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Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation

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The Ras and Raf1 proto-oncogenes transduce extracellular signals that promote cell growth. Cdc25 phosphatases activate the cell division cycle by dephosphorylation of critical threonine and tyrosine residues within the cyclin-dependent kinases. We show here that Cdc25 phosphatase associates with raf1 in somatic mammalian cells and in meiotic frog oocytes. Furthermore, Cdc25 phosphatase can be activated in vitro in a Raf1-dependent manner. We suggest that activation of the cell cycle by the Ras/Raf1 pathways might be mediated in part by Cdc25.

In eukaryotic cells, a family of related cyclin-dependent kinases (cdks) regulate progression through each phase of the cell division cycle (Heichman and Roberts 1994; Hunter and Pines 1994; King et al. 1994; Sherr 1994). These proteins are subject to multiple levels of control, including association with regulatory subunits known as cyclins (Evans et al. 1983; Swenson et al. 1986; Koff et al. 1991; Lew et al. 1991; Matsushime et al. 1991; Xiong et al. 1991) and activating phosphorylation by cdk-activating kinase [(CAK) Solomon et al. 1992]. Cyclin/cdk complexes are also negatively regulated by at least two different mechanisms. First, the recently identified cdk inhibitors (p21, p16, p15, p18, and p27; El-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Serrano et al. 1993; Xiong et al. 1993; Guan et al. 1994; Hannon and Beach 1994; Polyak et al. 1994a; Toyoshima and Hunter 1994; Guan et al. 1994) prevent cell cycle progression in the presence of DNA damage, contact inhibition, and senescence [(p21) Dulic et al. 1994; Noda et al. 1994], transforming growth factor-β1 TGF-β1 [(p15) Hannon and Beach 1994; (p27) Polyak et al. 1994b] or other unknown stimuli (p16, p18). Second, certain members of the cdk family (cdc2, cdk2) are inactivated by phosphorylation of threonine and tyrosine residues, usually at positions 14 and 15 (Draetta et al. 1988; Gould and Nurse 1989; Morla et al. 1989; Krek and Nigg 1991; Gu et al. 1992). These residues are conserved between fission yeast cdc2 and most of the human cdks (Meyerson et al. 1992).

In fission yeast, positive (cdc25) and negative (wee1 and mik1) regulators of mitosis have been identified (Russell and Nurse 1986, 1987; Lundgren et al. 1991). Biochemical analysis demonstrated that Wee1 and Mik1 are inhibitory tyrosine kinases that phosphorylate Tyr-15 and possibly Thr-14 (Parker et al. 1991, 1992; Featherstone and Russell 1991), whereas Cdc25 is a specialized dual specificity phosphatase capable of dephosphorylating the same residues and activating the cyclin kinases (Dunphy and Kumagai 1991; Galaktionov and Beach 1991; Gautier et al. 1991; Millar et al. 1991; Strausfeld et al. 1991). The role of the single fission yeast cdc25 gene in regulating the G_2/M transition is well documented (Russell and Nurse 1986; Millar et al. 1991). In humans, however, cdc25 consists of a multigene family, the A, B, and C isoforms, which share ~40%–50% amino acid identity (Sadhu et al. 1990; Galaktionov and Beach 1991; Nagata et al. 1991). Both Cdc25A and Cdc25C have some function in mitosis (Galaktionov and Beach 1991; Millar et al. 1991). However, Cdc25A has also been found to be essential for DNA replication following serum stimulation of quiescent rat fibroblasts (Jinno et al. 1994). The precise role of each member of the human Cdc25 family in cell cycle progression has yet to be determined.

In Xenopus oocytes, which are blocked in prophase of the first meiotic division Cdc25 function is rate limiting for entry into M-phase. Cdc2 kinase in oocytes is complexed with mitotic cyclins but is inactive, apparently because of phosphorylation of Thr-14 and Tyr-15. Upon stimulation with progesterone or insulin, oocytes undergo the transition into meiosis (maturation). The crucial step in this process, activation of maturation promoting factor (MPF) (cyclin B/Cdc2 kinase), is effected

by dephosphorylation of Cdc2 on Thr-14 and Tyr-15 by Cdc25. As a result of the inhibitory phosphate removal, Cdc2 histone H1 kinase is activated, causing the oocyte to progress into meiosis (Dunphy and Newport 1989; Gautier et al. 1989).

The *ras* and *raf1* proto-oncogenes are essential elements of several signal transduction pathways (Stacey et al. 1991; Thomas et al. 1992; Wood et al. 1992). *ras* and *raf1* become activated through various receptor and non-receptor tyrosine kinases in response to extracellular stimuli (for review, see Hunter 1995; Marshall 1995). It has been shown that the Ras protein binds GTP and functions in part to promote membrane localization and activation of the Raf1 protein kinase (Leevers et al. 1994; Stokoe et al. 1994). The exact mechanism of Raf1 activation is largely unknown (Marshall 1995) but might involve the 14-3-3 family proteins (Freed et al. 1994; Fu et al. 1994). Activated Raf1 kinase then phosphorylates and activates MEK (Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992), which results in MEK-dependent phosphorylation and activation of MAP kinases (MAPKs or ERKs; Ahn et al. 1991; Gomez and Cohen 1991; Kosako et al. 1992). These events constitute a signal transduction kinase "cascade," which is believed to be a central element of the cellular response to extracellular stimuli, including various mitogens (for review, see Herskowitz 1995; Marshall 1995). Current models suggest that the end result of this cascade is the phosphorylation and activation of transcription factors (for review, see Hill and Treisman 1995), which in turn activate cell proliferation. In *Xenopus* oocytes, however, the signal transduction cascade appears to activate meiotic maturation in the absence of new transcription. This suggests that a much more direct link between signal transduction and the cell cycle control machinery must exist.

In frog oocytes Raf1 has been implicated in meiotic maturation that is mediated by progesterone and receptor tyrosine kinases [(RTK) Fabian et al. 1993a; Muslin et al. 1993; for review, see Heidecker et al. 1991]. Introduction of oncogenic forms of either Ras or Raf1 causes precocious maturation of *Xenopus* oocytes, associated with activation of the cyclin B/Cdc2 kinase. Because the major event in oocyte maturation is thought to be the activation of Cdc25, we investigated the potential link between Raf1 and Cdc25 in mammalian cells and in *Xenopus* oocytes.

Results

Raf1/Cdc25 association in meiotic oocytes

To test for possible interaction between Raf1 and Cdc25 we first employed *Xenopus* oocytes, a well-defined biological system devoid of transcriptional control. Cytoplasmic extracts were prepared from prophase oocytes, progesterone-matured oocytes, eggs activated by Ca^{2+} in the presence of cycloheximide, cAMP-blocked oocytes, and insulin-like growth factor-1 (IGF-1)–matured oocytes (see legend to Fig. 1; Materials and methods). Levels of histone H1 kinase activity in prophase, inter-

phase, and metaphase extracts were found to be as described previously (Jessus and Beach 1992; Fig. 1A). Similar extracts were probed with antibodies against *Xenopus* Cdc25 (Kumagai and Dunphy 1992) or anti-human Raf1 antibodies that have been shown previously to cross-react with frog c-Raf1 (see Materials and methods). The relevant proteins were clearly detected (Fig. 1B,C). As described previously (Kumagai and Dunphy 1992), frog Cdc25 undergoes a change in mobility from 70 to 90 kD apparent molecular mass in matured oocytes. At the same time, Raf1 also shifts to a slightly higher molecular mass. In activated eggs (interphase) Cdc25 is present in mostly unphosphorylated form (Fig. 1B,D), whereas Raf1 displays an altered mobility (Fig. 1C,E). These observations prompted us to check the kinase activity of Raf1 in immunoprecipitates from prophase, metaphase, and activated egg (interphase) extracts. We found that Raf1 kinase activity toward exogenous, recombinant MEK is activated in metaphase ~5- to 10-fold, and the activity at interphase is equal to or even lower than that in prophase, despite the interphase shift of Raf1 (not shown).

