An NF-κB-Specific Inhibitor, IκBα, Binds to and Inhibits Cyclin-Dependent Kinase 4†

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ABSTRACT: IκBα, a protein composed of six ankyrin repeats, is a specific inhibitor of nuclear factor κB (NF-κB) and functions in signal transductions in many different cell types. Using both in vivo yeast two-hybrid assays and in vitro activity and binding assays, we showed that IκBα binds to cyclin-dependent kinase 4 (CDK4) specifically and inhibits its kinase activity. The potencies of binding and inhibition of IκBα are comparable to those of INK4 proteins, the specific CDK4 inhibitors that also contain ankyrin repeats. Furthermore, we showed that INK4 proteins and IκBα compete with each other for binding to CDK4. These results led us to propose a hypothesis that there is cross talk between the NF-κB/IκBα pathway and the p16/CDK4/Rb pathway in cells, and that IκBα could substitute for the CDK4-inhibiting function of p16, a tumor suppressor frequently inactivated in human tumors. To further understand the structural basis of IκBα-CDK binding, we used different mutants of CDK4 to show that there are notable differences between IκBα and INK4 proteins in CDK4 binding since the binding is affected differently by different CDK4 mutations. We also demonstrated that the interaction of IκBα with CDK4 is different from that with its NF-κB. While most of the contacts contributing to NF-κB binding are located within the last two C-terminal ankyrin repeats and the loop region bridging them, the first four ankyrin repeats at the N-terminus are responsible for CDK4 binding and inhibition.

Ankyrin repeats are a common motif of ∼34 amino acid residues (*1*). Its structure consists of two antiparallel α-helices, a *β*-turn, and a long loop that extends to the turn of the next repeat (*2*, *3*). Typically, ankyrin repeats stack in a linear fashion to form a right-handed solenoid structure with a hydrophobic core and a large solvent-accessible surface (*4*). To date, nearly 8000 ankyrin repeat sequences have been identified (*5*). Most of these sequences contain four to seven consecutive ankyrin repeats, though as many as 29 ankyrin repeats have been found in a single protein (*6*, *7*). Ankyrin repeats have been found to mediate protein−protein or protein−DNA interactions involved in diverse cellular events, such as cell cycling, signal transduction, and apoptosis (*1*, *4*). Two of the best-studied ankyrin repeat proteins are $p16^{INK4A}$ and IκBα.[1]

$p16^{INK4A}$ (hereafter p16) is a well-known tumor suppressor protein found to be inactivated in a variety of tumors and tumor cell lines (*8*). It is composed of four ankyrin repeats, and belongs to the INK4 class of cyclin-dependent kinase inhibitors which also include $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ (hereafter p15, p18, and p19, respectively). p16 functions in the G0-to-G1 and G1-to-S transitions through specific inhibition of cyclin-dependent kinase 4 and 6 (hereafter CDK4 and CDK6, respectively)-mediated phosphorylation of the retinoblastoma gene product (Rb). Phosphorylation of Rb leads to the release of E2F transcription factors from Rb−E2Fs complexes, and triggers the activation of genes required for S phase entry. Thus, by binding to and inhibiting CDK4 and 6, p16 impedes cell progression into the S phase (*1*, *8*, *9*).

IκBα consists of six ankyrin repeats, and acts as a specific inhibitor of NF-κB, a transcription factor controlling vital genes required for immune response and inflammation, cell growth and differentiation, cell adhesion, and apoptosis (*10*−*12*). Like other IκB family members (including IκB*β*, p105, p100, IκB*ϵ*, and Bcl-3), IκBα binds to NF-κB and sequesters the latter in the cytoplasm. Environmental stimuli trigger the sequence-specific phosphorylation of IκBα and disrupt the NF-κB−IκBα complex. Subsequently, NF-κB is translocated into the nucleus and activates the expression of specific genes to evoke appropriate cellular responses.

p16 and IκBα have been thought to function in independent pathways, and there is no evident similarity between these two proteins. An amino acid homology analysis seemingly supports this observation (*13*) (Figure 1). While the sequences of p16 and p18 are 40% identical, the level of identity between p16 and IκBα is only 16%, a level of identity present among most ankyrin repeat proteins. However, recent studies suggested that there are certain correlations between these two proteins and their functional pathways. First, it was reported that NF-κB transcription activation is coordinated with CDK activities (*14*). Second,

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[1] Abbreviations: AD, LexA activation domain; CDK4 and -6, cyclin-dependent kinase 4 and 6, respectively; DTT, dithiothreitol; GST, glutathione *S*-transferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HTLV-1, human T cell leukemia virus 1; IκBα, NF-κB inhibitor α; INK4, inhibitor of cyclin-dependent kinase 4; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Rb, human retinoblastoma susceptible gene product; tax, transactivation protein.

