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The Ras-GTPase-Activating Protein SH3 Domain Is Required for Cdc2 Activation and Mos Induction by Oncogenic Ras in *Xenopus* Oocytes Independently of Mitogen-Activated Protein Kinase Activation

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The Ras-GTPase-activating protein (RasGAP) is an important modulator of $p21^{ras}$-dependent signal transduction in *Xenopus* oocytes and in mammalian cells. We investigated the role of the RasGAP SH3 domain in signal transduction with a monoclonal antibody against the SH3 domain of RasGAP. This antibody prevented the activation of the maturation-promoting factor complex (cyclin B-$p34^{cdc2}$) by oncogenic Ras. The antibody appears to be specific because as little as 5 ng injected per oocyte reduced the level of Cdc2 activation by 50%, whereas 100 ng of nonspecific immunoglobulin G did not affect Cdc2 activation. The antibody blocked the Cdc2 activation induced by oncogenic Ras but not that induced by progesterone, which acts independently of Ras. A peptide corresponding to positions 317 to 326 of a sequence in the SH3 domain of human RasGAP blocked Cdc2 activation, whereas a peptide corresponding to positions 273 to 305 of a sequence in the N-terminal moiety of the SH3 domain of RasGAP had no effect. The antibody did not block the mitogen-activated protein (MAP) kinase cascade (activation of MAPK/ERK kinase [MEK], MAP kinase, and S6 kinase $p90^{rsk}$). Surprisingly, injection of the negative MAP kinase mutant protein ERK2 K52R (containing a K-to-R mutation at position 52) blocked the Cdc2 activation induced by oncogenic Ras as well as blocking the activation of MAP kinase. Thus, MAP kinase is also implicated in the regulation of Cdc2 activity. In this study, we further investigated the regulation of the synthesis of the c-*mos* oncogene product, which is necessary for the activation of Cdc2. We report that the synthesis of Mos protein induced by oncogenic Ras is prevented by injecting the antibody to the SH3 domain of RasGAP and by injecting the negative MAP kinase mutant protein ERK2 K52R. These results suggest that oncogenic Ras activates two signaling mechanisms: the MAP kinase cascade and a signaling pathway implicating the SH3 domain of RasGAP. These mechanisms might control Mos protein expression implicated in Cdc2 activation.

Ras proteins play a key role in the control of cell proliferation and differentiation (2). Growth factors and other stimuli that induce these processes promote accumulation of Ras-GTP. The level of Ras-GTP depends upon the activities of guanine nucleotide-releasing factors, which catalyze exchange of GDP-GTP (31), and of GTPase-activating proteins (GAPs), such as RasGAP and neurofibromatosis type I (33, 35). Signal transduction downstream of Ras implicates effector proteins such as the serine/threonine kinases, Rafs, which are only now beginning to be identified. There is also growing evidence that Ras regulates several signaling pathways involving protein kinases, the best studied of which is the mitogen-activated protein (MAP) kinase cascade (14). Ras activation leads to a sequential activation of Rafs or other MAPK/ERK kinase (MEK) kinases, MEKs, MAP kinases, and S6 kinase $p90^{rsk}$.

It has been suggested that RasGAP is also a Ras effector, especially for oncogenic Ras (38), although further evidence is needed to support this hypothesis. The NH_2-terminal domain of RasGAP contains the SH2 and SH3 domains, which are also found in many proteins involved in the control of cell proliferation and differentiation (24). A number of studies indicate that proteins with SH2 domains play an important role in the transduction of growth factor receptor signals (24). These proteins bind to the phosphorylated tyrosine growth factor receptors via a direct interaction between the SH2 domain and the phosphotyrosine-containing sequence. The presence of SH3 domains in proteins involved in signal transduction suggests that they act as regulators by interacting with a specific target(s). However, the binding interactions involving SH3 domains are less well described than those that involve SH2 domains (24). The SH3 domains of the Src and the RasGAP proteins have the same kind of spatial arrangement, but ^1H nuclear magnetic resonance studies indicate that they are not identical (37). The amino acid sequence from positions 317 to 326 lies at the surface of the RasGAP SH3 domain and is essential for RasGAP signaling (6). The antibody GP200, directed against the SH3 domain of the RasGAP protein (20), can block the maturation of *Xenopus* oocytes induced by oncogenic Ras or insulin (6). Other studies have shown that the GAP SH2-SH3 domains can regulate cytoskeletal structures and cell adhesion (18) and induce gene expression in a Ras-dependent manner (19).

Immature *Xenopus* oocytes are arrested in prophase of the first meiotic division. Upon stimulation with progesterone or insulin, *Xenopus* oocytes undergo the transition into meiosis

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(maturation). The events of maturation, including germinal vesicle breakdown (GVBD), chromosome condensation, and formation of the metaphase spindles, occur upon activation of the maturation-promoting factor (MPF). MPF is a complex consisting of $p34^{cdc2}$ (Cdc2) and cyclin B proteins regulated by phosphorylation-dephosphorylation processes (30). In *Xenopus* oocytes, maturation can also be induced by microinjection of the oncogenic form of Ras protein. We have established that the MAP kinase cascade is activated by oncogenic Ras in *Xenopus* oocytes (25), and we have shown that oncogenic Ras activates MAP kinase before activating Cdc2. MAP kinase and Cdc2 are activated at the same time in *Xenopus* oocytes treated with progesterone (7, 16, 21). However, a recent study showed that the activation of Cdc2 by progesterone requires the activation of the MAP kinase cascade (13). It is not clear whether Cdc2 activation depends on the MAP kinase pathway alone or if other signaling pathways are involved. Following progesterone exposure, many proteins are synthesized, such as the product of the c-*mos* proto-oncogene, a 39-kDa serine/threonine protein kinase. It has been shown by using antisense RNA of c-*mos* RNA that synthesis of the Mos protein is needed for the activation of Cdc2 and GVBD by progesterone (29) or by $p21^{ras}$ and insulin (3). The Mos protein alone can initiate meiosis in the absence of any added hormone (39). Recently, Mos has also been shown to be an upstream activator of MAP kinase by activating the MAP kinase kinase in *Xenopus* oocytes (26).

