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Novel Insights into the INK4-CDK4/6-Rb Pathway: Counter Action of Gankyrin against INK4 Proteins Regulates the CDK4-Mediated Phosphorylation of Rb[†]

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ABSTRACT: The newly discovered oncogenic protein gankyrin, which contains six ankyrin repeats, has been reported to be involved in the phosphorylation and degradation of the retinoblastoma gene product, Rb. Using in vitro systems, we have identified a peptide fragment of gankyrin, ^{176}LHLACDEERN^{185}, which is responsible for binding of gankyrin to Rb. We further demonstrated a different mechanism for gankyrin to facilitate the phosphorylation of Rb, by binding with cyclin-dependent kinase 4 (CDK4). This binding does not inhibit the Rb-phosphorylating kinase activity of CDK4, but it competes with p16 binding to CDK4 and counteracts the inhibitory function of p16. We then showed that binding of gankyrin to CDK4 and the consequent counter action of p16 function were not affected by the Rb-binding peptide ^{176}LHLACDEERN^{185}, indicating that the two mechanisms are independent. They also involve different structural regions of gankyrin. While the Rb-binding motif is located at the fifth ankyrin repeat, truncation mutants of gankyrin, with the first three or four ankyrin repeats remaining, are sufficient for binding to CDK4 and for counteracting the inhibitory function of p16. These results demonstrate the potential importance of gankyrin in cell cycle control and tumorigenesis and suggest an expanded INK4-CDK4/6-Rb pathway.

In the past decade, a great number of studies have demonstrated the important roles of the INK4-CDK4/6-Rb[1] pathway in cell cycle control and tumorigenesis. As key protein kinases functioning in the G0-to-G1 and G1-to-S transitions, cyclin-dependent kinases 4 and 6 (thereafter, CDK4 and 6) control cell progression through phosphorylating certain regulatory proteins, one of which is the retinoblastoma gene product (Rb) (*1*, *2*). Phosphorylation of Rb leads to the disruption of the Rb-E2F transcription factor complex, and the release of active E2Fs further triggers the activation of a number of genes required for the G0-to-G1 and G1-to-S transitions. Through inhibiting the CDK4/6 kinase activities, INK4 proteins (inhibitors of CDK4/6) are part of the cell cycle control machinery (*3*, *4*). The INK4 proteins, including p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D} (thereafter, p16, p15, p18, and p19), are a family of proteins with similar structural and functional properties. They are composed of a series of relatively conserved protein motifs called ankyrin repeats (four ankyrin repeats in p15 and p16 and five in p18 and p19) and act as specific inhibitors of CDK4 and 6 (*5*−*8*). Since DNA alterations in p16, such as homogeneous deletion, loss of heterogeneity, methylation, and promoter hypermethylation, have been found in more than 200 different cancers or cancer cell lines, p16 is well recognized as a tumor suppressor whose dysfunction may contribute to, or may even be, the primary cause of a variety of neoplasia (for review see ref *9*). To date, the focus of most p16 studies is on its genetic alterations and interactions with CDK4 and 6; therefore, little is known concerning the regulation of p16 at the protein level. The only report came from the study of human T-lymphocytic virus 1 (HTLV-1) tax protein (*10*, *11*). Tax protein has been found to bind to p16 and abolish p16 inhibition to CDK4 and 6, which may contribute to HTLV-related leukemia.

Recently, an oncogenic protein gankyrin (gann ankyrin-repeat protein; gann means cancer in Japanese) has been discovered and shown to be involved in the regulation of Rb (*12*). Gankyrin consists of six ankyrin repeats and a 38 amino acid N-terminal extension (Figure 1). As shown by Fujita and co-workers (*12*), the expression of gankyrin was increased in all studied hepatocellular carcinomas. Overexpression of gankyrin not only led to increased phosphorylation of Rb and activation of E2F-1 in U2-OS cells but also caused cell transformation in NIH3T3 cells. Further mutagenesis studies also demonstrated that gankyrin bound to Rb through a conserved Rb-binding motif LxCxE at its C terminus (*13*) and accelerated the degradation of Rb in vitro and in vivo. These results suggest that gankyrin is involved in the Rb pathway. However, the mechanism and importance of the correlation between gankyrin and Rb phosphorylation are yet to be established.

This work addresses the involvement of gankyrin in the INK4-CDK4/6-Rb pathway using both in vivo and in vitro systems. We demonstrated that, in addition to binding to Rb

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[1] Abbreviations: AD, LexA activation domain; BSA, bovine serum albumin; CDK4/6, cyclin-dependent kinase 4 and 6; DTT, dithiothreitol; *E. coli, Escherichia coli*; GST, glutathione *S*-transferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPV, human papillomavirus; INK4, inhibitor of cyclin-dependent kinase 4; IPTG, isopropyl 1-thio-*β*-D-galactopyranoside; Ni-NTA, nickel nitrilotriacetic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Rb, human retinoblastoma susceptible gene product; RT-PCR, retrotranscriptase polymerase chain reaction; tax, transactivation protein.