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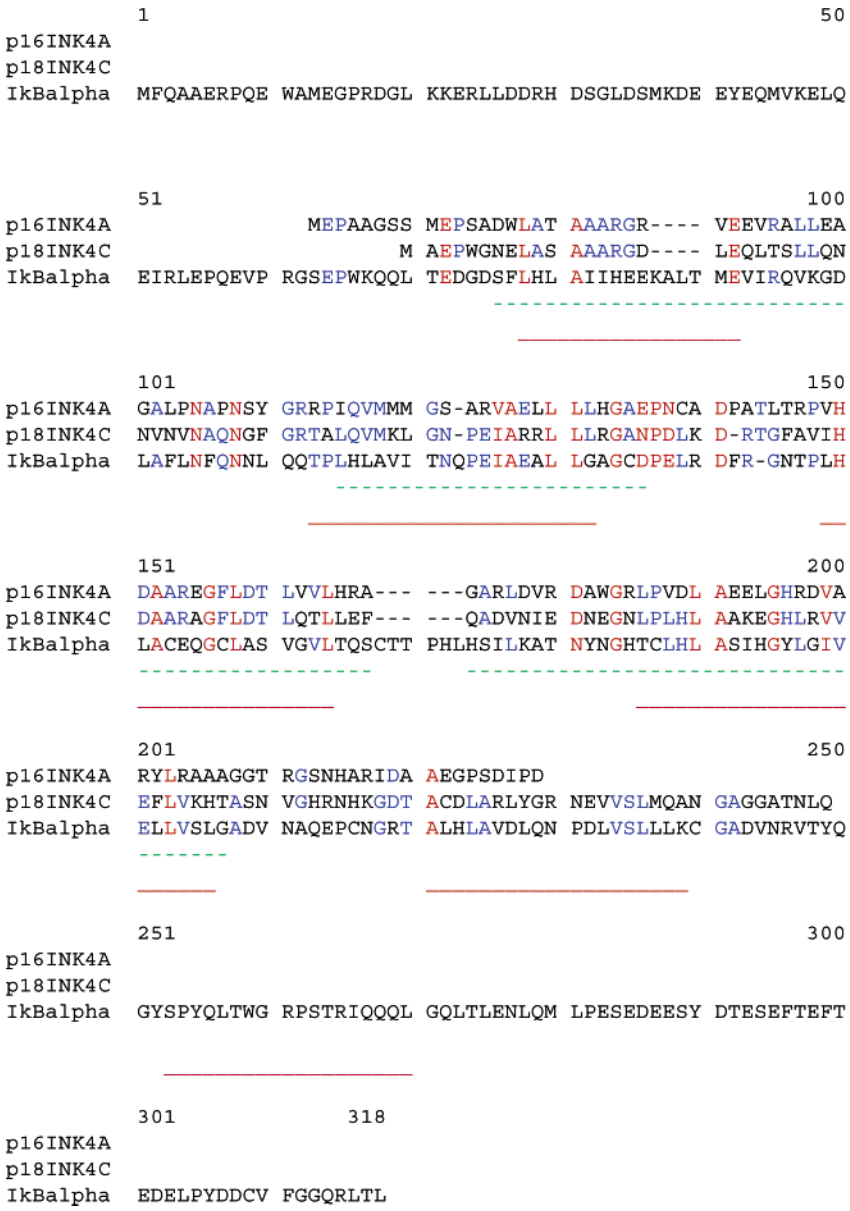

FIGURE 1: Sequence homology analysis. Antherprot 5.0 was used to align human p16 (AB060808), p18 (AF041248), and IκBα (AY033600) (*13*). Residues conserved in all three proteins are shown in red and in two proteins blue; those residues not conserved are shown in black. Green dashed lines below the sequence represent the four ankyrin repeats in human p16, and red solid lines mark the location of the six ankyrin repeats in IκBα. All ankyrin repeats were found by a Pfam HMM search.

both p16 and IκBα have been shown to interact with human T cell leukemia virus I (HTLV-1) Tax oncoprotein through ankyrin repeats, resulting in the loss of their inhibitory abilities (p16 vs CDK4, and IκBα vs NF-κB) (*15*−*20*). Moreover, it has been demonstrated that p16 binds to and suppresses the transactivational activity of NF-κB in vivo (*21*). These results taken together led us to speculate that IκBα may also interact with CDK4. If confirmed, it would suggest a possible cross talk between the NF-κB pathway and the Rb pathway; that is, IκBα and p16 may each have the potential to regulate two targets, namely, CDK4 (or CDK6) and NF-κB. This bifunctionality is potentially important for p16 since the p16 gene has been found to be either mutated or expressed at a very low level in many tumors or tumor cell lines (*18*). Inhibition of CDK4 by IκBα could potentially serve as a safety mechanism in the event that p16 is inactivated.

The goal of this work is to test the possibility of interactions between IκBα and CDK4. The approaches that were used were the in vivo yeast two-hybrid assay, binding and activity assays, site-directed mutagenesis, and rational construction of protein fragments. The results indicate that IκBα binds to and inhibits CDK4 with an affinity comparable to that of p16 (or INK4 proteins in general), and that there is competition between IκBα and INK4 proteins. We hypothesize that the interaction between IκBα and CDK4 also occurs in cells. The molecular basis of this interaction is similar, but not identical, to that of binding of p16 to CDK4. Furthermore, the CDK4 binding motif of IκBα is distinctive from its NF-κB binding motif.

