Characterization of p96^{h2bk}: immunoreaction with an anti-Erk (extracellular-signal-regulated kinase) peptide antibody and activity in *Xenopus* oocytes and eggs

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We have shown previously that oncogenic Ras induces cell cycle arrest in activated *Xenopus* egg extracts [Pan, Chen and Lin (1994) J. Biol. Chem. **269**, 5968–5975]. The cell cycle arrest correlates with the stimulation of a protein kinase activity that phosphorylates histone H2b *in vitro* (designated p96^{h2bk}) [Chen and Pan (1994) J. Biol. Chem. **269**, 28034–28043]. We report here that p96^{h2bk} is likely to be p96^{ram}, a protein of approx. 96 kDa that immunoreacts with a monoclonal antibody (Mk-1) raised against a synthetic peptide derived from a sequence highly conserved in Erk1/Erk2 (where Erk is extracellular-signal-regulated kinase). This is supported by two lines of evidence. First, activation/inactivation of p96^{h2bk} correlates with upward/downward bandshifts of p96^{ram} in polyacrylamide gels. Secondly, both p96^{h2bk} and p96^{ram} can be immunoprecipitated by antibody

Mk-1. We also studied the activity of p96^{h2bk}/p96^{ram} in *Xenopus* oocytes and eggs. p96^{h2bk}/p96^{ram} was inactive in stage 6 oocytes, was active in unfertilized eggs, and became inactive again in eggs after fertilization. Since stage 6 oocytes are at G2-phase of the cell cycle, unfertilized eggs arrest at M-phase and eggs exit M-phase arrest after fertilization, the results thus indicate that p96^{h2bk}/p96^{ram} activity is cell cycle dependent. Moreover, microinjection of oncogenic Ras into fertilized eggs at the one-cell stage arrests the embryos at the two-cell stage, and this induced arrest is correlated with an inappropriate activation of p96^{h2bk}/p96^{ram}. The data are consistent with the concept that inappropriate activation of p96^{h2bk}/p96^{ram} plays a role in the cell cycle arrest induced by oncogenic Ras.

INTRODUCTION

Ras protein has versatile biological functions; apparently it accomplishes a range of cellular functions by binding to several protein effectors that control diverse pathways [1–3]. In mammalian cells, these effectors include Raf (a serine/threonine kinase) [4], phosphoinositide 3-kinase [5] and RAL guanine nucleotide dissociation stimulator (RAL-GDS), a guanine nucleotide exchange factor for Ral-GTPase [2]. The Raf effector pathway regulates proliferation and differentiation, and the phosphoinositide 3-kinase effector pathway controls cytoskeletal organization [1,6]. However, it is less clear what cellular activity is controlled by the Ral-GDS effector pathway. Efficient transformation of cells by Ras requires more than one of these effector pathways [6].

Activation of Ras stimulates the Raf, phosphoinositide 3-kinase and Ral-GDS pathways, thereby regulating their downstream targets in order to achieve versatile cellular functions. In the Raf effector pathway, activation of Ras triggers a kinase cascade that leads to transcriptional activity [4,7–9]. Ras activates Raf kinase, which in turn activates Mek1/Mek2 [where Mek is mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (Erk) kinase], and Mek1/Mek2 then activates Erk1/Erk2 (MAPK) [7,8,10,11]. Mammalian Erk1 shares 90 % sequence identity with Erk2 [12], which was originally identified in rat cells by Sturgill and his group as a 42 kDa MAPK

(p42^{mapk}) [13]. Erk1/Erk2 then activates other protein targets, including protein kinases such as p90^{rsk} [14] and transcription factors [8,9], to regulate cellular proliferation and differentiation.

Oncogenic Ras can induce cell cycle arrest in mammalian cells [15,16]. In *Xenopus*, stage 6 oocytes arrest at G2-phase of the first meiotic cell cycle. Treatment of stage 6 oocytes with progesterone *in vitro* induces these oocytes to undergo maturation: the G2-arrested oocytes re-enter the cell cycle and then arrest at M-phase of the second meiotic cell cycle [17,18]. Microinjection of oncogenic Ras into stage 6 oocytes also induces the G2-arrested oocytes to undergo maturation [19–21]. Moreover, in fertilized *Xenopus* eggs, microinjection of oncogenic Ras causes mitotic cell cycle arrest [22]. Two lines of evidence support the concept that the Raf/Mek/Erk effector pathway is involved in the control of the cell cycle by oncogenic Ras in the *Xenopus* egg and oocyte system. First, microinjection into *Xenopus* oocytes or fertilized *Xenopus* eggs of constitutively active Raf, Mek or p42^{mapk} induces the maturation of oocytes [23–28] and cell cycle arrest of fertilized eggs [29] in the absence of oncogenic Ras. Secondly, induction of oocyte maturation by oncogenic Ras can be suppressed by inhibition of Raf activity by dominant-inhibitory Raf [23,30] or by the inhibition of Mek activity by a neutralizing antibody against Mek [31].

Using activated *Xenopus* egg extracts prepared at the interphase of the cell cycle, which are capable of undergoing cell-free cell

Abbreviations used: p96^{h2bk}, a 96 kDa kinase that is activated by oncogenic Ras to phosphorylate histone H2b *in vitro* (h2bk stands for histone H2b kinase); p96^{ram}, a 96 kDa protein that immunoreacts with Mk-1, an anti-MAPK peptide antibody (ram stands for recognized by an antibody against MAPK); MAPK, mitogen-activated protein kinase; Erk, extracellular-signal-regulated kinase; Mek, MAPK/Erk kinase; p97^{mapk}, a 97 kDa Erk protein; p42^{mapk}, a 42 kDa Erk protein; H2b, histone H2b; PP2A, protein phosphatase 2A; DTT, dithiothreitol; OA, okadaic acid; EB, extraction buffer; MBM, modified Barth medium; GVBD, germinal vesicle breakdown; RAL-DGS, RAL guanine nucleotide dissociation stimulator.

