The Nuclear Protein p34^{SEI-1} Regulates the Kinase Activity of Cyclin-Dependent Kinase 4 in a Concentration-Dependent Manner†

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ABSTRACT: Previous studies have shown that p34^{SEI-1}, also known as TRIP−Br1, is involved in cell cycle regulations by interacting with a number of important proteins including CDK4. However, the detailed mechanism and structural basis of the interaction remains to be determined. We report the use of in vitro studies to address these problems. First, it was shown that p34^{SEI-1} binds to CDK4 directly, and the binding does not compete directly with p16. In the presence of p16, a quaternary complex is formed between p34^{SEI-1}, CDK4, cyclin D2, and p16. Second, it was found that p34^{SEI-1} activates the kinase activity of CDK4 at lower concentrations (reaching the maximum at 500 nM) but inhibits the same activity at higher concentrations, implying that p34^{SEI-1}-mediated CDK4 activation is dose-dependent. Again, the effects of p34^{SEI-1} and p16 are independent of each other. Third, it was shown that p34^{SEI-1} possesses a LexA-mediated transactivation activity. Finally, a set of truncation mutants were used to dissect the structural elements responsible for the different functions of p34^{SEI-1}. The results indicate that the fragment 30−160 can bind, activate, and inhibit CDK4; the fragment 30−132 can bind and activate but does not inhibit CDK4, while the fragment 30−88 cannot bind, activate, or inhibit but retains the LexA-mediated transactivation activity.

Mammalian cyclin-dependent kinases (hereafter, CDKs)[1] are a group of conserved serine/threonine protein kinases driving the cell through most of the major cell cycle control points (*1*−*3*). In conjunction with different cyclins, CDKs phosphorylate specific substrates, which subsequently turn on/off downstream genes required for cell progression (*4*). For instance CDK4 and 6, in partnership with D cyclins, specifically phosphorylate the retinobalstoma susceptible gene product (Rb), thus regulating entry into the cell cycle and passage through the checkpoint in late G1 (*2*). As the central molecules of the machinery controlling cell progression, all CDKs are strictly controlled by multiple mechanisms, such as gene amplification and transcription, holoenzyme assembly, posttranslational modification, and protein/protein interactions (*5*). Many proteins have been found to act as negative or positive kinase regulators of CDK4 and 6 through protein/protein interactions. While binding of D cyclins is required for the full kinase activity (*4*), INK4 (including p16, p15, p18, and p19) (*6*−*11*) and KIP (including p21, p27, and p57) proteins (*5*, *12*−*14*) are inhibitors of CDK4 and 6. Moreover, the oncoprotein Tax from human T-cell leukemia virus 1 (HTLV-1) stimulates the CDK4 activity in infected cells through physical association (*15*, *16*), and the oncoprotein gankyrin affects CDK4 by counteracting against the inhibition of p16 and p18 (*17*). The intricate regulation of CDK4 and 6 activitied by these and possibly many other proteins is crucial for cell cycling and tumorigenesis.

p34^{SEI-1}, a nuclear protein originally cloned through a yeast two-hybrid approach using human p16 as bait, has been found to be involved in the regulation of CDK4 (*18*). Originally, a weak interaction between p34^{SEI-1} and p16 was observed in both yeast two-hybrid assay and in vitro pull-down assay. However, this interaction appeared much weaker than that between the CDK4 and INK4 proteins and was regarded as an artifact resulting from protein overexpression or the possible existence of a bridging molecule working between p34^{SEI-1} and p16 (*18*). Further binding and immunoprecipitation studies demonstrated that p34^{SEI-1} specifically binds to CDK4 (but not CDK6) in vitro and in vivo, and this binding appears to antagonize the function of p16, thus rendering the activity of CDK4/cyclin D resistant to the

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[1] Abbreviations: AEBSF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride; CDK, cyclin-dependent kinase; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycobis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GST, glutathione-*S*-transferase; GST-SEI1 proteins, recombinant human SEI1 proteins with a GST tag at the N terminus; HEPES, *N*-[2-hydroxyethel]piperazine-*N*′-[2-ethanesulfonic acid]; HTLV-1, human T-cell leukemia virus 1; INK4, inhibitor of cyclin-dependent kinase 4; IPTG, isopropyl-β-D-thiogalactopyranoside; KIP, cyclin-dependent kinase inhibitor protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; pRb or Rb, human retinoblastoma susceptible gene product; SEI1, CDK4-associated protein selected with INK4A as bait, also known as TRIP−Br1; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tax, HTLV-1 transcription activator; TRIP−Br1, transcriptional regulator interacting with the PHD-bromodomain.