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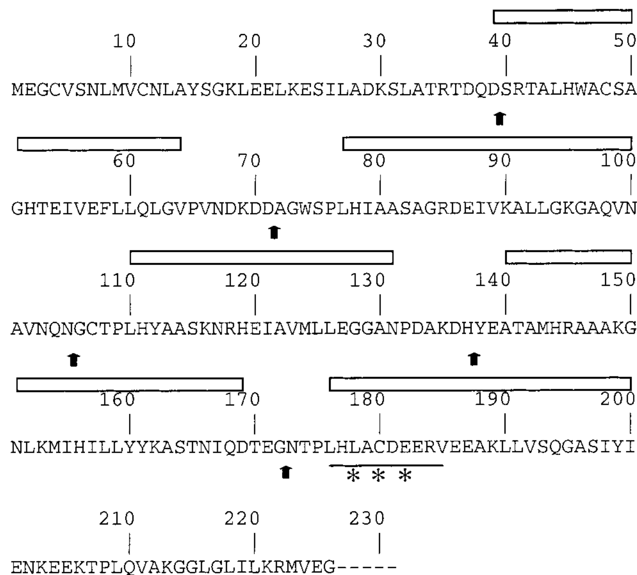

FIGURE 1: Sequence of human gankyrin and its putative secondary structure. Antherprot 5.0 was used to do the alignment. The boxes above the sequence represent the putative ankyrin repeats, and only five ankyrin repeats were found by Pfam HMM search. The sequence underlined is the putative Rb-binding motif in which the three conserved residues are labeled with asterisks. Arrows indicate the truncation mutation sites.

directly, gankyrin facilitates phosphorylation of Rb via a different mechanism: by competing with INK4 proteins for CDK4 binding. While this competition blocks the CDK4 inhibitory ability of the INK4 proteins, binding of gankyrin to CDK4 does not inhibit the kinase activity of CDK4. We further demonstrated that the two mechanisms of gankyrin function are independent of each other by peptide competition assays and by showing that different structural segments of gankyrin are involved in CDK4 binding and Rb binding.

MATERIALS AND METHODS

Protein Expression and Purification. Human gankyrin cDNA gene (GenBank No. H25881) was purchased from GenomicSystem Inc. (clone no. 1066950) (*14*) and was cloned into both pGEX-6p-2 (Pharmacia) and pET-21d (+) (Novagene) by PCR. Both plasmids were transformed into *E. coli* BL21(DE3) pLys S and expressed as tagged proteins. For N-terminal GST-tagged gankyrin, the cell lysate was purified on a glutathione−agarose column (Sigma) equilibrated with phosphate-buffered saline (PBS, pH 7.4). Bound GST−gankyrin protein was eluted out with reduced glutathione (20 mg/mL in PBS) and further purified by a Q Fastflow column (Pharmacia). To purify gankyrin without a GST tag, 100 units of PreScission protease (Pharmacia) was added to GST−gankyrin in PBS. After incubation at 4 °C for 24 h, the protein solution was loaded onto a glutathione−agarose column equilibrated with PBS. The flow-through was concentrated and further purified by a S100 column equilibrated with 5 mM HEPES, 1 μM EDTA, and 1 mM DTT (pH 7.4 at room temperature). For the C-terminal His×6-tagged gankyrin, the lysate from bacteria harboring the pET-21d (+)-gankyrin plasmid was loaded onto a Ni-NTA column (Qiagen) equilibrated with PBS and purified with a imidazole gradient of 80−500 mM. After SDS−PAGE analysis, fractions containing gankyrin−His×6 were pooled and dialyzed against PBS to remove imidazole.

All gankyrin truncation mutants were constructed by the QuickChange method (Stratagene) using pGEX-6p-2−gankyrin as template. These mutant proteins were expressed in *E*scherichia *coli* BL21(DE3) pLys S and purified as wild-type gankyrin.

Yeast ankyrin protein, Yar 1 cDNA, was cloned into pGEX-6p-2 and expressed as a GST fusion protein in *E. coli* BL21(DE3) as described (*18*). Purification of both GST-tagged and free Yar 1 proteins was similar to that of human gankyrin protein.

Genes encoding different parts of human Rb protein, Rb1−379, Rb379−787, and Rb787−928, were amplified by PCR using a whole Rb cDNA from ATCC (clone no. 65003) (*14*) as template and cloned into pGEX-6p-2 as N-terminal GST-fused proteins. The expression and purification procedures of these Rb proteins were similar to that of wild-type gankyrin. After the removal of the GST tag, the final products were aliquoted and stored at −80 °C for the pull-down assay.

The CDK4/cyclin D2 holoenzyme was expressed and purified as described (*6*). Briefly, human CDK4 cDNA, obtained by RT-PCR from total RNA of Hela cells, was subcloned into a pBacBAK8 phagemid (Clonetech) to generate the pBAC/CDK4 transfer vector, in which a His×6 tag was fused to the C terminus of CDK4 to facilitate the following purification. Meanwhile, cDNA for cyclin D2 was cloned into pBacPAK8 vector by PCR using human cyclin D2 cDNA as template DNA, which resulted in a pBAC/cyclin D2 transfer vector. Baculovirus was constructed using Autographa California nuclear polyhedrosis virus BacPAK6/Bsu 361 DNA and *Spodoptera frugipedra* Sf-9 cells following the manufacturer's instructions (Clonetech). Both baculovirus particles containing the above two transfer vectors were cotransfected into HighFive cells (Invitrogen), and the CDK4−cyclin D2 complex was purified by modifying a published procedure (*15*). Lysate from transfected insect cells was cleared by centrifugation and loaded on a TALON (Clonetech) resin column equilibrated with a washing buffer, 20 mM Tris-HCl (pH 7.4 at room temperature), 100 mM NaCl, 0.1 mM Na_3PO_4, 1 mM NaF, 10 mM β-glycerophosphate, 5 mM β-mercaptoethanol, 0.2 mM AEBSF, 5 mg/mL aprotinin, and 5 mg/mL leupeptin. After being washed with the washing buffer and the washing buffer with 10 mM imidazole, the column was eluted with the washing buffer containing 50 mM imidazole, and the eluent was dialyzed against a kinase buffer containing 50 mM HEPES (pH 7.5 at room temperature), 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. The eluent was further concentrated to approximately 0.3 mg/mL, and AEBSF, leupeptin, and aprotinin were added to the same concentrations as in the washing buffer. Aliquots were stored at −80 °C.