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cycling [32,33], we have shown previously that adding oncogenic Ras to these extracts induces cell cycle arrest [34]. The cell cycle arrest correlates with the activation of *Xenopus* p42^{mapk} [35] and with the activation of a protein kinase of approx. 96 kDa that phosphorylates histone H2b *in vitro* (designated p96^{h2bk}) [35]. p96^{h2bk} is also activated by a constitutively active Mek [36]. Moreover, the activation of p96^{h2bk} by oncogenic Ras is inhibited when endogenous Mek is suppressed by a specific chemical inhibitor of Mek, PD 098059 [36]. The data indicate that p96^{h2bk} is a potential downstream target/component of the Raf/Mek pathway. Like other known kinase components of the Raf effector pathway, activation of p96^{h2bk} by oncogenic Ras requires serine/threonine phosphorylation [36].

We report here that a monoclonal antibody (Mk-1) raised against a synthetic peptide derived from a highly conserved sequence of Erk1/Erk2 immunoreacts with a protein of approx. 96 kDa (designated p96^{ram}; ram stands for recognized by an antibody against MAPK). Our results indicate that p96^{ram} is likely to be p96^{h2bk}. Moreover, p96^{ram} did not immunoreact with three other antibodies against fragments of Erks. We also show that, although p96^{h2bk}/p96^{ram} was inactive in stage 6 oocytes, it became active in mature oocytes (or unfertilized eggs), and was inactive again after fertilization.

MATERIALS AND METHODS

Materials

Adult male and female frogs were obtained from NASCO, Fort Atkinson, WI, U.S.A. Pregnant-mare serum, chorionic gonadotropin, calcium ionophore A23107, cytochalasin B, Ficoll, leupeptin and protein A-agarose were purchased from Sigma. β -Mercaptoethanol was from Fisher. Okadaic acid (OA) was purchased from Gibco. Guanidine hydrochloride was purchased from Acros, NJ, U.S.A.. Amquel was purchased from a local store. Histones H2B and HF1 were purchased from Worthington Biochemical. Rabbit anti-(mouse IgG) was from Cappel, West Chester, PA, U.S.A. [¹²⁵I]-Protein A and [³²P]ATP were from ICN. Protein phosphatase 2A (PP2A) was from UBI (cat. no. 14-111). Bacterially expressed human [Leu⁶¹]RasH was purified as previously described [20]. Constitutively active [Asp²¹⁸, Asp²²²]Mek [27] was kindly donated by Dr. Barbara Brott and Dr. Raymond Erikson. Antibody Mk-1 (M37520; Transduction Laboratories) was raised against a synthetic peptide (TDEP-VAEPPFTFDMEDDLDPK) derived from residues 325–345 of rat Erk1 [36a]. The anti-Erk2 antibody was raised against bacterially produced, near-full-length human Erk2 [38], and was kindly donated by Dr. J. L. Bos. The anti-pan-Erk antibody was raised against a rat Erk2 fragment (residues 219–358), and was from Transduction Laboratories (E17120). The anti-p97^{mapk} antibody was raised against a synthetic peptide derived from residues 172–190 of human p97^{mapk}, an Erk3 protein [39], and was from Transduction Laboratories (P48920).

Preparation of activated *Xenopus* egg extracts

Activated egg extracts were prepared as previously described [33,34]. Briefly, adult female *Xenopus* frogs were primed with pregnant-mare serum (75–100 units per frog) for several days, followed by injection of human chorionic gonadotropin (750–1000 units per frog). Eggs were either squeezed from the frogs the next day or obtained by allowing the frogs to lay eggs in tap water containing 110 mM NaCl overnight. The tap water had been conditioned with 0.5 ml of Amquel/litre to neutralize any chloramine present. The eggs were washed in a modified

Barth medium (MBM) containing 110 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM NaHCO₃ and 10 mM Hepes, pH 7.8 [20]. After incubation with 0.33 × MBM for 15 min, the eggs were de-jellied with 2 % cysteine in 0.33 × MBM, pH 7.8, and then transferred to 0.33 × MBM containing 5 % Ficoll. The eggs were activated by treatment with 10 μ M calcium ionophore A23187 for 20 min.

After activation, eggs were washed several times with extraction buffer (EB) containing 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 50 mM sucrose and 10 mM Hepes, pH 7.8. After the wash, eggs were dripped into centrifuge tubes (5 mm × 41 mm or 13 mm × 51 mm; Beckman) containing 0.1 ml or 0.25 ml of Versilube F-50 oil (General Electric) underneath EB containing 10 μ g/ml leupeptin and 100 μ g/ml cytochalasin B. The eggs were packed in a clinical centrifuge for 2 min at 150 *g* and for 2 min at 600 *g*. After all buffer and oil on top of the eggs had been removed, eggs were centrifuged at 4 °C in a SW55Ti rotor at 15000 *g* for 20 min. The cytosolic layers were collected and re-centrifuged after the addition of 10 μ g/ml leupeptin, 10 μ g/ml cytochalasin B, 1 mM dithiothreitol (DTT) and 0.05 vol. of a mixture containing 150 mM phosphocreatine, 20 mM ATP, 2 mM EGTA and 20 mM MgCl₂. After re-centrifugation, the cytosolic layers were collected. Activated egg extracts were either used directly or frozen at –80 °C and used for experiments after thawing.