We have investigated the role of the SH3 domain of RasGAP in signal transduction by microinjecting the antibody GP200 (α-SH3 GAP), directed against the SH3 domain of the RasGAP protein into *Xenopus* oocytes, and assaying Cdc2 and MAP kinase activation by oncogenic Ras. A RasGAP SH3-dependent pathway seems to be required for Cdc2 activation by oncogenic Ras but not for MAP kinase activation. Experiments involving microinjection of a recombinant negative MAP kinase indicated that MAP kinase activation is also necessary for Cdc2 activation and Mos synthesis. We also show that activation of Cdc2 by oncogenic Ras requires protein synthesis, such as that of Mos, and that the SH3 domain of RasGAP is implicated in Mos induction.

MATERIALS AND METHODS

Expression and purification of recombinant Ras proteins. Ha-*ras* p21 was prepared as described previously (27). Ha-*ras* cDNA was cloned by PCR of DNA isolated from an amplified human B-lymphocyte cDNA library (gt10; Clontech). The p21 lys12 Ras mutant was obtained and purified by site-directed mutagenesis as described previously (25).

Expression and purification of recombinant MAP kinase mutant protein (ERK2 K52R). The conserved lysine at position 52 of ERK2 involved in phosphate transfer was mutated to arginine (K52R) to impair the catalytic efficiency of the enzyme. The K52R mutant cDNA encoding His_6 was ligated into NpT7-5, and the vector harboring the ERK2 K52R cDNA was transfected into the BL21-DE3 strain of *Escherichia coli*. This construct was generously given to us by M. Cobb (Dallas, Tex.). The *E. coli* strain was grown at 37°C in Luria broth, and ERK2 K52R was induced with isopropyl-β-D-thiogalactopyranoside. ERK2 K52R was purified from bacterial cultures by chromatography on a Ni^{2+}-nitrilotriacetic acid-agarose column as described by Robbins et al. (28).

Oocyte microinjection. *Xenopus laevis* frogs from South Africa were obtained from CRBM-Centre National de la Recherche Scientifique (Montpellier, France). *Xenopus* oocytes were removed under anesthesia and isolated by treatment with type II collagenase (0.2%; Sigma) in Barth buffer without Ca^{2+}. Stage VI *Xenopus* oocytes were selected for assays and incubated in Barth buffer containing 1 mM $CaCl_2$. Ras proteins or antibodies were diluted in Barth medium containing 1 mM $CaCl_2$, and 50-nl aliquots were microinjected into the cytoplasm. Peptides were dissolved in 5% dimethyl sulfoxide and centrifuged before injection. *Xenopus* oocytes were cultured at 18 to 19°C in Barth medium containing 1 mM $CaCl_2$. *Xenopus* oocytes were monitored for GVBD, which is indicated by the appearance of a white spot on the animal hemisphere and is confirmed by the disappearance of the germinal vesicle.

Oocyte extracts. For protein kinase assays, 15 *Xenopus* oocytes were homogenized in a Dounce homogenizer (20 strokes; pestle A) in 200 μl of a solution containing 80 mM glycerophosphate (pH 7.4), 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 15 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1-μg/ml trypsin inhibitor, 1-μg/ml leupeptin, and 1 mM Na_3VO_4. Homogenates were centrifuged at 100,000 × *g* for 30 min. The resulting supernatants were stored at −80°C.

Immunoprecipitation studies. Twenty-five microliters (50 γ proteins) of oocyte extract containing 0.5% Nonidet P-40 was precleared with Protein G Plus-Agarose (Oncogene Science) for 2 h at 4°C. Insoluble material was removed by centrifugation for 15 min at 10,000 × *g*. The supernatants were incubated for 4 h at 4°C with 15 μl of monoclonal anti-Cdc2 (3E1) given by T. Hunt (South Mimms, Hertfordshire, United Kingdom). Fifty microliters of Protein G Plus-Agarose was added to the immune complexes, and incubation was continued for an additional 60 min. The immunoprecipitates were collected and washed three times with H1 kinase assay buffer.

Immunoblotting studies. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BA 83; Schleicher & Schuell) by semidry transfer (40 mM aminocaproic acid, 300 mM Tris, 50% methanol) at 0.8 mA/cm^2 for 2 h. The membranes were blocked with 3% bovine serum albumin, and the blots were probed for 3 h with monoclonal antiphosphotyrosine antibody (Upstate Biotechnology Inc.) diluted 1:2,000, anti-MAP kinase antibody (Zymed Laboratories) diluted 1:2,000, or anti-Mos^{xe} antibody (Santa Cruz Biotechnology) diluted 1:250. The primary antibody was removed, and immunoreactive bands were visualized with an appropriate peroxidase-linked anti-immunoglobulin G (IgG) antibody (Bio Sys) diluted 1:25,000; this was followed by an enhanced chemiluminescence reaction and autoradiography.

MAP kinase assay. MAP kinase activity was measured by incubating 3 μl of oocyte extracts for 10 min at 30°C in a final volume of 50 μl containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 5 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 50 μM [γ-^{32}P]ATP (0.3 Ci/mmol; Amersham), 17 μg of myelin basic protein (Sigma), and 10 μg of the peptide inhibitor of cyclic AMP (cAMP)-dependent protein kinase (Sigma). The reaction was initiated by adding an aliquot of the extract and stopped by spotting 40 μl of the reaction mixture onto Whatman 3MM paper. The papers were washed once with 10% trichloroacetic acid containing 3 mM ATP (for at least 1 h) and three times with 5% trichloroacetic acid, dried with ethanol-ether and ether alone, and counted for radioactivity.

S6 kinase assay. 40S ribosomal subunits were isolated from rat liver according to the procedure of Martin and Wool (15). S6 kinase activity was assayed as described for the MAP kinase assay, except that the substrate was 20 μg of 40S ribosomal proteins and the incorporation of ^{32}P into S6 protein was determined as previously described (32).

