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Oncogenic Ras suppresses Cdk1 in a complex manner during the incubation of activated *Xenopus* egg extracts

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Abstract

The activity of Cdk1 is the driving force for entry into M-phase during the cell cycle. Activation of Cdk1 requires synthesis and accumulation of cyclin B, binding of cyclin B to Cdk1, and removal of the inhibitory tyr-15-Cdk1 phosphorylation. It was previously shown that oncogenic Ras suppresses Cdk1 activation during the incubation of activated *Xenopus* egg extracts. However, how oncogenic Ras suppresses Cdk1 remained unclear. Using the histone H1 kinase assay to follow Cdk1 activity and Western blot analysis to assess levels of both cyclin B2 and phosphorylated-tyr-15-Cdk1, how oncogenic Ras suppresses Cdk1 is studied. The results indicate that oncogenic Ras suppresses Cdk1 via induction of persistent phosphorylation of tyr-15-Cdk1. Interestingly, the results reveal that, compared with cyclin B2 in control activated egg extracts, which increased, peaked and then declined during the incubation, oncogenic Ras induced continuous accumulation of cyclin B2. The results also indicate that oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2, which is mediated by constitutive activation of the Raf-Mek-Erk-p90^{rsk} pathway. Taken together, these results indicate that oncogenic Ras suppresses Cdk1 in a complex manner: It induces continuous accumulation of cyclin B2, but also causes persistent inhibitory phosphorylation of tyr-15-Cdk1.

Keywords

Signal transduction; oncogenic Ras; Cdk1; Cell cycle arrest; *Xenopus* egg extracts

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Introduction

Oncogenic Ras plays a very important role in tumorigenesis (1). However, it has been shown that oncogenic Ras does not directly transform normal primary cells. Instead, it induces cell cycle arrest in these cells (2-6). Thus, understanding how oncogenic Ras regulates the cell cycle in normal cells is critical to understanding how oncogenic Ras induces transformation. It has been shown that oncogenic Ras induces the cell cycle to arrest at the G1, S, G2 and/or M-phases in different types of normal primary mammalian cells (3-6) and in fertilized *Xenopus* eggs (7), indicating that oncogenic Ras can suppress the entry into and/or progression through these stages of the cell cycle in normal primary cells. It has also been shown that oncogenic Ras induces G1-arrest in normal primary mammalian cells through induction of the G1 cyclin-dependent kinase inhibitors such as p16^{Ink4a} (5, 6, 8), which inhibit the activation of the G1 cyclin-dependent kinases (9) to prevent the cell cycle from entering S-phase. How oncogenic Ras suppresses the entry into and progression through M-phase remains unclear.

The activity of Cdk1 (formerly Cdc2) is the major driving force for the entry into and progression through M-phase during the cell cycle. Cdk1 is a cyclin B-dependent kinase (10-14). The activation of Cdk1 during the cell cycle proceeds through the following stages (10-14). The amount of Cdk1 is constant throughout the cell cycle. Cyclin B is synthesized and accumulated during interphase. After synthesis, cyclin B binds to Cdk1 to form the Cdk1/cyclin B complex. The thr-14 and tyr-15 residues of the Cdk1 are then phosphorylated by Wee1/Myt1 kinases (12, 15-17). The phosphorylation inhibits the activation of the Cdk1/cyclin B complex. When the Cdk1/cyclin B complex accumulates to a sufficient level, Cdc25 protein phosphatase is activated to dephosphorylate the thr-14 and tyr-15 residues of Cdk1 (12, 18-23), resulting in the final activation of the Cdk1/cyclin B complex and thus triggering the cell cycle to enter and progress through M-phase.

Activated *Xenopus* egg extracts are cytosolic extracts prepared from activated *Xenopus* eggs that are at the interphase stage of the first embryonic mitotic cell cycle (13). Activated *Xenopus* egg extracts are capable of undergoing cell-free cycling during the incubation at room temperature (13). Using activated *Xenopus* egg extracts, it was previously shown that oncogenic Ras suppresses Cdk1 activation and prevents the cell cycle from entering M-phase (24). Thus, activated *Xenopus* egg extracts are a suitable *in vitro* model for studying how oncogenic Ras suppresses Cdk1 and M-phase entry. Previous study also implied that the oncogenic Ras-induced suppression of Cdk1 involves neither the inhibition of cyclin B synthesis nor the inhibition of cyclin B binding to Cdk1 (24). Nonetheless, how oncogenic Ras suppresses Cdk1 remained unclear. Using the histone H1 kinase assay to assess Cdk1 activity and Western blot analysis to follow levels of both cyclin B2, one of the major cyclin B in *Xenopus* eggs (25), and phosphorylated-tyr-15-Cdk1 (p-tyr-15-Cdk1), we further pursue how oncogenic Ras suppresses Cdk1 during the incubation of activated *Xenopus* egg extracts. The results are reported here.

Materials and Methods

Preparation and incubation of activated *Xenopus* egg extracts

Amphibian frogs (*Xenopus laevis*) were used to produce eggs for the preparation of activated egg extracts. (The amphibian frogs were obtained, maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky. Approval was received from the IACUC.) Activation of unfertilized eggs and preparation of cytosolic extracts from activated eggs were performed as previously described (13, 24, 26). Briefly, unfertilized eggs were dejellied and 10 μ M calcium ionophore A23187 (Sigma) was used to activate the eggs to

enter the interphase of the first embryonic mitotic cell cycle. Cytosolic extracts were then prepared from these activated eggs. Freshly prepared cytosolic extracts (activated egg extracts), which remained in the interphase of the first embryonic mitotic cell cycle, were placed on ice and used for the incubation experiments as described below. Briefly, demembranated *Xenopus* sperm nuclei ($\sim 2.5 \times 10^5$ nuclei/ml extract), prepared as previously described (24), were added to aliquots (100–400 μ l) of freshly prepared activated egg extracts on ice. Then, to aliquots of sperm nuclei-containing activated egg extracts, either a buffer solution containing 1 mM DTT and 10 mM Hepes, pH 7.8, or a purified, bacterially expressed human oncogenic Ras^H (final ~ 5 μ g/ml extract) (24, 27) in the buffer solution was added. The total volumes of the buffer solution or Ras protein solution that were added to aliquots of activated egg extracts were less than one-tenth of the volumes of the original aliquots of activated egg extracts. The aliquots of activated egg extracts were then incubated at room temperature to initiate cell cycle progression. Samples were collected during the room temperature incubation and were frozen at -80°C for analysis.