Yeast Two-Hybrid Analysis. A MATCHMAKER LexA two-hybrid system (Clontech, catalog no. K1609-1) was used in this study. Human gankyrin and CDK4 genes were cloned into pLexA vector as binding domain fusion plasmids, and human gankyrin, p16, p18, and cyclin D2 genes together with yeast Yar 1 gene were cloned into pB42AD vector as activation domain fusion plasmids. All transformation, expression, and β-galactosidase activity liquid assay experiments were performed according to the manufacturer's instructions, while yeast strain EGY48 harboring p8op-lacZ was used as the host strain.

Pull-Down Assays. To investigate the interaction between N-terminal GST-tagged gankyrin proteins (including wild-type and truncated proteins) and the CDK4−cyclin D2 complex, 10 μg of the CDK4−cyclin D2 complex (*6*, *7*, *15*) and 25 μg of GST-tagged gankyrin proteins were incubated at 4 °C in 250 μL of PBS (pH 7.4) for 2 h. The concentrations of CDK4−cyclin D2 and gankyrin are 0.4 and 2.0 μM, respectively. Then 250 μL of fresh reduced glutathione−agarose beads (preequilibrated with PBS at 4 °C) was added to the reaction mixture, and the incubation was continued for another 1 h while the reaction mixture was gently agitated during the incubation. 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 further analyzed with a Western blot using anti-human CDK4 (Santa Cruz) and anti-human cyclin D2 antibodies (PharMingen). To investigate the competition between p16 and gankyrin, 1.0 mL of the reaction mixture including 0.1 μM CDK4−cyclin D2, 0.5 μM GST−gankyrin, and varying amounts of free p16 or 0.1 μM CDK4−cyclin D2, 0.5 μM GST−p16, and varying amounts of free gankyrin in PBS (pH 7.4) was incubated at 4 °C for 2 h. After addition of 250 μL of reduced glutathione−agarose, the mixture was incubated at 4 °C for another 2 h, and then the agarose-bound proteins were analyzed by Western blotting as above.

A similar approach was applied to investigate the interaction between gankyrin and different Rb proteins. The reaction mixture included certain amounts of GST-tagged Rb proteins (the final molar concentration is 0.4 μM) and 15 μg of free gankyrin (2.0 μM) in 250 μL of PBS (pH 7.4), and the proteins bound to reduced glutathione−agarose were assessed with Western blotting using anti-human gankyrin antibody (Affiniti, U.K.).

For peptide competition assay, varying amounts of synthesized peptides were added in 1.0 mL of the solution containing 0.1 μM Rb379−787 protein and 0.5 μM gankyrin−His×6 in PBS (pH 7.4). After incubation at 4 °C for 2 h, 250 μL of fresh TALON resin (preequilibrated with PBS at 4 °C) (Qiagen) was added, and the incubation was continued for another 2 h. The reaction mixture was loaded onto a spin column and after centrifugation at room temperature, 1500 rpm for 3 min, washed with PBS, PBS with 50 mM imidazole, and PBS with 100 mM imidazole. The column was eluted with 80 μL of PBS containing 1 M imidazole, and the elute was further analyzed with a Western blot.

In Vitro CDK4 Activity Assay. The in vitro CDK4 activity assay (*3*, *6*, *16*) involved 3 units of the CDK4−cyclin D2 complex (about 0.3 μg of protein) and varying concentrations of proteins under investigation in 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) supplemented with 0.2 mM AEBSF, 2.5 mg/mL leupeptin, and 2.5 mg/mL aprotinin in a total volume of 15.0 μL. One unit of CDK4 kinase is defined as the amount of CDK4 that catalyzes the incorporation of 1 μmol of phosphate into Rb at 30 °C within 15 min. These were preincubated for 30 min at 30 °C. Subsequently, GST−Rb379−928 (50 ng) and 5 μCi of [γ-^{32}P]ATP were added, and the reaction mixtures were incubated for another 15 min at 30 °C. After SDS−PAGE separation, the CDK4 activity was determined by ^{32}P incorporation into substrate Rb using a PhosphoImager (Molecular Dynamics, Inc.). Measurements were repeated in triplicate.

The substrate of CDK4, GST−Rb379−928, was prepared as follows: human "large pocket" Rb cDNA corresponding to residues 379−928 of wild-type Rb was cloned into pGEX-2T plasmid and expressed as a GST fusion protein in *E. coli.* BL21(DE3) pLys S. The cell lysate was purified on a glutathione−agarose column, and the bound protein was eluted out with 50 mM reduced glutathione in PBS and then dialyzed against the above kinase buffer.

To check the counter action between INK4 proteins and gankyrin, we first incubated the CDK4−cyclin D2 complex with different amounts of gankyrin at 4 °C for 30 min and added a fixed amount of p16 to the reaction mixture to measure the CDK4 kinase activity. The amount of p16 should be enough to inhibit 70%−90% of the CDK4 activity.

RESULTS AND DISCUSSION

Gankyrin Binds to CDK4−Cyclin D2 and Competes with p16. A previous study by Fujita and co-workers focused on the Rb-interacting ability of gankyrin (*12*) and indicated that the oncogenic activity of gankyrin was mediated by the interaction between gankyrin and Rb. However, their results did not rule out the possibility that the gankyrin−Rb correlation may involve other proteins. Despite the lack of direct evidence, we speculate that CDK4 might mediate the gankyrin-enhanced phosphorylation of Rb, since gankyrin has been found to increase phosphorylation of theronine and serine residues in Rb, and these residues are also the phosphorylation sites of CDKs. In addition, it has been reported that gankyrin exists in a multiprotein complex containing CDK4 (*12*).