Preparation of oocyte extracts

Xenopus oocyte extracts were prepared as follows. Oocytes that had been incubated with or without progesterone were washed in M-phase buffer containing 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 5 mM EGTA, 50 mM sucrose and 10 mM Hepes, pH 7.8 [33]. A total of 100 oocytes were homogenized in 50 μ l of M-phase buffer containing 10 μ g/ml cytochalasin B, 1 mM DTT, 10 μ g/ml leupeptin and 100 μ M PMSF. After centrifugation in an Eppendorf centrifuge at 5000 *g* for 10 min, the cytosolic layer was collected and re-centrifuged once. The cytosolic layers were then collected for analysis.

Preparation of extracts from fertilized and unfertilized eggs

Preparation of cytosolic extracts from de-jellied, unfertilized or fertilized eggs was similar to the method used for the preparation of activated extracts [34], except that de-jellied, unfertilized or fertilized eggs were washed and packaged in M-phase buffer instead of EB.

Preparation of fertilized *Xenopus* eggs

Ovulated *Xenopus* eggs were prepared as described above. To prepare fertilized eggs, eggs were pre-incubated in 0.33 × MBM for 10 min. Fragments of testes from adult *Xenopus* male frogs were added to the eggs and incubated for 10 min. The eggs were then de-jellied in 0.33 × MBM containing 2 % cysteine (pH 7.8) [37]. After removal of the jelly, the eggs were washed in 0.33 × MBM and were then placed in 0.33 × MBM containing 5 % Ficoll for microinjection.

Microinjection of fertilized *Xenopus* eggs

De-jellied fertilized *Xenopus* eggs prepared as described above were microinjected at the one-cell stage with 50 nl per egg of either oncogenic Ras protein solution (1 mg/ml in a buffer containing 1 mM DTT and 10 mM Hepes, pH 7.8) or buffer.

After microinjection, the eggs were placed at room temperature in $0.33 \times \text{MBM}$ containing 5% Ficoll.

Progesterone-induced oocyte maturation

Stage 6 *Xenopus* oocytes were isolated as previously described [20]. To induce oocyte maturation, stage 6 oocytes were incubated with 50 $\mu\text{g}/\text{ml}$ progesterone at room temperature. Germinal vesicle breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole of the oocyte [20].

Partial purification of p96^{h2bk} by SDS/PAGE

Active p96^{h2bk} was partially purified by SDS/PAGE from activated egg extracts pretreated with oncogenic Ras as previously described [36]. Briefly, oncogenic-Ras-treated egg extracts were centrifuged at 200 000 *g*. The supernatant was subjected to SDS/PAGE in 10–15% (w/v) polyacrylamide gels. Gels bands (without staining) in the 96 kDa region were sliced and the proteins were electroeluted in electrophoresis buffer. The eluted proteins were extensively dialysed in 0.05% β -mercaptoethanol/20 mM Hepes, pH 7.8. Alternatively, the eluted proteins in sealed dialysis tubings were further subjected to electroelution to remove SDS in SDS-free electrophoresis buffer containing 0.05% mercaptoethanol, and followed by dialysis in 0.05% mercaptoethanol/20 mM Hepes, pH 7.8. For protein phosphatase treatment, the eluted proteins were further dialysed in buffer containing 100 mM KCl, 1 mM MgCl_2 , 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ leupeptin and 20 mM Hepes, pH 7.8. For immunoprecipitation, the proteins were dialysed against buffer containing 100 mM NaCl, 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ leupeptin and 20 mM Tris, pH 7.4.

Immunoprecipitation

Active p96^{h2bk} was partially purified by SDS/PAGE as described above. Aliquots of 500 μl of p96^{h2bk}-containing solution were incubated with primary antibodies in a solution containing 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM DTT, 100 μM PMSF and 20 mM Tris, pH 7.4, at 4 °C overnight. After the incubation, 50 μl of Protein A beads pre-coated with rabbit anti-(mouse IgG) was added to each vial and the vials were incubated further. After washing, the immunoprecipitates were subjected to the in-the-gel histone H2b kinase assay and to Western blotting using the Mk-1 antibody (see below).

Western blot analysis

Western blotting was performed as previously described [34]. Briefly, samples were subjected to SDS/PAGE in 10–15% gradient gels. The proteins were blotted on to PVDF membranes. The membranes were blocked in buffer A containing 150 mM NaCl, 5% non-fat milk, 0.05% Tween 20, 0.02% sodium azide and 10 mM Tris, pH 7.4, followed by incubation at 4 °C overnight with various primary antibodies (1 $\mu\text{g}/\text{ml}$). After washing, the membranes were incubated further with rabbit anti-(mouse IgG) in buffer A at room temperature for 60 min. After washing, ^{125}I -Protein A (1 $\mu\text{Ci}/\text{ml}$) in buffer A was added, followed by further incubation at room temperature for 60 min. The membranes were washed and subjected to autoradiography.

In-the-gel H2b kinase assay

The in-the-gel H2b kinase assay was performed as previously described [35]. Briefly, samples were subjected to electrophoresis in 10% (w/v) polyacrylamide gels containing calf thymus histone

HF1 or HF2B fractions, which contained histone H2b, at a concentration of 0.2–0.4 mg/ml. After SDS/PAGE, the gels were treated at room temperature with 20% isopropyl alcohol/5 mM β -mercaptoethanol/50 mM Tris/HCl, pH 8.0, for 60 min, followed by treatment with 6 M guanidine hydrochloride/5 mM β -mercaptoethanol/50 mM Tris/HCl, pH 8.0, for 60 min. The gels were further treated with a buffer containing 0.04% Tween 40, 5 mM β -mercaptoethanol and 50 mM Tris/HCl, pH 8, at 4 °C overnight. For the kinase reaction, the treated gels were pre-incubated at 30 °C for 30 min with a kinase reaction buffer containing 10 mM MgCl_2 , 15 mM β -glycerophosphate, 0.1 mM EGTA, 2 mM DTT and 20 mM Tris, pH 7.2. The kinase reaction was initiated by replacing the pre-incubation buffer with buffer containing 50 μM ATP and 10 μCi of [^{32}P]ATP. The reaction was carried out at 30 °C for 30 min. The gels were washed extensively with 5% (w/v) trichloroacetic acid and 1% sodium pyrophosphate, and were dried for autoradiography.

