

## 14-3-3 proteins associate with cdc25 phosphatases

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**ABSTRACT** The cdc25 phosphatases play key roles in cell cycle progression by activating cyclin-dependent kinases. Two members of the 14-3-3 protein family have been isolated in a yeast two-hybrid screen designed to identify proteins that interact with the human cdc25A and cdc25B phosphatases. Genes encoding the human homolog of the 14-3-3 $\epsilon$  protein and the previously described 14-3-3 $\beta$  protein have been isolated in this screening. 14-3-3 proteins constitute a family of well-conserved eukaryotic proteins that were originally isolated in mammalian brain preparations and that possess diverse biochemical activities related to signal transduction. We present evidence that indicates that cdc25 and 14-3-3 proteins physically interact both *in vitro* and *in vivo*. 14-3-3 protein does not, however, affect the phosphatase activity of cdc25A. Raf-1, which is known to bind 14-3-3 proteins, has recently been shown to associate with cdc25A and to stimulate its phosphatase activity. 14-3-3 protein, however, has no effect on the cdc25A-kinase activity of Raf-1. Instead, 14-3-3 may facilitate the association of cdc25 with Raf-1 *in vivo*, participating in the linkage between mitogenic signaling and the cell cycle machinery.

In eukaryotic cells, the cyclin-dependent kinases (cdks) regulate cellular functions critical to passage through successive phases of the cell cycle (1). The activity of these kinases is regulated both by association with ancillary proteins and by phosphorylation. Foremost among cdk-associated proteins are the cyclins, which function as positive regulatory subunits for specific members of the cdk family. Several recently described cdk inhibitors, p15 (2), p16 (3), p21 (4–6), and p27 (7, 8), also physically interact with cdks. The expression of these proteins increases in response to cellular damage or extracellular growth inhibitory signals, resulting in the inhibition of kinase activity.

cdk activity is also regulated by phosphorylation. Phosphorylation of a threonine residue within the catalytic domain of cdks by the cdk-activating kinase (CAK) is required for kinase activity (9). Inhibitory phosphorylation of threonine and tyrosine residues in the ATP-binding site of most cdks inhibits kinase activity (10). These inhibitory sites are conserved between fission yeast and several metazoan cdks and are found near the N termini, usually at residues Thr-14 and Tyr-15 (11). The phosphorylation state of Thr-14 and Tyr-15 residues of fission yeast cdc2 is a major determinant of the onset of mitosis and is controlled by the opposing actions of the wee1/mik1 kinases and the cdc25 phosphatase (12–15). wee1 kinases are now known from many species (16–18) and have been shown to phosphorylate Tyr-15 (12, 13). cdc25 phosphatases, although weakly related to the main family of protein phosphatases, have been shown to specifically dephosphorylate Thr-14 and Tyr-15 (15, 19–23). Yeasts have a single *cdc25* gene (16, 24), whereas human cells possess three related *cdc25* genes: *CDC25A*, *-B*, and *-C* (25, 26).

The activity of the cdc25 phosphatases is also regulated by phosphorylation. In human cells, cdc25C is activated at the

onset of mitosis by cdc2–cyclin B phosphorylation (27). Since cdc25C phosphatase activity stimulates cdc2 kinase activity, phosphorylation by cdc2–cyclin B may result in the formation of an auto-amplification loop with increased cdc25C activity further activating cdc2 kinase activity. cdc25A, which plays a crucial role early in the cell cycle (28), has been shown to be phosphorylated by cdk2–cyclin E at the G<sub>1</sub>/S transition (29). Recently, the Raf-1 protooncogene kinase has been found to form a tight complex with cdc25 phosphatases both in somatic cells and in *Xenopus* oocytes (30). Raf-1-dependent kinase activity phosphorylates and stimulates cdc25A activity. This constitutes a major link between mitogenic signaling and cell cycle control.