To address this premise, we first examined the interaction between gankyrin and CDK4 using a yeast two-hybrid assay. Binding was determined by the activation of LexA-dependent *lacZ* reporter. The strength of the interactions was then quantified in a β-galactosidase assay. While both p16 and p18 were used as positive controls, the yeast protein Yar 1, which is composed of three ankyrin repeats and has been found to interact with yeast ribosomal protein YS3 (*17*, *18*), was used as a negative control. As shown in Figure 2A, like p16 and p18, human gankyrin interacted with CDK4. Meanwhile, no detectable interaction was observed between Yar 1 and CDK4, suggesting that the interaction between gankyrin and CDK4 is specific. In addition, no interaction was seen with p16 and cyclin D2, which eliminated the possibility that gankyrin is involved in the Rb pathway through interacting CDK4-regulatory proteins. Apparently, CDK4 interacted with p16, p18, and gankyrin with a rank order of binding strength as follows: p16 > p18 > gankyrin (Figure 2B).

We further confirmed these in vivo results using pull-down assays with glutathione *S*-transferase (GST)−gankyrin and the CDK4/cyclin D2 holoenzyme. Instead of free CDK4, the CDK4/cyclin D2 holoenzyme was used in this assay due to the following reasons. First, the holoenzyme, not CDK4 itself, is the biologically active form. Second, CDK4 itself is very unstable. As shown in lane 2 of Figure 3A, both CDK4 and cyclin D2 were detected as pull-down products,

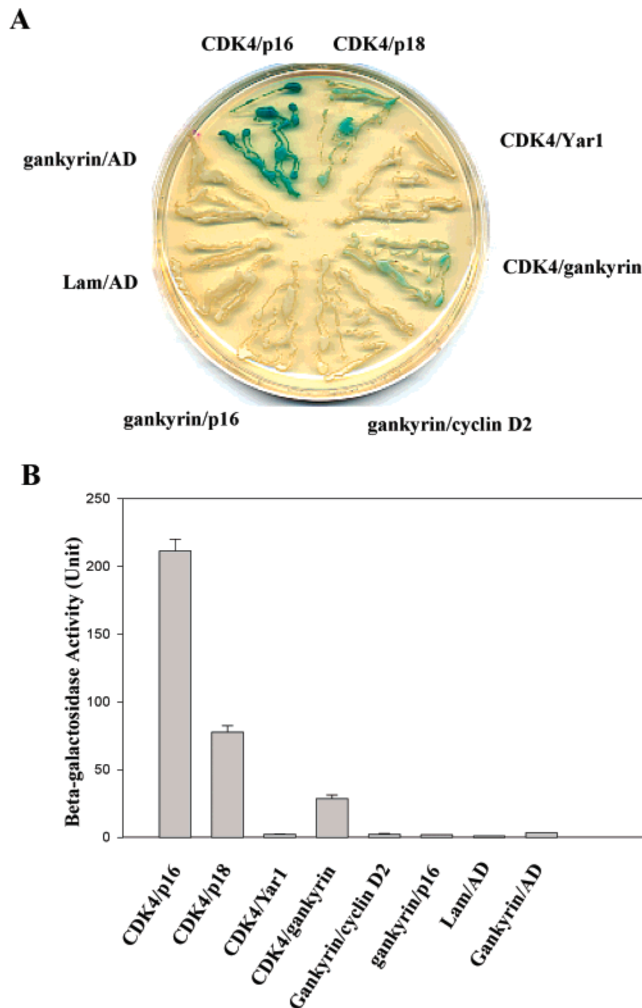

FIGURE 2: Analysis of two-hybrid interactions between gankyrin and other proteins. (A) LexA DNA binding domain and activation domain fusion plasmids were cotransformed into the yeast strain EGY48 harboring p8op-lacZ, and yeast transformants containing both LexA DNA binding and activation domains were selected on synthetic dropout (SD) medium deficient for uracil (Ura), tryptophan (Trp), and histidine (His). Ura/Trp/His positives were restreaked onto SD-Leu/-Trp/-His/X-gal/galactose/raffinose media. Protein−protein interactions were assessed by the blue color development on the above induction media. For each protein/protein pair, the first protein was fused to the LexA DNA binding domain, and the second protein was fused to the LexA activating domain. Lam is a negative control provided by the manufacturer, and AD stands for the LexA activation domain. (B) Semiquantitative analysis of the two-hybrid interactions based on the level of induction of the β-galactosidase gene was performed in a liquid β-galactosidase enzymatic activity assay using ONPG (Sigma) as the substrate. Assays were performed in triplicate.

suggesting that gankyrin binds to the CDK4−cyclin D2 complex to form the gankyrin−CDK4−cyclin D2 ternary complex. The result is similar to the previously established binding of p16 to the CDK4−cyclin D2 complex (*19*, *20*), which is shown in lane 3. For the control protein, Yar 1, there is no detectable interaction with the CDK4−cyclin D2 complex (lane 4), suggesting that not all ankyrin repeat proteins can bind to the CDK4−cyclin D2 complex.