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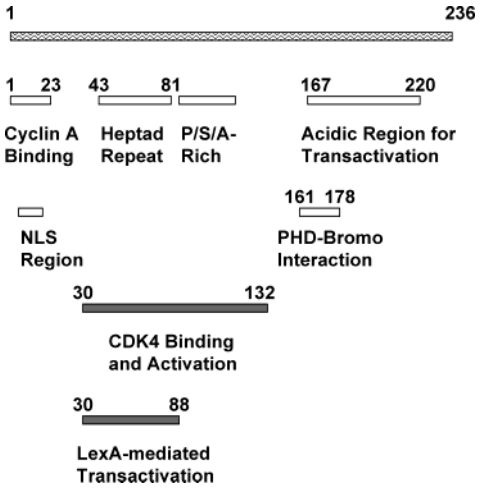

FIGURE 1: Schematic summary of the domain organization of p34^{SEI-1}. The figure was modified from reference 19. Filled boxes represent domains mapped out from this paper. NLS, nuclear localization sequence; P/S/A, proline-, serine-, and alanine-rich domain.

inhibitory effect of p16 during the later G1 phase (*18*). It has been further demonstrated that p34^{SEI-1} is identical to TRIP−Br1, a component of a multiprotein complex containing E2F1 and DP-1 (*19*). On one hand, TRIP−Br1 acts as a transcriptional regulator that interacts with the PHD-bromodomain of corepressors and coactivators. On the other hand, TRIP−Br1 functionally contacts DP-1 and stimulates E2F1/DP-1 transcriptional activity. These observations strongly suggest that p34^{SEI-1} or TRIP−Br1 may play a key role in cell cycle control.

However, many questions remain unanswered regarding how p34^{SEI-1} contributes to cell cycle regulation through the interaction with CDK4. First, it has not been shown how p34^{SEI-1} affects CDK4 activity in the absence and presence of p16. Second, p34^{SEI-1} has the transactivational activity (*19*), but it is unclear whether the transcriptional activating or repressing activity of p34^{SEI-1} affects the expression of CDK4-related proteins, such as INK4 and cyclins. The observed counteraction against p16 inhibition could result from both transactivation and CDK4 interaction. Third, although it was reported that there was no detectable interaction between p34^{SEI-1} and cyclin D1 (*18*), the existence of a cyclin A-binding motif (Figure 1) (*19*) provides p34^{SEI-1} the potential to interact with cyclins, thus indirectly affecting CDKs. Last, amplification of p34^{SEI-1} gene is a common event in human cancers such as ovarian tumors (*20*) and pancreatic tumors (*21*−*23*), in which p16 gene is inactivated or expressed at very low levels (*24*). If p34^{SEI-1} is indeed oncogenic, it is unclear how or whether this is caused solely by counteracting the CDK4-inhibitory activity of p16.

Using in vitro systems, we have studied the interactions between CDK4 and various proteins (p16, p18, Tax, and gankyrin), in various combinations (*16*, *17*, *25*−*27*). Here, we present biochemical and biophysical studies on p34^{SEI-1}/CDK4 interactions under a variety of conditions. Our results addressed some of the questions mentioned above and, in particular, led to a novel finding that p34^{SEI-1} directly interacts with CDK4 and affects the kinase activity in a concentration-dependent way. We also demonstrated that the CDK4-interacting motif is located within residues 30 and 160, and this motif (within residues 33−88) also retains LexA-mediated transactivation activity, which is different from the transactivation activity mediated by the acidic region located in the C terminus of p34^{SEI-1}.