In incubation experiments involving U-0126 (Sigma), cycloheximide or a constitutively active Cdc25B (28, 29), U-0126 (final 50 μ M), cycloheximide (final 150 μ M) or a constitutively active Cdc25B (final ~ 10 μ g/ml extract) was added at various time points to activated egg extracts that were incubated either in the absence or the presence of oncogenic Ras, as described above. The constitutively active Cdc25B, which was derived from the catalytic domain of human Cdc25B (28, 29) and bacterially expressed and purified, was a generous gift from Dr. Johannes Rudolph.

The precipitation of cyclin B2 by p13^{suc1}-agarose beads

The precipitation of cyclin B2 using p13^{suc1}-agarose beads was similarly performed as previously described (24, 30). Briefly, two aliquots of an activated egg extracts were incubated either in the absence (the control extract) or the presence (the oncogenic Ras extract) of oncogenic Ras at room temperature as described above. Aliquots collected from the extracts during the incubation were diluted 20-fold in a buffer containing 80 mM - glycerophosphate, 15 mM MgCl₂, 20 mM EGTA, 10% glycerol, 1 mM DTT, 10 μ g/ml leupeptin, and 10 mM Hepes pH 7.8. Each ~ 350 μ l of the diluted aliquots of extracts was then incubated with 25 μ l of p13^{suc1}-agarose beads (Sigma) at 4°C for 90 minutes. The mixtures were then centrifuged in an Eppendorf centrifuge. The supernatants were collected and the pellets were resuspended and washed twice in the buffer. The bound proteins were eluted from the pellets with 100 μ l of SDS-PAGE sampling buffer. Samples (15 μ l) from the diluted control extract and oncogenic Ras extract prior to p13^{suc1}-bead treatment and from the supernatants after p13^{suc1}-bead treatment and centrifugation, and 40 μ l of the eluates from the pellets were subjected to Western blot analysis using anti-cyclin B2 antibody (X121) (31, 32).

Immunodepletion of p90^{rsk2} from activated egg extracts

Immunodepletion of p90^{rsk2} from activated egg extracts was performed as previously described (33). Briefly, an anti-p90^{rsk2} antibody (Santa Cruz, sc-1430) or mock IgG was allowed to bind to Affi-gel protein A-agarose beads (Biorad). The anti-p90^{rsk2}-bound and mock-IgG-bound protein A-beads were then used to immunodeplete p90^{rsk2} from activated egg extracts. Two 200 μ l aliquots of an activated egg extract were added at 4°C to pellets of 50 μ l of anti-p90^{rsk2}-coated protein A-beads or 50 μ l of mock IgG-coated protein A-beads, respectively. The aliquots were incubated at 4°C for 90 min and then subjected to centrifugation in an Eppendorf centrifuge to remove the beads. After centrifugation, the supernatants from the anti-p90^{rsk2} beads-treated and the mock IgG-beads-treated aliquots were collected. Then, oncogenic Ras (final ~ 5 μ g/ml extract) was added as described above to the supernatant from the anti-p90^{rsk2} beads-treated aliquot, the supernatant from the

mock IgG-beads-treated aliquot, and an aliquot of the activated egg extract that was not treated with protein A-beads. These aliquots containing oncogenic Ras were then incubated at room temperature. Samples were collected during the incubation, frozen at -80°C for SDS-PAGE and Western blot analysis.

The histone H1 kinase assay

The histone H1 kinase assay was similarly performed as previously described (13, 24). Briefly, frozen activated egg extracts were thawed. Samples of thawed extracts were collected and diluted 20-fold in a buffer containing 80 mM γ -glycerophosphate, 15 mM $MgCl_2$, 100 mM KCl, 20 mM EGTA, 10% glycerol, 1 mM DTT, 10 μ g/ml leupeptin, and 10 mM Hepes pH 7.8. 10 μ l of the diluted extracts was mixed on ice with 10 μ l of an assaying buffer containing 0.5 mg/ml calf thymus histone H1 (Boehringer Mannheim), 0.1 mM ATP, 100 μ Ci/ml γ - ^{32}P -ATP (ICN), 80 mM γ -glycerophosphate, 15 mM $MgCl_2$, 100 mM KCl, 20 mM EGTA, 10% glycerol, and 10 mM Hepes pH 7.8. The mixtures were then incubated at 30°C for 10 minutes and the reaction was terminated by the addition of equal volumes of SDS-PAGE sampling buffer. 10 μ l of the mixtures (equivalent to 0.125 μ l of original activated egg extracts) was subjected to SDS-PAGE in 15% [w/v] polyacrylamide gel. After SDS-PAGE, gels were dried and subjected to autoradiography.

Quantitation of the autoradiograms from the histone H1 kinase assay

Quantitation of the autoradiograms from the results of the H1 kinase assay was performed as follows. The relative density of the H1 bands to the background in each autoradiogram was determined using NIH ImageJ program downloaded from website (<http://rsb.info.nih.gov/ij/>), and followed the protocol provided by Lukemiller. Org. The values were normalized to the value of the initial time-point in each autoradiogram and expressed as relative fold change in bar graphs.

Western blot analysis

Frozen samples from activated egg extracts during the incubation were thawed and then diluted 20-fold with SDS-PAGE sampling buffer. Unless otherwise specified, each 15 μ l of the mixtures (equivalent to 0.75 μ l of original activated egg extracts) was subjected to SDS-PAGE in 12.5% (w/v) polyacrylamide gel for Western blot analysis. The proteins on the gels were then electroblotted onto Immobilon-P membrane (Millipore) at 4°C. Blotted membranes were blocked in TBS buffer (150 mM NaCl, 0.05% Tween-20, 1mM EDTA and 20mM Tris, pH 7.4) containing 5% non-fat milk. The membranes were then incubated with dilutions of primary antibodies in TBS buffer containing 5% non-fat milk at room temperature for 1-3 hours or at 4°C overnight. The membranes were then washed with TBS buffer and re-incubated with dilutions of horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) at room temperature for 1-3 hours. After washing, either ECL or ECL Plus (Amersham Biosciences) was used for chemiluminescent detection. The primary antibodies used for the analysis were anti-cyclin B2 antibody (X121) (a generous gift from Drs. J. Gannon and T. Hunt), anti-PSTAIRE antibody (Santa Cruz, sc-53), anti-p-tyr-15-Cdk1 antibody (Santa Cruz, sc-7989R) and anti-p90^{rsk2} antibody (Santa Cruz, sc-1430).