MATERIALS AND METHODS

Yeast Two-Hybrid Analyses. A MATCHMAKER LexA Two-Hybrid system (Clontech, catalog no. K1609-1) was used to probe the interactions between human CDK4 and

other proteins. The human CDK4 gene was cloned into the pLexA vector as a binding domain fusion plasmid, and cDNA fragments encoding different domains of human IκBα were amplified by PCR using a pET-25b-IκBα template, and cloned into the pB42AD vector as activation domain fusion plasmids. While human p16 and p18 genes were cloned into pB42AD as positive controls, an activation domain fusing plasmid including the yeast ankyrin repeat protein, Yar-1, cDNA gene was constructed as a negative control. Yar-1 consists of three ankyrin repeats, and interacts with yeast ribosomal protein YS3 (*22*). Yeast strain EGY48 harboring p8op-lacZ was used as the host strain. Transformation, expression, and β-galactosidase activity liquid assay experiments were performed according to the manufacturer's instructions.

CDK4 mutants K22Q, R24C, and N41S were generated by the Quickchange method (Stratagene) using pLexA-CDK4 as a template.

Protein Expression and Purification. Genes encoding different domains of human IκBα were cloned into the pGEX-6p-1 (Pharmacia) vector and expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* BL21(DE3). After sonication and centrifugation, the cell lysate was purified on a reduced glutathione−agarose column (Sigma) equilibrated with a phosphate saline buffer (PBS, pH 7.4). For N-terminal GST-tagged IκBα proteins, bound proteins were eluted out of the column with reduced glutathione (20 mg/mL in PBS) and further purified with a Q Fastflow column (Pharmacia). To purify nontagged IκBα proteins, the above affinity resin was resuspended in 50 mL of PBS, and 100 units of PreScission protease (Pharmacia) was added to the suspension. After incubation at 4 °C for 24 h, the resin was repacked on a column, and the flowthrough was concentrated and further purified on an S100 column equilibrated with 5 mM HEPES, 1 μM EDTA, and 1 mM DTT (pH 7.4 at room temperature).

GST-tagged and nontagged p16, p18, p18 D76A, and Yar-1 proteins were expressed and purified as described previously (*3*, *23*, *24*).

The CDK4/cyclin D2 holoenzyme was prepared using a baculovirus/insect cell system as described previously (*3*). Briefly, human CDK4 and cyclin D2 cDNA genes were cloned into pBacBAK8 vectors, and baculovirus was constructed using Autographa California nuclear polyhedrosis virus BacPAK6/Bsu 361 DNA and Spodoptera frugipedra Sf-9 cells following the manufacturer's instructions (BD Clonetech). Of note, a His_6 tag was fused to the C-terminus of CDK4 to facilitate purification. Both baculovirus particles containing the above two pBacBAK8 transfer vectors were cotransfected into HighFive cells (Invitrogen), and the CDK4−cyclin D2 complex was purified using TALON affinity resin (BD Clontech).

Pull-Down Assays. To investigate the interaction between CDK4 and different ankyrin repeat proteins in vitro, different amounts of the CDK4−cyclin D2 complex and GST-tagged ankyrin repeat proteins were mixed and incubated at 4 °C in 250 μL of PBS (pH 7.4) for 2 h (*24*). The concentrations of the CDK4−cyclin D2 complex and the ankyrin repeat protein were 0.1 and 0.5 μM, respectively. Then 250 μL of fresh reduced glutathione−agarose beads (pre-equilibrated with PBS at 4 °C) was added to the reaction mixture; the incubation continued for an additional 1 h, while the reaction mixture was gently agitated. The reaction mixture was loaded onto a spin column (Fisher Scientific), centrifuged at room temperature (1500 rpm for 3 min), and washed with PBS. Subsequently, the column was eluted with 200 μL of PBS containing 20 mg/mL reduced glutathione (Sigma), and the elute was then analyzed with Western blot using anti-human CDK4 (Santa Cruz) and anti-human cyclin D2 antibodies (PharMingen).

In Vitro CDK4 Activity Assay. The in vitro CDK4 activity assay was performed as described previously (*3*) except that GST−Rb791−928, rather than GST−Rb379−928, was used as the substrate (*25*).

GST−Rb791−928 was prepared as follows. The cDNA gene encoding the C-terminal domain of human Rb (Rb791−928) was amplified by PCR using pGEX-2T-Rb379−928 as a template and cloned into pGEX-6p-2 (Pharmacia) to be expressed as a GST fusion protein in *E. coli* BL21(DE3) pLysS. The cell lysate was loaded on a reduced glutathione−agarose column. After being washed with PBS, bound proteins were eluted with 50 mM reduced glutathione in PBS, and then dialyzed against the kinase buffer, 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, and 1 mM DTT (pH 7.5).