Since both gankyrin and p16 bind to the CDK4−cyclin D2 complex, we then examined possible competition between gankyrin and p16 for CDK4 binding. As shown in Figure 3B, when p16 was present in a GST−gankyrin/CDK4−cyclin D2 mixture with a gankyrin:p16 molar ratio

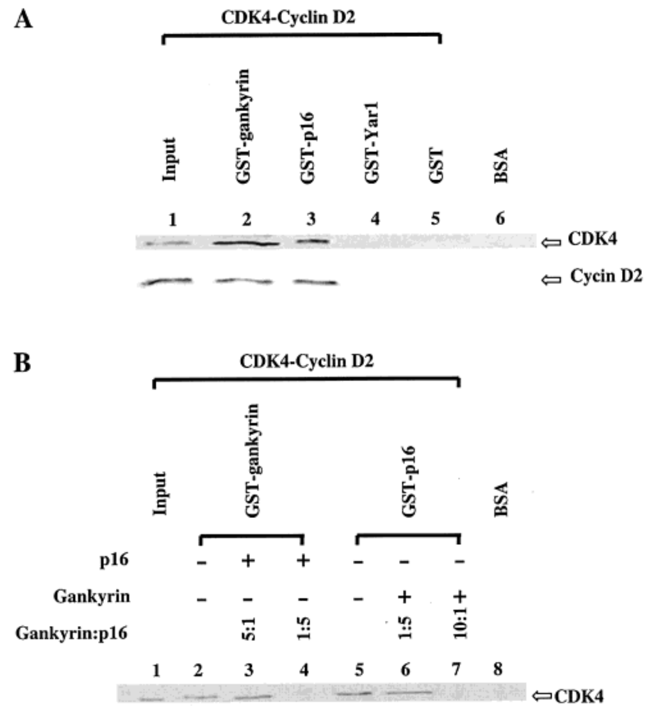

FIGURE 3: Pull-down assays to probe the interaction between gankyrin and the CDK4−cyclin D2 complex. (A) The reaction mixtures containing GST-tagged proteins and the CDK4/cyclin D2 holoenzyme were incubated with reduced glutathione−agarose, and after elution with reduced glutathione, the bound proteins were blotted with anti-human CDK4 antibody (Santa Cruz, C-22) and anti-human cyclin D2 antibody (PharMingen, 14821C) separately as indicated. Lanes: 1, the input only containing 5% of the amount of purified CDK4−cyclin D2 in other lanes; 2, GST−gankyrin (0.5 μM)/CDK4−cyclin D2 (0.1 μM); 3, GST−p16 (0.5 μM)/CDK4−cyclin D2 (0.1 μM); 4, GST−Yar 1 (0.5 μM)/CDK4−cyclin D2 (0.1 μM); 5, mock lane, GST (0.5 μM)/CDK4−cyclin D2 (0.1 μM); 6, bovine serum albumin (BSA) as negative control. (B) Competition between gankyrin and p16 for CDK4 binding as revealed by a blot using anti-human CDK4 antibody. Lanes 1 (input), 2 (GST−gankyrin/CDK4−cyclin D2), and 5 (GST−p16/CDK4−cyclin D2) correspond to lanes 1, 2, and 3, respectively, in (A). Lanes 3, 4, 6, and 7 are competition assays with addition of the competing protein (without GST tag): GST−gankyrin (0.5 μM)/p16 (0.1 μM) (5:1, lane 3); GST−gankyrin (0.5 μM)/p16 (2.5 μM) (1:5, lane 4); GST−p16 (0.5 μM)/gankyrin (0.1 μM) (5:1, lane 6); GST−p16 (0.5 μM)/gankyrin (5.0 μM) (1:10, lane 7).

of 5:1, CDK4 was still detected in the pull-down product (lane 3). However, when p16 was in excess (gankyrin:p16 = 1:5), no CDK4 was detected in the pull-down product (lane 4). Similar observations were obtained when gankyrin was used as a competitor in a GST−p16/CDK4−cyclin D2 pull-down reaction. The p16−CDK4−cyclin D2 ternary complex was disrupted by an excess amount of gankyrin (p16:gankyrin = 1:10) (lane 7). In addition, we also did not observe interaction between gankyrin and p16 or between gankyrin and cyclin D2 in pull-down assays (data not shown). Taken together, gankyrin and p16 are competitors for CDK4 binding.

CDK4 Binding and Rb Binding of Gankyrin Are Independent and Involve Distinct Structural Regions. Since gankyrin binds to both Rb (*12*) and CDK4 (previous section), we used pull-down assays and peptide competition assays to examine the relationship between the two binding functions. As shown in Figure 4A, gankyrin was detected in the pull-down products of GST−Rb1−928/gankyrin (lane 2), GST−Rb379−928/gankyrin (lane 3), and GST−Rb379−787/gankyrin (lane