MEK assay. MEK activity was measured directly in the oocyte extract with the negative MAP kinase mutant ERK2 K52R as the substrate (28). Aliquots (1 μl) of oocyte extract were incubated with 5 μg of recombinant ERK2 K52R for 15 min at 30°C in a final volume of 50 μl containing 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, and 50 μM [γ-^{32}P] ATP (0.3 Ci/mmol). The reaction was stopped by adding concentrated electrophoresis sample buffer. The reaction products were analyzed by SDS–12.5% PAGE, and the gel was autoradiographed.

H1 kinase assay. H1 kinase activity was assayed as described for the MAP kinase assay, except that the substrate was 15 μg of histone H1 (Boehringer). The reaction was stopped by adding concentrated electrophoresis sample buffer. Reaction products were analyzed by SDS–15% PAGE. After SDS-PAGE, the gel was dried and the ^{32}P-labelled histone H1 band was localized by autoradiography. Then the band was excised and counted for radioactivity.

RESULTS

Involvement of the SH3 domain of RasGAP in the activation of Cdc2. As it has recently been shown that a monoclonal antibody directed against the SH3 domain of RasGAP blocks the GVBD induced by oncogenic Ha-Ras lys12 in *Xenopus* oocytes (6), we examined the activation of Cdc2, a component of MPF (cyclin B-Cdc2) responsible for GVBD. *Xenopus* oocytes were microinjected with 10 ng of Ha-Ras lys12 or with 10 ng of Ha-Ras lys12 plus 100 ng of a monoclonal antibody to the SH3 domain of RasGAP (α-SH3 GAP). Figure 1A shows the GVBD at various times. The GVBD induced by Ha-Ras lys12 was maximal at 12 h and was completely inhibited by α-SH3 GAP. At the same times, cytosol extracts were obtained and the activity of Cdc2 was assayed with histone H1 as the substrate. Figure 1B shows the inhibition of H1 kinase activation by the antibody α-SH3 GAP. Ha-Ras lys12 stimulated H1 kinase activity after a 4-h lag, and H1 kinase activity reached a plateau 10 h after injection. Injection of α-SH3 GAP plus Ha-Ras lys12 totally inhibited the Cdc2 activity stimulated by

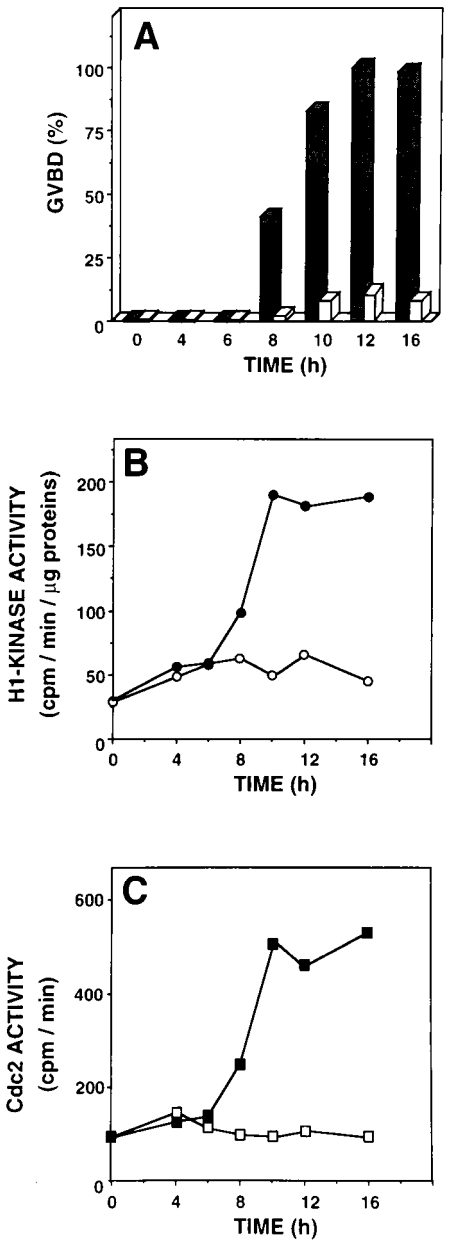

FIG. 1. Effect of microinjected α-SH3 GAP on GVBD and Cdc2-H1 kinase activity induced by Ha-Ras lys12. (A) Groups of 15 oocytes were microinjected with 10 ng of Ha-Ras lys12 (■) or with 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (□). At timed intervals, oocytes were monitored for their GVBD. GVBD is expressed as the percentage of each group of oocytes that underwent GVBD. (B) Groups of 15 oocytes were microinjected with 10 ng of Ha-Ras lys12 (●) or with 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (○). At timed intervals, oocytes were homogenized and H1 kinase activity was assayed in the 100,000 × *g* supernatants. H1 kinase activities are expressed as counts per minute of ^{32}P incorporated into histone H1 per minute of incubation per microgram of supernatant proteins. (C) Oocytes were microinjected with 10 ng of Ha-Ras lys12 (■) or with 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (□). *Xenopus* oocyte extracts were prepared at various times and H1 kinase activities were assayed after immunoprecipitation of Cdc2. Cdc2 activities are expressed as counts per minute of ^{32}P incorporated into histone H1 per minute of incubation.

Ha-Ras lys12 at any time. The measured H1 kinase activity corresponded to the Cdc2 activity, since the same time course of H1 kinase stimulation was obtained after immunoprecipitation of Cdc2 with a monoclonal antibody to the C-terminal 219 residues of *Xenopus* Cdc2 (Fig. 1C). The Cdc2 activity is well correlated with GVBD. As identical measurements of Cdc2 activity were obtained by immunoprecipitation and the assay of cytosol extracts, Cdc2 activity was routinely assayed on cytosol extracts with histone H1 as the substrate.