Results and Discussion

Oncogenic Ras induces continuous accumulation of cyclin B2 but suppresses Cdk1 activation during the incubation of activated *Xenopus* egg extracts

Activation of Cdk1 requires synthesis and accumulation of cyclin B (10-14). To study whether oncogenic Ras suppresses Cdk1 activation through inhibition of cyclin B synthesis or accumulation, we determined the effects of oncogenic Ras on the levels of the histone H1

kinase (Cdk1) activity, the levels of cyclin B2 and the levels of total Cdk1 during the incubation of activated *Xenopus* egg extracts. Two aliquots of a freshly prepared activated egg extract were respectively incubated either in the absence (designated as the control extract) or the presence of oncogenic Ras (designated as the oncogenic Ras extract) at room temperature, as described in Materials and Methods. Samples were collected during the incubation and then subjected to both the histone H1 kinase assay and Western blot analysis using anti-cyclin B2 antibody (X121) and anti-PSTAIRE (for total Cdk1).

Figure 1A show the results of the H1 kinase assay for the control extract and the oncogenic Ras extract. In the control extract, the H1 kinase activity increased, peaked at 180-min and then declined during the incubation (Figure 1A, panel a). In contrast, in the oncogenic Ras extract, the H1 kinase activity remained at low levels throughout the incubation period (Figure 1A, panel b). The comparison of the results thus shows that oncogenic Ras was able to suppress the activation of the histone H1 kinase (Cdk1). The results are consistent with previous report that oncogenic Ras suppresses Cdk1 activation during the incubation of activated *Xenopus* egg extracts (24). Figure 1B show the results of Western blot analysis using anti-cyclin B2 antibody. In the control extract, cyclin B2 increased from a low level, peaked at 180-min and then declined during the incubation (Figure 1B, panel a). In contrast, in the oncogenic Ras extract, cyclin B2 increased continuously throughout the incubation period (Figure 1B, panel b), reaching levels that were higher than the peak-level of the control extract (Figure 1B, compare lanes 240-min and 300-min in panel b with lane 180-min in panel a), indicating that oncogenic Ras was able to induce continuous accumulation of cyclin B2. Figure 1C shows the levels of total Cdk1, which remained constant in both the control extract and the oncogenic Ras extract throughout the incubation period. Taken together, the above results show that during the incubation of the control extract, the activity of the histone H1 kinase (Cdk1) was correlated with the levels of cyclin B2. In contrast, during the incubation of the oncogenic Ras extract, although cyclin B2 continuously accumulated to levels higher than the peak-level of the control extract, the histone H1 kinase (Cdk1) activity remained almost unchanged at low levels (Figures 1A and 1B, compare lanes 240-min and 300-min in panels b with lanes 180-min in panels a), indicating that the activation of the histone H1 kinase (Cdk1) was suppressed by oncogenic Ras and that the suppression was not due to the inhibition of the synthesis or accumulation of cyclin B2.

Cyclin B2 induced by oncogenic Ras binds to p13^{suc1}-beads

As the activation of Cdk1 requires the binding of cyclin B to Cdk1 (10-14), we thus further determined whether oncogenic Ras may suppress Cdk1 activation by inhibiting the binding of cyclin B2 to Cdk1. p13^{suc1}, the yeast *suc1* gene product, has a high affinity for Cdk1 (30). If cyclin B2 binds to Cdk1, it should be precipitated by p13^{suc1}-agarose beads through the Cdk1/cyclin B2 complex. We thus determined whether cyclin B2 in activated egg extracts containing oncogenic Ras may be precipitated by p13^{suc1}-agarose beads. Two portions of an activated egg extract were incubated at room temperature either in the absence or the presence of oncogenic Ras (designated as the control extract or the oncogenic Ras extract, respectively). Aliquots were collected at 30 and 240 minutes from the control extract and the oncogenic Ras extract during the incubation. The collected aliquots were diluted 20-fold, treated with p13^{suc1}-beads, and centrifuged, as described in Materials and Methods. Samples from the diluted extracts prior to p13^{suc1}-bead treatment, the supernatants after p13^{suc1}-bead treatment and centrifugation, and the eluates from the pellets of p13^{suc1}-beads were subjected to Western blot analysis using anti-cyclin B2 antibody X121. Figure 2 shows that almost all of the cyclin B2 presented in both the control extract and the oncogenic Ras extract prior to p13^{suc1}-bead treatment could be precipitated by p13^{suc1}-beads (Figure 2, panels a, b and c). Thus, the results indicate that almost all of the cyclin B2 induced by

oncogenic Ras was bound to Cdk1, further indicating that the suppression of Cdk1 activation by oncogenic Ras was not due to the inhibition of cyclin B2 binding to Cdk1.

Oncogenic Ras suppresses Cdk1 activation via induction of persistent inhibitory phosphorylation of Cdk1

The final activation of Cdk1 requires the removal of the inhibitory phosphorylation from the thr-14 and tyr-15 residues of Cdk1 in the Cdk1/cyclin B complex by Cdc25 phosphatase (12, 18-23). It was thus reasoned that oncogenic Ras may suppress Cdk1 activation through the induction of persistent phosphorylation on the thr-14 and tyr-15 residues of Cdk1. Moreover, it was also reasoned that if oncogenic Ras suppresses Cdk1 activation through such a mechanism, then the addition of a constitutively active Cdc25 to activated egg extracts pre-incubated with oncogenic Ras should remove the inhibitory phosphorylation and activate the suppressed Cdk1. To assess whether these speculations are true, we determined whether the suppression of Cdk1 by oncogenic Ras is correlated with persistent phosphorylation of tyr-15-Cdk1, and we also determined whether the addition of a constitutively active Cdc25B to activated egg extracts pre-incubated with oncogenic Ras may remove the phosphorylation of tyr-15-Cdk1 and activate Cdk1.