SDS/PAGE

SDS/PAGE was performed according to the method of Laemmli [40].

RESULTS

An anti-Erk antibody immunoreacts with a protein of approx. 96 kDa that migrates upward in a polyacrylamide gel in response to oncogenic Ras in activated *Xenopus* egg extracts

We analysed activated egg extracts incubated with or without oncogenic Ras by Western blotting using ^{125}I -labelled Protein A and a monoclonal antibody (Mk-1) raised against a synthetic peptide corresponding to a highly conserved sequence in mam-

Figure 1 Western blot analysis using antibody Mk-1 of oncogenic-Ras-treated extracts

Two 50 μl aliquots of an activated *Xenopus* egg extract were incubated with or without oncogenic Ras (1 $\mu\text{g}/\text{ml}$ of extract) at room temperature for 90 min. Samples (each 2.5 μl) were subjected to Western blot using antibody Mk-1 and ^{125}I -labelled Protein A. The membrane was subjected to autoradiography. Autoradiograms: (a) exposure for 8 h; (b) the same membrane exposed for 2 days. Incubation conditions are indicated above each lane. p42^{mapk} is indicated; the 96 kDa protein is indicated as p96^{am}. The results shown in this and other Figures are representative of those obtained in more than one similar experiments.

Figure 2 Activation of $p96^{h2bk}$ by oncogenic Ras, constitutively active Mek or OA is correlated with an upward bandshift of $p96^{ram}$

Aliquots of 150 μ l of an activated egg extract were incubated under mock conditions (control) or with oncogenic Ras (1 μ g/ml of extract), constitutively active Mek (5 μ g/ml) or 1 μ M OA at room temperature. At 0, 20, 60 and 120 min after the incubation, samples were subjected to the in-the-gel H2b kinase assay [34] (a) and to Western blot using antibody Mk-1 and 125 I-Protein A (b). $p96^{h2bk}$ and $p96^{ram}$ are indicated.

malian and *Xenopus* Erk1/Erk2 [12,41,42]. Oncogenic Ras induced an upward bandshift in the electrophoretic migration of a 42 kDa protein (presumably $p42^{mapk}$) (Figure 1a), consistent with our and others' previous reports that oncogenic Ras activates $p42^{mapk}$ in the *Xenopus* oocyte and egg system [35,43–45]. Interestingly, the antibody also immunoreacted with a protein of approx. 96 kDa (Figure 1b), although detection of the 96 kDa band by autoradiography required a longer period of exposure than did $p42^{mapk}$ detection. Moreover, like $p42^{mapk}$, this 96 kDa protein underwent an upward bandshift in response to oncogenic Ras (Figure 1b). Hereafter we designate this 96 kDa protein as $p96^{ram}$. In addition to $p42^{mapk}$ and $p96^{ram}$, antibody Mk-1 also immunoreacted with a protein with a molecular mass of approx. 36 kDa. However, unlike $p42^{mapk}$ and $p96^{ram}$, treatment of extracts with oncogenic Ras did not change the migration of this 36 kDa protein in the gel.

As discussed in the Introduction section, we previously showed that, in activated *Xenopus* egg extracts, oncogenic Ras activates $p96^{h2bk}$. We also showed that active $p96^{h2bk}$ can be inactivated by treatment with protein phosphatase 1 and PP2A [36], indicating that activation of $p96^{h2bk}$ requires serine/threonine phosphorylation [46,47]. Given that phosphorylation of a protein commonly results in an upward bandshift of the protein in polyacrylamide gels, $p96^{h2bk}$ may also undergo an upward bandshift after activation by phosphorylation. Since $p96^{ram}$ has a molecular size similar to that of $p96^{h2bk}$ and can undergo an upward bandshift in response to oncogenic Ras, this raised the possibility that $p96^{ram}$ could be $p96^{h2bk}$. In this scenario, the slower-migrating $p96^{ram}$ represents phosphorylated, active $p96^{h2bk}$, and the faster-migrating $p96^{ram}$ is unphosphorylated, inactive $p96^{h2bk}$. If this is true, we anticipate that activation of $p96^{h2bk}$ by means other than oncogenic Ras should correlate with the conversion of the fast-migrating $p96^{ram}$ into a slower-migrating $p96^{ram}$, whereas inactivation of $p96^{h2bk}$ by a protein phosphatase should correlate with the opposite change in the migration of $p96^{ram}$. Moreover, antibody Mk-1 should immunoprecipitate both $p96^{h2bk}$ and $p96^{ram}$.

Activation/inactivation of $p96^{h2bk}$ correlates with upward/downward bandshifts of $p96^{ram}$

First, we determined whether activation of $p96^{h2bk}$ by means other than oncogenic Ras resulted in an upward bandshift of $p96^{ram}$. We previously showed that, in addition to oncogenic Ras, $p96^{h2bk}$ can be activated by constitutively active Mek, as well as by OA [36].