To investigate a potential Raf1/Cdc25 interaction, equal amounts of extracts from interphase and activated oocytes and eggs were used for immunoprecipitations with anti-Cdc25 antibodies (Fig. 1D,E) or anti-Raf1 antibodies (Fig. 1F,G). These immunoprecipitates were then blotted with anti-Raf1 (Fig. 1E,F) or anti-Cdc25 antibodies (Fig. 1D,G), respectively. A significant reciprocal immunoprecipitation of Raf1 and Cdc25 was observed under each physiological condition (Fig. 1D–G).

Because Raf1 and Cdc25 apparently form a stable association, we determined what fraction of *Xenopus* Cdc25 protein exists in a complex with Raf1. Extracts prepared from prophase oocytes and progesterone-matured (metaphase II) oocytes were depleted with anti-Raf1 antibodies (see Materials and methods). The initial extracts, depleted extracts, and immunoprecipitates were probed with anti-Raf1 (Fig. 1H) or anti-Cdc25 antibodies (Fig. 1I). Under these conditions Raf1 kinase was almost fully depleted from extracts with the anti-Raf1 antibody. This also resulted in a clear codepletion (~75%) of Cdc25. Thus, the majority of the Cdc25 molecules in *Xenopus* oocytes is complexed with Raf1. By comparison, an 80-kD protein, which represented a nonspecific polypeptide that cross-reacts with the Cdc25 antisera on Western blots did not change in abundance following depletion with Raf1 antibody (Fig. 1H). The specificity of the observed interaction was also confirmed by the absence of any significant binding between Raf1 and cyclin B/Cdc2 (Fig. 1J).

Raf1/Cdc25 interaction in mammalian cells

We also investigated whether there might be a physical association between Cdc25 and c-Raf1 in mammalian tissue culture cells. Immunoprecipitates were prepared from human HeLa cell lysates using an antibody against the carboxy-terminal peptide of human Cdc25A protein and were blotted with antibodies against Cdc25A or Raf1

Figure 1. Raf1 and Cdc25 are associated in *Xenopus* oocytes. (*A*) Histone H1 kinase activity was assayed in anti-cyclin B2 immunoprecipitates from prophase oocytes (lane *1*), progesterone-matured oocytes (lane *2*), activated eggs (lane *3*), cAMP-blocked oocytes (lane *4*), and IGF-1-matured oocytes (lane *5*). Three oocyte equivalents were used per lane. (*B*) Western blot analysis using an anti-*Xenopus* Cdc25 antibody. (Lanes *1–5*) Same treatments as in *A*, using six oocyte equivalents per lane. (*C*) Western blot analysis using an anti-Raf1 antibody. (Lanes *1–5*) Same treatments as in *A*, using six oocyte equivalents per lane. (*D*) Anti-*Xenopus* Cdc25 antibody immunoprecipitates were analyzed by Western blot with the same anti-*Xenopus* Cdc25 antibody. (Lanes *1–5*): Same oocyte treatment as in *A*, using 25 oocyte equivalents per lane. (*E*) Anti-*Xenopus* Cdc25 antibody immunoprecipitates analyzed by Western blot using the anti-Raf1 antibody. Eighty oocyte equivalents were used per lane. (*F*) Anti-Raf1 antibody immunoprecipitates were analyzed by Western blot (with the same anti-Raf antibody). (Lanes *1–5*) Same oocyte treatment as in *A*, using 25 oocyte equivalents per lane. (*G*) Anti-Raf1 antibody immunoprecipitates analyzed by Western blot with the

anti-*Xenopus* Cdc25 antibody (80 oocyte equivalents per lane). (*H*) Western blot analysis using the anti-Raf antibody. (Lanes *1,3,5*) Prophase stage; (lanes *2,4,6*) metaphase stage. (Lanes *1,2*) Initial extracts, (lanes *3,4*) extracts after immunoprecipitation with the anti-Raf antibody; (lanes *5,6*) immunoprecipitates with anti-Raf1 antibody. (Lanes *1–4*) Six oocyte equivalents per lane; (lanes *5,6*) 35 oocyte equivalents per lane. (*I*) Western blot analysis with anti-*Xenopus* Cdc25 antibody of the same samples as in *H*. (Lanes *1–4*) Six oocyte equivalents per lane; (lanes *5–6*) 160 oocyte equivalents per lane. (*J*) (Lanes *1–4*) Extracts from prophase (lanes *1,2*) and metaphase (lanes *3,4*) oocytes (80 oocytes per lane) immunoprecipitated with anti-cyclin B2 (lanes *1,3*) or anti-Cdc25 (lanes *2,4*) antibodies and blotted with anti-Raf1 antibody.
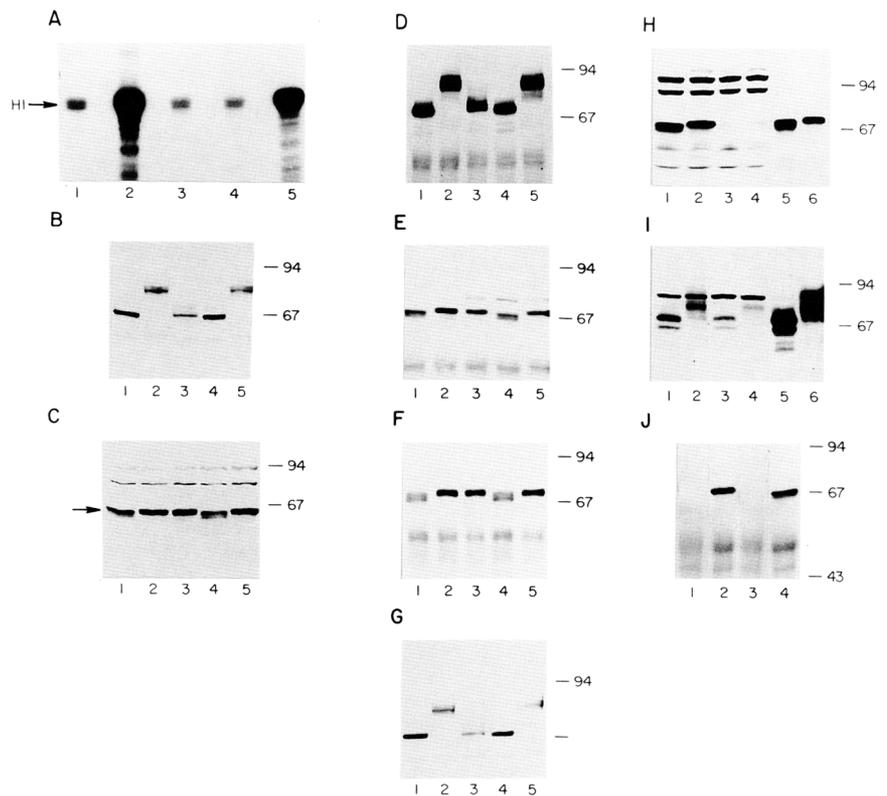
(Fig. 2A). Reciprocal coimmunoprecipitation of Raf1 and Cdc25A was detected using both high and low stringency buffers (Fig. 2A,C,D). In each experiment, both Cdc25A and Raf1 were detected in the Raf1 immune complexes. The specificity of all observed interactions was confirmed by competition with antigenic peptides that eliminated any specific signal from blots performed with anti-Cdc25A or Raf1 antibodies (Fig. 2A,C). Immunoprecipitation with antibodies specific to Cdc25 A, B, or C proteins indicated that the strongest interaction was between Cdc25A and Raf1 (Fig. 2D), but upon longer exposure we could detect some interaction of Raf1 and Cdc25B (not shown). We could not find any interaction between Raf1 and Cdc25C. Similar experiments were performed in mouse 3T3 cells under conditions optimized for detection of Raf1 protein complexes (Freed et al. 1994; Wartmann and Davis 1994). To ascertain what portion of the Cdc25A protein was present in a complex with Raf1, we depleted 3T3 cell extracts with anti-Raf1 antibodies in the absence or presence of the Raf1 antigenic peptide (Fig. 2B). We found that anti-Raf1 antibodies effectively cleared the extracts of Raf1 protein (at least 90%) and, at the same time, removed a majority

(60%–75%) of the 67- to 72-kD Cdc25A bands with slight preference toward slower migrating forms, indicating that in the extracts of the 3T3 cells a majority of the Cdc25A molecules are complexed with Raf1 kinase. Reprobing of the same blots with anti-Cdc25C antibodies showed no depletion of the Cdc25C protein (Fig. 2B). Similar results were obtained using the same procedure with extracts prepared from HeLa cells (not shown). A more substantial Raf1/Cdc25A interaction was observed when the extracts were prepared using hypotonic buffer (Freed et al. 1994; Fig. 2, cf. A and B). However, the association of Raf1 and Cdc25A was not sensitive to subsequent increase in ionic strengh of the extract (to 300 mM NaCl) or addition of 0.5% NP-40 (not shown).