An activated egg extract was incubated at room temperature in the presence of oncogenic Ras (designated as the oncogenic Ras extract). Samples were collected during the incubation. In addition, at 180 minutes during the room temperature incubation, a portion of the oncogenic Ras extract was collected and a constitutively active Cdc25B (~10 µg/ml extract) was added to this portion (designated as the oncogenic Ras + Cdc25B extract), which was then further incubated at room temperature. Samples were collected. The samples from both the oncogenic Ras extract and the oncogenic Ras + Cdc25B extract were subjected to both the H1 kinase assay and Western blot analysis using anti-cyclin B2 (X121), anti-p-tyr-15-Cdk1 and anti-PSTAIR antibodies. Figures 3A and 3B show the results. Consistent with the results from Figure 1A (panel b) and Figure 1B (panel b), respectively, the histone H1 kinase activity remained at low levels (Figure 3A, panel b; also see Figure 3B, panel e), but the levels of cyclin B2 increased continuously (Figure 3A, panel a) during the incubation of the oncogenic Ras extract. The low level of the H1 kinase activity was associated with persistent presence of p-tyr-15-Cdk1 throughout the incubation period (Figure 3A, panel c). However, following the addition of the constitutively active Cdc25B to the oncogenic Ras extract, p-tyr-15-Cdk1 became almost undetectable (Figure 3B, panel a; compare with Figure 3A, panel c) and the H1 kinase activity abruptly increased (Figure 3B, panel b; compare with Figure 3A, panel b; also see Figure 3B, panel f; compare panel f with panel e), indicating that the constitutively active Cdc25B dephosphorylated the p-tyr-15-Cdk1 to cause abrupt activation of the Cdk1. Note that the levels of total Cdk1 was almost constant during the incubations of both the oncogenic Ras extract (Figure 3A, panel d) and the oncogenic Ras + Cdc25B extract (Figure 3B, panel c). It should be noted that in Figure 3A (panel c), although tyr-15-Cdk1 remained phosphorylated in the oncogenic Ras extract, the level of p-tyr-15-Cdk1 was somewhat decreased during the incubation. However, in a separate set of experiments similar to those of Figure 3A (panels b and c) and Figure 3B (panels a and b) to determine the effects of the addition of constitutively active Cdc25B to another oncogenic Ras extract on the levels of p-tyr-15-Cdk1 and the histone H1 kinase activity, the results show that during the incubation of this oncogenic Ras extract, the level of p-tyr-15-Cdk1 did not decrease. Instead, it increased slightly during the incubation (Figure 3C, panel a). Moreover, similar to the results of Figure 3B (panels a and b), the addition of the constitutively active Cdc25B to this oncogenic Ras extract also abolished the p-tyr-15-Cdk1 (Figure 3C; compare panel b with panel a) and abruptly activated the histone H1 kinase (Figure 3C; compare panel d with panel c). What caused the difference in the levels of p-tyr-15-Cdk1 between these two oncogenic Ras extracts during the incubation was

unclear. Nevertheless, since tyr-15-Cdk1 remained phosphorylated during the incubation of these two oncogenic Ras extracts, and since the addition of a constitutively active Cdc25B, which has previously been shown to be able to dephosphorylate the inhibitory phosphorylation to activate Cdk1 (28, 29), to these oncogenic Ras extracts resulted in a complete removal of p-tyr-15-Cdk1 and an abrupt activation of the histone H1 kinase, these results are consistent with the concept that oncogenic Ras suppresses Cdk1 through induction of tyr-15-Cdk1 phosphorylation. It should be noted that since Ras is not a protein kinase, the induction of tyr-15-Cdk1 phosphorylation by oncogenic Ras is in an indirect manner.

Furthermore, in order to know whether the addition of the constitutively active Cdc25B to the oncogenic Ras extract may affect the oncogenic Ras-induced accumulation of cyclin B2, the samples from the oncogenic Ras + Cdc25B extract in Figure 3B were also subjected to Western blot analysis using anti-cyclin B2 (X121). The results (Figure 3B, panel d) show that as compared with cyclin B2 in the oncogenic Ras extract (Figure 3A, panel a), in which the cyclin B2 level increased continuously during the incubation, the cyclin B2 level remained almost constant after the addition of the Cdc25B to the oncogenic Ras extract. The result suggests that the addition of the constitutively active Cdc25B to the oncogenic Ras extract to activate the oncogenic Ras-suppressed Cdk1 could partially inhibit the oncogenic Ras-induced accumulation of cyclin B2.

Taken together, the results of Figures 3A-3C are consistent with the concept that oncogenic Ras suppresses Cdk1 activation through induction of persistent phosphorylation of tyr-15 Cdk1.

Oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2 mediated by constitutive activation of the Raf-Mek-Erk pathway

The results of Figure 1 show the intriguing observation that, during the incubation of activated egg extracts, oncogenic Ras was able to induce continuous accumulation of cyclin B2. Therefore, we wanted to know how oncogenic Ras induces the continuous accumulation, and addressed two questions. First, we wanted to know whether the oncogenic Ras-induced continuous accumulation of cyclin B2 involves the stabilization of cyclin B2 by oncogenic Ras. Second, because the Raf-Mek-Erk pathway is one of the well-characterized Ras-signaling pathways (34, 35, 36), and because it has been shown that oncogenic Ras can constitutively activate Raf, Mek and p42^{mapk} (a *Xenopus* Erk) in activated *Xenopus* egg extracts and/or in *Xenopus* oocyte extracts (37-41), we also wanted to know whether oncogenic Ras induces continuous accumulation of cyclin B2 through constitutive activation of the Raf-Mek-Erk pathway. In order to know whether oncogenic Ras induces continuous accumulation of cyclin B2 through stabilization of cyclin B2, we assessed the effect of oncogenic Ras on the stability of cyclin B2 in activated egg extracts that were incubated in the presence of cycloheximide, an inhibitor of protein synthesis. In order to know whether constitutive activation of the Raf-Mek-Erk pathway is responsible for the oncogenic Ras-induced continuous accumulation of cyclin B2, we determined the effect of U-0126, a specific inhibitor of Mek (42), on cyclin B2 levels in activated egg extracts incubated either in the absence or presence of oncogenic Ras. Briefly, two portions of an activated egg extract were incubated at room temperature either in the absence (the control extract) or presence of oncogenic Ras (the oncogenic Ras extract). Samples were collected during the incubation. In addition, at 30 minutes during the incubation, two aliquots each were collected from both the control extract and the oncogenic Ras extract. Then, cycloheximide (final 150 μ M) was added to one aliquot of the control extract and one aliquot of the oncogenic Ras extract. U-0126 (final 50 μ M) was added to the other remaining aliquots from the control extract and the oncogenic Ras extract. These four aliquots were

further incubated at room temperature. Samples were collected. All the samples were subjected to Western blot analysis using anti-cyclin B2 antibody X121.

Figure 4A shows the results with the control extract and the oncogenic Ras extract. Cyclin B2 increased, peaked at 150-min and then declined during the room temperature incubation of the control extract (Figure 4A, panel a), while cyclin B2 increased continuously in the oncogenic Ras extract (Figure 4A, panel b). The results are consistent with the results of Figure 1 showing that oncogenic Ras was able to induce continuous accumulation of cyclin B2. Figure 4B is the results from the control extract and the oncogenic Ras extract that contained cycloheximide. Panel a of Figure 4B shows the results from the control extract containing cycloheximide. Shortly after the addition of cycloheximide to inhibit new protein synthesis, cyclin B2 became almost undetectable during the subsequent incubation period (Figure 4B, panel a). This indicates that, in the control extract, almost all of the cyclin B2 synthesized prior to the addition of cycloheximide was degraded after the addition of cycloheximide. Panel b of Figure 4B shows the results from the oncogenic Ras extract containing cycloheximide. In contrast to the control extract containing cycloheximide, after the addition of cycloheximide to the oncogenic Ras extract, cyclin B2 did not decrease; instead it remained at the same level as when cycloheximide was added throughout the subsequent incubation period (Figure 4B, panel b). This indicates that cyclin B2 synthesized prior to the addition of cycloheximide was stable in the oncogenic Ras extract containing cycloheximide. Hence, the comparison of cyclin B2 levels between the oncogenic Ras extract and the control extract that were incubated in the presence of cycloheximide indicates that oncogenic Ras was able to stabilize cyclin B2.

