

## RHO-associated protein kinase $\alpha$ potentiates insulin-induced MAP kinase activation in *Xenopus* oocytes

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### SUMMARY

We recently identified *Xenopus* Rho-associated protein kinase  $\alpha$  (xROK $\alpha$ ) as a *Xenopus* insulin receptor substrate-1 binding protein and demonstrated that the non-catalytic carboxyl terminus of xROK $\alpha$  binds *Xenopus* insulin receptor substrate-1 and blocks insulin-induced MAP kinase activation and germinal vesicle breakdown in *Xenopus* oocytes. In the current study we further examined the role of xROK $\alpha$  in insulin signal transduction in *Xenopus* oocytes. We demonstrate that injection of mRNA encoding the xROK $\alpha$  kinase domain or full length xROK $\alpha$  enhanced insulin-induced MAP kinase activation and germinal vesicle breakdown. In contrast, injection of a kinase-dead mutant of xROK $\alpha$  or pre-incubation of oocytes with an xROK $\alpha$  inhibitor significantly reduced insulin-induced MAP kinase activation. To further dissect the mechanism by which xROK $\alpha$  may participate in insulin

signalling, we explored a potential function of xROK $\alpha$  in regulating cellular Ras function, since insulin-induced MAP kinase activation and germinal vesicle breakdown is known to be a Ras-dependent process. We demonstrate that whereas injection of mRNA encoding c-H-Ras alone induced xMAP kinase activation and GVBD in a very low percentage (about 10%) of injected oocytes, co-injection of mRNA encoding xROK $\alpha$  and c-H-Ras induced xMAP kinase activation and germinal vesicle breakdown in a significantly higher percentage (50-60%) of injected oocytes. These results suggest a novel function for xROK $\alpha$  in insulin signal transduction upstream of cellular Ras function.

Key words: Insulin, Protein serine/threonine kinase, Signal transduction, Ras, *Xenopus* oocyte

### INTRODUCTION

Rho-associated protein kinase  $\alpha$  (ROK $\alpha$ , also known as ROCKII) was first identified as a potential effector of the small monomeric GTPase Rho (Leung et al., 1995). ROK $\alpha$  and its isoform ROK (Rho-kinase, ROCKI) are serine/threonine protein kinases that contain an amino-terminal catalytic kinase domain, a central coiled coil domain within which Rho/GTP binds, and a carboxyl-terminal pleckstrin homology (PH) domain which is split by a cysteine-rich region (Leung et al., 1995, 1996; Matsui et al., 1996; Ishizaki et al., 1996). These relatively large proteins (molecular mass of about 160 kDa) appear to have diverse biological functions. For example, a specific chemical inhibitor of ROK kinases (Y-27632) is capable of inhibiting smooth muscle contraction both in vitro and in animals (Uehata et al., 1997), implicating ROK kinases in controlling muscle contraction. Using both Y-27632 and various mutants of ROK, studies have demonstrated a role of the kinase in regulating neurite retraction in neuroblastoma cells, a process involving both actin and the microtubule cytoskeleton (Hirose et al., 1998; Katoh et al., 1998). ROK kinases appear to play an essential role in mediating Rho

function during cytokinesis in cleaving *Xenopus* embryos (Yasui et al., 1998) but not in fibroblast cells in culture (Madaule et al., 1998). Instead, another kinase, citron (distantly related to ROK kinases), appears to mediate Rho function during cytokinesis in fibroblasts (Madaule et al., 1998). In addition to these physiological functions which involve actin and the microtubule cytoskeleton, ROK kinases have also been implicated in transcriptional activation of serum responsive element, perhaps through activation of the c-Jun kinase (Sahai et al., 1998; Chihara et al., 1997).

We have recently identified the *Xenopus* homolog of ROK $\alpha$  (xROK $\alpha$ ) as a *Xenopus* insulin receptor substrate 1 (xIRS-1) binding protein in a yeast two-hybrid screening (Farah et al., 1998). We further demonstrated that the non-catalytic carboxyl terminus of xROK $\alpha$  binds xIRS-1 and blocks insulin-induced MAP kinase activation in *Xenopus* oocytes. In the current study, we have examined the effect of injecting various xROK $\alpha$  constructs on insulin-induced xMAP kinase activation. We demonstrate that the wild-type xROK $\alpha$  or its kinase domain potentiated insulin-induced xMAP kinase activation, whereas a kinase-dead xROK $\alpha$  mutant or the ROK inhibitor Y-27632 reduced insulin-induced xMAP kinase activation. Finally, we

present evidence that xROK $\alpha$  might function upstream of Ras in insulin signal transduction.

## MATERIALS AND METHODS

### Materials

Y-27632 was a generous gift from Yoshitomi Pharmaceutical Industries, Ltd, and was dissolved in water as a 100 $\times$  stock and stored in aliquots at  $-70^{\circ}\text{C}$ . Aliquots of Y-27632 were thawed out before each use and any unused portion was discarded. Anti-xMAP kinase serum was produced by immunizing rabbits with a 15-amino acid peptide derived from the carboxyl terminus of Xp42MAPK (Posada and Cooper, 1992) covalently coupled to Keyhole limpet haemocyanin using glutaraldehyde according to the method of Harlow and Lane (1988). The serum was used at a 1:1000 dilution for immunoblotting or 5  $\mu\text{l}$  per 150  $\mu\text{l}$  oocyte lysate (from 15 oocytes) for immunoprecipitation (in the MAP kinase assay). Anti-phosphor MAP kinase antibodies were purchased from Upstate Biotechnology and were used at 1  $\mu\text{g/ml}$ . Anti-xMOS serum (Sagata et al., 1988) was from N. Sagata and was used at a 1:500 dilution. Anti-Myc ascites (used at a 1:1000 dilution) were produced in our laboratory using a hybridoma (9E10) provided by M. Tyers. All immunoblots were detected using an ECL kit (Amersham) together with the appropriate secondary antibodies.