To identify other potential regulators of cdc25, a yeast two-hybrid protein interaction screening was undertaken to identify proteins that interact with the human cdc25 phosphatases. Two interacting clones were found to encode 14-3-3 isoforms.<sup>†</sup> These proteins are members of a family of proteins that are known to associate with components of several signal transduction pathways, including the Raf-1 kinase cascade. This interaction is likely to play some role in linking mitogenic signaling to the regulation of cdc25 activity.

### MATERIALS AND METHODS

**Two-Hybrid Screens.** *Saccharomyces cerevisiae* strains HF7C (31) and L40 (32) were used as hosts for two-hybrid vectors. HeLa cell two-hybrid cDNA libraries were gifts of G. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). *cdc25A* and *cdc25B* were fused to the sequence encoding the GAL4 DNA-binding domain of pGBT9. Yeast cells were simultaneously transformed with GAL4 DNA-binding domain (GAL4<sup>BD</sup>) fusion plasmids and GAL4 transcriptional activation domain (GAL4<sup>AD</sup>) fusion plasmids. Leu<sup>+</sup> Trp<sup>+</sup> transformants, which contain both GAL4<sup>BD</sup> and GAL4<sup>AD</sup> fusion plasmids, were streaked on plates with or without histidine to determine whether the encoded proteins interacted. Yeast cells were grown in YPD (2% glucose/2% peptone/1% yeast extract) or synthetic minimal media (2% glucose/0.67% yeast nitrogen base, with appropriate auxotrophic supplements) by using standard techniques. Plasmids carrying sequences encoding Ras and Raf-1 fusions (33) were gifts of L. VanAelst (Cold Spring Harbor Laboratory).

**Binding Experiments.** A fusion of maltose-binding protein (MBP) and 14-3-3 $\beta$  for expression in bacteria was constructed by removing the open reading frame from pGBT9-14-3-3 $\beta$  by using PCR with primers to flanking DNA. The resulting fragment was inserted into the *EcoRI* and *Pst* I sites of pBluescript KS (–) (Stratagene), producing plasmid p14-3-3KS–. *In vitro* translation and DNA sequence analysis confirmed that the wild-type 14-3-3 $\beta$  open reading frame had been subcloned without the introduction of errors. The MBP-14-3-3 $\beta$  fusion was constructed by inserting the entire 14-3-3 $\beta$

Abbreviations: cdk, cyclin-dependent kinase; MBP, maltose-binding protein; GST, glutathione *S*-transferase.

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<sup>†</sup>The sequence reported in this paper has been deposited in the GenBank data base (accession no. U20972).

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open reading frame into pMAL-c2 (New England Biolabs). Construction of the glutathione *S*-transferase (GST)-*cdc25A* fusion has been described (25). Each protein was purified on either glutathione-Sepharose or amylose-Sepharose columns by using standard procedures. Purified proteins were incubated at approximate final concentrations of 500 nM in 50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40 for 30 min on ice. Complexes were subsequently precipitated by the addition of 50  $\mu$ l of a 1:1 slurry of glutathione-Sepharose and the above buffer. Beads were washed four times with the above buffer, heated to 95°C in sample buffer (55), and subjected to SDS/PAGE and immunoblotting. The immunoblot was probed with anti-MBP-antibody (New England Biolabs). Staphylococcal protein A coupled with horseradish peroxidase HRP (Amersham) and the ECL reagent (Amersham) were used to detect bound antibody in all immunoblots.

**Immunoprecipitation and Antibody Production.** HeLa cells, grown in suspension to a density of  $2 \times 10^6$  cells per ml, were pelleted by centrifugation at  $1000 \times g$  for 10 min, washed three times with phosphate-buffered saline, and lysed in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5/5 mM MgCl<sub>2</sub>/25 mM NaF/1 mM EGTA/1 mM dithiothreitol/1 mM sodium orthovanadate containing aprotinin at 10  $\mu$ g/ml, leupeptin at 10  $\mu$ g/ml, pepstatin at 0.5  $\mu$ g/ml, chymostatin at 1  $\mu$ g/ml, 1 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride). Soluble proteins from  $1.5 \times 10^9$  cells were subjected to immunoprecipitation with 5  $\mu$ g of affinity-purified anti-14-3-3 $\beta$  antibody, anti-*cdc25A* antibody, or preimmune rabbit IgG. Immunoprecipitations were carried out essentially as previously described (3).

