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INHIBITION OF *RAS*-INDUCED GERMINAL VESICLE BREAKDOWN IN XENOPUS OOCYTES BY *RAP*-1B

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A cDNA clone (K*rev*-1) has recently been identified that possesses the ability to reverse the transformed phenotype when introduced into a K-*ras*-transformed NIH/3T3 cell line. The K*rev*-1 protein, also known as *rap*-1A, was found to share 50% homology with the *ras* proteins. The *rap*-1A protein has also been shown to block the interaction of *ras* with its GTPase activating protein *in vitro*, leading to speculation regarding its role *in vivo*. A closely related protein, *rap*-1B, has also been identified in platelets, human erythroleukemia cells, neutrophils, and aortic smooth muscle cells. Unlike *rap*-1A, *rap*-1B has been shown to be phosphorylated in platelets. Given the high degree of similarity between the amino acid sequences of *rap*-1A and *rap*-1B, we sought to investigate the effect of microinjected *rap*-1B on H-*ras*(Val12)-induced germinal vesicle breakdown in Xenopus laevis oocytes. In this assay system, equimolar concentrations of *rap*-1B were found to block germinal vesicle breakdown triggered by the oncogenic *ras* protein. However, in the presence of IGF-1, this inhibition was not observed. Moreover, *rap*-1B is readily phosphorylated in the oocyte.

Although the precise role of *ras* proteins in metabolism has yet to be fully elucidated, their importance as key regulatory elements in cell growth and differentiation is demonstrated by the high incidence of mutated *ras* proteins in various forms of human cancer (1,2). In addition, cells in culture acquire a transformed phenotype after transfection with expression vectors bearing mutated *ras* sequences (for review, see ref. 3). Utilizing this latter characteristic of *ras* proteins, Kitayama and co-workers have identified a cDNA clone possessing the ability to reverse the *ras*-induced transformed phenotype of NIH/3T3 cells (4). This clone, termed K*rev*-1, was shown to code for a protein sharing 50% amino acid identity with *ras* proteins. Furthermore, a cDNA clone previously identified by hybridization of a Drosophila D*ras*3 probe with a human lymphoma cDNA library was shown to code for a protein identical in amino acid sequence to the K*rev*-1 protein (5). The lymphoma protein was named *rap*-1A. It is particularly notable that the region of *ras* proteins believed to interact with other proteins involved in *ras*-mediated signal transduction (i.e. the "effector" domain) is identical to the equivalent region in the K*rev*-1/*rap*-1A protein. Recently, Frech et al. (6) demonstrated the ability of the *rap*-1A protein to block *ras* interaction with *ras* GTPase activating protein (GAP) *in vitro* and have proposed a model by which *ras* action *in vivo* may be modulated by *rap*-1A.

Experiments involving site-directed mutagenesis of amino acid residue 61 suggest that this site may be important for the interaction of *ras* or *rap* proteins with *ras* GAP (6). Amino acids other than the native glutamine (e.g. histidine, leucine) at position 61 of H-*ras* decrease intrinsic and GAP-stimulated GTPase activity while increasing the affinity of *ras* for GAP (7). *Rap*-1A, possessing a threonine at position 61, binds *ras* GAP with 50- to 100-fold higher affinity than *ras* (6). The interaction of *rap*-1A and GAP, however, does not result in an increase in the GTPase activity of the

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rap protein (6). One can envision a mechanism by which *rap*-1A could thereby control those activities of *ras* which are dependent upon GAP by modulating the amount of GAP available for interaction with *ras*.

A protein closely related to *rap*-1A, *rap*-1B, has also been identified in the human lymphoma cell line (5). *Rap*-1B is approximately 95% identical to *rap*-1A, with 6 of the 8 amino acid differences occurring in the last 13 residues (5,8). Unlike *rap*-1A, *rap*-1B is phosphorylated in platelets and human erythroleukemia (HEL) cells (9-13).

Given the fact that the domains involved in guanine nucleotide binding and GAP interaction are identical in *rap*-1A and *rap*-1B, the potential exists for *rap*-1B to display anti-*ras* activity similar to that demonstrated for *rap*-1A. We have utilized the Xenopus oocyte system to investigate the potential *ras*-antagonizing activity of *rap*-1B. The microinjection into oocytes of oncogenic forms of *ras* protein, such as the valine-12 mutant, results in the triggering of meiotic maturation, also called germinal vesicle breakdown (GVBD) (14). Here we demonstrate the inhibition of H-*ras*(Val12)-mediated GVBD by *rap*-1B.