### Animal and oocyte manipulation

These procedures are essentially the same as described previously (Farah et al., 1998) with the following modifications. First, insulin stimulation was carried out in regular OR2 rather than potassium-free OR2 as described previously (Farah et al., 1998). Second, the concentrations of insulin and progesterone were 1  $\mu\text{M}$  unless otherwise stated. mRNA was transcribed using the MessageMachine kit from Ambion with SP6 polymerase. The resulting mRNA was resuspended in water to a final concentration of 1 mg/ml, as compared to RNA standards in electrophoresis on agarose gel. Unless otherwise indicated, 10 ng of mRNA were injected per oocyte. For DNA injection, DNA prepared by Qiagen's plasmid DNA isolation kit was diluted in water to 10 pg per nl; 5-15 nl (see Fig. 8) were injected directly into the nucleus of oocytes through the animal pole without actually visualizing the nucleus. Typically, our success rate, as assessed by lysing individual oocytes and immunoblotting for the expressed protein, was better than 75% (not shown).

### Construction of expression plasmids

The full-length xROK $\alpha$  was sub-cloned into the expression vector pCS2+ (Turner and Weintraub, 1994). The resulting plasmid contained the entire xROK $\alpha$  coding sequence (Farah et al., 1998) without an exogenous epitope tag. xROK-KD was identical to xROK $\alpha$  with the exception of a point mutation replacing the catalytically essential lys-107 with ala. xROK-K, which contains the catalytic kinase domain, was derived by inserting  $\lambda$ 63 (amino acids 32-837 of xROK $\alpha$ ; Farah et al., 1998) into the expression vector pCS2+MT (Turner and Weintraub, 1994) following appropriate manipulation of the cloning sites. The resulting plasmid encoded 6 copies of a Myc tag followed by LNSRPLEEIRGVS (an unrelated amino acid sequence resulting from vector manipulation) and then by amino acids 32-837 of xROK $\alpha$  (Farah et al., 1998). xROK-KA was identical to xROK-K with the exception of the same point mutation as xROK-KD (lys107ala).

The entire coding sequence of xIRS-1, except for the initiating ATG codon (Liu et al., 1995) was subcloned into pCS2+MT following appropriate manipulation. The resulting plasmid encodes 6 copies of the Myc tag followed by amino acids 2-1088.

The murine c-H-Ras cDNA (Ebinu et al., 1998) (provided by J. Stone) was amplified by polymerase chain reaction with Bg/III linkers and subcloned into the pSP64TM vector. pSP64TM is a modified

version of pSP64T (Krieg and Melton, 1984) which includes a translational initiation site (by I. MacLauglan). The resulting plasmid encoded an untagged version of murine c-H-Ras. v-H-Ras was a gift from M. Wigler (Birchmeier et al., 1985) and has previously been used in our laboratory (Farah et al., 1998).

A haemagglutinin (HA) tagged version of mouse RasGRF/CDC25<sup>Mm</sup> (Mattingly and Macara, 1996) was provided by Ian Macara and injected directly as DNA into the nuclei of oocytes.

### In vitro kinase assays

We used the same procedure for all of our in vitro kinase assays (xROK $\alpha$ , xMAP kinase and xIGF-1 receptor). Briefly, immunoprecipitates were washed twice with ice-cold PBS lysis buffer (Farah et al., 1998), twice with kinase buffer (50 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] or HEPES, pH 7.3, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 1 mM DTT, 0.05% Triton X-100). The washed beads were resuspended in 30  $\mu\text{l}$  kinase buffer. To initiate the kinase reaction, ATP (1  $\mu\text{Ci}$  [ $\gamma$ -<sup>32</sup>P]ATP, 10  $\mu\text{M}$ ) and, in the case of xROK $\alpha$  or xMAP kinase, myelin basic protein (Sigma, used at 100  $\mu\text{g/ml}$ ) was added. Following a 15 minute incubation at room temperature, the kinase reaction was stopped by the addition of 30  $\mu\text{l}$  of 2 $\times$  SDS sample buffer and boiling. The samples were then analysed by SDS-PAGE and autoradiography.