Interaction of recombinant Cdc25 and Raf1

Because Raf1 and Cdc25A proteins appeared to interact in cell extracts, we investigated further whether we could reconstruct this association using recombinant proteins expressed in insect cells. We coexpressed human Raf1 kinase (either wild type, a kinase inactive mutant (K375M) or an "activated" allele (Y340D) (Fabian et

tant and the activated allele of Raf1 was found to be similar. Reciprocal experiments using immmunoprecipitations with anti-Raf1 and blotting with Cdc25A antibodies confirmed the association between Cdc25A and Raf1 (not shown).
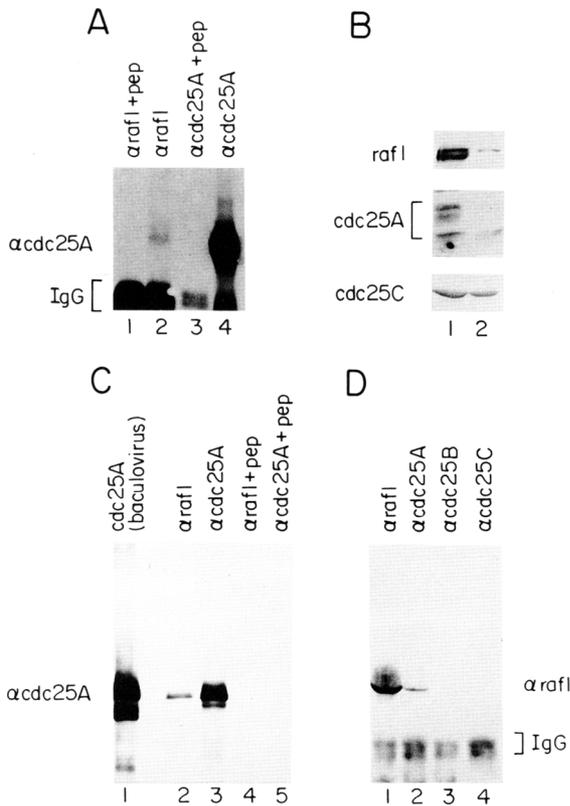

Figure 2. cdc25A associates with Raf1 in mammalian cells. (A) Immunoprecipitates of HeLa cell extracts using anti-Raf1 (lanes 1,2) or anti-Cdc25A (lanes 3,4) in the presence (lanes 1,3) or absence (lanes 2,4) of the relevant antigenic peptide were analyzed by SDS-PAGE, followed by immunoblotting with anti-Cdc25A antibody. (B) Extracts from the 3T3 cells, prepared as described (Freed et al. 1994), with some modifications were depleted with Raf1 antibodies in the presence (1) or absence (2) of competening antigenic peptide, analyzed by SDS-PAGE, followed by immunoblotting with anti-Raf1 antibody (top), anti-Cdc25A antibody (middle), or anti-Cdc25C antibody (bottom). (C) Extracts from insect Sf9 cells expressing Cdc25A protein were directly blotted with anti-Cdc25A antibodies (lane 1). HeLa cell extracts (lanes 2–5) were immunopecipitated with anti-Raf1 (lanes 2,4) or anti-Cdc25A (lanes 3,5) antibodies in the absence (lanes 2,3) or presence (lanes 4,5) of the relevant antigenic peptide, followed by SDS-PAGE and blotting with anti-Cdc25A antibody. (D) Immunoprecipitates from HeLa cell extracts using anti-Raf1 (lane 1), anti-Cdc25A (lane 2), anti-Cdc25B (lane 3) or anti-Cdc25C (lane 4) antibodies were separated by SDS-PAGE, followed by immunoblotting with anti-Raf1 antibody.

al. 1993b) along with human Cdc25A phosphatase (see legend to Fig. 3). Extracts made from these cells were either directly probed with antibodies against Raf1 or Cdc25A (Fig. 3A, top and bottom left) or immunopecipitated with antibodies against Cdc25A and then probed with anti-Raf1 antibodies (Fig. 3A, top right). We detected a significant interaction between Cdc25A and Raf1 (Fig. 3A). The extent of binding between Cdc25A and wild-type Raf1 kinase, the kinase inactive Raf1 mu-
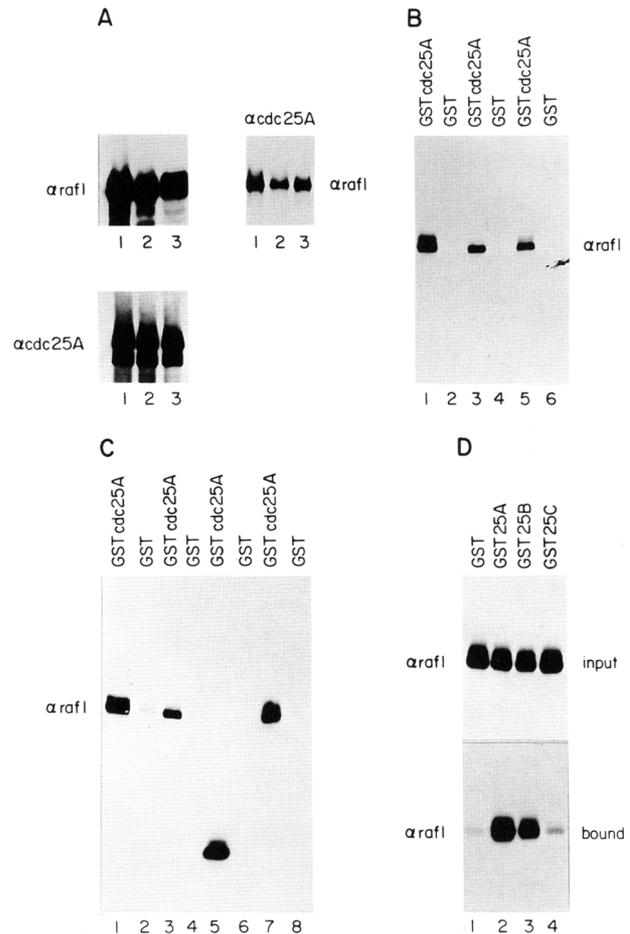
Figure 3. (A) Direct immunoblotting of the extracts from insect Sf9 cells cotransfected with wild-type raf1 (lane 1), kinase inactive mutant (K375M) (lane 2), or "activated" raf1 (Y340D) (lane 3) and cdc25A baculovirus (lanes 1–3) with anti-Raf1 (top left) or anti-Cdc25A antibodies (bottom left). (Right) Immunoprecipitates from the same extracts were made with anti-Cdc25A antibodies and blotted with anti-Raf1. (B) Extracts from insect cells, expressing wild-type Raf1 (lanes 1,2), kinase-inactive mutant (K375M; lanes 3,4), activated Raf1 (Y340D, lanes 5,6) were incubated with GST or GST–Cdc25A fusion protein and immunoblotted with anti-Raf1 antibodies. (C) Experiment similar to B. (Lanes 1,2) binding to wild-type Raf1 as a control; (lanes 3,4) kinase-inactive interfering mutant (K375W); (lanes 5,6) truncated Raf1 (22W); (lanes 7,8) Raf1 from cells cotransfected with ras and src viruses (hyperactivated Raf1). Binding of Raf1 variants was detected by immunoblotting with anti-Raf1 antibodies. (D) Association of various human Cdc25 fusion proteins with wild-type Raf1. (Lane 1) Binding to GST; (lane 2) binding to GST–Cdc25A; (lane 3) binding to GST–Cdc25B; (lane 4) binding to GST–Cdc25C. (Top) Input control. Binding of Raf1 is assayed by blotting with anti-Raf1 antibody.