Figure 4C shows the results from the control extract and the oncogenic Ras extract that contained U-0126. Panel b of Figure 4C shows the results from the oncogenic Ras extract containing U-0126. Following the addition of U-0126 at the 30 min-time point to the oncogenic Ras extract, the cyclin B2 level abruptly reduced to a barely detectable level (Figure 4C, panel b). However, cyclin B2 then slightly increased during the subsequent incubation period, although it did not increase back to the 30 min-time point level. This profile of U-0126 effect has the following implications concerning the Raf-Mek-Erk pathway's role in the oncogenic Ras-induced continuous accumulation of cyclin B2. First, because the addition of U-0126 to the oncogenic Ras extract could nearly (but not completely) eliminate the oncogenic Ras-induced accumulation of cyclin B2 (compare panel b in Figure 4C with panel b in Figure 4A), it indicates that the oncogenic Ras-induced continuous accumulation of cyclin B2 was primarily mediated by the Raf-Mek-Erk pathway. Second, the addition of U-0126 to the oncogenic Ras extract caused an abrupt decrease in the cyclin B2 level (Figure 4C, panel b, compare lane 70-min with lane 30-min). This finding suggests that activity of the Raf-Mek-Erk pathway was required for the stabilization of cyclin B2 that was synthesized prior to the addition of U-0126 to the oncogenic Ras extract, and therefore also suggests that activity of the Raf-Mek-Erk pathway was required for the stabilization of cyclin B2 in the oncogenic Ras extract. Together with the results of Figure 4B which show that oncogenic Ras was able to stabilize cyclin B2, this result supports the notion that activity of the Raf-Mek-Erk pathway mediates the oncogenic Ras-induced stabilization of cyclin B2. Third, although the cyclin B2 level initially decreased after the addition of U-0126 to the oncogenic Ras extract, the level then slightly increased, which suggests that activity of the Raf-Mek-Erk pathway might not be necessary for the synthesis of new cyclin B2 in the oncogenic Ras extract. Taken together, these results support the notion that the oncogenic Ras-induced continuous accumulation of cyclin B2 is primarily mediated by activity of the Raf-Mek-Erk pathway, which induces the accumulation of cyclin B2 mainly by stabilizing cyclin B2.

Panel a of Figure 4C shows the results from the control extract containing U-0126. The profile of the U-0126 effect on cyclin B2 in the control extract was similar to that of the oncogenic Ras extract containing U-0126. Thus, following the addition of U-0126 at the 30-min time point to the control extract, the cyclin B2 level abruptly dropped to an almost undetectable level. Cyclin B2 then slightly increased, although it did not return to the 30-min time point level (Figure 4C, panel a). This U-0126 profile has the following implications concerning the Raf-Mek-Erk pathway's potential role in the accumulation of cyclin B2 to the peak-level in the control extract. First, as compared with cyclin B2 levels in the control extract, which peaked at 150-min (Figure 4A, panel a), the addition of U-0126 to the control extract prevented cyclin B2 from accumulating to the 150-min peak-level during the subsequent incubation period (compare panel a in Figure 4C with panel a in Figure 4A). This implies that activity of the Raf-Mek-Erk pathway might be required for the accumulation of cyclin B2 to the peak-level during the incubation of the control extract. Second, the initial abrupt drop in cyclin B2 level following the addition of U-0126 to the control extract (Figure 4C, panel a, compare lane 70-min with lane 30-min) implies that, in the control extract, activity of the Raf-Mek-Erk pathway might be required for the stabilization of cyclin B2 that was synthesized prior to the addition of U-0126 to the control extract. Third, the increase in cyclin B2 level that occurred after the initial drop following the addition of U-0126 to the control extract (Figure 4C, panel a, compare lane 360-min with lane 70-min) implies that activity of the Raf-Mek-Erk pathway might not be necessary for the synthesis of new cyclin B2 in the control extract. These results together imply that activity of the Raf-Mek-Erk pathway, via stabilization of cyclin B2, might also be required for the accumulation of cyclin B2 to the peak-level during the incubation of the control extract.

Taken together, the results from Figures 4A, 4B and 4C are consistent with the following concept. During the incubation of activated egg extracts, the activity of the Raf-Mek-Erk pathway, via stabilization of cyclin B2, is required for the continuous accumulation of cyclin B2 in the oncogenic Ras extract and for the accumulation of cyclin B2 to the peak-level in the control extract. It is presumed that because oncogenic Ras can constitutively activate the Raf-Mek-Erk pathway, oncogenic Ras is able to induce continuous accumulation of cyclin B2. It is also presumed that because U-0126 can block the oncogenic Ras-induced constitutive activation of the Raf-Mek-Erk pathway, U-0126 is able to nearly eliminate the oncogenic Ras-induced accumulation of cyclin B2. If this concept is true, it is expected that the Raf-Mek-Erk pathway should be constitutively active in the oncogenic Ras extract, but not in the control extract, and that this oncogenic Ras-induced constitutive activation should be blocked in the oncogenic Ras extract containing U-0126. To assess this concept, we determined whether the Raf-Mek-Erk pathway was constitutively active in the oncogenic Ras extract and whether the addition of U-0126 to the oncogenic Ras extract could block the constitutive activation of the Raf-Mek-Erk pathway. Because p90^{rsk} is a physiological substrate of Erk (36, 43) and p90^{rsk2} is the predominant isoform of p90^{rsk} in *Xenopus* eggs (44), and because activated p90^{rsk} is phosphorylated and undergoes electrophoretic mobility up-shift in polyacrylamide gel, we thus assessed the activation state of the Raf-Mek-Erk pathway by determining the mobility up-shift of p90^{rsk2} by Western blot analysis using an anti-p90^{rsk2} antibody. Samples from Figures 4A (the control extract and the oncogenic Ras extract) and 4C (the control extract containing U-0126 and the oncogenic Ras extract containing U-0126) were subjected to Western blot analysis. Figures 4D and 4E show the results with the samples from Figures 4A and 4C, respectively. As compared with the control extract (Figure 4D, panel a), p90^{rsk2} underwent mobility up-shift during the incubation of the oncogenic Ras extract (Figure 4D, panel b), indicating that the Raf-Mek-Erk pathway was constitutively active in the oncogenic Ras extract. As compared with the oncogenic Ras extract (Figure 4D, panel b), the addition of U-0126 to the oncogenic Ras extract inhibited the oncogenic Ras-induced mobility up-shift of p90^{rsk2} (Figure 4E, panel

b), indicating that U-0126 blocked the oncogenic Ras-induced constitutive activation of the Raf-Mek-Erk pathway. Therefore, the results of Figures 4D and 4E confirm that the Raf-Mek-Erk pathway was constitutively active in the oncogenic Ras extract, but not in the control extract, and that the addition of U-0126 to the oncogenic Ras extract blocked the oncogenic Ras-induced constitutive activation of the Raf-Mek-Erk pathway.