Specificity of the effect of the antibody raised against the RasGAP SH3 domain. The specificity of the effect of α-SH3 GAP on Cdc2 activity was examined by injecting *Xenopus* oocytes with oncogenic Ras in the presence of 100 ng of either nonimmune IgG or α-SH3 GAP. Cdc2 activities were assayed 12 h later as described in Materials and Methods. The effect of oncogenic Ras was not blocked by nonimmune IgG, whereas it was strongly inhibited by α-SH3 GAP (Fig. 2).

The dependence of Ha-Ras lys12-stimulated Cdc2 activity on the α-SH3 GAP concentration was studied by microinjecting 10 ng of Ha-Ras lys12 plus various amounts of α-SH3 GAP into *Xenopus* oocytes. Injected *Xenopus* oocytes were incubated in Barth buffer for 12 h and then homogenized. The Cdc2 activities of 100,000 × *g* supernatants were assayed with histone H1 as the substrate (Fig. 3). Injection of 50 ng of α-SH3 GAP completely inhibited Cdc2 activity, while 5 ng of α-SH3 GAP produced a 50% inhibition of Cdc2 activity. The GVBD correlated completely with Cdc2 activity (Fig. 3, inset). Half-maximal inhibition of GVBD induced by Ha-Ras lys12 was obtained with 5 ng of α-SH3 GAP, and 50 ng of α-SH3 GAP produced maximal inhibition of GVBD.

In addition, α-SH3 GAP also blocked the Cdc2 activity induced by insulin but did not block the Cdc2 activity induced by progesterone (data not shown), which is in agreement with the effects of these hormones on GVBD (6).

Region of RasGAP SH3 implicated in the mechanism of Ras-induced Cdc2 activity. We used peptides, corresponding

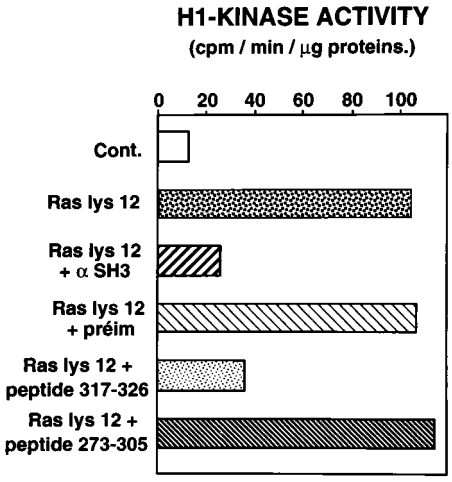

FIG. 2. Effects of α-SH3 GAP, nonimmune IgGs, and GAP-SH3 peptides on the Cdc2-H1 kinase activity induced by Ha-Ras lys12. Groups of 15 oocytes were microinjected with buffer (Cont.), with 10 ng of Ha-Ras lys12, or with a combination of 10 ng of Ha-Ras lys12 and either 100 ng of monoclonal α-SH3 GAP, 100 ng of nonimmune IgG (préim), 80 ng of GAP peptide from 317 to 326 (peptide 317–326), or 80 ng of GAP peptide from 273 to 305 (peptide 273–305). Peptides were dissolved in 5% dimethyl sulfoxide and centrifuged before injection. Oocytes were incubated for 12 h, homogenized, and assayed for H1 kinase activities. H1 kinase activities are expressed as counts per minute of ^{32}P incorporated into histone H1 per minute of incubation per microgram of 100,000 × *g* supernatant proteins.

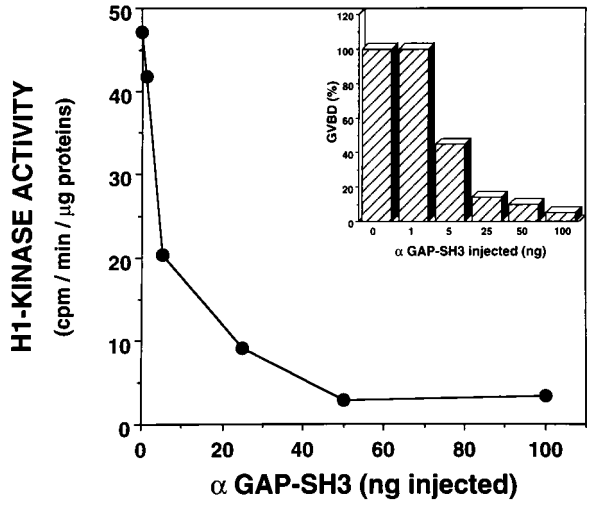

FIG. 3. Relationship among Ha-Ras lys12-stimulated Cdc2, GVBD, and amount of α-SH3 GAP injected. Groups of 15 oocytes were microinjected with 10 ng of Ha-Ras lys12 plus various amounts of α-SH3 GAP and incubated in buffer for 12 h. Oocytes were monitored for their GVBD and homogenized, and the 100,000 × *g* supernatant proteins were assayed for H1 kinase activity. H1 kinase activities are expressed as counts per minute of ^{32}P incorporated into histone H1 per minute of incubation per microgram of supernatant proteins. GVBD is expressed as the percentage of each group of oocytes that underwent GVBD (inset).

to different regions of RasGAP, which had been tested for their ability to inhibit Ras-induced GVBD (6). *Xenopus* oocytes were injected with oncogenic Ras in the presence of 80 ng of various peptides, and Cdc2 activities were assayed 12 h later as described in Materials and Methods. The peptide corresponding to positions 317 to 326 of the RasGAP SH3 domain has been shown to block Ras-induced GVBD (6). The sequence (317 to 326) forms an accessible lipophilic zone followed by a short hydrophilic domain compatible with a putative binding site for a candidate effector (37). As determined by its effect on GVBD, the peptide corresponding to positions 317 to 326 of the human sequence of RasGAP blocked the activation of Cdc2 stimulated by Ha-Ras lys12, as did α-SH3 GAP (Fig. 2). In contrast, the peptide corresponding to positions 273 to 305, which does not encompass the sequence from 317 to 326, had no effect.