METHODS AND MATERIALS

Protein Purification and Expression. DNA fragments encoding full-length and truncated human p34^{SEI-1} genes were amplified by PCR using pcDNA3.0-p34^{SEI-1} as the template and cloned into pGEX-6p-1 (Amersham Pharmacia) at *Eco*R I and *Xho* I sites. The resultant constructs were transformed and expressed in *E. coli* BL21 (DE3) CodonPlus cells as glutathione-*S*-transferase (GST)-fusion proteins. Bacteria harboring these constructs were incubated in Luria−Bertani media at 27 °C until OD_{600} reached 0.6. After IPTG induction at the final concentration of 0.1 mM, bacteria were further incubated at 27 °C for 12 h. To purify GST-tagged p34^{SEI-1} proteins, the cell lysate was loaded on a reduced glutathione-agarose column (Sigma) preequilibrated with the above PBS buffer. After washing with PBS buffer, bound proteins were eluted with PBS buffer containing 20 mg/mL of freshly prepared reduced glutathione. After addition of ATP and $MgSO_4$ to the final concentrations of 100 mM and 10 mM, respectively, the eluent was incubated at 25 °C for 24 h (*28*). Subsequently, the eluent was dialyzed against PBS buffer to remove extra salts and glutathione and further purified on a Q Fastflow sepharose column (Pharmacia) with a NaCl gradient from 0 to 600 mM in 50 mM Tris-HCl (pH 8.4). After analysis by SDS−PAGE, the fractions containing GST-CDK4 were dialyzed against 4 mM HEPES (pH 7.4), 1 μM EDTA, 1 mM DTT, and 5% glycerol (v/v). To remove the GST tag, GST-tagged p34^{SEI-1} proteins were incubated with PreScission protease (2 units/mL) at 4 °C for 16 h and then loaded on a preequilibrated reduced glutathione-agarose column. The flow-through was collected and concentrated.

The preparation of CDK4/cyclin D2 holoenzyme, GST-p16, GST-Rb791−928, free p16, and p18 D76A was previously described (*17*, *25*, *27*, *29*).

Pull-down Assays. To investigate the interaction between GST-tagged p34^{SEI-1} proteins and CDK4/cyclin D2 holoenzyme, a 2.5 mL assay mixture containing 0.1 μM p34^{SEI-1} proteins and 0.05 μM CDK4/cyclin D2 in PBS buffer was incubated at 4 °C for 12 h and then loaded on a 200 μL reduced glutathione-agarose column, which had been equilibrated with PBS buffer. Subsequently, the column was washed with 1.0 mL of PBS buffer and then eluted with 100 μL of PBS buffer with reduced glutathione (20 mg/mL). The eluent was analyzed by Western blot using an antihuman CDK4 antibody (Santa Cruz) and an antihuman cyclin D2 antibody (BD Pharmingen).

Pull-down assays in the presence of free p16 were conducted in a manner analogous to the above assays except that free p16 was included in the reaction mixtures and an antihuman p16 antibody (BD Pharmingen) was used to probe the presence of p16 in the pull-down products.

To investigate the possible competition between the p34^{SEI-1} and INK4 proteins for CDK4 binding, various amounts of p18 D76A, a mutant retaining CDK4 binding ability, unperturbed global structure, but no kinase inhibitory activity, were included into the above pull-down mixtures containing 0.1 μM GST-tagged p34^{SEI-1} proteins, 0.05 μM

CDK4/cyclin D2, and 0.1 μM free p16. After incubation, the pull-down mixtures were loaded onto a mini column containing 200 μL PBS-equilibrated glutathione-agarose. Subsequently, the column was washed with 1.0 mL of PBS, and bound proteins were eluted out with 100 μL of PBS buffer with reduced glutathione (20 mg/mL). The presence of CDK4, cyclin D2, and p16 was assessed by Western blotting as above.

In Vitro Kinase Assay. The in vitro kinase assay involved 1.5 units of the CDK4/cyclin D2 complex (about 0.15 μg of protein) and varying concentrations of the proteins under investigation in kinase buffer (50 mM HEPES at 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 of leupeptin, and 2.5 mg/mL of aprotinin in a total volume of 15.0 μL. The final concentration of CDK4-cyclin D2 in the reaction mixture was about 0.15 μM. One unit of CDK4 kinase is defined as the amount of CDK4 that catalyzes the incorporation of 1 μmol of phosphate into pRb at 30 °C within 15 min. After preincubation at 30 °C for 30 min, GST-Rb791−928 (100 ng) and 5 μCi of γ-^{32}P-ATP were added into each reaction mixture. After incubation at 30 °C for another 15 min, the reaction mixtures were separated by SDS−PAGE and the CDK4 activity was determined by quantifying the incorporation of ^{32}P into substrate pRb using a PhosphorImager (Molecular Dynamics, Inc.). Measurements were repeated in duplicates.