Antibody against human 14-3-3 $\beta$  was raised against purified MBP-14-3-3 $\beta$  fusion protein in rabbits and affinity purified on a column containing Sepharose 4B-coupled, hexahistidine-tagged human 14-3-3 $\beta$  (see below). Antibodies against Raf-1 (C20) were purchased from Santa Cruz Biotechnology. Antibodies against *cdc25A* have been described (30). Samples for detection of Raf-1 and *cdc25A* were analyzed on 7.5% polyacrylamide gels. Samples for detection of 14-3-3 were analyzed on 12.5% gels.

**Baculovirus Infections.** A baculovirus which directs the expression of the entire 14-3-3 $\beta$  protein was constructed with the vector pLV1393 and the Baculo-gold kit (PharMingen). This was used to infect *Spodoptera frugiperda* (Sf9) cells grown in monolayer at a multiplicity of infection of 5, either alone or in combination with viruses directing the expression of *cdc25A* and Raf-1. Labeling with [<sup>35</sup>S]methionine indicated that approximately equal amounts of each protein were produced in both doubly and triply infected cells. Insect cells were lysed at 60–72 h after infection in an NP40 lysis buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/1 mM dithiothreitol containing 1 mM sodium orthovanadate, aprotinin at 10  $\mu$ g/ml, leupeptin at 10  $\mu$ g/ml, pepstatin at 0.5

$\mu$ g/ml, chymostatin at 1  $\mu$ g/ml, 1 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride). A soluble extract was prepared by repeated passage of cells through a 26 gauge needle followed by the removal of cellular debris by centrifugation. Hexahistidine-tagged human 14-3-3 $\beta$  was prepared from insect cells infected with a baculovirus expressing the entire 14-3-3 $\beta$  protein fused in frame to the six-histidine tag sequence of pAcSGHisNT-A (PharMingen).

## RESULTS

**Isolation of *cdc25A*-Binding Proteins.** A two-hybrid screening was undertaken to identify proteins that physically interact with the human *cdc25* phosphatases. Human *cdc25A* and *cdc25B* genes, fused in frame to the GAL4 DNA-binding domain vector pGBT9, were each cotransformed into yeast with a HeLa cell-derived cDNA library contained in the GAL4 transcriptional activation domain vector, pGADGH. Thirty-four clones interacted with the *cdc25* constructs sufficiently to allow growth of the transformed host on media lacking histidine and to express detectable levels of  $\beta$ -galactosidase activity.

Among these positives were two members of the 14-3-3 family of proteins. One of 10 interaction-positive clones found with *cdc25A* encoded the previously identified (34) 14-3-3 $\beta$  isoform (Fig. 1). Eight of 24 interaction-positive clones found with *cdc25B* encoded the human homolog of the mouse 14-3-3 $\epsilon$  isoform (35). The complete nucleotide sequence of the cDNA corresponding to the human 14-3-3 $\epsilon$  gene is shown in Fig. 2. The deduced human 14-3-3 $\epsilon$  protein sequence is identical to the mouse 14-3-3 $\epsilon$  sequence. Despite originating in different screenings, both 14-3-3 clones interacted with either *cdc25* construct (Fig. 1). The interactions between 14-3-3 and *cdc25* constructs were judged to be specific, since interactions between the GAL4<sup>AD</sup>-14-3-3 clones and GAL4<sup>DB</sup> gene fusions with the human lamin gene or the *S. cerevisiae* *SNF1* gene were not observed.