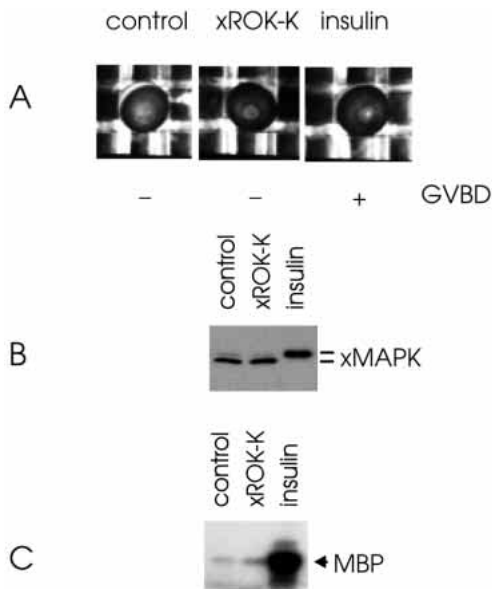
## RESULTS

### xROK $\alpha$ causes depigmentation in *Xenopus* oocytes

We injected mRNA encoding the N-terminal kinase domain and part of the coiled coil domain (xROK-K) into stage VI *Xenopus* oocytes. Following an overnight incubation to allow for protein synthesis, we observed depigmentation of the animal pole in the majority (typically between 80-100%) of injected oocytes (Fig. 1A, xROK-K). The pattern of depigmentation was strikingly similar to that observed following an overnight incubation of uninjected oocytes with insulin (Fig. 1A, insulin). However, upon dissection of the oocytes, we found that unlike insulin-treated oocytes which had undergone germinal vesicle breakdown (GVBD), oocytes injected with xROK-K contained intact GV's. To confirm that the xROK-K mRNA-injected oocytes had not undergone resumption of meiosis, we tested for the activation of *Xenopus* MAP kinase, a hallmark kinase involved in resumption of meiosis (Gebauer and Richter, 1997; Sagata, 1997). As shown in Fig. 1B, xMAP kinase from control oocytes were mostly in the unphosphorylated (inactive) form (Posada and Cooper, 1992). In contrast, xMAP kinase from insulin-incubated oocytes, which had undergone GVBD (Fig. 1A), were mostly in the phosphorylated (activated) form (Posada and Cooper, 1992). Injection of xROK-K mRNA did not result in phosphorylation (activation) of xMAP kinase (Fig. 1B), consistent with a lack of GVBD response (Fig. 1A). To confirm the kinase activity of xMAP kinase in the various samples, we performed immune kinase assays using myelin basic protein as a substrate. Fig. 1C shows that indeed xMAP kinase from insulin treated oocytes were active whereas that from either control oocytes or oocytes that had been injected with xROK-K were inactive.

### xROK-K potentiates insulin-induced xMAP kinase activation and GVBD

Typically we incubated oocytes overnight in the presence of 1  $\mu\text{M}$  insulin before assessing GVBD or xMAP kinase



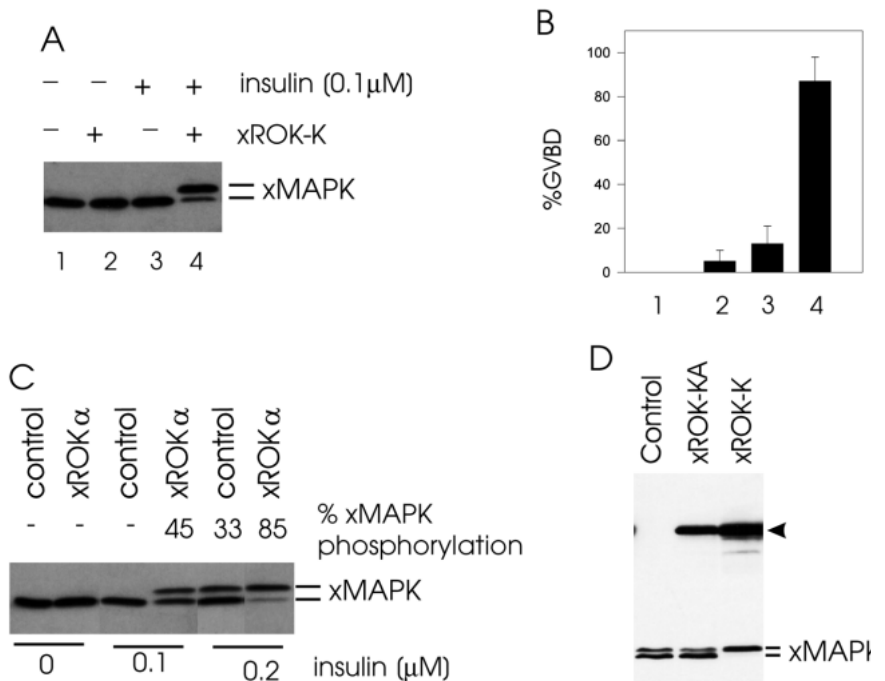
**Fig. 1.** Effect of xROK-K on oocyte morphology. (A) Stage VI *Xenopus* oocytes were incubated overnight in OR2 (control) or OR2 containing 1  $\mu$ M insulin or injected with 5 ng of x-ROK-K mRNA followed by an overnight incubation in OR2 (xROK-K). A typical oocyte from each group was photographed. The GVBD status was confirmed by dissection. (B) 20-30 oocytes from each group (as in A) were lysed and a fraction of the lysate (usually representing 1/4 of one oocyte) were analysed by SDS-PAGE followed by immunoblotting with anti-xMAP kinase antibodies which recognize both the phosphorylated (upper) and unphosphorylated (lower) forms. (C) The same lysates (each representing about 15 oocytes) were immunoprecipitated with anti-xMAP kinase antibodies followed by an immune kinase assay using myelin basic protein (MBP) as a substrate. The kinase reactions were analysed by SDS-PAGE followed by autoradiography. Shown are phosphorylated MBP.

activation. However, to determine whether xROK-K had any influence on insulin-induced xMAP kinase activation in *Xenopus* oocytes, we employed a sub-optimal concentration of insulin (0.1  $\mu$ M) which, following an overnight incubation at 18°C, only induced low percentages (0-20%, Fig. 2B) of control oocytes to undergo GVBD, with little xMAP kinase activation (Fig. 2A, lane 3). A prior injection of xROK-K mRNA increased the GVBD response to >80% (Fig. 2B), with correspondingly high levels of xMAP kinase activation (Fig. 2A, lane 4). The GVBD status of xROK-K mRNA-injected

oocytes was assessed by dissection following fixation with trichloroacetic acid. Similar to xROK-K, the full-length xROK $\alpha$  also enhanced insulin-induced xMAP kinase activation at both 0.1 and 0.2  $\mu$ M insulin (Fig. 2C).