To check for potential competition between IκBα and INK4 proteins, we developed a rescue assay on the basis of the above in vitro CDK4 activity assay. Three units of the CDK4−cyclin D2 complex (~0.3 μg of protein) was incubated with a fixed amount of p16 or IκBα protein at 30 °C for 30 min. The amount of p16 or IκBα protein used should be enough to inhibit 70−90% of the CDK4 activity. Subsequently, various amounts of p18 D76A protein, which retains the CDK4 binding ability but exhibits no CDK4 inhibitory activity (*23*), were added to the reaction mixtures in a total volume of 15 μL. After incubation at 30 °C for 30 min, 50 ng of GST−Rb and 5 μCi of [γ-^{32}P]ATP were added followed by incubation for additional 15 min at 30 °C. After SDS−PAGE separation, the phosphorylation of Rb was visualized with a PhosphorImager (Molecular Dynamics, Inc.). Yeast ankyrin protein Yar-1 was used as a negative control in this rescue assay.

Sequence Alignment and Structure Reconstruction. Antheprot 5.0 software was used to analyze the sequence homology among human p16 (AB060808), p18 (AF041248), and IκBα (AY033600) (*13*), while the localization of ankyrin repeats was determined with the Pfam program. The tertiary structures of p16 and IκBα were reconstructed using coordinates from PDB entries 1BI7 (*26*) and 1NFI (*11*).

RESULTS AND DISCUSSION

IκBα Binds to CDK4 and Inhibits the Kinase Activity. We first examined the potential interaction between IκBα and CDK4 in vivo. Both IκBα and CDK4 were expressed in a yeast two-hybrid system, and protein−protein association was assessed via the activation of the Lex A-dependent *lacZ* reporter. The strength of the interactions was then quantified by the β-galactosidase assay. Instead of the whole molecule of IκBα, which contains a signal receiving domain for phosphorylation and ubiquitination at the N-terminus, a centrally located ankyrin repeat domain, and a PEST segment at the C-terminus for protease degradation, only the ankyrin

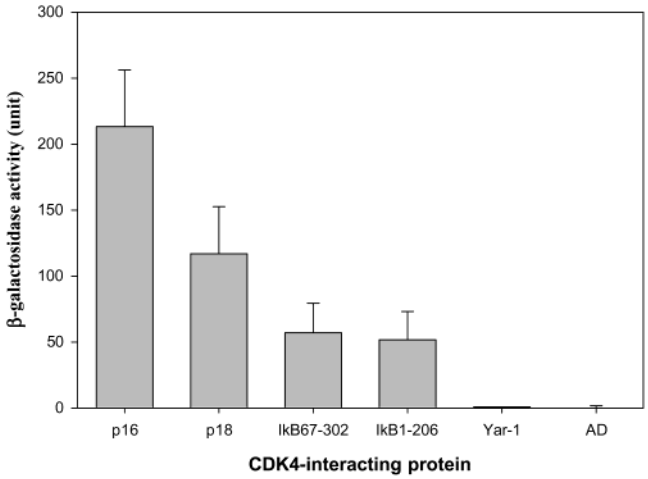

FIGURE 2: Analysis of two-hybrid interactions between CDK4 and ankyrin repeat proteins. pLexA-CDK4 and different activation domain fusion plasmids were cotransformed into yeast strain EGY48 harboring p8op-lacZ, and yeast transformants containing both LexA DNA binding and activation domains were selected on synthetic dropout (SD) medium deficient for uracil (Ura), tryptophan (Trp), and histidine (His). The strength of protein−protein interactions was semiquantitatively assessed using a liquid β-galactosidase enzymatic activity assay using ONPG (Sigma) as the substrate. Assays were performed in triplicate. AD, the activation domain, only was used as a negative control. Error bars represent standard deviations.