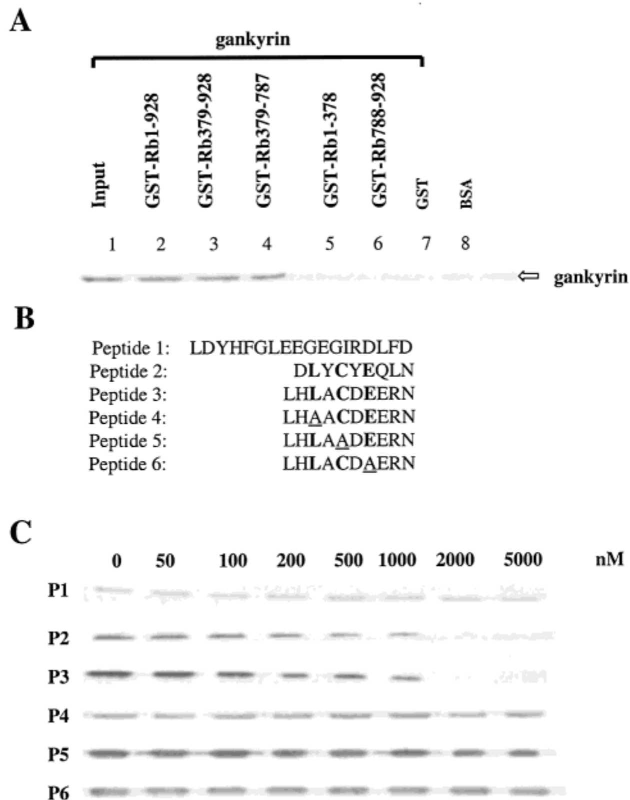

FIGURE 4: Domain mapping with pull-down assay and peptide competition assay. (A) Mapping of the gankyrin-binding domain of Rb. 0.25 mL of the mixture containing 0.4 μM GST−Rb proteins and 2.0 μM free gankyrin in PBS was incubated with reduced glutathione−agarose, and the bound proteins were blotted with anti-human gankyrin antibody (Infiniti). (B) Sequence of synthesized peptides. Peptide 1 is from E2F1 as negative control. Peptides 2 and 3 are peptides containing the LxCxE Rb-binding motif from HPV E7 and human gankyrin, respectively. Peptides 4, 5, and 6 contain the LxCxE motif of human gankyrin with alanine substitutions at different residues. Conserved residues in the Rb-binding motif are in bold, while residues with alanine substitutions are underlined. (C) Peptide competition showing the sequence specificity of the Rb-binding motif of gankyrin. 1.0 mL of the reaction mixture containing 0.1 μM Rb379−787, 0.5 μM gankyrin−His×6, and varying amounts of synthetic peptides were pulled down using Talon affinity gel, and anti-human Rb antibody (PharMingen, 14061A) was used in the blotting of pull-down products. P1, P2, etc. represent synthetic peptides 1, 2, etc., and the concentrations listed above are peptide concentrations in the competition assay.

4) while no binding was observed between gankyrin and both the N- and C-terminal fragments of Rb (Rb1−379 and Rb787−928, respectively) (lanes 5 and 6). Rb379−787, sometimes called the "pocket" region, contains the conserved A and B boxes and is where most of the Rb-binding cellular and viral proteins make their primary contacts (*21*). Our results suggest the same for gankyrin. This fragment was used for the rest of the binding experiments. To investigate the role of the LxCxE motif of gankyrin in binding to Rb, we synthesized a series of peptides (Figure 4B) and studied their competition with gankyrin protein. As shown in Figure 4C, two peptides encompassing the LxCxE Rb-binding motif, D**L**Y**C**Y**E**QLN (peptide 2) from HPV E7 viral protein and LH**L**A**C**D**E**ERN (peptide 3) spanning residues 176−185 of gankyrin, disrupted the gankyrin−Rb binding, while one peptide containing the Rb-binding motif from E2F1 (peptide 1) did not affect the gankyrin−Rb binding. While the peptide from HPV E7 (peptide 2) has been shown previously to bind to the pocket region of Rb (*13*, *21*), our results suggested that the LxCxE motif of gankyrin is also involved in binding to Rb. Furthermore, peptides with substitution of one of the three conserved residues in the LxCxE motif by Ala (peptides 4, 5, and 6) lost their ability to disrupt the binding, as shown in Figure 4 (L178A, peptide 4; C180A, peptide 5; E182A, peptide 6). These results suggest that the peptide LHLACDEERN of gankyrin is sufficient for Rb binding and that the binding mode between gankyrin and Rb is similar to that between peptide 2 and Rb. The latter has already been elucidated by Pavletich and co-workers (*13*). In the crystal structure of the Rb pocket domain bound to a peptide from HPV E7, the peptide DLYCYEQLN bound to a highly conserved groove of the B-box portion in the pocket. The side chains of the conserved Leu, Cys, and Glu residues of the peptide pointed into the B-box groove and made intermolecular contacts with Rb, while the rest of the residues pointed away toward the solvent and did not directly participate in binding.

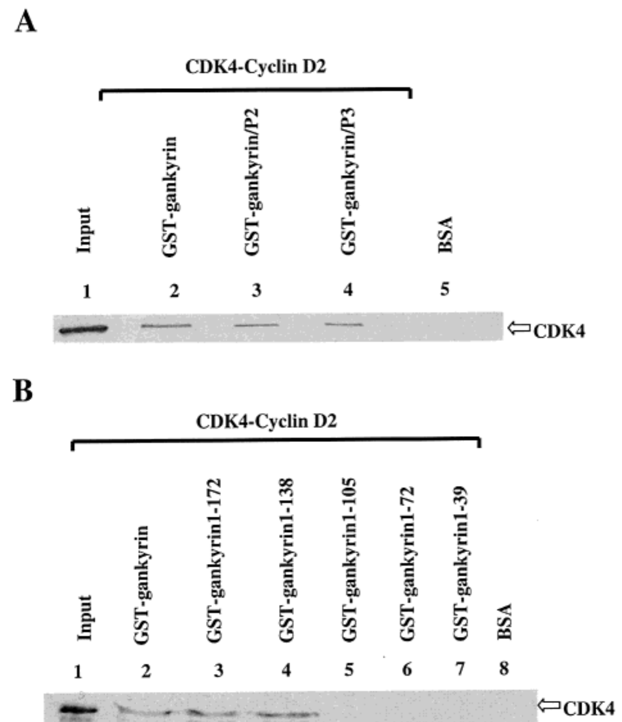

FIGURE 5: Mapping of the CDK4-binding domain of gankyrin. (A) Peptide competition assay. 1.0 mL of the reaction mixture containing 0.1 μM CDK4/cyclin D2 holoenzyme, 0.5 μM GST−gankyrin, and 2.0 μM synthetic peptides was pulled down using reduced glutathione−agarose. P1, P2, etc. represent different peptides as described in the text. (B) 0.25 mL of the reaction mixture containing 0.4 μM CDK4/cyclin D2 holoenzyme and 2.0 μM GST−gankyrin proteins was incubated with reduced glutathione−agarose. In both (A) and (B), anti-human CDK4 antibody was used to blot the pull-down products, while purified CDK4−cyclin D2 and bovine serum albumin were used as positive and negative controls, respectively. For each set, the input lane only contains 5% of the amount of proteins used in other corresponding reactions.