To investigate how INK4 binding of CDK4 affects the p34^{SEI-1} function, a fixed amount of p18 D76A (100 μM) and varied amounts of p34^{SEI-1} proteins were included in the reaction mixtures. Similarly, a fixed amount of p34^{SEI-1} protein (100 μM) and various amounts of free p16 were included in the reaction mixtures to study the potential effect of p34^{SEI-1}/CDK4 binding on p16 inhibition.

Transactivation Assay. A modified yeast one-hybrid system (BD Clontech) was used to evaluate the basic transactivation activities of full-length and truncated p34^{SEI-1} proteins as previously described (*16*). Full-length or truncated p34^{SEI-1} genes were cloned into a pLexA vector at the *Eco*R I and *Xho* I sites as binding domain fusion plasmids. The host strain, EGY48 harbored p8op-*lac Z*, a reporter plasmid carrying the *lac Z* reporter gene under the control of the LexA operator and the minimal TATA region from the *GAL1* promoter. A pLexA-Tax plasmid was used as a positive control.

RESULTS AND DISCUSSIONS

p34^{SEI-1} Directly Binds to CDK4 in the Absence and Presence of INK4 Proteins. We first examined the potential interaction between p34^{SEI-1} and CDK4 using an in vitro pull-down system containing GST-tagged p34^{SEI-1} proteins and the CDK4/cyclin D2 complex. The CDK4/cyclin D2 complex was used because the complex, not free CDK4, is the biologically functional form. As shown in lane 3 of Figure 2, both CDK4 and cyclin D2 were detected in the pull-down product (the upper and bottom rows, respectively), suggesting that p34^{SEI-1} binds to the CDK4/cyclin D2 complex. To determine whether p34^{SEI-1} binds directly to CDK4, we checked the interaction between the CDK4/cyclin D2 complex and SEI1 30−236, a truncated version of p34^{SEI-1} without the N-terminal cyclin A binding domain. The pull-down results (lane 4 of Figure 2) show that SEI1 30−236 retains the ability to bind to the CDK4/cyclin D2 complex, suggesting that the association between p34^{SEI-1} and the CDK4/cyclin D2 complex results from a direct binding of p34^{SEI-1} to CDK4.

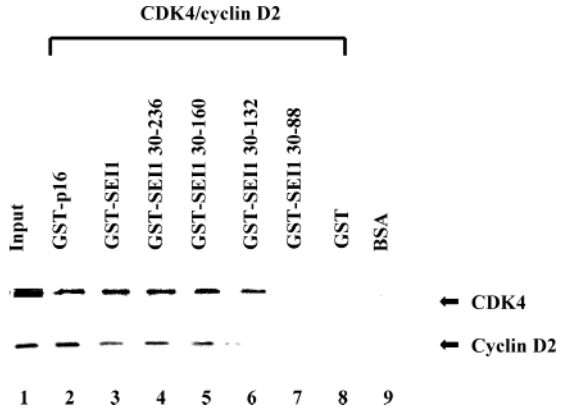

FIGURE 2: Pull-down assays to investigate the binding of p34^{SEI-1} proteins to CDK4. A total of 2.5 mL of assay mixture containing 0.1 μM GST-p34^{SEI-1} proteins and 0.05 μM CDK4/cyclin D2 was incubated at 4 °C for 12 h and then loaded on a mini column with 200 μL of reduced glutathione agarose. After the column was washed with 1.0 mL of PBS buffer, bound proteins were eluted with 100 μL of PBS buffer containing reduced glutathione (20 mg/mL) and blotted with antihuman CDK4 antibody (sc-260, Santa Cruz) and antihuman cyclin D2 antibody (14821C, BD Pharmingen): lane 1, the input containing 10% of the amount of CDK4/cyclin D2 used in other lanes; lane 2, 0.1 μM GST-p16 with 0.05 μM CDK4/cyclin D2 as a positive control; lane 3, 0.1 μM GST-p34^{SEI-1} with 0.05 μM CDK4/cyclin D2; lane 4, 0.1 μM GST-SEI1 30−236 with 0.05 μM CDK4/cyclin D2; lane 5, 0.1 μM GST-SEI1 30−160 with 0.05 μM CDK4/cyclin D2; lane 6, 0.1 μM GST-SEI1 30−132 with 0.05 μM CDK4/cyclin D2; lane 7, 0.1 μM GST-SEI1 30−88 with 0.05 μM CDK4/cyclin D2; lane 8, 0.1 μM GST with 0.05 μM CDK4/cyclin D2; lane 9, BSA as a negative control.