Implication of the RasGAP SH3 domain in the MAP kinase cascade. It has been reported that the MAP kinase cascade is required for the maturation of *Xenopus* oocytes induced by progesterone (13). Therefore, we studied the effect of the SH3 domain of RasGAP on MAP kinase activity. *Xenopus* oocytes were injected with 10 ng of Ha-Ras lys12 with or without 100 ng of α-SH3 GAP. *Xenopus* oocyte extracts were prepared after various times and assayed for MAP kinase activity. Figure 4A shows the time course of MAP kinase activation under these conditions. Ten nanograms of Ha-Ras lys12 alone activated MAP kinase after a lag and attained maximum activation at 9 h. Injection of α-SH3 GAP with Ha-Ras lys12 did not alter the MAP kinase activity stimulated by Ras at any incubation time.

As the p42 of *Xenopus* MAP kinase requires tyrosine phosphorylation for its activation, we performed immunoblotting studies of the cytosolic proteins with a monoclonal antiphosphotyrosine antibody (Fig. 4B). *Xenopus* oocytes were injected with 10 ng of Ha-Ras lys12 with or without 100 ng of α-SH3 GAP. *Xenopus* oocyte extracts were prepared after 6 and 12 h, and 75 μg of protein from each extract was subjected to immunoblotting with the antiphosphotyrosine antibody. Oncogenic Ras promoted phosphorylation of tyrosine residues in p42 MAP kinase, as previously described (25). *Xenopus* oocytes injected with Ha-Ras lys12 plus α-SH3 GAP showed that oncogenic Ras always promoted tyrosine phosphorylation in MAP kinase, confirming that α-SH3 GAP did not prevent the activation of MAP kinase by oncogenic Ras.

We confirmed that the stimulation of MAP kinase by Ha-Ras lys12 was not prevented by α-SH3 GAP by examining the effect of α-SH3 GAP on the activation of several components of the MAP kinase cascade. The activities of MAP kinase, MEK (the activator of MAP kinase), and S6 kinase p90^{rsk}, a substrate of MAP kinase, were measured in the same extracts (Fig. 5). *Xenopus* oocytes were injected with 10 ng of Ha-Ras lys12 with or without 100 ng of α-SH3 GAP. *Xenopus* oocyte extracts were prepared 12 h later. MEK activity was measured by the in vitro phosphorylation of the recombinant MAP kinase ERK2 K52R by the *Xenopus* oocyte supernatants. The MEK activity in the extracts from Ha-Ras lys12-injected *Xenopus* oocytes was the same as the activity in the supernatants from *Xenopus* oocytes injected with Ha-Ras lys12 plus α-SH3 GAP (Fig. 5A).

As it has been reported that the electrophoretic mobility of MAP kinase decreases after its phosphorylation and activation, we checked the shift in the mobility of MAP kinase following electrophoresis and immunoblotting with an anti-MAP kinase antibody (Fig. 5B). A 40-kDa protein reacting with anti-MAP kinase antibody was detected in control *Xenopus* oocyte ex-

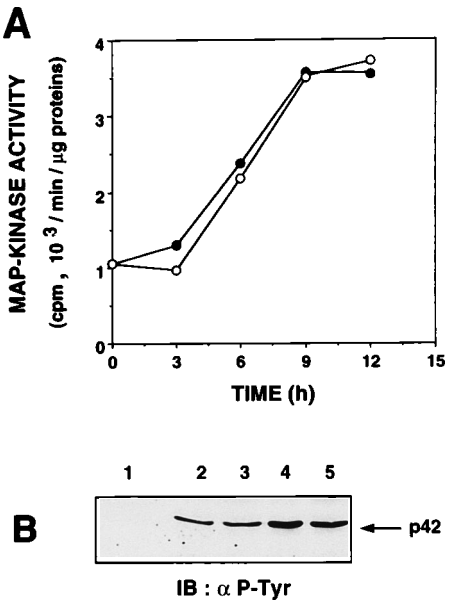

FIG. 4. Effect of microinjected α-SH3 GAP on MAP kinase stimulated by Ha-Ras lys12. (A) Oocytes were microinjected with 10 ng of Ha-Ras lys12 (●) or with 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (○). At timed intervals, 15 oocytes were homogenized in 200 μl of extraction buffer and centrifuged at 100,000 × *g*. MAP kinase activities were assayed with myelin basic protein as the substrate and are expressed as counts per minute of ^{32}P incorporated into myelin basic protein per minute of incubation per microgram of supernatant proteins. (B) Groups of 15 oocytes were microinjected with Barth buffer (lane 1), 10 ng of Ha-Ras lys12 for 6 h (lane 2) or for 12 h (lane 4), or 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP for 6 h (lane 3) or for 12 h (lane 5). Oocytes were homogenized, and 50 μg of protein from the supernatants was analyzed by SDS–12% PAGE. Proteins were transferred to nitrocellulose and blotted with antiphosphotyrosine antibody (1:2,000 dilution). Immunoreactive bands (IB) were visualized by an enhanced chemiluminescence reaction with an anti-horseradish peroxidase mouse antibody (1:25,000 dilution).

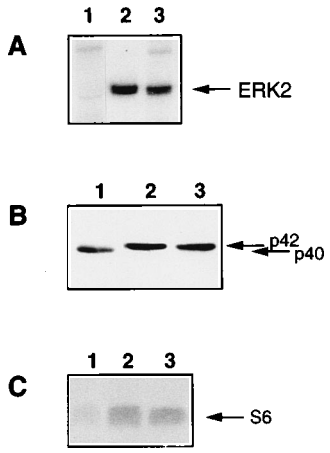

FIG. 5. Effect of microinjected α-SH3 GAP on MAP kinase cascade stimulated by Ha-Ras lys12. Groups of 30 oocytes were microinjected with Barth buffer (lane 1), 10 ng of Ha-Ras lys12 (lane 2), or 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (lane 3) and incubated for 12 h. They were homogenized, and 100,000 × *g* supernatants were prepared. (A) MEK activity. Aliquots (1 μl) of supernatants were incubated with 5 μg of recombinant ERK2 K52R as a substrate plus [γ-^{32}P]ATP for 15 min at 30°C. The reaction products were analyzed by SDS–10% PAGE and the gel was autoradiographed. (B) MAP kinase activation. Fifty micrograms of supernatant proteins was analyzed by SDS–12% PAGE, transferred to nitrocellulose, and blotted with anti-MAP kinase antibody (1:2,000 dilution). Immunoreactive bands were visualized by an enhanced chemiluminescence reaction with an anti-horseradish peroxidase mouse antibody (1:30,000 dilution). (C) S6 kinase activity. Aliquots (3 μl) of supernatants were incubated with 15 μg of 40S ribosomal subunits as the substrate plus [γ-^{32}P]ATP for 10 min at 30°C. The reaction products were analyzed by SDS–15% PAGE and the gel was autoradiographed.