To determine whether activation of $p96^{h2bk}$ by constitutively active Mek or by OA causes an upward bandshift of $p96^{ram}$, we treated activated egg extracts with mock treatment (control), oncogenic Ras (1 μ g/ml of extract), constitutively active Mek (5 μ g/ml of extract) or 1 μ M OA. These extracts were incubated at room temperature. At 0, 20, 60 and 120 min after the incubation had started, samples were subjected to the in-the-gel H2b kinase assay for $p96^{h2bk}$ activity, as described previously [35], and to Western blotting using Mk-1 to assess the $p96^{ram}$ bandshift, as described above. Figure 2(a) shows that, in comparison with the control, $p96^{h2bk}$ was activated by oncogenic Ras, constitutively active Mek or OA. Activation was detectable 20 min after the incubation was started. Figure 2(b) shows that, in comparison with the control, incubation with oncogenic Ras, constitutively active Mek or OA all caused an upward bandshift of a fast-migrating $p96^{ram}$. The upward bandshift was also detectable at 20 min. Thus the results of Figures 2(a) and 2(b) show that activation of $p96^{h2bk}$ by oncogenic Ras, by constitutively active Mek or by OA is correlated with an upward bandshift of $p96^{ram}$.

We then determined whether inactivation of active $p96^{h2bk}$ by PP2A also correlates with conversion of slow-migrating into fast-migrating $p96^{ram}$. Active $p96^{h2bk}$ was partially purified by SDS/PAGE, as described previously [36], from egg extracts pre-incubated with oncogenic Ras. Two aliquots of a $p96^{h2bk}$ -containing solution were incubated at 30 °C with or without PP2A. After various incubation periods, samples were subjected to analyses for $p96^{h2bk}$ and $p96^{ram}$, as described above. For comparison, an additional aliquot of $p96^{h2bk}$ solution was treated with both PP2A and 50 nM OA and was similarly analysed.

Figure 3 Inactivation of $p96^{h2bk}$ by PP2A is correlated with a downward bandshift of $p96^{ram}$

Active $p96^{h2bk}$ was partially purified from oncogenic-Ras-treated extracts by SDS/PAGE, as described in the Materials and methods section. Portions of 200 μ l of a $p96^{h2bk}$ -containing solution (each from 100 μ l of egg extract) composed of 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10 μ g/ml leupeptin and 20 mM Hepes, pH 7.8, were incubated at 25 $^{\circ}C$ with 2 μ l of 0.2 unit/ μ l PP2A, with PP2A plus 50 nM OA, or in the absence of PP2A and OA (control). After 0, 30, 60 and 120 min of incubation, samples were subjected to the in-the-gel H2b kinase assay (a) and to Western blot using Mk-1 (b). $p96^{h2bk}$ and $p96^{ram}$ are indicated.

Figure 4 Immunoprecipitation of $p96^{h2bk}$ and $p96^{ram}$ by antibody Mk-1

Aliquots of 500 μ l of a $p96^{h2bk}$ -containing solution (each from approx. 125 μ l of oncogenic-Ras-treated activated egg extract), purified as described in the Materials and methods section, were subjected to immunoprecipitation using Mk-1, anti-pan-Erk antibody or mouse non-specific IgG, as indicated. The immunoprecipitates were then subjected to the in-the-gel H2b kinase assay (a) and to Western blot using Mk-1 (b). $p96^{h2bk}$ and $p96^{ram}$ are indicated.

Inactivation of $p96^{h2bk}$ by PP2A (Figure 3a) was correlated with a downward bandshift of $p96^{ram}$ (Figure 3b). Moreover, co-incubation with OA to prevent the inactivation of $p96^{h2bk}$ by PP2A (Figure 3a) blocked the downward bandshift of $p96^{ram}$ (Figure 3b). These results demonstrate that inactivation of $p96^{h2bk}$ correlates with the conversion of a slow-migrating into a fast-migrating $p96^{ram}$. Altogether, the above results demonstrate that the activation and inactivation of $p96^{h2bk}$ are correlated respectively with upward and downward bandshifts of $p96^{ram}$. Moreover, the observation that treatment of $p96^{ram}$ with PP2A induced a downward bandshift which was blocked by co-

Figure 5 Western blot analysis of OA-treated egg extracts with various anti-Erk antibodies

Activated egg extracts were incubated with or without 1 μ M OA at room temperature for 90 min. Samples were then subjected to Western blot using ^{125}I -Protein A and Mk-1 (a), an anti-Erk2 antibody (b), anti-pan-Erk antibody (c) or an anti- $p97^{mapk}$ antibody (d). For comparison, a Jurkat cell lysate, which was provided with the purchased anti- $p97^{mapk}$ antibody as a positive control for $p97^{mapk}$, was also included in the analysis using the anti- $p97^{mapk}$ antibody (lane 'mammalian cells'). $p96^{ram}$ and $p42^{mapk}$ are indicated; the 95 kDa protein is indicated by an arrow. Positions of molecular mass markers are indicated to the right of the gels (k = kDa).

incubation with OA indicates that the $p96^{ram}$ bandshift is a result of a serine/threonine phosphorylation/dephosphorylation reaction.