We then checked the interactions between SEI1 30−236 and the CDK4/cyclin D2 complex in the presence of free p16. As shown in lanes 3 and 4 of Figure 3, both CDK4 and cyclin D2 were present in the pull-down products even when free p16 was in excess in the pull-down mixtures (the upper and middle rows, respectively), suggesting that the presence of p16 does not affect the binding of p34^{SEI-1} to CDK4/cyclin D2. Furthermore, the presence of p16 in the pull-down product (the bottom row) indicated that a quaternary complex is formed among SEI1 30−236, CDK4/cyclin D2, and p16. This observation distinguishes p34^{SEI-1} from other CDK4-interacting proteins such as Tax and gankyrin. In the presence of p16, Tax forms a binary complex with p16 and/or a ternary complex with CDK4/cyclin D2 (*15*, *16*, *30*, *31*), while gankyrin competes with p16 and only forms a ternary complex with CDK4/cyclin D2 (*17*). Moreover, the result suggests that p34^{SEI-1} and INK4 proteins are not competitors for binding to CDK4.

To further verify the above interpretations, a competition experiment was performed by using p18 D76A, a p18 mutant known to compete with p16 for binding to CDK4 but does not inhibit the kinase activity of CDK4 (*29*). In the pull-down mixtures containing SEI1 30−236, CDK4/cyclin D2, p16, and p18 D76A, as the concentration of p18 D76A increased, the amount of p16 in the pull-down products decreased (lanes 5, 6, and 7 of Figure 3). When the ratio between p18 D76A and p16 was 10:1 (1.0 versus 0.1 μM)

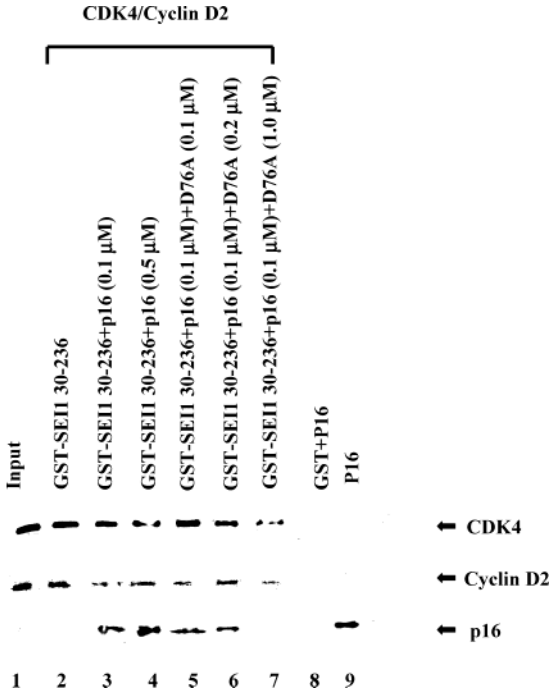

FIGURE 3: Pull-down assays to investigate the interactions between p34^{SEI-1} proteins and CDK4/cyclin D2 in the presence of INK4 proteins. The experiments were performed as described in Figure 2, except that varying amounts of p16 and/or p18 D76A were included in the reaction mixtures: lane 1, the input containing 10% of the amount of CDK4/cyclin D2 used in other lanes; lane 2, 0.1 μM GST-SEI1 30−236 and 0.05 μM CDK4/cyclin D2; lane 3, 0.1 μM GST-SEI1 30−236, 0.05 μM CDK4/cyclin D2, and 0.1 μM p16; lane 4, 0.1 μM GST-SEI1 30−236, 0.05 μM CDK4/cyclin D2, and 0.5 μM p16; lane 5, 0.1 μM GST-SEI1 30−236, 0.05 μM CDK4/cyclin D2, 0.1 μM p16, and 0.1 μM p18 D76A; lane 6, 0.1 μM GST-SEI1 30−236, 0.05 μM CDK4/cyclin D2, 0.1 μM p16, and 0.2 μM p18 D76A; lane 7, 0.1 μM GST-SEI1 30−236, 0.05 μM CDK4/cyclin D2, 0.1 μM p16, and 1.0 μM p18 D76A; lane 8, 0.1 μM GST and 0.1 μM p16; lane 9, p16 as a positive control.