All together, the results from Figure 4 are consistent with the concept that oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2, which is mediated by constitutive activation of the Raf-Mek-Erk pathway. If this concept is true, it is further expected that if U-0126 and cycloheximide are co-added to activated egg extracts containing oncogenic Ras to block both activity of the Raf-Mek-Erk pathway and synthesis of new cyclin B2, it should abolish the oncogenic Ras-induced stabilization of cyclin B2 and result in the complete elimination of cyclin B2 accumulation in activated egg extracts containing oncogenic Ras. We then determined whether this is true. An activated egg extract containing oncogenic Ras (the oncogenic Ras extract) was prepared. Four aliquots of the oncogenic Ras extract were incubated at room temperature. Samples were collected at 40 min and 160 min. After sampling at 160 min, mock (a buffer containing 1 mM DTT and 10 mM Hepes, pH 7.8), U-0126 (final 50 μ M), cycloheximide (final 150 μ M), or both U-0126 (final 50 μ M) and cycloheximide (final 150 μ M) was respectively added to the remaining portions of the four aliquots of the oncogenic Ras extract, which were then further incubated at room temperature. Samples were collected. All samples were subjected to Western blot analysis using anti-cyclin B2 antibody (X121). Figure 5 shows the results. In the oncogenic Ras extract with mock (Figure 5, panel a), the cyclin B2 level increased continuously during the incubation. In the oncogenic Ras extract with U-0126 alone (Figure 5, panel b), after the addition of U-0126, cyclin B2 initially decreased abruptly but then slightly increased, although it did not increase back to the level at the time of U-0126 addition. In the oncogenic Ras extract with cycloheximide alone (Figure 5, panel c), after the addition of cycloheximide cyclin B2 remained stable during the subsequent incubation period. These results are thus consistent with the results of panels b of Figures 4A, 4B and 4C. Panel d of Figure 5 shows the results from the oncogenic Ras extract with both U-0126 and cycloheximide. As compared with panel c of Figure 5, which shows that cyclin B2 was stable in the oncogenic Ras extract with cycloheximide alone, the co-addition of U-0126 and cycloheximide to the oncogenic Ras extract now completely abolished the otherwise stable cyclin B2 (Figure 5, compare panel d with panel c), confirming that activity of the Raf-Mek-Erk pathway was responsible for the oncogenic Ras-induced stabilization of cyclin B2. Moreover, as compared with panel a of Figure 5, which shows that cyclin B2 accumulated continuously in the oncogenic Ras extract with mock, the co-addition of U-0126 and cycloheximide to the oncogenic Ras extract now also completely eliminated the accumulation of cyclin B2 in the oncogenic Ras extract (Figure 5, compare panel d with panel a). Therefore, Figure 5 further supports the concept that oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2, which is mediated by constitutive activation of the Raf-Mek-Erk pathway.

Immunodepletion of p90^{rsk2} from activated egg extracts prevents oncogenic Ras from inducing cyclin B2 accumulation

As mentioned above, p90^{rsk} is a physiological substrate for Erk (36, 43) and p90^{rsk2} is the predominant isoform of p90^{rsk} in *Xenopus* eggs (44). In order to know whether p90^{rsk2} is responsible for the oncogenic Ras-induced, the Raf-Mek-Erk pathway-mediated continuous accumulation of cyclin B2, we assessed the effect of p90^{rsk2} depletion from activated egg extracts on the oncogenic Ras-induced continuous accumulation of cyclin B2. The depletion of p90^{rsk2} from activated *Xenopus* egg extracts was performed as previously described (33). Three aliquots of an activated egg extract were untreated, treated with mock IgG-coated

protein A-beads or with anti-p90^{rsk2} antibody-coated protein A-beads, respectively, as described in the Materials and Methods. After the removal of the beads, the untreated, mock IgG-beads-treated, and anti-p90^{rsk2}-beads-treated extracts were incubated at room temperature in the presence of oncogenic Ras. Samples were subjected to Western blot analysis using anti-p90^{rsk2} and anti-cyclin B2 (X121) antibodies. The results show that during the incubation of the untreated extract, oncogenic Ras induced both a mobility up-shift of p90^{rsk2} and continuous accumulation of cyclin B2 (Figure 6, panel a). Similar results were obtained during the incubation of the mock IgG-beads-treated extract (Figure 6, panel b). In contrast, in the anti-p90^{rsk2}-beads-treated extract, p90^{rsk2} was depleted and little cyclin B2 was detected (Figure 6, panel c). Hence, the results show that the depletion of p90^{rsk2} from activated egg extracts could prevent oncogenic Ras from inducing cyclin B2 accumulation. The results thus indicate that p90^{rsk2} was responsible for the oncogenic Ras-induced continuous accumulation of cyclin B2. This finding, together with the results from Figures 4 and 5, is consistent with the concept that oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2, which is mediated by constitutive activation of the Raf-Mek-Erk-p90^{rsk} pathway.

In conclusion, our results indicate that, during the incubation of activated *Xenopus* egg extracts, oncogenic Ras suppresses Cdk1 activation through induction of persistent inhibitory phosphorylation of tyr-15-Cdk1. Our results also reveal that oncogenic Ras is able to induce continuous accumulation of cyclin B2. Our results further indicate that oncogenic Ras induces continuous accumulation of cyclin B2 primarily through stabilization of cyclin B2, which is mediated by constitutive activation of the Raf-Mek-Erk-p90^{rsk} pathway. Because cyclin B2 is stimulatory to Cdk1 activation, while tyr-15-Cdk1 phosphorylation is inhibitory to Cdk1 activation, our results thus indicate that oncogenic Ras suppresses Cdk1 in a complex manner during the incubation of activated *Xenopus* egg extracts. The results will provide important insight into understanding how oncogenic Ras suppresses M-phase entry/progression in normal primary cells. How oncogenic Ras induces persistent inhibitory phosphorylation of tyr-15-Cdk1 remains unclear; however, there is evidence suggesting that activity of Erk and p90^{rsk} is involved (45-49). How p90^{rsk2} stabilizes cyclin B2 is also unclear, although there is evidence suggesting that it involves the inhibition of ubiquitin-mediated degradation of cyclin B (50-53). In the future, it will be interesting to further study the mechanisms by which oncogenic Ras induces persistent inhibitory phosphorylation of tyr-15-Cdk1 and stabilizes cyclin B2.