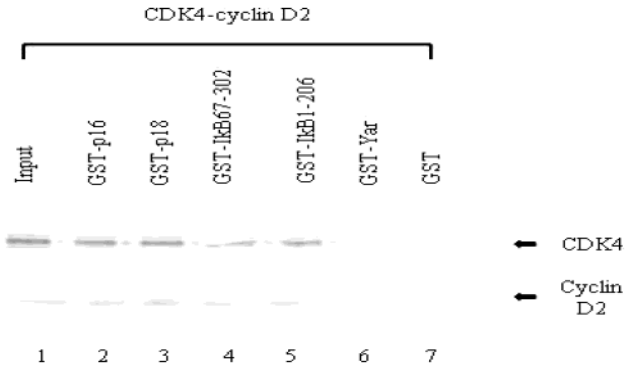

FIGURE 3: Pull-down assays for probing the interaction between ankyrin repeat proteins and the CDK4−cyclin D2 complex. The reaction mixtures containing GST-tagged proteins and the CDK4−cyclin D2 complex were incubated with reduced glutathione−agarose beads. After elution with reduced glutathione, the bound proteins were blotted with anti-human CDK4 antibody (Santa Cruz, C-22) and anti-human cyclin D2 antibody (PharMingen, 14821C) separately as indicated: lane 1, input only containing 5% of the amount of the purified CDK4−cyclin D2 complex in other lanes; lane 2, 0.5 μM GST−p16 and 0.1 μM CDK4−cyclin D2; lane 3, 0.5 μM GST−p18 and 0.1 μM CDK4−cyclin D2; lane 4, 0.5 μM GST−IκBα67−302 and 0.1 μM CDK4−cyclin D2; lane 5, 0.5 μM GST−IκBα1−206 and 0.1 μM CDK4−cyclin D2; lane 6, 0.5 μM GST−Yar-1 and 0.1 μM CDK4−cyclin D2; and lane 7, 0.5 μM GST and 0.1 μM CDK4−cyclin D2.

repeat domain, IκBα67−302, was used in this study. This domain was used in previous crystal structure studies, and the structural results indicate that this domain is responsible for protein−protein interactions (*10*). INK4 proteins p16 and p18 were used as positive controls, and the yeast protein Yar-1, which is composed of three ankyrin repeats and has been found to interact with yeast ribosomal protein YS3 but not with CDK4 (*22*), was used as a negative control. As shown in Figure 2, like p16 and p18, human IκBα67−302 interacts with CDK4 with the following rank order of binding strength: p16 > p18 > IκBα67−302. Meanwhile, no detectable interaction was observed between Yar-1 and CDK4, suggesting that the interaction between IκBα67−302 and CDK4 is specific. The result for IκBα1−206 will be addressed later.

The above results were further supported by in vitro pull-down assays with glutathione *S*-transferase (GST)-IκBα67−302 and the CDK4−cyclin D2 holoenzyme. The CDK4−cyclin D2 complex was used because the complex, not free CDK4, is the biologically active form. As shown in the upper row, lane 4 of Figure 3, CDK4 was detected in the pull-down product (eluent), suggesting that IκBα67−302 binds to the CDK4−cyclin D2 complex. Moreover, cyclin D2 was detected in the same pull-down product (the bottom row, lane 4 of Figure 3), indicating that IκBα67−302 binding does not disrupt the CDK4−cyclin D2 complex. Similar results were observed in the bindings of p16 and p18 to the CDK4−cyclin D2 complex (lanes 2 and 3, respectively). Therefore, like that of p16 and p18 (*23*, *26*), binding of IκBα67−302 to the CDK4−cyclin D2 complex resulted in the formation of a ternary complex. In addition, neither CDK4 nor cyclin D2 was detected in the pull-down product with Yar-1 (lane 6), again indicating that binding to CDK4 is specific and that not all ankyrin repeat proteins can bind to the CDK4−cyclin D2 complex.

We then used an in vitro assay to evaluate the kinase activity of CDK4 in response to binding of IκBα67−302. As more IκBα67−302 was added to the kinase reaction mixture, the inhibition of CDK4 kinase activity increased accordingly as shown in Figure 4A. The IC_{50} value, or the concentration of IκBα67−302 required to achieve 50% of the maximum inhibitory activity, is 85 ± 18 nM, which is almost identical to the corresponding values of p16 and p18 (60 ± 20 and 80 ± 25 nM, respectively), suggesting that IκBα67−302 is a CDK4 inhibitor that is as effective as INK4 proteins. The negative controls with Yar-1 and p18 D76A are addressed below.

IκBα and INK4 Proteins Compete for CDK4 Binding. Since our results so far suggest that IκBα67−302 binds to the CDK4−cyclin D2 complex in a manner similar to that of INK4 proteins, the potential competition between IκBα67−302 and INK4 proteins was investigated using a rescue assay. In this assay, the p18 D76A mutant protein was used to compete with IκBα67−302 and p16 for CDK4 binding, thereby counteracting the inhibition of IκBα67−302 and p16. Analogous to the p16 D84A mutant found in human tumors, p18 D76A retains an unperturbed global structure and comparable CDK4 binding ability, but exhibits little kinase inhibitory activity (Figure 4A) (*2*, *23*). As shown in Figure 4B, the CDK4 kinase activity (i.e., the phosphorylation of Rb) in the presence of IκBα67−302 (the top row) and p16 (the bottom row) increased with increasing concentrations of p18 D76A (as a rescuer). This suggests that p18 D76A counteracts the inhibition of both IκBα67−302 and p16. In the control experiment where p18 D76A is replaced with yeast ankyrin protein Yar-1, the CDK4 kinase activity remained unchanged (*24*), indicating that the inhibition of IκBα67−302 or p16 cannot be counteracted by Yar-1; hence, the counteraction by p18 D76A is specific. It has been demonstrated that protein−protein interactions involve multiple patches of residues in both ankyrin repeat proteins and