in the pull-down mixture, no p16 was detected in the pull-down products while CDK4 and cyclin D2 were still present, suggesting that p18 D76A competes with p16, not p34^{SEI-1}, for CDK4 binding. When taken together, our results suggest that p34^{SEI-1} and INK4 proteins bind to distinct sites of CDK4. This conclusion agrees with the following previous reports from our lab and others, when considered together: (i) Using an in vitro binding assay, Hara and his colleagues demonstrated that the middle domain of CDK4 encompassing residues 79−245 is responsible for binding to p34^{SEI-1} (*18*). (ii) We have reported that a fragment containing the first 52 residues at the N terminus of CDK4 binds to p16 in vitro (*25*). (iii) Studies of CDK6, a close homologue of CDK4, further supports this observation. As revealed in the crystal structures of the p16/CDK6 and p19/CDK6 complexes (*32*, *33*), domains at the N and C termini (the N and C lobes) of CDK6 contribute the most to INK4 binding.

p34^{SEI-1} Affects the CDK4 Kinase Activity in a Concentration-Dependent Manner. In vitro kinase activity assays were then performed to evaluate the functional effect of p34^{SEI-1}/CDK4 interaction. To our great surprise, it was found that the effect of p34^{SEI-1} on the kinase activity is concentration-dependent as shown in Figure 4 (curves A and B). As the concentration of p34^{SEI-1} (or SEI1 30−236) increased, CDK4 was activated. When p34^{SEI-1} and SEI1 30−236 concentrations reached 500 nM, CDK4 kinase activity increased to

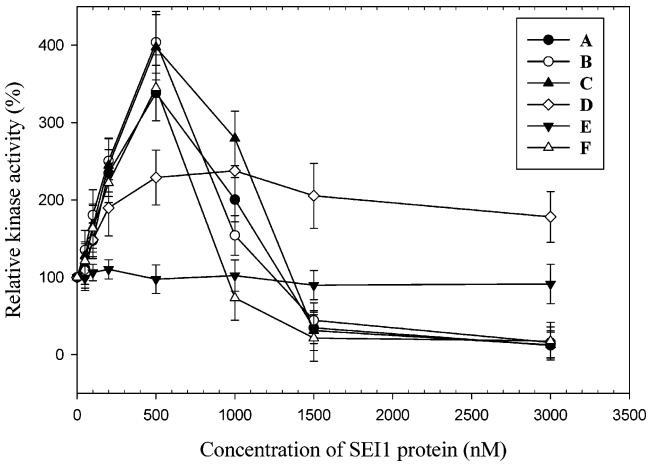

FIGURE 4: In vitro kinase activity assay to examine p34^{SEI-1} protein-mediated activation of CDK4. Each assay mixture contained 1.5 units of CDK4/cyclin D2 (ca. 0.15 μM in the reaction mixture), 100 ng of GST-Rb791−928, 5 μCi of γ-^{32}P-ATP, and a varying amount of SEI1 protein. The reaction was performed at 30 °C, and the incorporation of ^{32}P into GST-Rb791−928 was quantified with a PhosphorImager. A, p34^{SEI-1}; B, SEI1 30−236; C, SEI1 30−160; D, SEI1 30−132; E, SEI1 30−88; F, SEI1 30−236 in the presence of 100 nM p18 D76A. All measurements were performed in duplicates, and changes higher than 50% were regarded as significant. The kinase activity in the absence of effectors is used as the reference point 100%.

about 350% and 400%, respectively. However, as the concentrations of p34^{SEI-1} and SEI1 30−236 further increased, the kinase activity decreased. When the concentrations of p34^{SEI-1} and SEI1 30−236 reached 3.0 μM, the kinase activity was completely inhibited. Note that this assay was done in the absence of p16; thus, the effect on CDK4 activity can only be attributed to the interaction between p34^{SEI-1} and CDK4.