Antibody Mk-1 immunoprecipitates both $p96^{h2bk}$ and $p96^{ram}$

Next we set out to determine whether antibody Mk-1 can immunoprecipitate both $p96^{h2bk}$ and $p96^{ram}$. Active $p96^{h2bk}$, purified as described above, was incubated with both Mk-1 and Protein A beads. The immunoprecipitate was then subjected to both the in-the-gel H2b kinase assay and Western blotting using Mk-1, as described above. For comparison, similar experiments were performed using an anti-Erk antibody raised against residues 219–358 of rat Erk2 (anti-pan-Erk antibody) or non-specific IgG. Mk-1 was able to immunoprecipitate both $p96^{h2bk}$ (Figure 4a) and $p96^{ram}$ (Figure 4b). However, neither the anti-pan-Erk antibody nor the non-specific IgG was able to immunoprecipitate $p96^{h2bk}$ (Figure 4a) or $p96^{ram}$ (Figure 4b). These results suggest that $p96^{ram}$ is $p96^{h2bk}$. However, we cannot exclude the possibility that $p96^{ram}$ and $p96^{h2bk}$ are related proteins sharing immunoreactivity with Mk-1, or that $p96^{h2bk}$ is different from $p96^{ram}$ but forms a complex with it so that immunoprecipitation of $p96^{ram}$ by Mk-1 results in co-precipitation of $p96^{h2bk}$.

$p96^{h2bk}$ does not immunoreact with some other antibodies against Erk1, Erk2 and Erk3

Our observation that $p96^{ram}$ immunoreacted with Mk-1, a monoclonal antibody against a synthetic peptide derived from a sequence highly conserved in Erk1/Erk2, raised the possibility that $p96^{ram}$ is closely related to Erk1/Erk2. For example, $p96^{ram}$ might be a dimer of $p42^{mapk}$. We reasoned that, if $p96^{ram}$ is a

Figure 6 p96^{h2bk}/p96^{ram} activity in *Xenopus* oocytes during progesterone-induced maturation and in unfertilized and fertilized eggs

(a, b) p96^{h2bk}/p96^{ram} activity in *Xenopus* oocytes during progesterone-induced maturation. Stage 6 *Xenopus* oocytes were isolated as previously described [20]. Oocytes were incubated with 50 µg/ml progesterone in four separate groups, each containing 100 oocytes. At 0, 2, 4 and 18 h after the incubation, one group was examined for the appearance of mature oocytes and was frozen at -80 °C. The percentage of mature oocytes is expressed as % GVBD, given below each lane. Cytosolic extracts were then prepared from each group. Samples were subjected to the in-the-gel H2b kinase assay (a) and to Western blot using Mk-1 antibody (b). (c, d) p96^{h2bk}/p96^{ram} activity in unfertilized and fertilized *Xenopus* eggs. Ovulated *Xenopus* eggs were obtained and fertilized. Cytosolic extracts were prepared from unfertilized *Xenopus* eggs and from fertilized eggs at 20 and 50 min after fertilization. Samples were subjected to the in-the-gel H2b kinase assay (c) and to Western blot using Mk-1 (d). p96^{h2bk} and p96^{ram} are indicated.

dimer of p42^{mapk} or if it is closely related to Erk1/Erk2, then it should also immunoreact with other anti-Erk1/Erk2 antibodies. To test this possibility, we performed Western blots on activated egg extracts incubated with or without OA, which can induce an upward bandshift of p96^{ram} (Figure 2b), using Mk-1 as well as two other anti-Erk1/Erk2 antibodies: an anti-Erk2 antibody raised against a near-full-length product of human Erk2 [38] and the anti-pan-Erk antibody described above. Mammalian Erk1/Erk2 share 80% identity in amino acid sequence with *Xenopus* p42^{mapk} [12,41,42]. The results are shown in Figure 5. Both the anti-Erk2 antibody and the anti-pan-Erk antibody recognized a 42 kDa protein (presumably p42^{mapk}), which underwent an upward bandshift in response to OA (Figures 5b and 5c). In addition, both the anti-Erk2 antibody and the anti-pan-Erk antibody immunoreacted with a protein of approx. 95 kDa (indicated by arrows in Figures 5b and 5c). However, unlike p96^{ram}, which immunoreacted with Mk-1 and underwent an upward bandshift in response to OA (Figure 5a), this 95 kDa protein did not change its electrophoretic mobility in the presence of OA (Figures 5b and 5c), indicating that it is distinct from p96^{ram}. Taken together, these results showed that the anti-Erk2 and anti-pan-Erk antibodies immunoreacted with p42^{mapk} but

they did not immunoreact with p96^{ram}, indicating that p96^{ram} is not a potential dimer of p42^{mapk}.

Erk3 is a member of the Erk protein family [12,39]. In mammalian cells, a 97 kDa Erk3 protein (p97^{mapk}) has been identified [39]. Erk3 shares high sequence identity with Erk1/Erk2 in its N-terminus (residues 1–350) and is primarily differentiated from Erk1/Erk2 by the C-terminal extension [39]. The similarity in size between p97^{mapk} and p96^{ram} raised the possibility that p96^{ram} is a *Xenopus* homologue of mammalian p97^{mapk}. We thus investigated whether an antibody against mammalian p97^{mapk} immunoreacts with p96^{ram}. Extracts incubated with or without OA were subjected to Western blot using an anti-(human p97^{mapk}) antibody (Transduction Laboratories). For comparison, a Jurkat cell lysate was also similarly analysed. The anti-p97^{mapk} antibody recognized a protein of approx. 97 kDa (presumably p97^{mapk}) in the Jurkat cell lysate (Figure 5d; p97^{mapk} is indicated). The antibody also immunoreacted with a protein in activated *Xenopus* egg extracts that co-migrated with the p97^{mapk} of Jurkat cells (Figure 5d), suggesting that the *Xenopus* protein is a potential homologue of mammalian p97^{mapk}. However, unlike p96^{ram} (Figure 5a), this potential *Xenopus* p97^{mapk} did not undergo a bandshift in response to OA (Figure 5d), suggesting that this potential *Xenopus* p97^{mapk} is distinct from p96^{ram}.