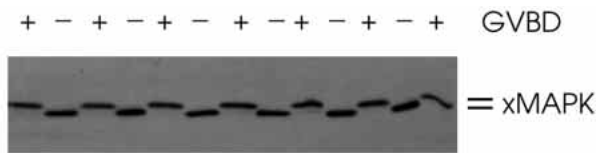
To ensure that the effect of xROK-K was due to the kinase activity of xROK $\alpha$  and not to other sequence contained within the xROK-K construct, we changed the catalytically essential lysine residue (lys-107) (Farah et al., 1998) to alanine. Injection of the mutant (xROK-KA) mRNA did not induce depigmentation of oocytes (not shown), nor did it enhance insulin-induced MAP kinase activation (Fig. 2D) or GVBD (not shown). In multiple experiments involving oocytes injected with xROK-KA, we did not observe any inhibition or delay in insulin response when compared with oocytes injected with water (not shown). This was in contrast to the full-length

**Fig. 2.** xROK enhances insulin-induced MAP kinase activation and GVBD. (A and B) Oocytes injected with water (-) or xROK-K mRNA (+) were incubated in OR2 until about 50% of the xROK-K-injected oocytes exhibited depigmentation (Fig. 1A) (usually 5-8 hours were required). Oocytes were divided into groups of at least 25 oocytes each and incubated in OR2 (-) or OR2 containing 0.1  $\mu$ M insulin (+) overnight. Oocytes were lysed for xMAP kinase immunoblotting (A) or were assessed for GVBD following fixation with 5% trichloroacetic acid (B). (B) A summary of three similar experiments with standard errors. (C) Oocytes injected with water (control) or full-length xROK mRNA were incubated in OR2 until about 50% of the xROK-injected oocytes exhibited depigmentation (Fig. 1A) (usually 6-10 hours were required). Oocytes were then divided into groups of at least 25 and incubated overnight in OR2 (0) or OR2 containing the indicated concentrations of insulin. Oocytes were then lysed for anti-xMAP kinase immunoblotting. Shown is a representative of several similar experiments. The % xMAP kinase phosphorylation was calculated as the intensity ratio of upper band vs the sum of upper and lower bands based on scanning of the X-ray film. The - sign indicates a negligible amount of the upper form. (D) Groups of 30 or more oocytes injected with water (control), xROK-K, or xROK-KA mRNA were incubated in OR2 until about 50% of the xROK-K-injected oocytes exhibited depigmentation (Fig. 1A). Insulin was added to a final concentration of 0.1  $\mu$ M and the oocytes were incubated overnight. Oocytes were lysed for anti-xMAP kinase immunoblotting. Expression of the xROK-K and xROK-KA (approximately 80 kDa) was detected by anti-Myc western blotting (arrowhead).



Group	%GVBD
1 (Control, -)	~0
2 (xROK-K, -)	~5
3 (Control, +)	~15
4 (xROK-K, +)	~85

Insulin ( $\mu$ M)	Control	xROK $\alpha$
0	-	-
0.1	45	33
0.2	85	-



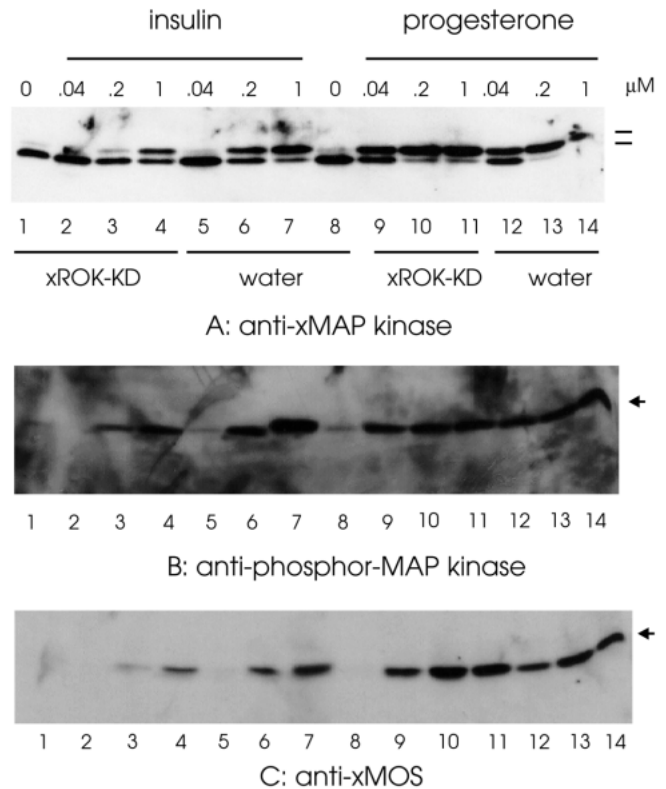
**Fig. 3.** All-or-none xMAP kinase activation in insulin-stimulated oocytes. Oocytes were incubated overnight in OR2 containing 0.2  $\mu$ M insulin. Oocytes with clear GVBD status (+; Fig. 1A) and GVBD negative oocytes (-) were lysed individually for anti-xMAP kinase immunoblotting.

xROK $\alpha$  bearing the same point mutation (see below) or a construct containing the carboxyl terminus of xROK $\alpha$  (Farah et al., 1998).

We noticed that the ratio of the upper, phosphorylated xMAP kinase vs the lower, unphosphorylated xMAP kinase in a given experiment always closely resembled the ratio of GVBD positive vs GVBD negative oocytes. To further explore the relationship between xMAP kinase activation and GVBD, we assessed xMAP kinase phosphorylation (activation) in individual oocytes following an overnight incubation with a sub-optimal concentration (0.2  $\mu$ M) of insulin. Fig. 3 shows that oocytes that had undergone GVBD (+) contained only the upper, phosphorylated form of xMAP kinase whereas the GVBD negative oocytes contained only the lower, unphosphorylated xMAP kinase. This all-or-none biochemical conversion of xMAP kinase in *Xenopus* oocytes, which was first reported by Ferrell and Machleder (1998) with progesterone as a stimulus, was also true with oocytes which underwent v-Ras-induced GVBD (not shown). Therefore, when a group of oocytes had reached maximum response to hormones (usually an overnight incubation with insulin or progesterone), the ratio of the upper form of xMAP kinase vs the lower form of xMAP kinase represented that of GVBD positive vs GVBD negative oocytes. This correlation was particularly important in the current study since injection of xROK-K or xROK $\alpha$  induced depigmentation (Fig. 1A) which interfered with visual assessment of the GVBD status of the injected oocytes.