tracts. The apparent molecular mass of MAP kinase changed from 40 to 42 kDa in an identical manner in supernatants from *Xenopus* oocytes injected with Ha-Ras lys12 and from *Xenopus* oocytes injected with Ha-Ras lys12 plus α-SH3 GAP.

S6 kinase activity was also stimulated by injection of oncogenic Ras (Fig. 5C), as has been shown by others (3, 12). Injection of *Xenopus* oocytes with Ha-Ras lys12 plus α-SH3 GAP also caused stimulation of the S6 kinase activity identical to that produced by Ha-Ras lys12 alone. These results indicate that the SH3 domain of RasGAP is not implicated in the regulation of the MAP kinase cascade.

Effect of ERK2 K52R on Ha-Ras lys12-stimulated Cdc2. The fact that the SH3 domain of RasGAP was not implicated in the activation of the MAP kinase cascade but was involved in the activation of Cdc2 raised the question of the role of MAP kinase in the activation of Cdc2. This point was tested by stimulating *Xenopus* oocytes with Ha-Ras lys12, injecting ERK2 K52R, and measuring Cdc2 activity. The MAP kinase activity stimulated by Ha-Ras lys12 was totally inhibited by injection of ERK2 K52R (data not shown). Cdc2 activity was stimulated by Ha-Ras lys12, and this activity was inhibited by injection of ERK2 K52R, whereas injection of ERK2 K52R alone had no effect (Fig. 6). These results suggest that MAP kinase participates in the activation of Cdc2.

Effect of the RasGAP SH3 domain on Mos synthesis. Since α-SH3 GAP did not affect the MAP kinase cascade, the effect of this antibody on other proteins required for Cdc2 activation was examined. As Mos synthesis is required for the activation of Cdc2 by progesterone and insulin, we investigated the implication of the SH3 domain of RasGAP for the regulation of Mos expression. The dependence of Cdc2 activation by oncogenic Ras on protein synthesis was checked, since it has been reported that Ras lys12-induced maturation is not blocked by protein synthesis inhibitors (1). *Xenopus* oocytes were preincubated in Barth medium with or without 70 μM cycloheximide and were injected or not with 10 ng of Ha-Ras lys12. *Xenopus* oocytes were homogenized 12 h later, and the 100,000 × *g* supernatants were assayed for their MAP kinase and Cdc2 activities. Figure 7 illustrates the effect of cycloheximide on MAP kinase and Cdc2 activities. The MAP kinase activity stimulated by oncogenic Ras was not dependent on protein synthesis (Fig. 7A), since the MAP kinase activation remained unchanged in the presence of cycloheximide at every time point (25). By contrast, Cdc2 activity stimulated by oncogenic Ras was dependent on protein synthesis, since Cdc2 activation was also completely inhibited by the cycloheximide treatment (Fig. 7B), as was the GVBD induced by Ha-Ras lys12 (data not shown).

The induction of Mos, which is known to be necessary for the activation of Cdc2, by oncogenic Ras in *Xenopus* oocytes was then examined, and the capacity of α-SH3 GAP to prevent the induction of Mos by oncogenic Ras was checked. *Xenopus* oocytes either were injected with 10 ng of Ha-Ras lys12 with or without 100 ng of α-SH3 GAP or were injected with 10 ng of Ha-Ras lys12 and incubated with cycloheximide or progesterone (as a positive control). At the times indicated in the legend to Fig. 8, *Xenopus* oocytes were removed and homogenized. The extracts were analyzed by immunoblotting with an antibody to Mos^{xe} (Fig. 8). Mos was expressed when *Xenopus* oocytes were treated with progesterone. Ha-Ras lys12 promoted an expression of Mos that was very low 6 h after injection but was clearly visible 12 h after stimulation. Mos expression was correlated with Cdc2 activity. By contrast, there was no Mos produced in *Xenopus* oocytes injected with Ha-Ras lys12 and incubated in cycloheximide or in *Xenopus* oocytes injected with Ha-Ras lys12 plus α-SH3 GAP. Injection of α-SH3 GAP did not modify the total amount of proteins in the oocyte extract. However, the expression of Mos also required MAP kinase, since injection of negative MAP kinase (ERK2 K52R) blocked the production of Mos induced by oncogenic Ras. The 39-kDa immunoreactive protein detected in *Xenopus* oocytes incubated with progesterone, and in *Xenopus* oocytes injected with Ha-Ras lys12, was not detected when the anti-

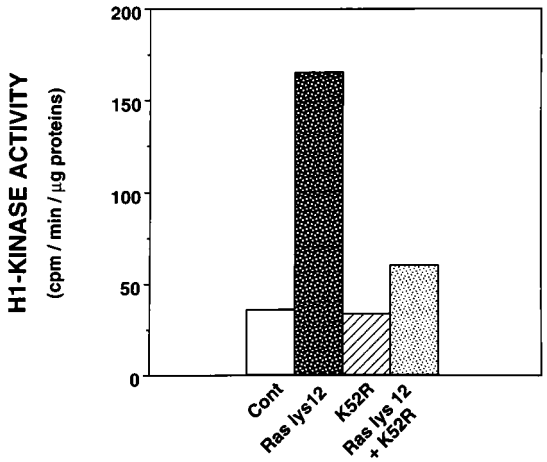

FIG. 6. Effect of a dominant negative MAP kinase mutant (ERK2 K52R) on Ha-Ras lys12-stimulated Cdc2 activity. Groups of 15 oocytes were microinjected with buffer (Cont), 5 ng of Ha-Ras lys12, 10 ng of ERK2 K52R, or 5 ng of Ha-Ras lys12 plus 10 ng of ERK2 K52R and incubated for 12 h. They were homogenized, and 100,000 × *g* supernatants were assayed for H1 kinase activities. H1 kinase activities are expressed as counts per minute of ^{32}P incorporated into histone H1 per minute of incubation per microgram of supernatant proteins.