14-3-3 proteins have recently been implicated in a number of mitogenic signaling pathways, including the kinase cascade that contains Raf-1 (36–38). In *Xenopus* oocytes, increased 14-3-3 expression affects the Raf-1-dependent activation of *cdc2* during *Xenopus* maturation. We therefore tested for other potential interactions between 14-3-3 clones and other players in Raf-1 signal transduction and cell cycle control. The 14-3-3 clones and Raf-1, Ras, *cdc2*, *cdk2*, *cdk4*, and *cdc25* proteins were tested in pairwise combinations for protein–protein interactions by using the two-hybrid assay. Both 14-3-3 clones were capable of interacting with full-length Raf-1 as well as an N-terminal deletion protein lacking amino acids 26–302 (Table 1). Both clones also interacted with a C-terminal deletion protein lacking amino acids 380–648, although growth on plates lacking histidine was not as strong as in the case of

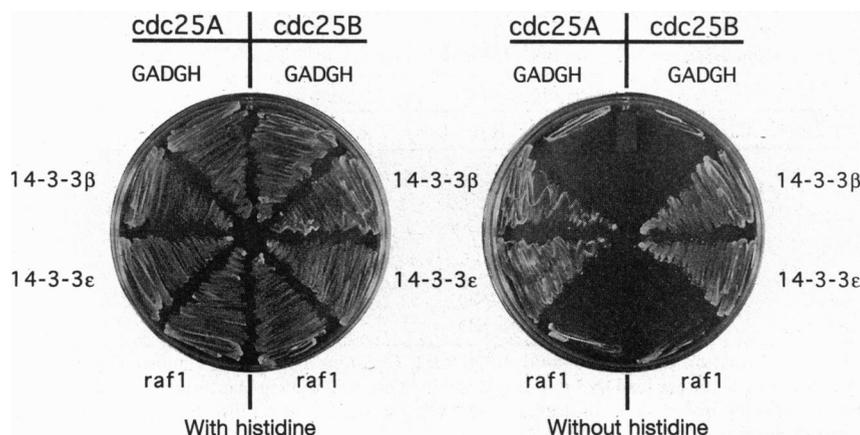


FIG. 1. *cdc25* proteins interact with 14-3-3 proteins in the two-hybrid screen. GAL4<sup>BD</sup>-*cdc25* fusion proteins and GAL4<sup>AD</sup>-14-3-3 fusion proteins interact in yeast cells, reconstituting functional GAL4 transcriptional activator. The expression of *HIS3* from a *GAL4* promoter allows the *his3* host to grow on media lacking histidine. A GAL4 transcriptional activation domain-Raf-1 fusion or a vector control does not allow cells to grow.