#### A dominant negative mutant of xROK $\alpha$ or a ROK-specific inhibitor interferes with insulin-induced xMAP kinase activation

Over-expression of the non-catalytic carboxyl terminus of xROK $\alpha$  specifically inhibits insulin-induced xMAP kinase activation (Farah et al., 1998) whereas over-expression of the xROK $\alpha$  kinase domain (xROK-K) potentiates it (Fig. 2). These data suggest that endogenous xROK $\alpha$  may be required for insulin signalling in *Xenopus* oocytes. To test this further, we employed two approaches. In the first approach, we introduced lys107ala mutation into the full-length xROK $\alpha$ . The resulting mutant (xROK-KD) significantly reduced xMAP kinase activation at both 1  $\mu$ M insulin (an optimal concentration; Fig. 4A, comparing lane 7 to lane 4) and at 0.2  $\mu$ M insulin (a sub-optimal concentration; comparing lane 6 to lane 3). In contrast, xROK-KD did not affect progesterone-induced xMAP kinase activation, at either an optimal concentration (1  $\mu$ M; lane 11 vs lane 14) or a sub-optimal concentration (0.04  $\mu$ M, lane 9 vs lane 12). Fig. 4B shows only the upper (phosphorylated) form of xMAP kinase as it was recognized by anti-phosphor-MAP kinase antibodies, designed against the mammalian p42 and

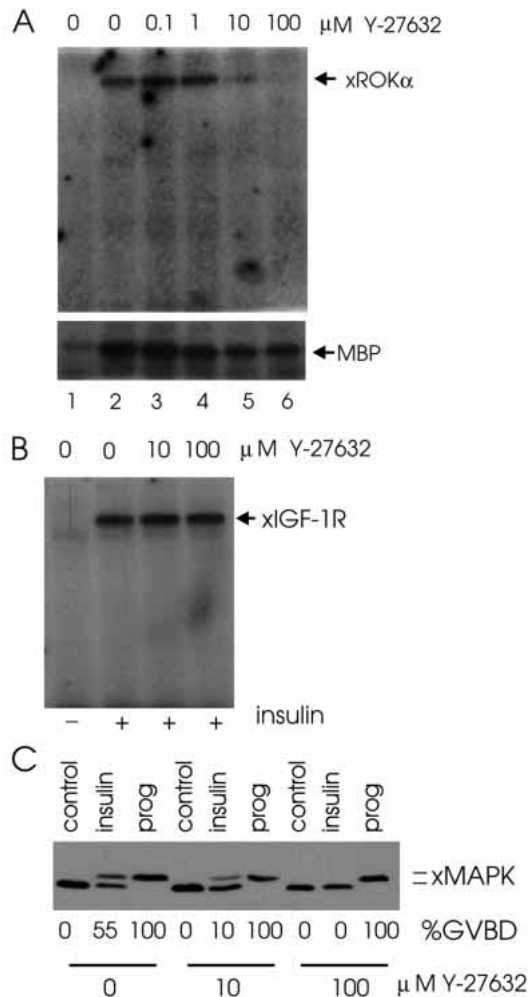


**Fig. 4.** xROK-KD inhibits insulin-induced xMOS synthesis and xMAP kinase activation. Oocytes injected with water or xROK-KD mRNA were incubated overnight in OR2. Water-injected or xROK-KD-injected oocytes were then divided into groups of 20 oocytes each and further incubated with the indicated concentrations of insulin or progesterone for an additional 16-24 hours. Following this incubation, oocytes were lysed and samples were analysed by SDS-PAGE and immunoblotting with the indicated antibodies.

p44 MAP kinases (Upstate Biotechnology). As is evident from the comparison, anti-xMAP kinase immunoblotting (Fig. 4A), which revealed the ratio of phosphorylated (activated) vs unphosphorylated (inactive) forms of xMAP kinase and was therefore not influenced by sample loading, had the advantage of more accurately reflecting the degree of xMAP kinase activation in a given sample.

Unlike insulin-induced MAP kinase activation in somatic cells which occurs rapidly (within minutes) (De Vries-Smiths et al., 1992), xMAP kinase activation in oocytes occurs several hours following insulin stimulation. The delayed xMAP kinase activation in oocytes is due to the requirement of *Xenopus* MOS (xMOS) synthesis (Sagata et al., 1988). To determine whether xROK-KD also inhibited insulin-induced xMOS synthesis, we immunoblotted the same samples with anti-xMOS antibodies. In Fig. 4C it can be seen that xROK-KD significantly reduced insulin-induced xMOS synthesis (compare lane 7 to lane 4 or lane 6 to lane 3). As might be expected, xROK-KD did not affect progesterone-induced xMOS synthesis (Fig. 4C, lanes 9 through 14). These data further demonstrated the specificity of xROK-KD as an inhibitor of insulin signalling. Moreover, they indicate that endogenous xROK $\alpha$  functions upstream of xMOS synthesis in the insulin signalling pathway.

The involvement of endogenous xROK $\alpha$  in insulin



**Fig. 5.** Y-27632 inhibits xROK $\alpha$  kinase activity in vitro and blocks insulin signalling in vivo. (A) Uninjected and untreated oocytes were lysed. Oocyte lysates (each sample was 200  $\mu$ l, from 20 oocytes) were immunoprecipitated with either pre-immune serum (lane 1) or anti-ROK $\alpha$  serum (Farah et al., 1998). The immunoprecipitates were pre-incubated for 30 minutes (at room temperature) with the indicated concentrations of Y-27632. Immune kinase assays were performed (Farah et al., 1998). Indicated are autophosphorylated xROK $\alpha$  and phosphorylation of myelin basic protein (MBP). (B) xIGF-1 receptor was purified from unstimulated oocytes and incubated with 1  $\mu$ M insulin to achieve receptor activation (Cummings et al., 1996). Activated receptors were then pre-incubated with 10 or 100  $\mu$ M Y-27632 for 30 minutes before being subjected to an autophosphorylation assay. Indicated are autophosphorylated receptors. (C) Groups of at least 25 oocytes each were incubated in OR2 (0) or OR2 containing 10 or 100  $\mu$ M Y-27632 for 2 hours. Insulin (1  $\mu$ M) or progesterone (prog, 1  $\mu$ M) was added and incubation continued overnight. Oocytes were lysed and lysates were subjected to western blotting with anti-xMAP kinase.