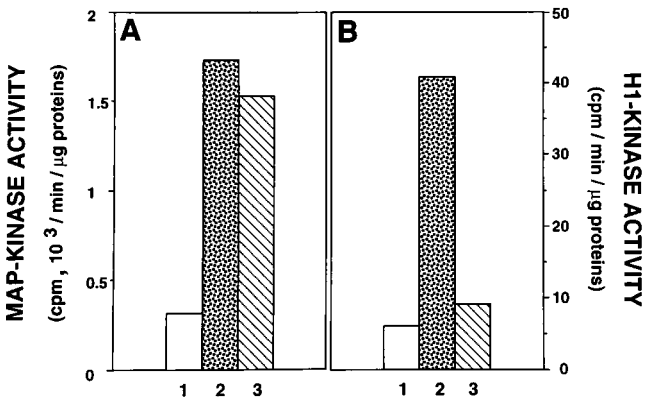

FIG. 7. Effect of cycloheximide treatment on oocyte MAP kinase and Cdc2 activities stimulated by Ha-Ras lys12. Groups of 15 oocytes were microinjected with buffer (bar 1) and with 10 ng of Ha-Ras lys12 (bar 2) and incubated in buffer for 12 h. Another group of 15 oocytes were preincubated in 70 μM cycloheximide in buffer for 2 h. They were then microinjected with 10 ng of Ha-Ras lys12 (bar 3) and incubated in buffer containing cycloheximide for 12 h. All oocytes were homogenized, and 100,000 × *g* supernatants were prepared. MAP kinase and Cdc2 activities were assayed with myelin basic protein or histone H1 as the substrate. Kinase activities are expressed as counts per minute of ^{32}P incorporated in substrate per minute of incubation per microgram of soluble protein.

body anti-Mos^{xe} was preincubated with the peptide including the C-terminal region of Mos^{xe} (results not shown). This demonstrates that the Mos^{xe} antibody detected a band with an apparent molecular mobility of 39 kDa, which is the Mos protein. Thus, the SH3 domain of RasGAP was implicated in Mos synthesis.

DISCUSSION

The present study investigated the molecular mechanisms of the inhibition by a monoclonal antibody directed against the SH3 domain of RasGAP (α-SH3 GAP). This antibody blocks the GVBD induced by oncogenic Ras in *Xenopus* oocytes (6). Cdc2 activity, which corresponds to the activation of the MPF complex, is increased after injection of oncogenic Ras. We show that Cdc2 activity stimulated by oncogenic Ras is blocked by the antibody directed against the SH3 domain of RasGAP. α-SH3 GAP is very specific; its specificity was demonstrated by checking that (i) nonspecific IgGs do not block the activation of Cdc2 by oncogenic Ras, (ii) α-SH3 GAP does not block *Xenopus* oocyte maturation or the Cdc2 activity induced by progesterone, which are independent of Ras and probably of RasGAP, and (iii) α-SH3-GAP does not recognize any other SH3 domains (6). α-SH3 GAP recognizes a p100 protein in oocyte lysates which is also recognized by another antibody raised against another epitope of RasGAP protein (data not shown). The fact that α-SH3 GAP recognizes a p100 protein is possible because the cDNA of *Xenopus* GAP was not cloned. A p95-100 GAP was also characterized and purified from human placental tissue (9, 40). The region of the SH3-GAP domain implicated in the molecular mechanism of Ras-induced Cdc2 activation is the region that is implicated in Ras-induced GVBD, since a peptide corresponding to positions 317 to 326 of the human sequence suppressed the two effects.

To understand the mechanism of Cdc2 activation, a fundamental issue to determine is whether MAP kinase activation precedes Cdc2 activation. In fact, the components involved in the MAP kinase cascade appear to lie downstream of several proto-oncogene products, such as Ras, Raf, and Mos, and it has been proposed that the MAP kinase cascade may function as a key step in a wide variety of intracellular signal transductions. In *Xenopus* oocytes, progesterone-induced activation of MPF and progesterone-induced activation of MAP kinase occur almost simultaneously (7, 16, 21), and it is difficult to demonstrate whether MAP kinase activation precedes Cdc2 activation. In Ras-injected *Xenopus* oocytes, activation of the MAP kinase cascade could be involved just before Cdc2 activation (22, 25). This is supported by experiments of others showing that a MEK-blocking antibody prevents the activation of Cdc2 (13) and that an activating mutation of MEK-1 activated both MAP kinase and H1 kinase and induced GVBD (10). Haccard et al. (8) have also shown that injection of constitutive active MAP kinase is able to activate MPF and promote GVBD. The antibody α-SH3 GAP may block the activation of MAP kinase, thereby preventing Cdc2 activation. But our findings demonstrate that injection of α-SH3 GAP did not prevent the activation of the components of the MAP kinase pathway by oncogenic Ras. MEK, MAP kinase, and S6 kinase p90^{rsk} activities were not affected by injection of α-SH3 GAP. c-Raf1 activity could also be implicated in the MAP kinase cascade, but we detected no c-Raf1 activity stimulated by Ha-Ras lys12 in *Xenopus* oocytes. White et al. (34) have shown that the activation of p42 MAP kinase by Ha-Ras lys12 is probably due to a MEK kinase different from the c-Raf1 identified by Itoh et al. (11) as REKS (Ras-dependent ERK kinase stimulator). Recently, it has been reported that this Ras-dependent ERK kinase stimulator might be a complex of B-Raf and 14-3-3 proteins (36). Regardless of the identity of the MEK kinase, activation of MAP kinase is required for the activation of Cdc2, since the negative MAP kinase ERK2 K52R, which blocks MAP kinase activation, also blocks the activation of Cdc2. Thus, MAP kinase seems to be necessary, but not sufficient, for Cdc2 activation.