We also assayed whether bacterially produced Cdc25–glutathione S-transferase (GST) fusion protein could form complexes with the Raf1 kinase. Extracts prepared from insect cells expressing various forms of Raf1 were incubated with GST–Cdc25A or GST alone and then incubated with glutathione–agarose beads. The recovered material was probed with antibodies against Raf1. We observed binding of Cdc25A to the wild-type Raf1, the kinase inactive mutant of Raf1 (both K375M and interfering K375W), and Raf1 kinase activated by coinfection with *ras* and *src* baculoviruses (Fig. 3B,C). Interestingly, an amino-terminal deletion of 303 amino acids that produces a hyperactivated Raf1 kinase (22W) did not abolish the interaction with Cdc25A (Fig. 3C, lane 5), indicating that Cdc25A can associate with the carboxy-terminal kinase domain of the Raf1 protein [(CR3) Rapp et al. 1988].

We also tested the ability of each human Cdc25 protein to interact with Raf1 in vitro (Fig. 3D). Equivalent amounts of extracts from cells infected with Raf1 baculovirus were incubated with equal amounts of GST, GST–Cdc25A, GST–Cdc25B or GST–Cdc25C proteins (Fig. 3,D). Material recovered on glutathione beads was probed with anti-Raf1 antibody. The Raf1 kinase preferentially interacted with Cdc25A, to a lesser extent with Cdc25B, and barely with Cdc25C (Fig. 3D), essentially mirroring the results of in vivo analysis.

To test whether the Cdc25/Raf1 interaction is direct, we expressed the full-length Raf1 and amino-terminally truncated Raf1 (amino acids 304–648) as GST fusion proteins in bacterial cells and assessed binding of these proteins to bacterially expressed, purified Cdc25A (Fig. 4A,B; see Materials and methods). Strong binding of Cdc25A to full-length or truncated Raf1–GST fusions, but not to GST, was detected (Fig. 4A, bottom). Binding was not sensitive to the presence of 0.5% NP-40 or to addition of a carrier protein (BSA), suggesting a strong and specific direct interaction between Raf1 and Cdc25A in vitro.

Raf1-dependent activation of cdc25

Since we observed in vivo and in vitro interaction between Raf1 and Cdc25, we evaluated the potential significance of these phenomena with respect to the phosphatase activity of human Cdc25A. In initial experi-
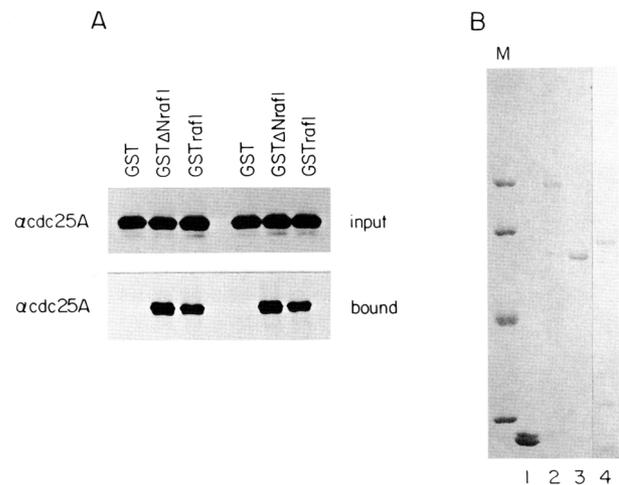

Figure 4. (*A*) Binding of bacterial Cdc25A to bacterially produced GST–Raf1 variants in the absence (*left*) or presence (*right*) of the carrier protein (BSA). (*B*) Coomassie-stained gel showing purified GST (lane *1*), GST–Raf1 (lane *2*), GSTΔNRaf1 (304–648) (lane *3*) and thrombin-cleaved, FPLC-purified Cdc25A (lane *4*). Note that thrombin-cleaved, purified Cdc25A runs at 65–66 kD in comparison with 90 kD for GST–Cdc25A fusion protein (Galaktionov and Beach 1991). (Lane M) Low molecular weight markers (Pharmacia), as indicated from the *top*: phosphorylase (94 kD), BSA (69 kD), ovalbumin (45 kD), and carbonic anhydrase (30 kD).

ments, we prepared Raf1 immunoprecipitates from HeLa cells or from insect Sf9 cells coinfected with *raf1*, *ras*, and *src* baculoviruses to yield maximally active Raf1 kinase (Williams et al. 1992) and assayed the ability of this kinase to use GST–Cdc25A as a substrate. We observed incorporation of radioactive phosphate into Cdc25A, and this reaction was negated by competition with antigenic Raf1 peptide in the initial immunoprecipitation (Fig. 5A). To test the specificity of phosphorylation with respect to different members of the Cdc25 family, we assayed the ability of Raf1 immunoprecipitates from HeLa cells to phosphorylate equal amounts of GST, GST–Cdc25A, GST–Cdc25B, and GST–Cdc25C. Phosphorylation of the Cdc25A fusion protein was strongest (Fig. 5B),

Figure 5. Phosphorylation and activation of Cdc25 by Raf1 immuno- and protein complexes. (*A*) Immunoprecipitates from HeLa (lanes *1,2*) or Sf9 cells triply transfected with *raf1*, *ras*, and *src* (lanes *3,4*) performed in the absence (lanes *1,4*) or presence of Raf1 antigenic peptide were incubated with GST–Cdc25A in a kinase buffer with [γ-³²P]ATP, followed by SDS-PAGE. (*B*) Immunoprecipitates from HeLa cells were incubated with GST (lane *1*), GST–Cdc25A (lane *2*), GST–Cdc25B (lane *3*), GST–Cdc25C (lane *4*) (using the same conditions as in *A*), followed by SDS-PAGE. (*C*) Time course of the phosphatase reaction using paranitrophenylphosphate (PNPP) as a substrate was followed with Cdc25A preincubated with mock control (extract prepared from Sf9 cells infected with a wild-type baculovirus) and hyperactive Raf1 (22W). (*D*) GST–Cdc25A phosphatase activity against PNPP was measured after incubation with mock control (*1*), Raf1 in the absence (*2*) or presence of ATP (*3*), or kinase inactive mutant of Raf1 (*4*). Experiments were done in triplicate. Error bars show two standard deviations from the mean. (*E*) Phosphatase activity of Cdc25A against tyrosine-phosphorylated inactive cyclin A/cdk2 kinase was measured using histone H1 as a substrate. Each point (from *1* to *4*) correspond to threefold increase in amount of added Cdc25 with the same amount of substrate (largely inactive cyclin A/cdk2). Hatched bars correspond to control Cdc25; solid bars correspond to Cdc25 activated by Raf1 complexes.

followed by phosphorylation of Cdc25B. The Cdc25C protein was phosphorylated at least 100 times less effi-