Importantly, peptides 2 and 3 failed to disrupt the interaction between gankyrin and the CDK4−cyclin D2 complex (Figure 5A, lanes 3 and 4), suggesting that different motifs of gankyrin are involved in Rb binding and CDK4 binding. To map out the residues responsible for CDK4 binding, a series of truncated mutants of gankyrin were designed (Figure 1A) and used to probe the interactions with

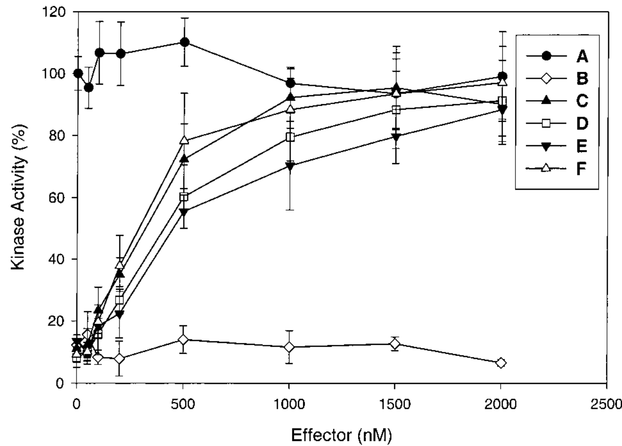

FIGURE 6: In vitro CDK4 kinase activity assay. The reaction mixtures included 3 units of CDK4−cyclin D2, 50 ng of GST−Rb379−928, 5 μCi of [γ-^{32}P]ATP, and varying amounts of effector proteins. The incorporation of ^{32}P was quantitated using a PhosphoImager. (A) Gankyrin only, without p16. (B−F) 100 nM p16 plus different effector protein or peptide present: (B) Yar 1 as a control protein; (C) gankyrin; (D) gankyrin 1−172; (E) gankyrin 1−138; (F) gankyrin and 2.0 μM peptide 3. All measurements were repeated in triplicate, and only those changes higher than 30% were regarded as significant.

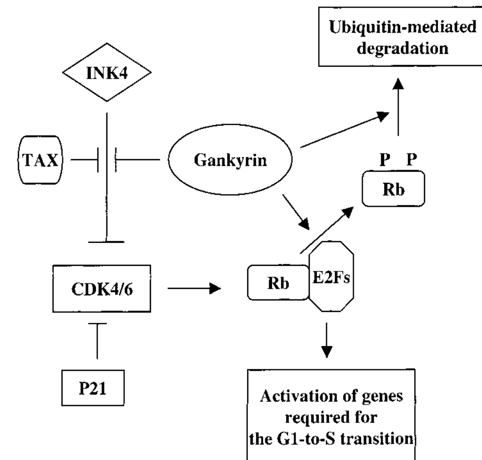

FIGURE 7: Model of the expanded INK4-CDK4/6-Rb pathway demonstrating the involvement of gankyrin. Arrows represent positive effect, and cross bars indicate negative regulation. P represents phosphorylation.

the CDK4−cyclin D2 complex. Unlike the previous findings that Rb-binding ability of a truncated gankyrin containing only the first four ankyrin repeats was abolished (*12*), we found that truncated gankyrin proteins containing the first three and four ankyrin repeats (gankyrin 1−138 and gankyrin 1−172) still retain comparable wild-type CDK4-binding ability (Figure 5B, lanes 3 and 4). Our NMR data also showed that these two truncated mutants were well structured (data not shown). Taken together, gankyrin apparently uses the first three or four ankyrin repeats for CDK4 binding and the fifth one for Rb binding.

Gankyrin Does Not Inhibit CDK4 but Counteracts Inhibition by INK4 Proteins. To understand the biological significance of the competition between gankyrin and INK4 proteins, an in vitro assay was developed to evaluate the kinase activity of CDK4 in the presence of gankyrin and p16 (*3*, *6*, *16*). As shown in Figure 6 (curve A), gankyrin did not cause any detectable change in the CDK4 kinase activity, suggesting that gankyrin does not inhibit the activity of CDK4. As expected, the CDK4 activity dropped approximately 90% in the presence of 100 nM p16, which is the starting point (0 nM effector) for all of the other curves. Gradual increments of gankyrin in the reaction mixtures led to parallel recovery of CDK4 activity (curve C). When gankyrin was added to a final concentration of 1.0 μM, 90% of CDK4 activity was regained, suggesting that the inhibitory effect of p16 could be counteracted by gankyrin. The control protein Yar 1 did not cause detectable recovery in kinase activity even at a concentration as high as 2.0 μM (curve B), which supports the fact that gankyrin binding to CDK 4 and counter action against INK4 proteins is not a nonspecific function for all ankyrin-repeat proteins. Additional experiments showed that gankyrin also counteracted against the inhibition of p18 to CDK4 (data not shown).

The counter action of gankyrin against p16 is different from that of Tax protein (*10*, *11*). Tax binds to p16 and acts as a competitor of CDK4, whereas gankyrin binds to CDK4 and acts as a competitor of p16. Interestingly, both counter actions could lead to the same consequence, which is increasing the phosphorylation of Rb.

The Counter Action between Gankyrin and INK4 Proteins Is Independent of Binding of Gankyrin to Rb. In this section we further used functional assays to support the conclusion reached from pull-down assays that binding of gankyrin to Rb and to CDK4/CyinD2 are independent processes. As shown in Figure 6, curves D and E, truncation mutants of gankyrin containing the first three or four ankyrin repeats behave similarly to native gankyrin (curve C) in countering against the inhibitory activity of p16. Furthermore, addition of 2.0 μM peptide 3, which has been demonstrated to disrupt the binding between gankyrin and Rb (Figure 4C), did not affect the ability of gankyrin to counteract the activity of p16 (curve F). These results suggested that the counter action between gankyrin and INK4 proteins is not related to binding of gankyrin to Rb.