Dissecting the Structural Elements for the Different Functions of SEI1. To further analyze the phenomenon of bifunctionality, we designed a series of truncated mutants to map out the structural elements responsible for the different functions of p34^{SEI-1}. As shown in lanes 5, 6, and 7 of Figure 2, CDK4 and cyclin D2 were detected in the pull-down products of SEI1 30−160 and SEI1 30−132, but not SEI1 30−88, suggesting that SEI1 30−160 and SEI1 30−132, but not SEI1 30−88, retain the CDK4-binding ability.

Although both SEI1 30−160 and SEI1 30−132 can bind to CDK4 as shown above, we further showed that the latter has lost the inhibitory activity. As shown in Figure 4, SEI1 30−160 (curve C) behaves as full-length p34^{SEI-1} (activation at lower concentrations and inhibition at higher concentrations), SEI1 30−132 (curve D) can only activate CDK4 throughout the concentration range, and SEI1 30−88 (curve E) does not affect the activity of CDK4 likely because of the lack of binding. In summary, our results have clearly dissected the structural elements of the different functions of p34^{SEI-1}. The fragment 30−160 can bind, activate, and inhibit CDK4; fragment 30−132 can bind and activate but does not inhibit CDK4, while fragment 30−88 cannot bind, activate, or inhibit. The role of the motif 30−88 will be addressed later.

Effects of p34^{SEI-1} and p16 to the Activity of CDK4 Are Additive. Our results so far have demonstrated that in the absence of p16, p34^{SEI-1} itself can activate (at low concentra-

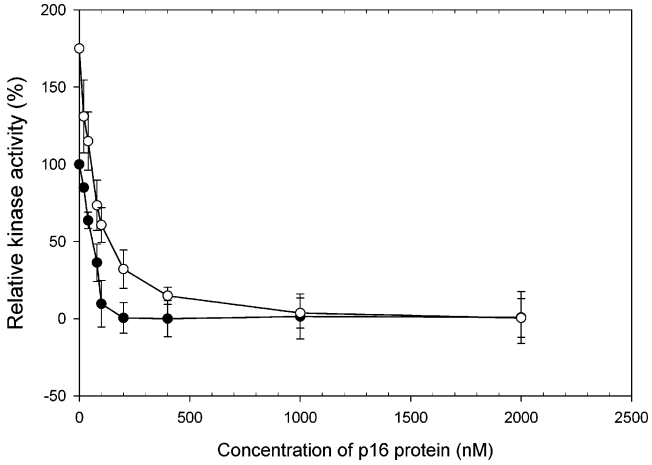

FIGURE 5: Cooperation between p34^{SEI-1} and p16 on regulating the CDK4 kinase activity. Experiments were performed as described in Figure 4 except that the effector protein in each reaction mixture is different. Solid circles represent p16 only, and empty circles represent p16 in the presence of 100 nM SEI1 30−236.

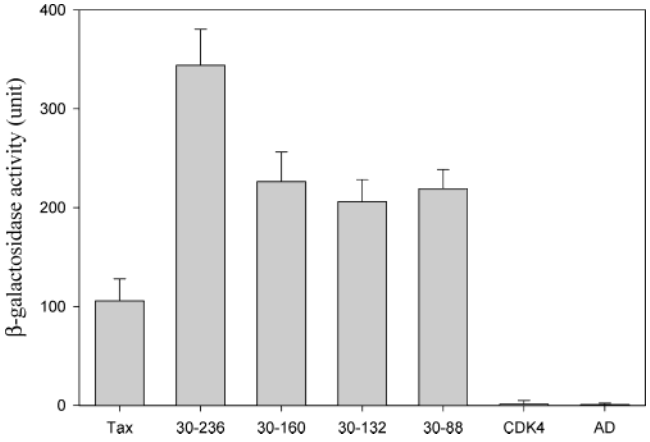

FIGURE 6: Transactivation assays. pLexA plasmids with p34^{SEI-1} genes were transformed into yeast EGY48 harboring the reporter plasmid, p8op-*lac Z*, and transformants containing both pLexA and p8op-*lac Z* were selected on synthetic dropout (SD) medium deficient for uracil (Ura) and histine (His). Ura/His positives were restreaked onto SD-Ura/-His/X-gal/galactose/raffinose media. The β-galactosidase activity of the *lac Z* gene product, which represented the strength of p34^{SEI-1} proteins in transactivation was determined in a liquid β-galactosidase enzymatic activity assay using ONPG (Sigma) as the substrate. Tax oncogene was used a positive control, while CDK4 was used as a negative control. Assays were performed in triplicates.