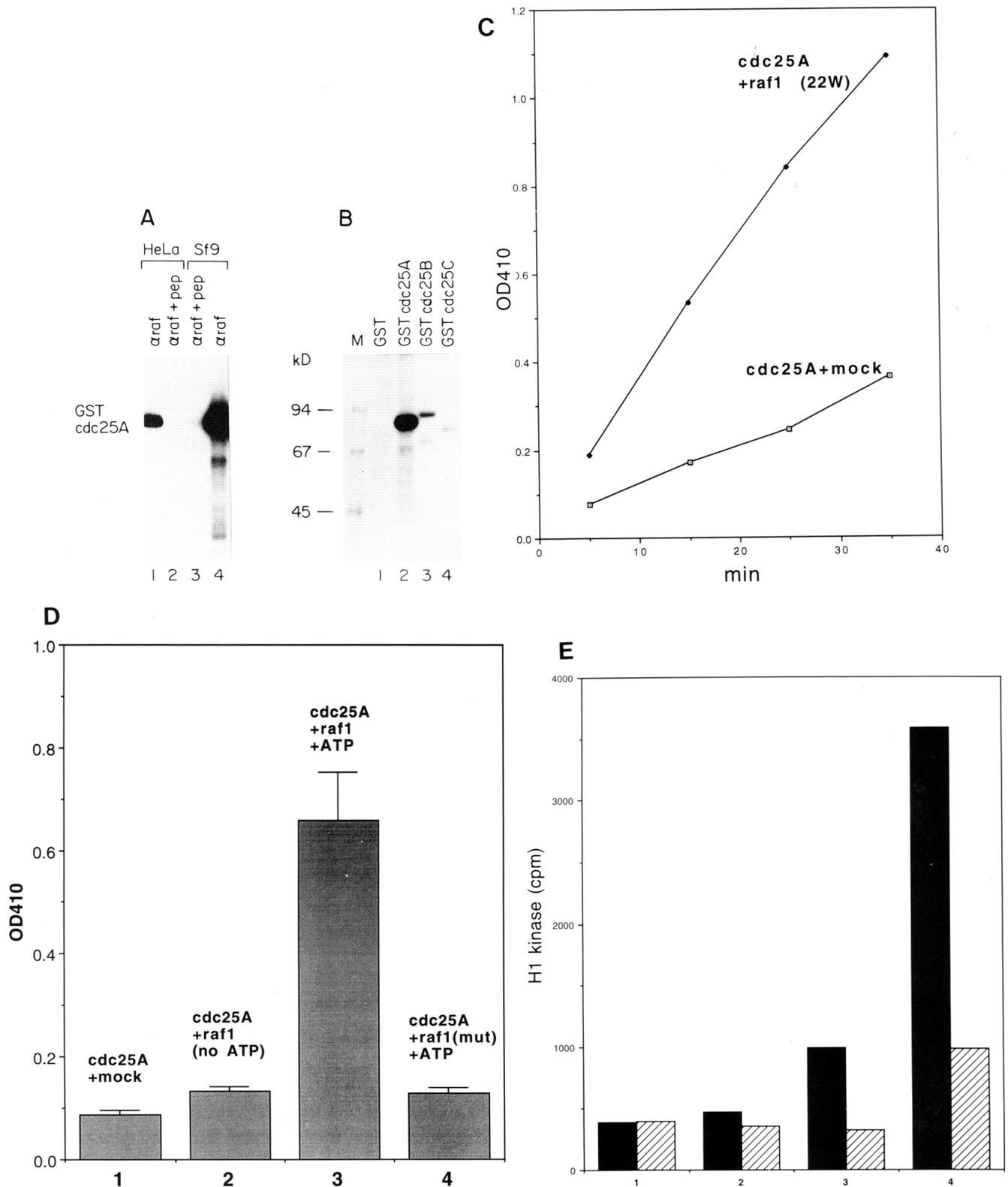ciently than Cdc25A, and no phosphorylation of GST was detected.

Figure 5. (*See facing page for legend.*)

To assess the effect of phosphorylation of Cdc25A on its intrinsic phosphatase activity we incubated GST–Cdc25A or GST with cell extracts from Sf9 cells infected with various baculoviruses, including wild-type and mutant Raf1 (see legend to Fig. 5; Materials and methods). After incubation with glutathione–agarose beads the resulting complexes were washed, retaining Raf1 kinase associated with Cdc25A. Complexes were then incubated in kinase buffer (in the presence or absence of ATP). Thereafter, ATP was washed away and Cdc25 phosphatase activity was measured using paranitrophenylphosphate (PNPP), a synthetic substrate that is structurally similar to phosphotyrosine (Dunphy and Kumagai 1991; Galaktionov and Beach 1991). Experiments described above were done using either hyperactive *raf1* (22W; Fig. 5C), or *raf1* kinase activated by cotransfection with *ras* and *src* (Fig. 5D,E). A three- to fourfold increase in Cdc25A phosphatase activity was detected (Fig. 5D,E). Activation was dependent on the addition of ATP and was negligible with a kinase-inactive (K375M) *raf1* mutant (Fig. 5D, lane 4). Similar activation of Cdc25A phosphatase activity was seen using a Cdc25 assay that measured dephosphorylation and activation of GST–cyclin A/cdk2 (inhibited in vitro by Wee1 kinase; see legend to Fig. 5; Fig. 5E). In both experiments a three- to fourfold activation of Cdc25 phosphatase activity was observed (Fig. 5C–E). We have been unable to determine the sites of Raf1-dependent phosphorylation on Cdc25A, as the amount of ^{32}P-labeled Cdc25A produced in vivo in unsynchronized 3T3 or HeLa cells was insufficient for mapping purposes.

Membrane colocalization of Cdc25 with Ras and Raf1

It has been shown previously that some fraction of Raf1 and Ras colocalize in the cell membrane (Traverse et al. 1993). Colocalization was enhanced in cells transformed with oncogenic Ras and was abolished in cells transformed with interfering Ras mutants (Leevers et al. 1994; Stokoe et al. 1994). To test whether Cdc25 might also colocalize with Ras and Raf1, we took advantage of mouse fibroblasts stably transfected with oncogenic Ha-*ras* (V12) and *cdc25A* (see Materials and methods). Cells were stained with monoclonal antibodies against Ras or Raf1 and with affinity-purified polyclonal antibodies against Cdc25A or Cdc25B. As expected, we could detect cytoplasmic and membrane staining with both anti-Raf1 and anti-Ras antibodies (Fig. 6B,D,F). We could also clearly observe membrane staining with both anti-Cdc25A and -Cdc25B antibodies (Fig. 6A,C,E). Using double immunofluorescence, we observed significant colocalization of Ras or Raf1 and Cdc25 at the cell membrane (Fig. 6, arrows). Areas of cellular membrane "ruffles," representing the moving edge of the cell, were stained most prominently with anti-Cdc25A and -Cdc25B antibodies, as well as anti-Ras and anti-Raf1 antibodies. Both anti-Cdc25 antibodies also stain the nucleus; however, nuclear staining was not observed with anti-Raf1 and anti-Ras antibodies. Some immunofluorescence with anti-Cdc25A antibodies was found in cyto-

plasmic "dots" and anti-Cdc25B-stained cytoskeletal structures (Fig. 6A,C,E). Preincubation of antibodies with antigenic peptides abolished all staining observed with anti-Cdc25A and anti-Cdc25B antibodies (Fig. 6G,H).

To extend our observations on Cdc25/Raf1 colocalization, we fractionated 3T3 and mouse fibroblast cells into nuclear, S100, and P100 fractions (Freed et al. 1994; Leevers et al. 1994; Wartmann and Davis 1994). These were directly blotted with anti-Raf1 or anti-Cdc25A antibodies. As expected, Raf1 localized to S100 and P100 fractions with very little in the nuclear fraction, whereas Cdc25A was found in all three fractions (Fig. 6I). The Cdc25A present in S100 fraction was readily immunoprecipitated by anti-Cdc25A antibodies (Fig. 6I, middle) which also brought down Raf1 in a complex (Fig. 6I, bottom). Following serum stimulation Cdc25A associates exclusively with the phosphorylated (upshifted) species of Raf1 (Fig. 6I, bottom). The nuclear and membrane-bound Cdc25A (fractions N and P100) was not extracted by 1% NP-40 (Fig. 6I), suggesting a tight association with cytoskeleton and/or nuclear matrix structures. We estimate, after correcting for the protein content of the S100, P100, and nuclear fractions, that 5%–10% of Cdc25A is associated with the membrane/cytoskeletal fraction (P100), 40%–50% is cytoplasmic, and ~40%–50% is nuclear.

Discussion

The existence of a link between mitogenic signal transduction and the cell cycle machinery has long been anticipated. Previous work has shown that D-type cyclins are induced in response to growth factor stimulation (Matsushime et al. 1991; Won et al. 1992). Induction of the D-type cyclins, however, corresponds in time to passage through restriction point (Rossow et al. 1979; for review, see Pines and Hunter 1994; Sherr 1994), representing a downstream effect of the signal transduction cascade. The results presented here suggest that one aspect of a link between signal tranduction and cell cycle is surprisingly direct. We demonstrate that Raf1 kinase forms complexes with a cell cycle activator, the Cdc25 phosphatase. This interaction was observed in human and mouse somatic cells and in frog oocytes. Furthermore, bacterially produced human Cdc25A could associate with recombinant Raf1 produced in insect cells. The association of Raf1 and Cdc25A involves the carboxy-terminal CR3 (kinase) domain of the Raf1 protein, a region that is indispensable for Raf1-transforming activity (Stanton et al. 1989; Heidecker et al. 1990). Raf1 and Cdc25A expressed in bacteria also bind in vitro, suggesting a direct protein–protein interaction.