It remains to be established how gankyrin could compete with p16 for binding to CDK4−cyclin D2, yet counteracts the inhibitory activity of p16. It is possible that the gankyrin-binding and the p16-binding sites of CDK4 partially overlap in such a way that gankyrin does not block the kinase site while p16 does. The structures of the p16−CDK6 binary complex and the p18−CDK6−viral D type cyclin ternary complex have been reported recently (*19*, *20*), and the corresponding structures for gankyrin complexes will be of great interest. Furthermore, since gankyrin binds to both CDK4 and Rb, studies on the molecular basis and the dynamic nature of the multiprotein complex containing CDK4, cyclin D, gankyrin, and Rb will provide more insights on signal transduction.

Our Results May Expand the INK4-CDK4/6-Rb Pathway. Taking advantage of reconstructed in vitro systems, we have demonstrated that gankyrin competes with p16 for CDK4 binding and counteracts against the inhibition of p16 to CDK4. If verified by in vivo studies, these findings may not only represent new cellular functions of gankyrin but also reveal a novel mechanism in cell cycle control, that is, "silencing" of INK4 proteins at the protein level rather than at DNA level. Along with its ability to bind Rb directly, gankyrin plays dual roles in the INK4-CDK4/6-Rb pathway (*2*, *22*, *23*) as shown in Figure 7. On one hand, gankyrin

binds to Rb through the Rb-binding motif at its C terminus, and this binding may facilitate the ubiquitin-mediated degradation of Rb. On the other hand, gankyrin binds to CDK4 through its first three ankyrin repeats and abolishes the inhibition of INK4 proteins. To date, gankyrin is the only protein that has been found to have these two independent functions. In this respect, the regulation of gankyrin expression and the intricate coordination between gankyrin and INK4 proteins could very well be the key element in Rb-mediated cell cycle control and tumorigenesis. For instance, a cell without any p16 alteration but with increased gankyrin expression may lead to cell transformation, whereas a cell with high expression of both gankyrin and p16 may resume normal cell progression. In depth investigation of the status of gankyrin at the cellular level is essential to untangle the involvement of the INK4-Rb pathway in carcinogenesis or cell regulation in general.

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REFERENCES

1. Matsushime, H., Ewen, M., Strom, D. K., Kato, J.-Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992) *Cell 71*, 323−334.
2. Weinberg, R. A. (1995) *Cell 81*, 323−330.
3. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature 366*, 704−707.
4. Kamb, A., Gruis, N., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavigian, S., Stockert, E., Day, R., III, Johnson, B., and Skolnick, M. (1994) *Science 264*, 436−440.
5. Luh, F. Y., Archer, S. J., Domaille, P. J., Smith, B. O., Owen, D., Brotherton, D. H., Raine, A. R. C., Xu, X., Brizuela, L., Brenner, S. L., and Laue, E. D. (1997) *Nature 389*, 999−1003.
6. Byeon, I.-L., Li, J., Ericson, K., Selby, T. L., Tevlev, A., Kim, H. J., O'Maille, P., and Tsai, M.-D. (1998) *Mol. Cell 1*, 421−431.
7. Li, J., Byeon, I.-J., Ericson, K., Poi, M., O'Maille, P., Selby, T. L., and Tsai, M.-D. (1999) *Biochemistry 38*, 2930−2940.
8. Yuan, C., Selby, T. L., Li, J., Byeon, I.-J., and Tsai, M.-D. (2000) *Protein Sci. 9*, 1120−1128.
9. Li, J., Byeon, I.-J., Poi, M., Ericson, K., Selby, T. L., O'Maille, P., Qin, D., and Tsai, M.-D. (2000) in *DNA Alterations in Cancer: Genetic and Epigenetic Changes* (Ehrlich, M., Ed.) pp 71−83, Eaton Publishing, Natick, MA.
10. Suzuki, T., Kitao, S., Matsushime, H., and Yashida, M. (1996) *EMBO J. 15*, 1607−1614.
11. Suzuki, T., Narita, T., Uchida-Toita, M., and Yashida, M. (1999) *Virology 529*, 384−391.
12. Higashitsuji, H., Itoh, K., Nagao, T., Dawson, S., Nonoguchi, K., Kido, T., Mayer, J. R., Arii, S., and Fujita, J. (2000) *Nat. Med. 6*, 96−99.
13. Lee, H.-O., Russo, A. A., and Pavletich, N. P. (1996) *Nature 391*, 859−865.
14. Lennon, G. G., Auffray, C., Polymeropoulos, M., and Soares, M. B. (1996) *Genomics 33*, 151−152.
15. Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. (1995) *Nature 375*, 506−510.
16. Hannon, G. J., and Beach, D. (1994) *Nature 371*, 257−261.
17. Lycan, D. E., Stafford, K. A., Bollinger, W., and Breeden, L. (1996) *Gene 17*, 33−40.
18. Sagerson, H. J., and Lycan, D. E. (1999) *FASEB J. 13*, A1493.
19. Russo, A. A., Tong, L., Lee, J., Jeffrey, P. D. E., and Pavletich, N. P. (1998) *Nature 395*, 237−243.
20. Jeffrey, P. D., Tong, L., and Pavletich, N. P. (2000) *Genes Dev. 14*, 3115−3125.
21. Jones, R. E., Wegizyn, R. J., Patrick, D. R., Balishin, N. L., Vuocolo, G. A., Riemen, M. W., Defeo-Jones, D., Garsky, V. M., Heimbrook, D. C., and Oliff, A. (1990) *J. Biol. Chem. 265*, 12782−12785.
22. Sherr, C. J. (1996) *Science 274*, 1672−1677.
23. Serrano, M. (1997) *Exp. Cell Res. 237*, 7−13.

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