tions) or inhibit (at high concentrations) CDK4. We also showed that in the presence of p16, p34^{SEI-1} forms a quaternary complex with CDK4/cyclin D2 and p16. In this section, we show that the effects of p34^{SEI-1} and p16 on the activity of CDK4 are independent of each other (and thus additive when both are present). As shown in curve F of Figure 4, in the presence of 100 nM p18 D76A (which is known to bind but not inhibit CDK4), SEI1 30−236 affected CDK4 as in the absence of INK4 proteins (curve B of Figure 4), suggesting that the function of p34^{SEI-1} on CDK4 is independent of INK4 proteins. Similarly, the inhibition of INK4 proteins is also independent of p34^{SEI-1} binding to CDK4. Figure 5 shows the inhibitory effect of p16 to CDK4 in the absence and presence of SEI1 30−236 (100 nM). The CDK4 activity is higher in the latter case as expected, but p16 inhibits CDK4 in both cases with nearly identical values of IC_{50} (65 ± 17 nM and 80 ± 20 nM, respectively). Therefore, in the presence of both p34^{SEI-1} and p16 (or INK4 proteins, in general), changes in CDK4 kinase activity reflect the total effect resulting from both p34^{SEI-1}/CDK4 and p16/CDK4 interactions. This result could explain the previous observation that the inhibition of p16 appears to be counteracted by p34^{SEI-1} (*18*), because at low concentrations of p34^{SEI-1}, the effect of p34^{SEI-1} is activating, while the effect of p16 is inhibiting.

CDK4-Binding Domain of p34^{SEI-1} Retains LexA-Mediated Tansactivation Activity. We used a yeast one-hybrid assay to evaluate the LexA-mediated transactivation activity (*16*). As shown in Figure 6, p34^{SEI-1} exhibited a transactivation activity three times higher than that of oncoprotein Tax, a positive control, while the negative control, CDK4, did not show any detectable activity. Domain mapping results showed that SEI1 30−88 retained a transactivation activity comparable to intact p34^{SEI-1}, suggesting that this LexA-mediated transactivation is different from that mediated by the acidic region at the C terminus of p34^{SEI-1} (*19*). It has been reported that p34^{SEI-1} or TRIP−Br1 interacts with corepressors of KRIP-1 and TIF1α as well as the coactivator/adaptor p300/CBP (*19*). However, we still do not know how many genes are transcriptionally repressed or activated by p34^{SEI-1}, and it is possible that p34^{SEI-1} is involved in the CDK4/Rb pathway by transcriptionally activating or repressing some CDK4-related genes.

Potential Biological Significance. Under our in vitro assay conditions, p34^{SEI-1} acts as both an activator and inhibitor of CDK4, which distinguishes p34^{SEI-1} from other CDK4-binding proteins. INK4 (*5*−*11*) and KIP proteins (*12*−*14*) only inhibit CDK4; gankyrin functions as a competitor to antagonize INK4 proteins (*17*), while Tax is competent in activating CDK4 (*15*, *16*), quenching p16 (*32*, *33*), and affecting cyclin Ds (*34*). It is important to point out that most of these studies were performed by in vitro or cell-based studies, and their biological relevance remains to be established. However, the in vitro results, as reported here, provide potentially important leads for biological studies and for further structural analyses.

Although we have dissected the structural segments of p34^{SEI-1} responsible for different functions, the structural basis of CDK4 binding by p34^{SEI-1} is totally unclear. One hypothetical possibility is that CDK4 possesses two p34^{SEI-1} binding sites, one with high affinity and the other with low affinity. At low concentrations of p34^{SEI-1}, only the high-affinity site is saturated, and the effect is activating; at higher concentrations, the low-affinity site is also occupied, and the overall effect is inhibiting. In any case, our results have provided new insight and a novel finding for the biochemical properties of p34^{SEI-1} in the CDK4−Rb pathway, which can serve as the basis for much more extensive biological and structural studies in the future.

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