We observed interaction of Raf1 with human Cdc25A and, to some extent, with Cdc25B but not with Cdc25C. Raf1 also associates with *Xenopus* Cdc25, which has been classified as a C-type Cdc25 phosphatase (Kumagai and Dunphy 1992). However, human Cdc25C is 80 amino acids shorter than *Xenopus* Cdc25 and shares
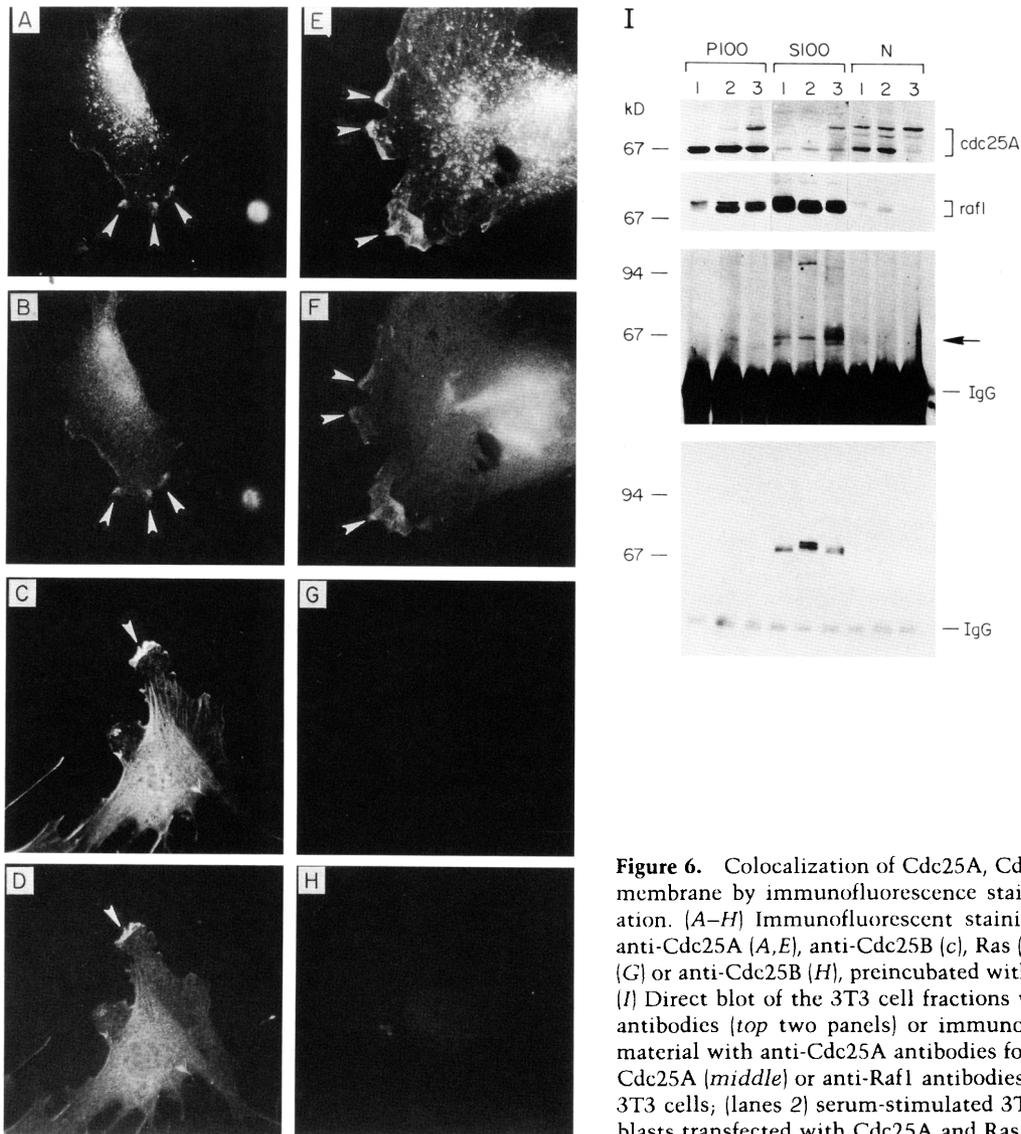
Figure 6. Colocalization of Cdc25A, Cdc25B, Ras, and Raf1 at the cell membrane by immunofluorescence staining and subcellular fractionation. (A–H) Immunofluorescent staining of mouse fibroblasts with anti-Cdc25A (A,E), anti-Cdc25B (c), Ras (B,D), Raf1 (f) and anti-Cdc25A (G) or anti-Cdc25B (H), preincubated with competing antigenic peptide. (I) Direct blot of the 3T3 cell fractions with anti-Cdc25A or anti-Raf1 antibodies (top two panels) or immunoprecipitation of the extracted material with anti-Cdc25A antibodies followed by detection with anti-Cdc25A (middle) or anti-Raf1 antibodies (bottom). (Lanes 1) Quiescent 3T3 cells; (lanes 2) serum-stimulated 3T3 cells; (lanes 3) mouse fibroblasts transfected with Cdc25A and Ras.

rather limited homology to it (Galaktionov and Beach 1991; Kumagai and Dunphy 1992), complicating classification of the known *Xenopus* enzyme with respect to the human proteins.

The significance of the Raf1/Cdc25 interaction is suggested by the fact that Cdc25 can serve as a substrate of the Raf1 kinase in vitro. The ability of Raf1 immunocomplexes to phosphorylate different human Cdc25 proteins correlates with their Raf1-binding properties. This has also been shown for MEK (van Aelst et al. 1993), a previously described physiological substrate of Raf1 (Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992). Raf1-dependent phosphorylation of Cdc25A is associated with activation of Cdc25A phosphatase activity. One explanation for our results is that Cdc25A is phosphorylated directly by Raf1. However, our findings are also consistent with the possibility that a Raf1-dependent kinase, present in the same protein complex, phosphory-

lates Cdc25. One candidate is MEK, but we cannot exclude that another uncharacterized kinase that is present both in HeLa and Sf9 cells binds to Raf1 and phosphorylates Cdc25 proteins in a Raf1-dependent manner.

Physical and functional interaction of Cdc25 with Raf1 was initially surprising, as membrane translocation of Raf1 has been recently shown to be important for its activation (Leevers et al. 1994; Stokoe et al. 1994). However, using double immunofluorescence microscopy, we have shown that Ras and Raf1 colocalize in part with Cdc25A and Cdc25B at the cell membrane. Biochemical fractionation further supported immunofluorescence data. It has been shown that in HeLa cells Cdc25A is largely nuclear and is phosphorylated at the G_1/S border presumably by the cyclin E/Cdk2 kinase (Hoffman et al. 1994). Our data on the localization of the Cdc25A in mammalian cells show that although Cdc25A is partially localized to the nucleus in 3T3 cells, we can also

detect cytoplasmic and presumably membrane- or cyto-skeleton-bound Cdc25A. Because Cdc25A is phosphory-lated by Raf1 complexes in vitro and is associated with upshifted (activated) Raf1 shortly after serum stimula-tion (Fig. 6I), we believe that it may be phosphorylated and activated earlier in the cell cycle than G_1/S border. It is possible, however, that both suggestions are correct and that Cdc25A has functions in several points in the cell cycle. Both Cdc25A and Cdc25B have been shown to dephosphorylate and activate cdc2 and cdk2; however, it is possible that cdks are not the only substrates of Cdc25 phosphatases. It is unlikely that Raf1 phosphoprotein it-self is such a substrate, since dephosphorylation of ac-tive, phosphorylated Raf1 was not observed during incu-bation with Cdc25A phosphatase in vitro.