	GGCACGAGGGAGCGAGAGGCTGAG	
	AGAGTCGGAGCACTATCCGCTTCCATCCGTCGCGCAGACCCCTGCCGAGCCGCTGCCGCT	
1	ATGGATGATCGAGAGGATCTGGTGTACCAGGCGAAGTTGGCCGAGCAGGCTGAGCGATAC	60
1	M D D R E D L V Y Q A K L A E Q A E R Y	20
61	GACGAAATGGTGGAGTCAATGAAGAAAGTAGCAGGGATGGATGTGGAGCTGACAGTTGAA	120
21	D E M V E S M K K V A G M D V E L T V E	40
121	GAAAGAAACCTCCTATCTGTTCATATAAGAATGTAATTGGAGCTAGAAGACCTCCTGG	180
41	E R N L L S V A Y K N V I G A R R A S W	60
181	AGAATAATCAGCAGCATTTGAACAGAAAGAAGAAAACAAGGGAGGAGAAGACAAGCTAAAA	240
61	R I I S S I E Q K E E N K G G E D K L K	80
241	ATGATTCGGGAATATCGGCAAAATGGTTGAGACTGAGCTAAAGTTAATCTGTGTGACATC	300
81	M I R E Y R Q M V E T E L K L I C C D I	100
301	CTGGATGTACTGGACAACACCTCATTCCAGCAGCTAACACTGGCAGTCCAAGGTTTTTC	360
101	L D V L D K H L I P A A N T G E S K V F	120
361	TATTATAAAATGAAAGGGGACTACCACAGGTATCTGGCAGAATTTGCCACAGGAAACGAC	420
121	Y Y K M K G D Y H R Y L A E F A T G N D	140
421	AGGAAGGAGGCTGCGGAGAACAGCCTAGTGGCTTATAAAGCTGCTAGTGATATTGCAATG	480
141	R K E A A E N S L V A Y K A A S D I A M	160
481	ACAGAACTTCCACCAACGCATCCTATTTCGCTTAGGCTTGTCTCAATTTTCCGTATTC	540
161	T E L P P T H P I R L G L A L N F S V F	180
541	TACTACGAAATCTTAATCCCCTGACCGTGCCTGCAGGTTGGCTAAAGCAGCTTTTGAT	600
181	Y Y E I L N S P D R A C R L A K A A F D	200
601	GATGCAATTCAGAACTGGATACGCTGAGTGAAGAAAGCTATAAGGACTCTACACTTATC	660
201	D A I A E L D T L S E E S Y K D S T L I	220
661	ATGCAGTTGTTACGTGATAATCTGACACTATGGACTTCAGACATGCAGGGTGACGGTGAA	720
221	M Q L L R D N L T L W T S D M Q G D G E	240
721	GAGCAGAATAAAGAAGCGCTGCAGGACGTTGAAGACGAAAATCAGTGAGACATAAGCCAA	780
241	E Q N K E A L Q D V E D E N Q *	260
781	CAAGAGAAACCATCTCTAACCACCCCTCCTCCCATCCCACCCCTTTGGAACTCCCCAT	840
841	TGTCACGTGAGAACCAAACTGTACTTTACATTTGGTCTCAGAAATTTAGGTTCTCTGCC	900
901	CTGTTGGTTTTTTTTTTTTTTTTTAAAGTTTTCAAAAGTCTTAAAGGCAAGAGTGAA	960
961	TTT 963	

FIG. 2. Sequence of the human 14-3-3 $\epsilon$  cDNA. The DNA sequence of both strands was determined by using an Applied Biosystems fluorescent DNA sequencer. The deduced protein sequence is shown below the nucleotide sequence.

full-length Raf-1 or the N-terminal Raf-1 deletion. In general, these results are similar to those previously reported for 14-3-3-Raf-1 interactions and indicates that the 14-3-3 $\epsilon$  isoform is similar to the  $\beta$  and  $\zeta$  isoforms in terms of its ability to interact with Raf-1 (36–38). None of the 14-3-3 clones or cdc25 clones interacted with Ras. Interactions between 14-3-3 proteins and the human cdk2 also were not observed. Although physical interaction between cdc25A or cdc25B and Raf-1 has been observed in other experimental systems, we did not detect an interaction between these proteins in the two-hybrid assay. Taken together, these results indicate that only indirect interactions were observed between the cell cycle control components and the Ras pathway signaling components that we tested with the two-hybrid assay. Although 14-3-3 interacted with both Raf-1 and cdc25, we did not observe an

interaction between the cdc25s and Raf-1 even when human 14-3-3 was expressed in these cells.

**14-3-3 Associates with cdc25A *in Vitro* and *in Vivo*.** To confirm the existence of a direct interaction between 14-3-3 and cdc25 proteins, the association was studied in a cell-free system. Bacterially produced GST-cdc25A and MBP-14-3-3 $\beta$  fusion proteins were purified and found to associate *in vitro* (Fig. 3A). Interactions between the GST and MBP moieties were not observed. This indicates that the two proteins are capable of directly interacting without the Ras/mitogen-activated protein kinase signaling pathway components that are present in yeast cells (39).