p96^{h2bk}/p96^{ram} activity during progesterone-induced oocyte maturation

Stage 6 *Xenopus* oocytes arrest at G2-phase of the first meiotic cell cycle. Treatment of stage 6 oocytes *in vitro* with progesterone induces oocyte maturation: the oocytes are induced to re-enter the cell cycle and eventually arrest at M-phase of the second meiotic cell cycle [17,18]. A white spot appears in the animal pole of the mature oocyte (indicating GVBD). To determine the activity of p96^{h2bk}/p96^{ram} during progesterone-induced oocyte maturation, stage 6 oocytes were treated with 50 µg/ml progesterone. At various times after treatment, the oocytes were examined for the appearance of the white spot. Cytosolic extracts were then prepared from these oocytes and subjected to analyses for p96^{h2bk} activity and for p96^{ram} bandshift using Mk-1, as described above. Figure 6(a) shows that p96^{h2bk} remained inactive at 0, 2 and 4 h after progesterone treatment, but became active after 18 h. Consistent with this, Figure 6(b) shows that the migration of p96^{ram} (the fast-migrating form) remained unchanged at 0, 2 and 4 h after progesterone treatment, but it had undergone an upward bandshift at 18 h. None of the oocytes at 0, 2 and 4 h after progesterone treatment showed the white spot in their animal poles (0% GVBD), but all oocytes exhibited the white spot at 18 h after treatment (100% GVBD), indicating that the oocytes remained at G2-phase of the cell cycle at 0, 2 and 4 h after progesterone treatment, but were at M-phase of the second meiotic cell cycle at 18 h. These results are consistent with the concept that p96^{h2bk}/p96^{ram} is inactive at G2-phase of the meiotic cell cycle but becomes active at M-phase of the second meiotic cell cycle during *in vitro* oocyte maturation.

p96^{h2bk}/p96^{ram} activity in unfertilized and fertilized eggs

Unfertilized *Xenopus* eggs arrest at M-phase of the second meiotic cell cycle. After fertilization, the eggs exit the M-phase arrest and enter interphase of the mitotic cell cycle [37]. We determined the activity of p96^{h2bk}/p96^{ram} in unfertilized and fertilized *Xenopus* eggs. We prepared extracts from unfertilized eggs and from eggs at 20 min and 50 min after fertilization. Samples were subjected to analyses for p96^{h2bk} activity and for p96^{ram} bandshift. Figure 6(c) shows that p96^{h2bk} was active in

Figure 7 $p96^{h2bk}/p96^{ram}$ is activated by oncogenic Ras in fertilized *Xenopus* eggs

(a) Fertilized *Xenopus* eggs were microinjected at the one-cell stage with oncogenic Ras (50 nl per egg of a 1 mg/ml solution of oncogenic Ras) or a control solution containing 1 mM DTT and 10 mM Hepes, pH 7.8. Cytosolic extracts were prepared 30 min after microinjection from both groups of 40 eggs. Samples were subjected to the in-the-gel H2b kinase assay (left panel) and to Western blot using Mk-1 antibody (right panel). As a control, a sample from cytosolic extract of unfertilized eggs was also similarly analysed. $p96^{h2bk}$ and $p96^{ram}$ are indicated. (b) Oncogenic Ras induces cleavage arrest of fertilized eggs. Fertilized eggs were microinjected with oncogenic Ras (right panel) or a control solution (left panel) as described above. The injected eggs were then incubated at room temperature for several hours.

unfertilized eggs, but it became inactive after fertilization. Consistent with this, Figure 6(d) shows that slow-migrating $p96^{ram}$ present in unfertilized eggs underwent a downward bandshift after fertilization. These results demonstrate that $p96^{h2bk}/p96^{ram}$ was active in the unfertilized eggs, but became inactive after fertilization, consistent with the concept that $p96^{h2bk}/p96^{ram}$ is active at M-phase of the second meiotic cell cycle but becomes inactive after the exit from the M-phase arrest.

$p96^{h2bk}/p96^{ram}$ is activated by oncogenic Ras in fertilized eggs

We have shown that oncogenic Ras activates $p96^{h2bk}/p96^{ram}$ in activated egg extracts. To determine whether oncogenic Ras also activates $p96^{h2bk}$ in fertilized eggs, we microinjected oncogenic Ras or a control solution into one-cell-stage fertilized eggs. At 30 min after injection, cytosolic extracts were prepared from the injected eggs for the determination of $p96^{h2bk}$ activity and $p96^{ram}$ bandshift. For comparison, a sample of cytosolic extract from unfertilized eggs was also similarly analysed. Compared with fertilized eggs injected with control solution, $p96^{h2bk}$ was activated in fertilized eggs injected with oncogenic Ras (Figure 7a, left panel). The level of activity was comparable with that of unfertilized eggs (Figure 7a, left panel). In keeping with this

result, $p96^{ram}$ underwent an upward bandshift in response to oncogenic Ras (Figure 7a, right panel). Since $p96^{h2bk}/p96^{ram}$ was active in fertilized eggs injected with oncogenic Ras but remained inactive in the controls, the data demonstrate that oncogenic Ras activates the otherwise inactive $p96^{h2bk}/p96^{ram}$ in fertilized eggs. Moreover, fertilized eggs that were microinjected with oncogenic Ras at the one-cell stage remained arrested at the two-cell stage several hours after injection (Figure 7b, right panel), while the control eggs continued to divide (Figure 7b, left panel), suggesting that the inappropriate or premature activation of $p96^{h2bk}/p96^{ram}$ may play a role in the cleavage arrest induced by oncogenic Ras.