signalling in oocytes was also tested using a recently described chemical inhibitor of ROK kinases (Uehata et al., 1997). Y-27632 is one of a series of pyridine derivatives that cause smooth muscle relaxation by specifically inhibiting ROK $\alpha$  and ROK $\beta$  (Uehata et al., 1997). To test whether Y-27632 also inhibits xROK $\alpha$ , we carried out immune kinase assays in the presence of increasing concentrations of Y-27632. Pre-

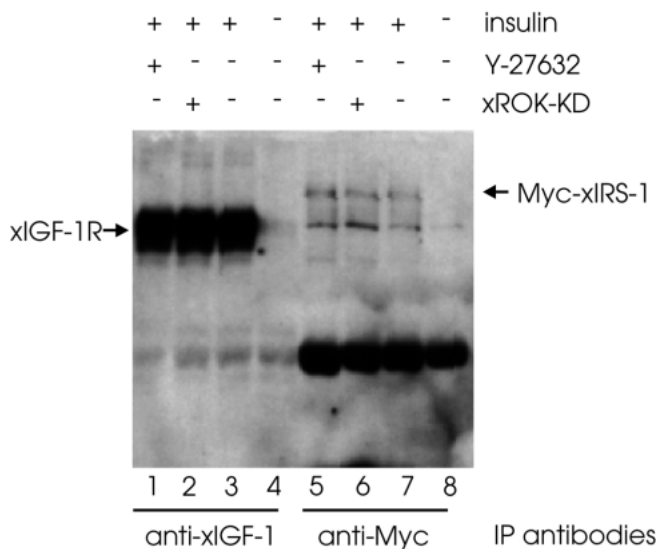
incubation of xROK $\alpha$  immunoprecipitates with Y-27632 resulted in inhibition of xROK $\alpha$  kinase activity, with 50% inhibition occurring between 1 and 10  $\mu$ M (Fig. 5A). By way of comparison, Y-27632 causes 50% inhibition of mammalian ROK $\alpha$  or in vitro at 1  $\mu$ M (Uehata et al., 1997). As a control, autophosphorylation of purified xIGF-1 receptor (Cummings et al., 1996) was not affected by 10 or 100  $\mu$ M Y-27632 (Fig. 5B). Incubation of oocytes with 10  $\mu$ M of Y-27632 caused significant reduction of insulin-induced xMAP kinase activation (Fig. 5C). At 100  $\mu$ M Y-27632, insulin-induced xMAP kinase activation was abolished (Fig. 5C). In contrast, neither 10 nor 100  $\mu$ M Y-27632 had any effect on progesterone-induced MAP kinase activation (Fig. 5C). Significantly, the concentration (10  $\mu$ M) of Y-27632 used to inhibit insulin signalling in *Xenopus* oocytes is identical to that required to inhibit mammalian ROK $\alpha$  in culture cells, where the specificity of this compound was previously established (Hirose et al., 1998; Uehata et al., 1997).

Since xROK $\alpha$  was identified as an xIRS-1 binding protein, we wished to determine whether inhibition of endogenous xROK $\alpha$  function (by xROK-KD or Y-27632) would interfere with xIRS-1 tyrosine phosphorylation or xIGF-1 receptor autophosphorylation. We have previously reported that although endogenous xIRS-1 protein was readily detectable in *Xenopus* oocytes, we were unable to detect its tyrosine phosphorylation before or after insulin stimulation when oocyte extracts were analysed by antibodies against phosphotyrosine (Liu et al., 1995). A lack of antibodies that efficiently immunoprecipitate endogenous xIRS-1 has so far prevented us from carrying out immunoprecipitation experiments prior to anti-pTyr immunoblotting. To overcome this, we injected Myc-tagged xIRS-1 into oocytes and tested its tyrosine phosphorylation using anti-Myc antibodies for immunoprecipitation. Tyrosine phosphorylation of Myc-xIRS-1, which was not detectable prior to insulin stimulation (Fig. 6, lane 8), was evident following insulin stimulation (Fig. 6, lane 7). However, under conditions that insulin-induced xMAP kinase activation was reduced (Figs 4 and 5), xROK-KD injection or Y-27632 had no effect on insulin-induced autophosphorylation of xIGF-1 receptor or tyrosine phosphorylation of Myc-xIRS-1 (Fig. 6). These data suggest that endogenous xROK $\alpha$  functions downstream from xIRS-1 tyrosine phosphorylation.

#### xROK-K and c-H-Ras act synergistically to induce xMAP kinase activation in *Xenopus* oocytes

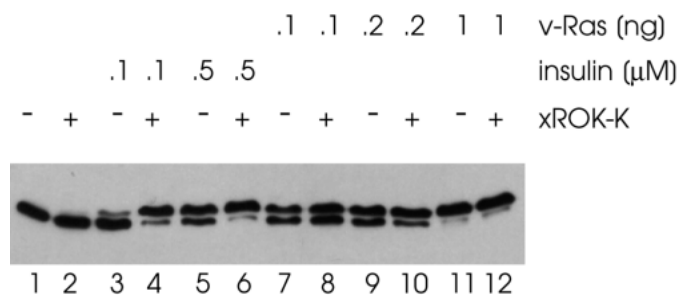
We have previously shown that the non-catalytic carboxyl terminus of x-ROK $\alpha$  (xROK-C) inhibits insulin-induced, but not v-Ras-induced, xMAP kinase activation (Farah et al., 1998), suggesting that xROK $\alpha$  may function upstream of cellular Ras. Consistent with this notion, injection of xROK-K mRNA enhanced the ability of sub-optimal concentrations of insulin to activate xMAP kinase (Fig. 7, comparing lane 6 to lane 5 or lane 4 to lane 3), while it did not similarly enhance xMAP kinase activation in the presence of several concentrations of v-Ras tested (Fig. 7).