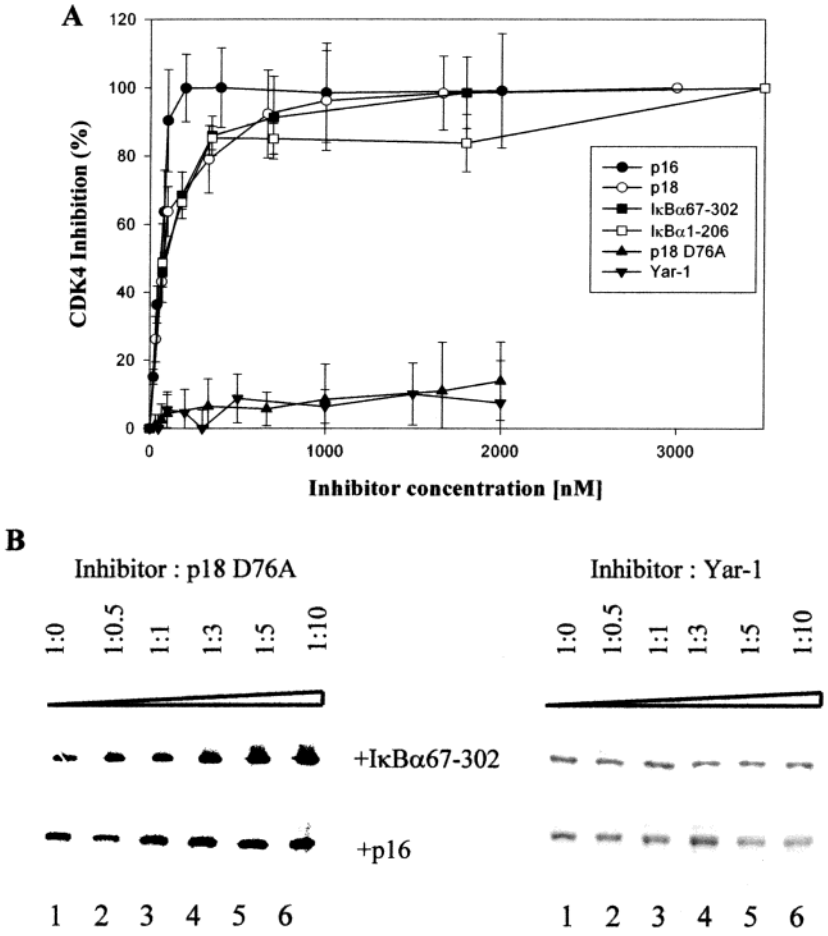

FIGURE 4: (A) In vitro CDK4 kinase assay for determining the inhibitory activities of ankyrin repeat proteins. The reaction mixtures included 3 units of the CDK4−cyclin D2 complex, 50 ng of GST−Rb791−928, 5 μCi of [γ-^{32}P]ATP, and varying amounts of ankyrin repeat proteins. The incorporation of ^{32}P was quantified using a PhosphorImager. All measurements were repeated in triplicate. Error bars represent standard deviations. (B) Inhibition rescue. All experiments were performed as in the above CDK4 kinase assays except that a fixed amount of p16 or IκBα67−302 (at a final concentration of 150 nM) and varied amounts of p18 D76A or Yar-1 were included in the reaction mixtures. The ratio of each lane indicates the ratio between the CDK4 inhibitor (p16 or IκBα67−302) and the rescuer (p18 D76A or Yar-1).

their targets (*23*, *27*). On the basis of the results of competition experiments, the IκBα67−302 binding site of CDK4 should overlap, at least partially, with the INK4 binding site of CDK4.

Differences between CDK4 Binding by IκBα and INK4 Proteins. Despite the fact that IκBα67−302 competes with INK4 proteins for CDK4 binding, there are notable differences between them. As shown in Figure 5, IκBα67−302 has six helix−turn−helix motifs that are stacked more compactly to form a helical bundle than those in p16. Moreover, the sequence homology analysis shows that some residues of p16 that are important for CDK4 binding are not conserved in IκBα67−302 (Figure 1). For example, D84 of p16, a residue conserved among all INK4 proteins, has been found to play a key role in CDK4 inhibition. The electrostatic interaction between D84 and R24 of CDK4 is crucial for INK4 inhibition since mutations in either residue eliminate the inhibition (*2*, *26*, *28*). In contrast, the corresponding residue in IκBα67−302 is L150, a residue that is not able to form an electrostatic interaction with R24 of CDK4. To further address this issue, we investigated the interactions between these inhibitors (including IκBα67−302, p16, and p18) and three CDK4 mutants. Of note, all three CDK4 mutants have been found in human cancers (*28*). As shown in Figure 6, all three inhibitors interact with CDK4 K22Q in a manner similar to that of wild-type CDK4. This is consistent with the observation that K22 of CDK4 is involved in cyclin D binding, but not in INK4 binding (*29*). However, interactions between INK4 and CDK4 R24C dropped by ~50%, while the interaction between IκBα67−302 and CDK4 R24C remained unchanged from the interaction between IκBα67−302 and wild-type CDK4. This is consistent with the difference in the sequence (D84 of p16 vs L150 of IκBα) mentioned above. Furthermore, while the interactions between INK4 proteins and CDK4 N41S were almost identical to the corresponding interactions with wild-type CDK4, the level of interaction between IκBα67−302 and CDK4 N41S increased by 150%, suggesting that N41 of CDK4 contributes negatively to IκBα67−302 binding. Clearly, the interaction between IκBα and CDK4 needs to be further investigated, but the results presented here are sufficient to indicate that the mode of interaction between IκBα and CDK4 differs from that between p16 and CDK4, even though there are competition and partial overlap of the binding sites as suggested in the previous section. Further detailed studies on the dual specificities of p16 and IκBα will enhance our understanding of the structure−function relationship of not only p16 and IκBα but also ankyrin repeat proteins in general.