Both proteins were also found to associate *in vivo*. Fig. 3B shows that cdc25A protein is precipitated from the soluble fraction of HeLa cells with anti-cdc25A antibody or anti-14-

Table 1. Summary of interactions between cdc25 phosphatases, 14-3-3 proteins, and Raf-1 domains in the two-hybrid screen

Activation domain	Binding domain								
	cdc25A	cdc25B	cdc2	cdk2	cdk4	RAF-1	RAFAN1	RAFAC1	RASC186R
GADGH	–	–	–	–	–	–	–	–	–
cdc25A	ND	ND	–	–	–	–	–	–	–
cdc25B	ND	ND	–	+	–	–	–	–	–
14-3-3 $\beta$	+	+	–	–	–	+	+	+	–
14-3-3 $\epsilon$	+	+	–	–	–	+	+	+	–
Raf-1	–	–	–	–	–	ND	ND	ND	+
Raf K375W	–	–	–	–	–	ND	ND	ND	+

Rows list coding regions fused to the GAL4 DNA activation domain plasmid pGADGH. Columns list coding regions fused to the GAL4 DNA-binding domain plasmids except for Raf-1 deletions, which were lexDNA-binding domain fusions. + indicates interaction of the two constructs as evidenced by the ability to form colonies on plates lacking histidine; – indicates no growth and therefore no interaction; and ND signifies not determined. Plasmids carrying Ras and Raf-1 fusions were gifts of L. VanAelst (33).

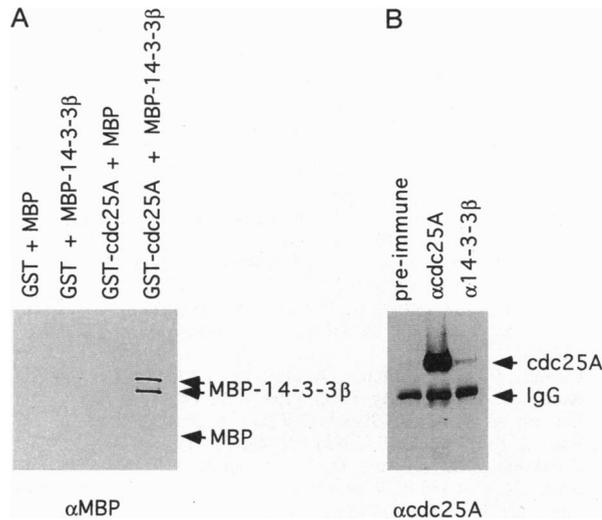


FIG. 3. Interaction between 14-3 $\beta$  and cdc25A proteins. (A) Purified bacterially produced MBP-14-3 $\beta$ , which migrates as a doublet, is precipitated by purified bacterially produced GST-cdc25A and glutathione-Sepharose beads *in vitro*.  $\alpha$  indicates an antibody. (B) Co-immunoprecipitation of 14-3-3 and cdc25 from the soluble fraction of HeLa cells with anti-14-3 $\beta$  antibody. Protein preparations and immunoprecipitations were as described in the text. Equal volumes of sample from equal volumes of protein A-Sepharose beads were loaded in each lane. The gel was blotted and probed with anti-cdc25A antibody.

3-3 $\beta$  antibody. Co-immunoprecipitation of human cdc25A with anti-14-3 $\beta$  antibody indicates that the two proteins are associated in human cells. Immunoprecipitation with anti-14-3 $\beta$  antibody depleted  $\approx$ 5% of the soluble cdc25A from HeLa lysates. This was similar to the amount of Raf-1 protein that we could precipitate from HeLa lysates under the same conditions with the anti-14-3 $\beta$  antibody (data not shown). Although Raf-1-14-3-3 interactions have been observed in membrane fractions of cells (38), the existence of cdc25A-14-3-3 membrane-bound complexes could not be addressed due to a difficulty inherent in the immunoprecipitation of cdc25A from membrane preparations (30).