To test directly whether xROK-K can act in concert with Ras in *Xenopus* oocytes, we employed murine c-H-Ras (Ebinu et al., 1998). As expected (Birchmeier et al., 1985), injection of c-H-Ras mRNA into oocytes only weakly induced xMAP kinase activation, even at 10 ng mRNA per oocyte (Fig. 8A). This was in contrast to v-Ras mRNA which was very effective

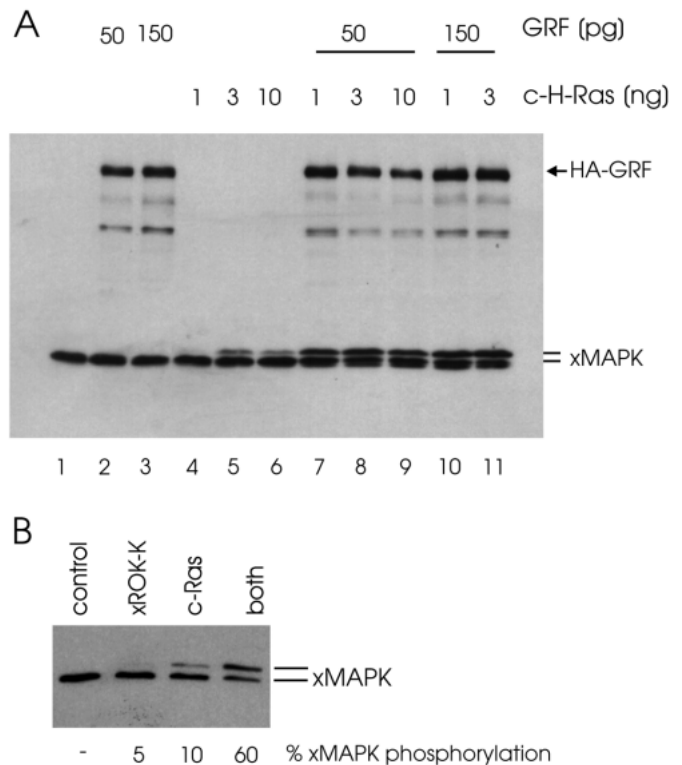


**Fig. 6.** Autophosphorylation of xIGF-1 receptor and tyrosine phosphorylation of xIRS-1. Oocytes injected with mRNA for Myc-xIRS-1 (10 ng per oocyte) were further injected with xROK-KD mRNA (10 ng per oocyte) or treated with 100  $\mu$ M Y-27632. Groups of at least 50 oocytes were then left unstimulated or stimulated with 1  $\mu$ M insulin for 10 minutes. Oocyte lysates were prepared from each group and split for immunoprecipitation with anti-xIGF-1 receptor or anti-Myc followed by immunoblotting with anti-pTyr. A much lighter exposure revealed no detectable difference in autophosphorylation of xIGF-1 receptor among lanes 1, 2 and 3 (not shown).

even at 0.1 ng per oocyte (Fig. 7, lane 7). Immunoblotting (by the monoclonal anti-Ras antibody Y13-259) confirmed the relative intensities of the p21 Ras protein in both cases (not shown). Unlike v-Ras protein which is constitutively active in cells, c-H-Ras requires an upstream activator which promotes GDP/GTP exchange. Several mammalian Ras activators, or guanine nucleotide releasing factor (GRF) have been identified. Co-injection of c-H-Ras and a mammalian GRF, RasGRF/CDC25<sup>Mm</sup> (Mattingly and Macara, 1996; Shou et al.,



**Fig. 7.** xROK-K does not influence v-Ras-induced xMAP kinase activation. Oocytes were either injected with water (-) or xROK-K mRNA (+). The injected oocytes were incubated in OR2 until about 50% of the xROK-K-injected oocytes exhibited depigmentation (Fig. 1A). Water-injected or xROK-K mRNA-injected oocytes were divided into groups of 20-30 oocytes and the individual groups were either further injected with the indicated amounts of v-Ras mRNA (lanes 7 to 12), or incubated with the indicated concentrations of insulin (lanes 3-6), or left untreated (lanes 1 and 2). Following an overnight incubation, each group was lysed for xMAP kinase immunoblotting.



**Fig. 8.** xROK-K potentiates c-H-Ras-induced xMAP kinase activation. (A) Oocytes were first injected with the indicated amounts of GRF cDNA (nuclear injection). The DNA injected oocytes or control oocytes were further injected with the indicated amount of c-H-Ras mRNA. Each group (of at least 20 oocytes) were incubated overnight in OR2 before being lysed for anti-xMAP kinase immunoblotting or anti-HA immunoblotting. Lane 1 represents uninjected oocytes. The % xMAP kinase phosphorylation was calculated as the intensity ratio of upper band vs the sum of upper and lower bands based on scanning of the X-ray film. The - sign indicates a negligible amount of the upper form. (B) Groups of 30 oocytes each were injected with xROK-K mRNA (5 ng/oocyte), c-H-Ras mRNA (5 ng/oocyte), or both (5 ng of each per oocyte). Following an overnight incubation in OR2, oocytes were lysed for xMAP kinase immunoblotting. Shown here is a representative of several similar experiments.

1992), resulted in activation of xMAP kinase (Fig. 8A), indicating that the GRF was capable of activating c-H-Ras in *Xenopus* oocytes. Unlike c-H-Ras which usually induced low percentages of oocytes to undergo GVBD (or xMAP kinase activation), we never observed activation of xMAP kinase or GVBD in oocytes injected with RasGRF/CDC25<sup>Mm</sup> alone (lanes 2 and 3 and data not shown). This is intriguing given that oocytes are known to contain an endogenous Ras protein (Davis and Sadler, 1992). This phenomena is under investigation but will not be further discussed here.

