The transcriptional cross-coupling between AP-1 and NF-κB proteins at the cyclin D1 promoter ATF-2 site may serve to integrate and potentiate their individual biological activities.

In addition to their role in cytokine signaling and cell survival, NF-κB proteins have also been implicated in regulating cell cycle progression and transformation. NF-κB activity is induced as cells pass from the G<sub>0</sub> into the G<sub>1</sub> phase of the cell cycle (29), and lymphocytes of mice homozygously deleted of NF-κB/Rel genes exhibited defective mitogen-induced proliferative responses (30, 31). NF-κB activity is induced by the <i>Bcr/Abl</i> oncogene and is constitutively overexpressed in breast cancer and Hodgkin’s disease (32–34). The inhibition of aberrant NF-κB overexpression contributed to a reversal of transformed phenotype by <i>Bcr/Abl</i> and of the tumor cells derived from patients (32–34). Because the abundance of cyclin D1 is rate-limiting in cellular transformation by Ras (10, 25) and we have identified a critical role for NF-κB in Rac1-induced cyclin D1 abundance, these studies raise the possibility that cyclin D1 may play a role in NF-κB proliferative/oncogenic signaling events.
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