Our studies show that GVBD and Cdc2 activation by oncogenic Ras requires protein synthesis, in contrast to the results of others (1, 4). Therefore, we also looked for other components implicated in the activation of Cdc2, such as Mos, and we examined whether the SH3 domain of RasGAP was involved

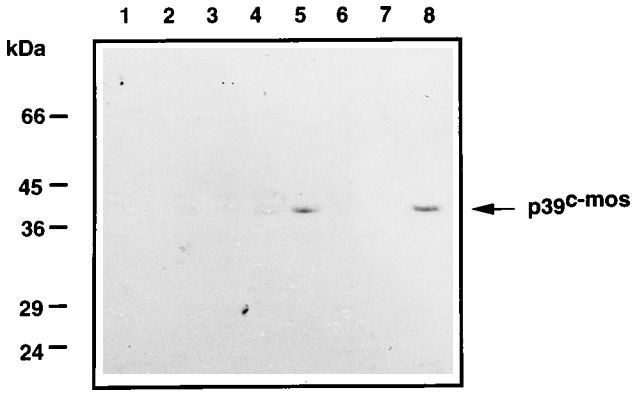

FIG. 8. Effect of microinjected α-SH3 GAP on Mos synthesis induced by Ha-Ras lys12. Groups of 15 oocytes were microinjected with buffer (lane 1), 10 ng of Ha-Ras lys12 (lanes 2 and 5), 10 ng of Ha-Ras lys12 plus 100 ng of monoclonal α-SH3 GAP (lanes 3 and 6), 10 ng of Ha-Ras lys12 plus 10 ng of ERK2 K52R (lane 4), 10 ng of Ha-Ras lys12 after preincubation in 20 μg of cycloheximide per ml, as described in the legend for Fig. 7 (lane 7), or 10 ng of Ha-Ras lys12 and incubated with 1 μM progesterone (lane 8). After 6 h (lanes 2, 3, and 8) or 12 h (lanes 1, 4, 5, 6, and 7), oocytes were homogenized and 100,000 × *g* supernatants were prepared. Aliquots (75 μg) of supernatant proteins were analyzed by SDS–12.5% PAGE, transferred to nitrocellulose, and blotted with polyclonal anti-Mos^{xe} antibody (1:250 dilution). Immunoreactive bands were visualized by an enhanced chemiluminescence reaction with an anti-horseradish peroxidase rabbit antibody (1:20,000 dilution).

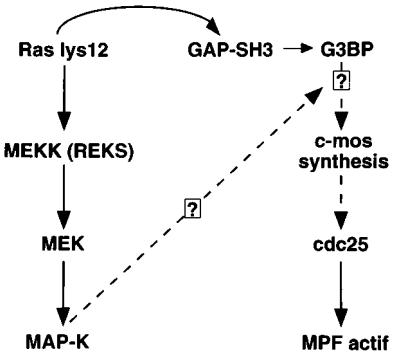

FIG. 9. Diagram showing the points of action of oncogenic Ras and the SH3 domain of RasGAP. Arrows indicate the direct targets identified in signal transduction. Dashed arrows indicate incomplete information or unknown steps. G3BP, GAP SH3-binding protein; REKS, Ras-dependent ERK kinase stimulator.

in Mos expression. Barrett et al. (3) reported that Mos synthesis is required for the activation of Cdc2 induced by oncogenic Ras, in disagreement with the results of Daar et al. (5). We found that Mos is induced by oncogenic Ras and is blocked by cycloheximide. We did not detect Mos during the first 6 h following Ras injection. Its expression is correlated with the activation of Cdc2 and GVBD. This expression is blocked when Ha-Ras lys12 is injected together with α-SH3 GAP, suggesting that the SH3 domain of RasGAP is implicated in a signaling mechanism which regulates Mos expression and which must be distinct from the MAP kinase cascade.

A RasGAP-SH3-associated protein has been identified recently in mammalian cells (23). This protein shows homology with heterogeneous nuclear ribonucleoprotein. Such a protein might exist in *Xenopus* oocytes and might play a role in the regulation of Mos expression.

Synthesis of additional proteins also seems necessary to complete oocyte maturation, since injection of recombinant Mos protein induces GVBD and the activation of MPF but the oocytes do not proceed into meiosis II (39). It is also possible that additional pathways regulating components other than Mos take part in the activation of Cdc2. For example, a second MAP kinase pathway activated by oncogenic Ras in mammalian cells has been described (14). This pathway does not implicate p42 or p44 MAP kinase but involves other proteins of the MAP kinase family, such as the Jun kinases (JNKs/stress-activated protein kinases). It will be interesting to look for the existence of this pathway in *Xenopus* oocytes and to test the effect of α-SH3 GAP on this pathway.

In summary, according to the proposed model outlined in Fig. 9, oncogenic Ras stimulates at least two pathways. One is the pathway involving the SH3 domain of GAP, which can be associated with a GAP-SH3 binding protein and which regulates expression of Mos and activation of MPF. Matten et al. (17) have suggested that Mos activates Cdc2 by regulating cdc25 phosphatase in *Xenopus* oocytes stimulated with progesterone. They have also shown that cAMP acts as a negative regulator of Mos expression. It is tempting to speculate that RasGAP might regulate protein kinase A or another protein kinase implicated in the regulation of Mos expression. The second pathway is the MAP kinase cascade, which converges with the pathway implicating the SH3 domain of RasGAP to activate MPF. Convergence can take place before Mos expression, since injection of negative MAP kinase mutant protein prevents Mos expression. Thus, Mos plays a fundamental role in the activation of MPF in *Xenopus* oocytes and its expression is regulated by the MAP kinase and the SH3 domain of RasGAP.

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