Interestingly, co-injection of xROK-K with c-H-Ras also resulted in xMAP kinase activation to a significantly greater extent than injection of c-H-Ras alone (Fig. 8B).

## DISCUSSION

### ROK kinases in insulin signalling

We originally identified xROK $\alpha$  as an xIRS-1-binding protein

in a yeast two-hybrid screening (Farah et al., 1998) using a bait construct containing the amino terminal PH and PTB domains of xIRS-1 (Liu et al., 1995). Further analyses in yeast suggested that the PTB domain of xIRS-1 was the primary binding site for xROK $\alpha$  (Farah et al., 1998). Although the biochemical mapping of a potential PTB binding motif in xROK $\alpha$  represents an interesting on-going project, the current study focuses on the role of xROK $\alpha$  in insulin signal transduction in *Xenopus* oocytes. We primarily used xMAP kinase activation, in particular phosphorylation of xMAP kinase, as our measure of insulin signalling activity throughout the study. As demonstrated in Fig. 3, the ratio of phosphorylated xMAP kinase vs unphosphorylated xMAP kinase accurately reflects that of GVBD-positive vs GVBD negative oocytes.

We demonstrated that injection of the kinase active xROK $\alpha$  (full-length or xROK-K) enhanced insulin-induced xMAP kinase activation (Fig. 2) whereas injection of a kinase deficient mutant (xROK-KD) significantly reduced it (Fig. 4). Furthermore, we demonstrated that a previously characterized inhibitor of ROK kinases (Y-27632) inhibited xROK $\alpha$  in vitro and reduced insulin-induced xMAP kinase activation in *Xenopus* oocytes. These results suggest that endogenous xROK $\alpha$  is required for insulin-induced xMAP kinase activation. While it is tempting to suggest that insulin might positively regulate xROK $\alpha$  kinase activity in *Xenopus* oocytes, we have not been able to demonstrate any insulin-related changes in xROK $\alpha$  kinase activity by immune kinase assays using MBP as a substrate (not shown). These negative results, however, do not eliminate the possibility, and indeed the likelihood, that the ability of xROK $\alpha$  to phosphorylate physiological substrate(s) in *Xenopus* oocytes might be capable of being regulated by insulin, nor do they exclude the possibility that an activating factor associated with xROK $\alpha$ , and hence the activation of xROK $\alpha$ , was lost in the immunoprecipitation procedure.

Clearly, the kinase activity of xROK $\alpha$  has a dramatic effect on the oocyte cytoskeleton, as indicated by the characteristic depigmentation in xROK-K mRNA-injected oocytes (Fig. 1A). This depigmentation is quite distinct from that caused by injection of an activated RhoA mutant (V14-RhoA; Mohr et al., 1990; our unpublished data). In addition, V14-RhoA did not enhance insulin-induced xMAP kinase activation (not shown). Therefore it appears that xROK $\alpha$  may function independently of Rho GTPases in *Xenopus* oocytes. ROK kinases were first identified as effectors of Rho GTPases (Leung et al., 1995). A possible role in insulin signalling, as identified here, may represent the first Rho-independent function of ROK kinases.

### xROK-K potentiates Ras activity in *Xenopus* oocytes

We wondered whether xIRS-1 (Liu et al., 1995), or xIGF-1 receptor (Zhu et al., 1998), might be a physiological substrate for xROK $\alpha$ , since both mammalian insulin receptor and IRS-1 are known to be phosphorylated by serine/threonine kinases (Sun et al., 1991). We did not observe any significant increase in the overall serine/threonine phosphorylation of either xIGF-1 receptor or xIRS-1 in oocytes over-expressing xROK $\alpha$  or its kinase domain, as determined by metabolic labelling of oocytes with [<sup>32</sup>P]orthophosphate (not shown), suggesting that neither xIGF-1 receptor nor xIRS-1 is a direct substrate of

xROK $\alpha$ . Consistent with this notion, we also failed to detect any alteration in insulin-induced tyrosine phosphorylation of either the xIGF-1 receptor or xIRS-1 in oocytes over-expressing xROK-KD or treated with the ROK kinase inhibitor Y-27632 (Fig. 6). Therefore, xROK $\alpha$  is distinct from a tumour necrosis factor (TNF)  $\alpha$ -regulated serine/threonine kinase which is known to phosphorylate IRS-1 and negatively regulate insulin receptor-dependent tyrosine phosphorylation of IRS-1 (Hotamisligil et al., 1996).

Insulin-induced GVBD in *Xenopus* oocytes is known to be dependent on an endogenous Ras protein (Deshpande and Kung, 1987; Korn et al., 1987). Injection of oncogenic Ras (v-Ras) induces GVBD (Birchmeier et al., 1985) and xMAP kinase activation (Chesnel et al., 1997; Farah et al., 1998) in *Xenopus* oocytes. However, unlike insulin-induced xMAP kinase activation which was enhanced by a prior injection of xROK-K, v-Ras-induced xMAP kinase activation was not influenced by xROK-K injection (Fig. 7). These results suggest that endogenous xROK $\alpha$  may function upstream of Ras in insulin signal transduction. Consistent with this notion, xROK-K significantly enhanced the ability of c-H-Ras to induce xMAP kinase activation (Fig. 8B). We do not yet know how xROK-K regulates c-H-Ras. However, it is tempting to speculate that xROK-K might phosphorylate, and activate, an endogenous RasGRF protein. RasGRF/CDC25<sup>Mm</sup> is known to be phosphorylated, and activated, by an as yet unidentified serine/threonine kinase (Mattingly and Macara, 1996). Whatever the mechanism might be, data presented in this study suggests that xROK $\alpha$  may represent a novel link to the Ras signalling pathway.

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