DISCUSSION

We have shown previously that oncogenic Ras stimulates a protein kinase activity, designated $p96^{h2bk}$, in activated *Xenopus* egg extracts. We report here that $p96^{h2bk}$ is likely to be $p96^{ram}$, a protein of approx. 96 kDa that immunoreacts with a monoclonal antibody (Mk-1) raised against a synthetic peptide derived from a sequence highly conserved in Erk1/Erk2. Two lines of evidence support this conclusion. First, activation of $p96^{h2bk}$ by oncogenic Ras, by constitutively active Mek or by OA is correlated with an upward bandshift of $p96^{ram}$ in polyacrylamide gels. In addition, inactivation of $p96^{h2bk}$ by PP2A is correlated with a downward bandshift of $p96^{ram}$, consistent with our previous result that activation of $p96^{h2bk}$ requires serine/threonine phosphorylation. Secondly, both $p96^{h2bk}$ and $p96^{ram}$ could be specifically immunoprecipitated by antibody Mk-1. The results indicate that $p96^{h2bk}$ and $p96^{ram}$ are likely to be identical, although we cannot exclude the possibility that they are two structurally related proteins sharing immunoreactivity with Mk-1 or that they are two different proteins that form a complex with each other.

Our observation that $p96^{ram}$ immunoreacts with Mk-1 but not with two other antibodies against fragments of Erk1/Erk2 suggested that $p96^{h2bk}/p96^{ram}$ is not a potential dimer of $p42^{mapk}$. It is worthwhile to discuss further the anti- $p97^{mapk}$ immunoblots. It is possible that the anti- $p97^{mapk}$ antibody might recognize only the unphosphorylated form, but not the putative phosphorylated form, of $p97^{mapk}$. Thus the fact that the anti- $p97^{mapk}$ antibody detected only one form of putative *Xenopus* $p97^{mapk}$ (presumably the unphosphorylated form) (Figure 5d) could not exclude the possibility that the putative *Xenopus* $p97^{mapk}$ is $p96^{ram}$. However, the combined results from Figures 5(a) and 5(d) argue against this possibility. Figure 5(a) shows that the fast-migrating (presumably unphosphorylated) $p96^{ram}$ appeared to be completely converted into the slow-migrating (presumably phosphorylated) $p96^{ram}$ in the presence of OA. Therefore, if the putative $p97^{mapk}$ is $p96^{ram}$, then the former should also be completely converted into a slow-migrating form (phosphorylated form) and become undetectable by the anti- $p97^{mapk}$ antibody in the extract incubated with OA. However, in Figure 5(d), both the migration and the amount of the putative *Xenopus* $p97^{mapk}$ were not significantly changed by OA, arguing against the possibility that the putative $p97^{mapk}$ is $p96^{ram}$.

Erks other than Erk1, Erk2 and Erk3 have been identified or predicted [9,48,49]. It is possible that $p96^{h2bk}/p96^{ram}$ is related to one of these Erks. Alternatively, $p96^{h2bk}/p96^{ram}$ may not be related to Erks, but may simply share an epitope with Erk1/Erk2. Whether or not $p96^{h2bk}/p96^{ram}$ is related to Erks requires further analysis.

In *Xenopus*, stage 6 oocytes arrest at G2-phase of the first meiotic cell cycle. These oocytes can be induced *in vitro* to undergo maturation by progesterone. Like ovulated eggs, these mature oocytes arrest at M-phase of the second meiotic cell cycle.

p96^{h2bk}/p96^{ram} remained inactive before GVBD but became active after GVBD during progesterone-induced oocyte maturation. This is consistent with the concept that p96^{h2bk}/p96^{ram} activity is cell-cycle dependent during oocyte maturation: it is inactive at G2-phase of the first meiotic cell cycle, but is active at M-phase of the second meiotic cell cycle. Ovulated, unfertilized *Xenopus* eggs arrest at M-phase of second meiotic cell cycle. After fertilization, the eggs exit M-phase and enter the mitotic cell cycle. p96^{h2bk}/p96^{ram} was active in ovulated, unfertilized eggs, but became inactive after fertilization. Thus p96^{h2bk}/p96^{ram} was inactive in G2-phase oocytes, became active in M-phase-arrested mature oocytes or unfertilized eggs, and was inactive again after exit from the M-phase arrest after fertilization. These data are consistent with the concept that activation of p96^{h2bk}/p96^{ram} plays a physiological role in triggering or maintaining the M-phase arrest of unfertilized eggs.

Oncogenic Ras activates p96^{h2bk}/p96^{ram} in activated egg extracts; such an effect was detectable at 20 min after oncogenic Ras was added to the extracts, while in control extracts p96^{h2bk}/p96^{ram} remained inactive (Figure 2). Microinjection of fertilized eggs at the one-cell stage with oncogenic Ras induced p96^{h2bk}/p96^{ram} activation, while in control fertilized eggs p96^{h2bk}/p96^{ram} remained inactive. Thus our results demonstrate that oncogenic Ras inappropriately activates p96^{h2bk}/p96^{ram} both *in vitro* and *in vivo*. It has been reported [22] that microinjection of oncogenic Ras into fertilized eggs arrests the division of the embryos. Consistent with this, microinjection of one-cell-stage fertilized eggs with oncogenic Ras induced the eggs to arrest at the two-cell stage, while the control embryos continued to divide. As discussed in the Introduction section, the Raf/Mek pathway is responsible for the cell cycle arrest induced by oncogenic Ras. We have shown previously that p96^{h2bk} is a potential downstream target of the Raf/Mek pathway [36]. Thus our data are consistent with the concept that inappropriate or premature activation of p96^{h2bk}/p96^{ram} by oncogenic Ras through the Raf/Mek pathway plays a role in induced cell cycle arrest.

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