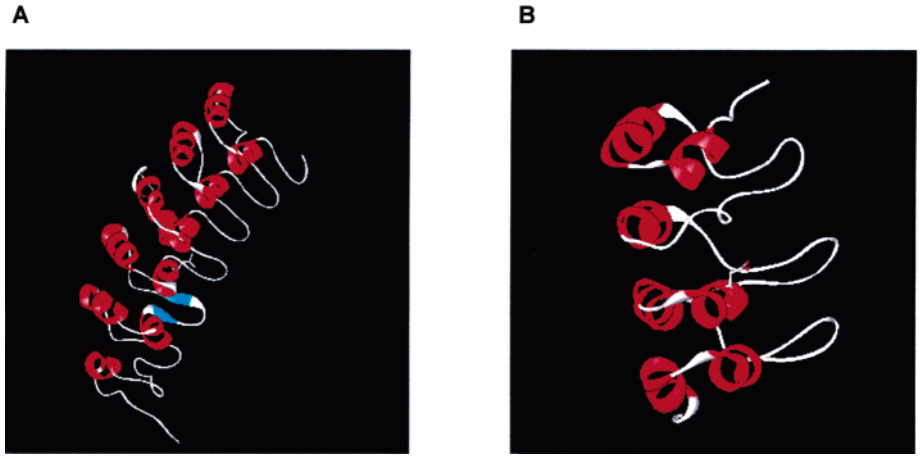

FIGURE 5: Structures of human IκBα67−302 and p16. Coordinates from PDB entries 1NFI and 1BI7 were used to reconstruct the tertiary structures of human IκBα67−302 (A) and p16 (B). In both structures, the helical regions are shown in red and the β-sheets are in blue. The N-termini are oriented at the top, while the C-termini are at the bottom. The side chains of two corresponding residues, L150 of IκBα and D84 of p16, are shown in panels A and B, respectively. Note that the structure of IκBα67−302 is taken from the structure of its complex with NF-κB.

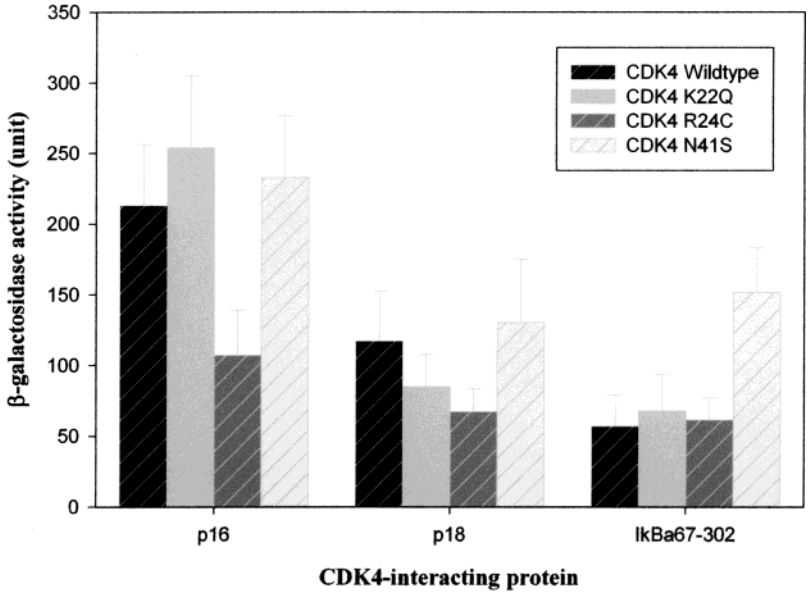

FIGURE 6: Yeast two-hybrid analysis for determining the interactions between ankyrin repeat proteins and CDK4 mutants. The experiments were performed as described in the legend of Figure 2, and error bars represent standard deviations.