Our results suggest that direct physical interaction of Cdc25 and Raf1 may play a key role in activation of the cell cycle in response to mitogens. However, ras and Raf1 almost certainly control mitogenic response to ex-tracellular stimuli via multiple pathways (Herskowitz 1995; Marshall 1995). For example, Ras is required for the Raf/MEK/ERK pathway, for PI3–kinase activation (Rodriguez-Viciana et al. 1994) and for a MEKK-1/SEK-1/JNK(SAPK) kinase pathway (Hibi et al. 1993; Lange-Carter and Johnson 1994; Minden et al. 1994; Sanchez et al. 1994; Yan et al. 1994) induced in response to osmotic shock, UV irradiation, and cytokines (for review, see Marshall 1995). In 3T3 cells, activated MEK, which does not require Raf1, causes induction of DNA replication and oncogenic transformation in parallel with MAP ki-nase activation (Cowley et al. 1994). Alternatively, in Rat1 cells, Ras and activated Raf1 cause oncogenic trans-formation without constitutively activating MEK and MAP kinases (ERKs), further underlying emerging com-plexity of the signal transduction pathways.

The Raf1 kinase is also an essential element of meiotic maturation, where it has been shown to function down-stream of Mos, possibly activating MEK (Muslin et al. 1993). The downstream target of MEK, MAP kinase, is not, however, involved in the maturation of *Xenopus* oocytes but, instead, displays cytostatic factor (CSF) ac-tivity (Haccard et al. 1993), posing an apparent paradox for interpretation of the role of Raf1 in meiosis. As a resolution to this paradox we propose that at the level of Raf1 or MEK Ras/Raf1 signal transduction pathways bi-furcate with at least two major downstream targets: MAP kinases and Cdc25 cell cycle phosphatases. Acti-vation of Cdc25 by the Ras/Raf1 pathway is proposed to lead to cell cycle stimulation.

Stimulation of Raf1 by Ras is certainly a major event in Ras-mediated signal transduction, but because Ras has been shown to directly activate phosphatidylinosi-tol-3 kinase activity (Rodriguez-Viciana et al. 1994), it is possible that inositol metabolites and, perhaps, protein kinase C are involved in an additional pathway that leads to the activation of Cdc25 or other cell cycle com-ponents after mitogenic stimuli. The possible involve-ment of the newly discovered Ras/MEKK-1/SEK1/JNK-(SAPK) pathway in the activation of Cdc25 awaits fur-ther investigation.

It may seem paradoxical that Cdc25 is apparently in-volved in the signal transduction pathways. To date, Cdc25 proteins have been shown to activate Cdc2 and Cdk2; however, the possibility remains that other cdks, namely cdk4 and cdk6, that function in G_1 could be un-der some Wee1/Cdc25 control because they both con-tain the evolutionarily conserved Tyr residue in the nu-cleotide-binding domain. Activation of the Cdc25A by Ras/Raf1 signal transduction pathway could ensure that all cdk/cyclin complexes in G_1 are active in the presence of growth factors and that their activity depends only on the balance of relevant cyclins/cdks and cdk inhibitors (pending continuous presence of the active cdk-activat-ing kinase). Withdrawal of the growth factors in G_1 pre-ceding the restriction point (Rossow et al. 1979) could abort the cell cycle via rapid inactivation of Cdc25. In possible agreement with this hypothesis it has been shown that Cdc25A is essential in G_1 (Jinno et al. 1994).

In summary, the results presented here constitute a direct link between cell cycle control and mitogenic sig-nal transduction pathways in higher eukaryotes and es-tablish Cdc25 phosphatases as key molecules in this pro-cess.

Materials and methods

Oocytes

Xenopus leavis prophase oocytes were prepared as described previously (Jessus et al. 1987; Jessus and Beach 1992). Proges-terone-induced meiotic maturation and egg activation were per-formed as described in Jessus and Beach (1992), and IGF-1-in-duced meiotic maturation as described in Sadler and Maller (1989). cAMP-blocked oocytes were pretreated for 1 hr with 1 mM IBMX and 0.1 µg/ml of cholera toxin before adding proges-terone. Oocytes were rinsed extensively in extraction buffer [(EB) 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl$_2$, 1 mM DTT, 25 mM NaF, and 1 mM orthovanadate] and lysed in 5 volumes of EB with protease inhibitors (1 mM PMSF, 25 µg/ml of leupeptin, 25 µg/ml of aprotinin, 1 mM benzamidine, 10 µg/ml of N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and 70 µg/ml of tosyl-L-phenylalanine chloromethyl ketone (TPCK). Insoluble material and lipids were separated by centrifugation at 13,000g for 15 min at 4°C, and the supernatant was used for immunoprecipitations and Western blot analysis. Nitrocellu-lose filters were incubated for 4 hr with primary antibody di-luted in 1% milk–TBS with 0.1% Tween 20 (TBST). The anti-Raf1 carboxy-terminal antipeptide antibody (C-20, Santa Cruz Biotechnology) and the anti-*Xenopus* Cdc25 antibody (described in Kumagai and Dunphy 1992; gift of Dr. W. Dunphy) were used at concentrations of 10 µg/10 ml, and 2.5 µg/10 ml, respec-tively. After washing, filters were incubated for 1 hr with HRP-coupled protein A (GIBCO) at a 1:5000 dilution in 1% milk–TBST and developed using enhanced chemiluminescence (ECL) (Amersham). Immunoprecipitations were performed by incu-bating oocyte lysates with the anti-Raf1 antibody (1 µg of C-20, Santa Cruz Biotechnology), with an anti-*Xenopus* Cdc25 anti-body [1:100 dilution (described in Izumi et al. 1992; gift of Dr. J. Maller)] or with anti-cyclin B2 antibody (described in Izumi and Maller 1991; gift of Dr. J. Maller) for 4 hr at 4°C. Protein A–Sepharose beads were used to collect the immune com-plexes. To estimate Cdc2 histone H1 kinase activity, immuno-precipitations were performed with an anti-*Xenopus* cyclin B2 antibody [dilution 1:100 (described in Izumi and Maller 1991;

gift of Dr. J. Maller]] for 1 hr at 4°C. Protein G–Sepharose beads were used to collect the immune complexes; these were washed with kinase buffer (Jessus and Beach 1992) and assayed for histone H1 kinase activity (Jessus and Beach 1992).

Mammalian cells

HeLa cells grown in suspension were obtained from Cold Spring Harbor Laboratory Tissue Culture Facility and used at the cell density of 0.5×10^6 to 0.8×10^6 cells/ml. Cells were centrifuged for 10 min at 1000g, washed with PBS three times, and lysed in 5 volumes of the lysis buffer. In some experiments (Fig. 2A,C) a standard buffer was used (Xiong et al. 1993). In other experiments (B,D) we used low ionic strength buffer with 1% NP-40 (D) or without it (B) (Freed et al. 1994; Wartmann and Davis 1994). 3T3 cell fractionation was performed essentially as described (Freed et al. 1994; Stokoe et al. 1994; Wartmann and Davis 1994), using hypotonic buffer [10 mM Tris-HCl at pH 7.5, 5 mM MgCl$_2$; 25 mM NaF, 1 mM EGTA, 1 mM orthovanadate, supplemented with aprotinin (10 µg/ml), leupeptin (10 µg/ml), soybean trypsin inhibitor (20 µg/ml), pepstatin (1 µg/ml), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] for cell lysis as described in Freed et al. (1994) and buffer A [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, and 10% glycerol, supplemented with PMSF (0.5 mM), leupeptin (10 µg/ml) and aprotinin (10 µg/ml), Wartmann and Davis 1994)] with 1% NP-40 for extraction of the membrane and nuclear fraction from confluent quiescent 3T3 cells before or after 15 min of stimulation with 10% calf serum. The same procedure was applied to unsynchronized mouse fibroblasts, cotransfected with RcCMV–cdc25A and Ha-ras (Val12) in pNV, obtained from Dr. D. Bar-Sagi (Cold Spring Harbor Laboratory). Immunoprecipitation from S100 fraction was optimal for detecting Cdc25A/Raf1 interaction in all mammalian cell lines tested. Equal amounts (50 µg) of proteins were loaded in each lane. Antibodies were raised against a peptide representing the eight carboxy-terminal residues of Cdc25A (CMYSRLKKL), and another, representing seven carboxy-terminal amino acids of Cdc25B (CSRLQDQ). Both peptides were cross-linked with keyhole limpet hemocyanin (KLH) and used to immunize rabbits. Antiserum was collected and affinity purified using the peptides cross-linked with SulphoLink beads (Pierce). GST–fusions with Cdc25A, Cdc25B, and Cdc25C were described previously (Galaktionov and Beach 1991). Proteins were purified as described (Galaktionov and Beach 1991) with some modifications and used to immunize rabbits. Antisera against GST fusions were depleted extensively on GST–Sepharose beads followed by Sepharose beads with nonspecific Cdc25 fusion (we used GST Cdc25B to remove cross-reacting species from anti-Cdc25A antibodies and vice versa). Finally, specific antibodies were affinity purified using the relevant fusion protein attached to the Sepharose beads. We routinely used carboxy-terminal peptide antibodies for immunoprecipitation, followed by Western blotting with affinity-purified antibodies against the full-length protein. No cross-reactivities were detected between any of the affinity-purified antibodies against Cdc25A, Cdc25B, and Cdc25C (both carboxy-terminal and full length). Antibodies against the carboxyl terminus of Cdc25C were described previously (Hoffmann et al. 1993) and kindly provided to us by Dr. G. Draetta (Mitotix) or purchased from Santa Cruz Biotechnology. Antibodies against Raf1 (C20) were purchased from Santa Cruz Biotechnology. All antibodies were used at 0.5–1 µg per immunoprecipitation (except for Fig. 2A, lanes 1 and 2, where 5 µg was used). Antibodies were typically incubated with cell extracts for 4–6 hr, followed by a 1 hr incubation with protein A or protein G beads (Pierce,