**14-3-3 Associates with cdc25A and Raf-1 in Insect Cells.** We also tested the interaction between cdc25A and 14-3 $\beta$  by using baculovirus-produced proteins in insect cells, where interactions with Raf-1 could also be assessed. All pairwise associations between Raf-1, cdc25A, and 14-3 $\beta$  proteins were observed. 14-3 $\beta$  could be co-immunoprecipitated with antibodies to either Raf-1 (Fig. 4,  $\alpha$ 14-3-3 panel, lanes 3 and 4) or cdc25A (Fig. 4,  $\alpha$ 14-3-3 panel, lanes 7 and 8). Similarly, Raf-1 and cdc25 could be precipitated with anti-human 14-3 $\beta$  antibody. All possible combinations of co-immunoprecipitation were also observed in triple infections. However, it was not possible to determine whether co-immunoprecipitation of all three proteins represented precipitation of triple complexes (14-3 $\beta$ -Raf-1-cdc25A) or multiple pairwise complexes (14-3 $\beta$ -Raf-1, Raf-1-cdc25A, or 14-3 $\beta$ -cdc25A). We consistently observed an approximate 2.5-fold increase in the amount of Raf-1-cdc25A precipitable complex in cells infected with 14-3 $\beta$  construct versus cells infected with only Raf-1 and cdc25A (Fig. 4,  $\alpha$ Raf-1 panel, lane 9 vs. lane 6;  $\alpha$ cdc25A panel, lane 5 vs. lane 2). This may represent an underestimate of the increase in binding between Raf-1 and cdc25A, since Raf-1-cdc25A complex formation in doubly infected cells may be stimulated by insect cell 14-3-3 protein. Insect cell 14-3-3 protein, which cross-reacted with the anti-14-3 $\beta$  antibody, was also observed binding to Raf-1 (Fig. 4,  $\alpha$ 14-3-3 panel, lanes 2 and 5; refs. 38 and 40) and could

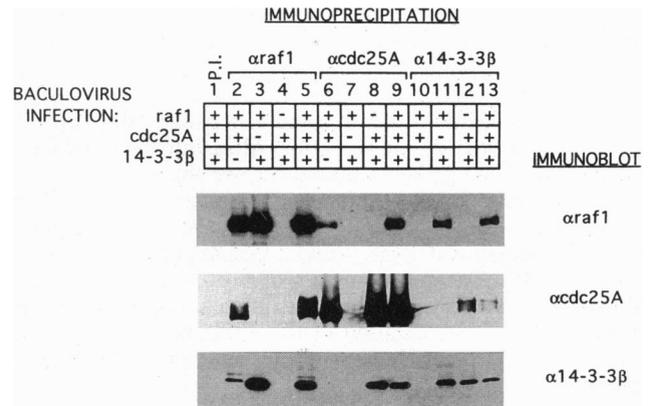


FIG. 4. Associations between recombinant human Raf-1, 14-3-3, and cdc25A proteins in insect cells. Cell lysate preparation and immunoprecipitation were carried out as described in the text. Equal volumes of sample from equal volumes of protein A-Sepharose beads were loaded in each lane. Gels were blotted and probed with antibodies ( $\alpha$ ) indicated. P.I., preimmune serum. Only blot regions corresponding to proteins of interest are shown. Signal found in lanes 2 and 5 of the  $\alpha$ 14-3-3 panel corresponds to insect cell 14-3-3 protein (38, 40).

potentially assist in complex formation in the absence of baculovirus-expressed 14-3-3.