CDK4 and NF-κB Binding Sites of IκBα Are Different. To map out the CDK4 binding domain of IκBα, we constructed a series of truncated IκBα compounds and analyzed their abilities in CDK4 binding and inhibition. While most of the truncated IκBα proteins exhibited poor solubility and stability, a truncated IκBα encompassing residues 1−206 was folded well with good solubility and stability as revealed by NMR analyses (unpublished data). This truncated IκBα protein interacts with CDK4 in vivo (Figure 2) and in vitro (Figure 3, lane 5), and its IC_{50} value is 100 ± 20 nM, a value identical to that of IκBα67−302 (Figure 4). Since IκBα1−206 consists of the N-terminal signal receiving domain and the first four ankyrin repeats while IκBα67−302 consists of the six ankyrin repeats, our results taken together suggest that the first four ankyrin repeats (I−IV) of IκBα are responsible for CDK4 binding. This is different from its interaction with NF-κB. As revealed in the crystal structure of the IκBα67−302−NF-κB complex, ankyrin repeats V and VI of IκBα contribute most to binding to the NF-κB p65 subunit, while loops between ankyrin repeats IV and V, and V and VI, are involved in binding to the NF-κB p50 subunit (*10*, *11*). In addition, these domain mapping data further support the fact that IκBα binds to CDK4 in a manner similar to that of INK4 proteins. In the crystal structures of p16−CDK6 and p19−CDK6 complexes (*26*, *30*), most contacts are within ankyrin repeats II and III and the loop between them, while ankyrin repeats I and IV contribute little to CDK4 interaction. No interaction has been found between ankyrin repeat V of p19 and CDK6. Taken together, these results suggest that the mechanism of IκBα−CDK4 binding is quite different from that of IκBα−NF-κB binding.

Potential Cross Talk between the NF-κB Pathway and the Rb Pathway. Using both in vivo yeast two-hybrid assays and in vitro binding and activity assays, we have demonstrated that IκBα specifically binds to and inhibits CDK4. Together, with the results from previous studies (*15*, *20*, *21*), these findings led us to propose a hypothesis for a possible new

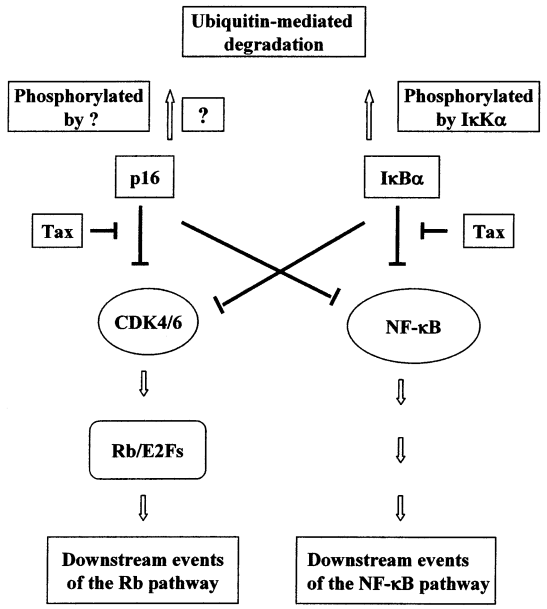

FIGURE 7: Model showing the potential cross talk between the NF-κB pathway and the Rb pathway. The stop bar between IκBα and CDK4 is proposed on the basis of the results of this work. The question marks indicate that no evidence is yet available.

mechanism for coordinating cell progression and signal transduction. As shown in Figure 7, both p16 and IκBα could have dual specificities and function in both NF-κB and Rb pathways. This mechanism could be extended to other IκB family members if further verified in cellular studies. On one hand, inducers of NF-κB, including bacterial and viral products, inflammatory cytokines, reactive oxygen, and ultraviolet light bring about the inactivation of IκBs through phosphorylation and subsequently activate both gene transcription (through NF-κB) and cell cycling (through CDK4) (*31*). On the other hand, IκBs could act as functional alternatives of INK4 proteins and bypass frequent inactivation or low-level expression of p16 in many cells, which is believed to be the primary cause for a variety of neoplasia (*8*, *18*). In addition, the similarities between p16 and IκBα may aid in understanding the regulation of p16. For IκBα regulation, IκBα-specific kinase (IKKα) triggers the sequence-specific phosphorylation of IκB, resulting in the rapid degradation of IκB through a ubiquitin-mediated pathway (*12*, *16*, *32*). Binding of Tax to IκBα increases the extent of phosphorylation and ubiquitination of IκBα (*15*, *19*). Although no experimental evidence is yet available, it is possible that a similar phosphorylation and ubiquitination process contributes to the rapid degradation of p16 in vivo. A very recent report demonstrating that p16 is phosphorylated by an unknown kinase in human fibroblasts at Ser152 further supports this hypothesis (*33*).

Conclusion. Our biochemical studies demonstrate that IκBα competes with INK4 proteins for CDK4 binding and inhibition. These results led us to propose a hypothesis that there is crossing between the NF-κB/IκBα pathway and the p16/CDK4/Rb pathway in cells, and that IκBα could substitute for the CDK4-inhibiting function of the tumor suppressor p16. Distinct differences at the structural level have been demonstrated for the binding of CDK4 to IκBα and p16, as well as for the binding of IκBα to NF-κB and CDK4. A detailed structural basis for the dual specificities of both p16 and IκBα, and the biological significance of the proposed potential cross talk between the NF-κB pathway and the Rb pathway, remain to be established by future structural and cellular studies.

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