Pharmacia). Immunoprecipitates were recovered by low speed centrifugation and washed four to five times in the lysis buffer. Samples for 8.5% SDS-PAGE were prepared by treatment of the recovered immune complexes with sample buffer at 95°C for 5–10 min as described (Laemmli 1970). Immunoblotting analysis was performed as described. Positive signals were detected using protein A–horseradish peroxidase (HRP) (at 1:2000 dilution) and ECL (Amersham) according to instructions provided by the manufacturer.

Insect cells

Spodoptera frugiperda (Sf9) cells grown in monolayer were infected at 5× multiplicity with recombinant baculoviruses encoding Cdc25A alone or in a combination with viruses encoding wild-type Raf1, kinase inactive Raf1 (K375M or K375W), truncated Raf1 (22W) or "activated" raf1 (Y340D) (Fabian et al. 1993b; Williams et al. 1993). In some cases, triple infection with raf1, ras, and src baculoviruses was performed as described (Williams et al. 1992). At 60–72 hr postinfection, cells grown on 100- or 150-mm plates were washed with PBS, scraped from the plates, washed two more times in PBS, and lysed in kinase buffer (KB), containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl$_2$, 0.1 mM EDTA, 5 mM EGTA, supplemented with 1 mM DTT, 1 mM orthovanadate, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin, 0.5 µg/ml of pepstatin, 1 µg/ml chymostatin, 1 mM benzamidine, 0.5 mM PMSF. Cells were disrupted by passing six times through a 26½-gauge needle. Extracts were cleared by centrifugation twice at 15,000g for 15 min each. Cleared lysates were supplemented with glycerol to 20% and stored frozen at −70°C in aliquots. Immunoprecipitations were performed as described in the legend to Figure 1. GST–fusion proteins and GST were purified as described (Galaktionov and Beach 1991) on glutathione–Sepharose columns (Pharmacia) and eluted with 10 mM glutathione in GT buffer (50 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, supplemented with 0.5 mM PMSF, 1 mM benzamidine, 10 µg/ml of leupeptin, and 10 µg/ml of aprotinin. GST or GST–fusion proteins were diluted at least 10-fold in KB, mixed with insect cell extracts (typically 2 µg of the GST fusion and 20–50 µl of the extract), and incubated on ice for 2–4 hr. Glutathione–agarose beads, equilibrated in KB buffer, were added (30 µl of a 1:1 slurry), rotated at 4°C for 1 hr and washed four to five times in KB. Samples were separated by 8.5% SDS-PAGE, electrotransferred onto nitrocellulose, and probed with anti-Raf1 antibodies (0.2 µg/ml), followed by protein A–HRP (1:2000–1:5000; Amersham). Positive signals were detected using ECL (Amersham).

Bacterial proteins

To purify bacterial Cdc25A protein, GST–Cdc25A (Galaktionov and Beach 1991) was digested with thrombin. Cdc25A, GST–Raf1 and GSTΔNRaf1 (amino acids 304–648) were purified on FPLC (mono Q) and checked by 10% PAGE (Fig. 4). Proteins (1 µg each) were mixed in 50 µl of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl with 10 µl of glutathione–agarose beads (with or without 5 mg/µl of BSA), incubated for 1 hr at 4°C, and washed four times with the same buffer with 0.5% NP-40. Samples were analyzed further by immunoblotting with anti-Cdc25A as described above.

Kinase reactions

Immunoprecipitates of HeLa cell extracts with anti-Raf1 antibodies were done under standard conditions (Xiong et al. 1993). In some cases, antigenic peptide was added at 2–3 µg/µg of

antibody. Immunoprecipitates were washed twice in KB buffer, and 1–2 μg of GST or GST–fusion proteins was added. Reactions were supplemented with 50 μM ATP, 10 μCi of [γ-^{32}P]ATP, incubated at 30°C for 10 min, and terminated by the addition of equal volumes of 2× Laemmli sample buffer (Laemmli 1970). Proteins were resolved by 8.5% SDS-PAGE, and phosphorylated products were detected by autoradiography at −70°C on Kodak X-OMAT film. GST–Cdc25A protein or GST was purified as described previously (Galaktionov and Beach 1991). Typically, 5–10 μg of Cdc25A fusion or GST was incubated with 400–800 μl of the extracts from Sf9 cells coinfected with raf1 (wild type or K375M mutant), ras, and src baculoviruses for 4–8 hr at 4°C, followed by addition of 40 μl of glutathione–sepharose slurry (1:1) for an additional 1–2 hr incubation. Beads were washed four times with KB and resuspended in 50 μl of KB. ATPγS was added to 2 mM, and samples were incubated for an additional 30 min at 30°C. Beads were washed with cold phosphatase buffer (PB), containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM DTT and resuspended in PB, supplemented with 200 mM PNPP. Reaction mixtures were incubated at 30°C for 15–30 min (Fig. 5D) or 5–35 min (Fig. 5C); and phosphatase activity was assayed by measuring OD$_{410}$. For the experiment described in (Figure 5E); extracts from Sf9 cells infected with Wee1 and GST–cyclin A/cdk2 baculoviruses were mixed, ATP regeneration system was added, and incubation continued for 30 min at 30°C. Largely inactive GST–cyclin A/cdk2 complexes were recovered on glutathione–Sepharose beads and eluted with 10 mM glutathione in KB (without vanadate). This material was mixed with GST–Cdc25A, treated as described above, eluted with 10 mM glutathione [each point represents threefold subsequent dilution of the eluted GST–Cdc25A starting from 1:27 (Fig. 5E,1) to 1:1 (Fig. 5E,4)], and incubated for 10 min. Histone H1 and ATP/[γ-^{32}P]ATP was added to 1 μg and 50 μM/10 μCi, and incubation continued for another 10 min at 30°C. The supernatant was recovered, mixed with 2× Laemmli sample buffer, and run on an 8.5% SDS–polyacrylamade gel. The gel was dried and processed on Fuji Imager BAS2000 to quantitate the results of the kinase reaction.

Indirect immunofluorescence

Mouse embryo fibroblasts (MEF) stably transfected with cdc25A and Ha-ras (V12) were fixed with 3.7% formaldehyde for 10 min, permeabilized by 0.1% NP-40 for 10–20 min, treated with 3% BSA for 30–60 min, and stained with affinity-purified antibodies against the carboxy-terminal peptide of Cdc25A or Cdc25B (at 30 μg/ml) and monoclonal antibodies against Ras (Y13-238) (Oncogene Sciences) or monoclonal antibodies against Raf1 (Transduction Laboratories) at the same concentration. Indirect double immunofluorescence staining was done using FITC-conjugated goat anti-mouse or anti-rat secondary antibodies and Texas Red-conjugated goat anti-rabbit antibodies (Cappell). Coverslips were mounted in Immunomount with 1 μg/ml of paraphenylendiamine. Microphotographs were taken on Zeiss Axiophot fluorescent microscope using PlanApochromat 63× objective.

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