## DISCUSSION

14-3-3 proteins have been isolated in a yeast two-hybrid screen designed to identify proteins that interact with human cdc25 phosphatases. The 14-3-3 $\beta$  isoform has been found to physically associate with cdc25A both *in vivo* and *in vitro*. The consequence of the observed interaction between 14-3-3 and cdc25, however, is not fully understood. Although once believed to be a nuclear protein present in cells only after mitogenic stimulation (28, 29), cdc25A has been found to associate with Raf-1 at or near the plasma membrane immediately after the stimulation of quiescent cells (30). Since Raf-1-dependent kinase activity also phosphorylates and stimulates cdc25A activity, cdc25A is thought to mediate, at least in part, cell cycle activation by the Ras/Raf-1 pathway (30). 14-3-3 proteins, including the 14-3-3 $\beta$  isoform which interacts with the cdc25s, have, in some cases, been shown to stimulate Raf-1 kinase activity (40, 41). We did not, however, observe consistent 14-3-3-dependent stimulation of cdc25A phosphorylation by Raf-1 which was prepared by immunoprecipitation from insect cells triply infected with Raf-1, Ras, and Src baculoviruses (42). We were also unable to detect any effect of 14-3-3 on cdc25A phosphatase activity *in vitro*.

That 14-3-3 proteins have been found to physically interact with a cell cycle regulatory element is not surprising. 14-3-3 proteins have been implicated in the regulation of cell cycle control in *Xenopus* oocytes and in fission yeast. A direct physical interaction between 14-3-3 and cdc25 proteins is generally consistent with the observed effects of 14-3-3 proteins on cell cycle control. In *Xenopus* oocytes, 14-3-3 proteins have a positive effect on the Raf-1-dependent activation of cdc2 (37). We did not observe interaction between 14-3-3 proteins and cyclin-dependent kinases in two-hybrid assays. Since cdc25 is also well established as a primary regulator of cdc2 activity in *Xenopus*, it is possible that the stimulatory effect of 14-3-3 on cdc2 activity may be mediated by Raf-1-dependent stimulation of cdc25 phosphatase activity. In fission yeast, 14-3-3 proteins are required for a DNA damage-induced cell cycle checkpoint (43) and for the correct timing of mitosis. Although a direct connection between 14-3-3 and cdc25 proteins has not been established genetically in fission yeast, the

cdc25 gene has been firmly established as a regulator of the timing of mitosis (44) and has been implicated in checkpoint control (45). It should be noted, however, that 14-3-3 proteins appear to have a positive effect on cell cycle progression in higher cells (37, 38, 41, 46, 47) and a negative effect on cell cycle progression in fission yeast (43). More information on the function of 14-3-3 proteins should resolve this paradox.

14-3-3 proteins have been shown to associate with a number of proteins involved in the cellular response to mitogenic factors, including Raf-1 (38, 40, 41), Bcr-Abl (46), and middle T antigen (47). The consequences of 14-3-3 binding by these mitogenic signal transduction proteins are not fully known. Moreover, the determination of the effect of 14-3-3 binding has been hampered by the bewildering number of biochemical activities related to signal transduction that 14-3-3 proteins have been shown to possess (48). These include ADP-ribosyltransferase activity (49), phospholipase A<sub>2</sub> activity (50), Ca<sup>2+</sup> binding (50), activation and inhibition of protein kinase C (51), activation of amino acid hydroxylases in neurotransmitter synthesis (52), and stimulation of secretion (53). At present, it seems difficult to attribute all of these activities to a single 30-kDa protein, or even to a subset of this highly conserved family.

On the basis of the studies of interaction between 14-3-3 proteins and the Raf-1, Bcr-Abl, and middle T antigen proteins, it has been suggested that 14-3-3 proteins play an organizational role in mitogenic signal transduction (36). The modest increase in Raf-1-cdc25A complex formation observed in insect cells expressing high levels of 14-3-3 is consistent with this hypothesis. That we did not observe increased Raf-1-cdc25A interaction *in vitro* in the presence of either bacterially produced or baculovirus-produced 14-3-3 protein suggests that 14-3-3 may require other cellular components such as membranes or submembranous cytoskeletal structures similar to those containing Raf-1 (54), to stimulate Raf-1-cdc25A complex formation. Whether 14-3-3 stimulates Raf-1-cdc25A complex formation in human cells is not known. Nevertheless, the physical association of 14-3-3 proteins with cdc25 phosphatases offers further evidence for the interaction between mitogenic signaling and cdc25-dependent cell cycle control.

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