Finally, it is interesting to discuss whether the oncogenic Ras-induced persistent phosphorylation of tyr-15-Cdk1 and the oncogenic Ras-induced accumulation of cyclin B2 are functionally related. It has been known that cyclin B is degraded by the ubiquitin-mediated proteolytic process (51-53). In this process, cyclin B is first ubiquitinated by the anaphase promoting complex /cyclosome (APC/C) (51, 54, 55). The ubiquitinated cyclin B is then degraded by the proteasome (51, 56). It has also been shown that the activity of Cdk1 is critical to the activation of APC/C, through phosphorylation of components of the APC/C (57-60). It thus suggests that the suppression of Cdk1 by oncogenic Ras through persistent phosphorylation of tyr-15-Cdk1 would inhibit the activation of APC/C and thus decrease the degradation of cyclin B2 (or increase the accumulation of cyclin B2). On the contrary, the activation of the oncogenic Ras-suppressed Cdk1 by Cdc25B would stimulate APC/C and hence increase the degradation of cyclin B2 (or decrease the oncogenic Ras-induced accumulation of cyclin B2). Our results (Figure 3B, panel d), which show that activation of the oncogenic Ras-suppressed Cdk1 by constitutively active Cdc25B was able to partially inhibit the oncogenic Ras-induced accumulation of cyclin B2, is consistent with this concept.

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Highlights

We study how oncogenic Ras suppresses Cdk1

Oncogenic Ras suppresses Cdk1 via induction of persistent inhibitory phosphorylation of Cdk1

Oncogenic Ras stabilizes cyclin B2

The stabilization of cyclin B2 is mediated by the Raf-Mek-Erk-p90^{rsk} pathway

Our results indicate that oncogenic Ras suppresses Cdk1 in a complex manner

Figure 1. Oncogenic Ras induces continuous accumulation of cyclin B2 but suppresses the activation of the histone H1 kinase (Cdk1) during the incubation of activated egg extracts

To two 400 μ l aliquots of an activated egg extract, either a buffer solution containing 1 mM DTT and 10 mM Hepes, pH 7.8, or a purified, bacterially expressed human oncogenic Ras^H in the buffer solution (final $\sim$ 5 μ g/ml extract) was added respectively (designated as the control extract or the oncogenic Ras extract, respectively), and the aliquots were then incubated at room temperature, as described in the Materials and Methods. Samples were collected during the incubation and subjected to both the histone H1 kinase assay (**1A**) and Western blot analysis using anti-cyclin B2 antibody (X121) (**1B**) and anti-PSTAIRE antibody (**1C**), as described in the Materials and Methods. Autoradiograms: Panels a of 1A, 1B and 1C, the control extract; panels b of 1A, 1B and 1C, the oncogenic Ras extract. Conditions are indicated on each panel. Note that in 1A, the relative density of H1 bands to the background in each autoradiogram was also quantitated as described in the Materials and Methods. The values were normalized to the value of the initial time-point of the autoradiogram, and expressed as relative fold change in bar graphs.

Figure 2. Cyclin B2 binds to p13^{suc1}-agarose beads

Two aliquots of an activated egg extract were incubated at room temperature in either the absence or the presence of oncogenic Ras (designated as the control extract or the oncogenic Ras extract, respectively) as described in Figure 1. Aliquots were collected at 30 minutes and 240 minutes during the incubation, diluted 20-fold with a buffer, and then treated with p13^{suc1}-beads as described in the Materials and Methods. After the treatment, the p13^{suc1}-beads were precipitated by centrifugation. Equal volumes of samples from the diluted extracts prior to p13^{suc1}-bead treatment (panel a) and from the supernatants after p13^{suc1}-bead treatment and centrifugation (panel b), and equal volumes of the eluates from pellets of the p13^{suc1}-beads (panel c) were subjected to SDS-PAGE and Western blot analysis using anti-cyclin B2 antibody (X121), as described in the Materials and Methods. Autoradiogram: Conditions are indicated on each panel. The band of cyclin B2 is indicated. Almost all of the cyclin B2 in both the diluted control extract and the diluted oncogenic Ras extract prior to the p13^{suc1}-bead treatment is removed by p13^{suc1}-beads (compare panel b with panel a). Note that the bands below cyclin B2 in panels a and b are non-specific.

A **B** **B**

Figure 3. Oncogenic Ras suppresses the histone H1 kinase (Cdk1) via persistent phosphorylation of tyr-15-Cdk1

(3A) The suppression of the histone H1 kinase is correlated with persistent phosphorylation of tyr-15-Cdk1. A 200 μ l aliquot of an activated egg extract was incubated at room temperature in the presence of oncogenic Ras (final ~ 5 μ g/ml extract) (designated as the oncogenic Ras extract). Samples were collected and subjected to the histone H1 kinase assay and Western blot analysis using anti-cyclin B2 (X121), anti-PSTAIRE and anti-p-tyr-15-Cdk1 antibodies. Autoradiograms: Conditions are indicated on each panel. (3B, 3C) The addition of a constitutively active Cdc25B dephosphorylates p-tyr-15-Cdk1 and abruptly activates the histone H1 kinase. (3B) A portion of the oncogenic Ras extract described in 3A was collected at 180 minutes during the room temperature incubation. Then a constitutively active Cdc25B (final ~ 10 μ g/ml extract) was added to this portion (designated as the oncogenic Ras + Cdc25B extract), which was then further incubated at room temperature. Samples were collected and subjected to both the histone H1 kinase assay and Western blot analysis as described in 3A. Autoradiograms: Conditions are indicated on each panel. Note that Figure 3B (panels e and f) are the same results as Figure 3A (panel b) and Figure 3B (panel b), respectively. In Figure 3B (panels e and f), the relative density of H1 bands to the background in each autoradiogram was quantitated. The values were normalized to the value of the initial time-point in the autoradiogram, and expressed as relative fold change in bar graphs. Also note that the addition of the Cdc25B to the oncogenic Ras extract

dephosphorylates p-tyr-15-Cdk1 (compare panel a in 3B with panel c in 3A) and abruptly activates the histone H1 kinase (compare panel b in 3B with panel b in 3A). **(3C)** An aliquot from another activated egg extract was similarly incubated in the presence of oncogenic Ras as described in 3A. A portion of the oncogenic Ras extract was collected at 180 minutes during the incubation and constitutively active Cdc25B was added to this portion (designated as the oncogenic Ras + Cdc25 B extract) as described in 3B. The oncogenic Ras + Cdc25B extract was further incubated at room temperature. Samples collected from the oncogenic Ras + Cdc25B extract were similarly analyzed as described in 3B.

Autoradiograms: Conditions are indicated on each panel. Note that like the results in Figure 3B, the addition of the Cdc25B to the oncogenic Ras extract also dephosphorylates p-tyr-15-Cdk1 (3C; compare panel b with panel a) and abruptly activates the histone H1 kinase (3C; compare panel d with panel c). The total Cdk1 is unchanged (3C, panels e and f). It should also be noted that the experiments for 3C are originally a part of the experiments with Figure 1. The autoradiograms of Figure 3C (panel c and panel e), which show the histone H1 kinase activity and total Cdk1 level during the incubation of the oncogenic Ras extract, are from Figure 1A (panel b) and Figure 1C (panel b), respectively.

Figure 4. The effects of cycloheximide and U-0126 on cyclin B2 levels in activated egg extracts incubated either in the absence or presence of oncogenic Ras

(4A) Oncogenic Ras induces continuous accumulation of cyclin B2. Two 400 μ l aliquots of an activated egg extract were incubated at room temperature either in the absence (the control extract) or the presence of oncogenic Ras (the oncogenic Ras extract) as described in Figure 1. Samples were collected and subjected to Western blot analysis using anti-cyclin B2 (X-121). Autoradiograms: Panel a, the control extract; panel b, the oncogenic Ras extract. Conditions are indicated on each panel. The band of cyclin B2 is indicated. Note that the band below cyclin B2 is a non-specific band, and its level does not significantly change during the incubation. (4B) The effect of cycloheximide on cyclin B2 levels: Oncogenic Ras stabilizes cyclin B2 in the presence of cycloheximide. Cycloheximide (final 150 μ M) was added to a 100 μ l aliquot that was collected from either the control extract or the oncogenic Ras extract described in 4A at 30 minutes during the room temperature incubation. Both the cycloheximide-containing aliquot of the control extract (panel a) and the cycloheximide-containing aliquot of the oncogenic Ras extract (panel b) were further incubated at room temperature. Samples were collected and subjected to Western blot analysis using anti-cyclin B2 (X121). Autoradiograms: Conditions are indicated on each

panel. The band of cyclin B2 is indicated. Note that cyclin B2 synthesized prior to the addition of cycloheximide is stable in the oncogenic Ras extract but not in the control extract (compare panel b with panel a). The band below cyclin B2 is a non-specific band, and its level does not significantly change during the incubation. (4C) The effect of U-0126 on cyclin B2 levels: U-0126 dramatically reduces cyclin B2 accumulation in the oncogenic Ras extract. U-0126 (final 50 μ M) was added to another 100 μ l aliquot that was also collected from either the control extract or the oncogenic Ras extract described in 4A at 30 minutes during the room temperature incubation. Both the U-0126-containing aliquot of the control extract (panel a) and the U-0126-containing aliquot of the oncogenic Ras extract (panel b) were further incubated at room temperature. Samples were collected and subjected to Western blot analysis using anti-cyclin B2 (X121). Autoradiograms: Conditions are indicated on each panel. The band of cyclin B2 is indicated. Note that the addition of U-0126 to the oncogenic Ras extract nearly completely eliminates the oncogenic Ras-induced accumulation of cyclin B2 (compare panel b of 4C with panel b of 4A). The band below cyclin B2 is a non-specific band, and its level does not significantly changed during the incubation. (4D and 4E) Oncogenic Ras induces mobility up-shift of p90^{rsk2} and U-0126 blocks the oncogenic Ras-induced mobility up-shift. Samples from 4A and 4C were also subjected to Western blot analysis using an anti-p90^{rsk2} antibody. Autoradiograms: The conditions for 4D and 4E were as Figure 4A and 4C, respectively. Bands of p90^{rsk2} and pp90^{rsk2} (up-shifted p90^{rsk2}) are indicated. Lanes with pp90^{rsk2} are indicated by asterisks.

Figure 5. U-0126 completely blocks the oncogenic Ras-induced stabilization of cyclin B2

Four 100 μ l aliquots of an activated egg extract were incubated in the presence of oncogenic Ras (final ~ 5 μ g/ml extract) (designated as the oncogenic Ras extract) as described in Figure 1. Samples were collected at 40 min and 160 min. After sampling, mock (a buffer containing 1 mM DTT and 10 mM Hepes, pH 7.8), U-0126 (final 50 μ M), cycloheximide (final 150 μ M), or both U-0126 (final 50 μ M) and cycloheximide (final 150 μ M) was respectively added to the remaining portions of the four aliquots of the oncogenic Ras extract (designated as the oncogenic Ras + mock extract, the oncogenic Ras + U-0126 extract, the oncogenic Ras + cycloheximide extract, and the oncogenic Ras + U-0126 + cycloheximide extract, respectively). The aliquots were then further incubated at room temperature. Samples were collected and subjected to Western blot analysis using anti-cyclin B2 antibody (X121). Autoradiograms: Panel a, the oncogenic Ras + mock extract; panel b, the oncogenic Ras + U-0126 extract; panel c, the oncogenic Ras + cycloheximide extract; and panel d, the oncogenic Ras + U-0126 + cycloheximide extract. Conditions are indicated on each panel. The band of cyclin B2 is indicated. Note that the co-addition of U-0126 and cycloheximide to the oncogenic Ras extract completely abolishes the oncogenic Ras-induced stabilization of cyclin B2 (compare panel d with panel c) and eliminates all cyclin B2 accumulation in the oncogenic Ras extract (compare panel d with panel a). The band below cyclin B2 is a non-specific band, and its level does not significantly change during the incubation.

Figure 6. Immunodepletion of p90^{rsk2} from activated egg extracts prevents oncogenic Ras from inducing cyclin B2 accumulation

Immunodepletion of p90^{rsk2} from activated egg extracts was performed as described in the Materials and Methods. Untreated, mock IgG-beads-treated and anti-p90^{rsk2}-beads-treated activated egg extracts were incubated at room temperature in the presence of oncogenic Ras. Samples were collected and subjected to Western blot analysis using anti-p90^{rsk2} and anti-cyclin B2 (X121) antibodies. Autoradiograms: Conditions are indicated on each panel. The bands of cyclin B2, p90^{rsk2} and pp90^{rsk2} are indicated; lanes with pp90^{rsk2} are indicated by asterisks.