EXPERIMENTAL PROCEDURES

Oocyte isolation: Xenopus laevis oocytes were isolated as described previously (15). Frogs were primed with 35 to 50 I.U. pregnant mare's serum gonadotropin 24 to 48 hours prior to surgery. Following manual separation from surrounding membranes and prior to microinjection, oocytes were incubated overnight at 19°C in modified Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 µg/ml penicillin, 10 µg/ml streptomycin sulfate, 15 mM HEPES, pH 7.6).

Oocyte microinjection and GVBD: Oocytes were microinjected using a Drummond 10 µl digital microdispenser adjusted to deliver 50 nl of solution into each oocyte. Following microinjection, oocytes were periodically observed for signs of germinal vesicle breakdown (GVBD). In most cases, GVBD could be readily recognized by the appearance of a circular unpigmented area at the animal pole. In situations where GVBD was not clear, oocytes were fixed in 10% (w/v) trichloroacetic acid and dissected in order to verify the presence or absence of the germinal vesicle.

Protein purification: The valine-12 mutant of the H-*ras* protein was purified from *E. coli* bearing the expression plasmid pRS3430. After induction of *ras* protein synthesis with isopropylthio-β-galactoside, cells were grown overnight, harvested by centrifugation, and lysed by freezing and thawing in 20 mM Tris, pH 7.4, 1 mM EDTA, 2 µg/ml bovine pancreatic trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, and 100 µg/ml lysozyme. The suspension was then treated with 10 µg/ml DNase and 5 mM $MgCl_2$ for 20 min. Following centrifugation (47,000 x g, 4°C, 30 min), the cleared extract was subjected to successive rounds of column chromatography over Q-sepharose (Pharmacia), S-300, and butyl-sepharose. *Ras*-containing fractions were identified by SDS-PAGE. The purity of the final product was estimated to be 90%.

Rap-1B cDNA was isolated from a human platelet expression library and expressed in *E. coli* as described (Winegar, Ohmstede, Chu, Reep, and Lapetina, manuscript in preparation). Details of the purification of the recombinant protein are to be published elsewhere.

SDS-polyacrylamide gel electrophoresis: Prior to microinjection, defolliculated oocytes were incubated for 16 h at room temperature in modified Barth's medium containing 2 mCi/ml carrier-free $H_3[^{32}P]O_4$. Oocytes were then microinjected with *rap*-1B as described previously. Following a 30-min incubation, the oocytes were homogenized in NP-40 buffer (155 mM Nacl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM sodium ortho-vanadate, 0.5% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin) in the ratio of 25 µl buffer per oocyte. Homogenates were then centrifuged for 10 min at 4°C in a microcentrifuge. Aliquots of the infranatant fraction (between the pellet and lipid layer) were then subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (16) (12% (w/v) acrylamide, 1.5 mm thick). The proteins in the gel were then transferred to PVDF membrane (Immobilon) and probed with an anti-H-*ras* antibody (M90) which cross-reacts strongly with *rap*-1B

(12). Detection of immunoreactive peptides was by alkaline phosphatase-linked secondary antibody. Radiolabelled proteins were detected by fluoragraphy.

RESULTS and DISCUSSION

Inhibition of H-*ras*(Val12)-mediated GVBD by *rap*-1B: Stage 6 Xenopus laevis oocytes can be induced to proceed through meiotic maturation (GVBD) *in vitro* by the action of progesterone, insulin, or insulin-like growth factor 1 (IGF-1). In 1985, Birchmeier et al. (14) demonstrated that an oncogenic form of the H-*ras* protein could also trigger GVBD when microinjected into oocytes. The wild-type protein was found to be much less potent in the same assay. Moreover, the inhibition of insulin-induced GVBD by microinjected antibodies to the *ras* gene product suggest that *ras* is a key member of the insulin-mediated signal transduction pathway (17). In our system, the microinjection of the H-*ras*(Val12) protein (75 ng per oocyte) consistently induced meiotic maturation. Germinal vesicle breakdown induced by *ras*, however, was effectively blocked by the co-injection of *rap*-1B (100 ng per oocyte) (Figure 1). Co-injection of purified recombinant *rap*-2B, a *ras*-related protein first identified in human platelets (18), or bovine serum albumin (100 ng each per oocyte), however, resulted in negligible inhibition of GVBD. In 4 separate experiments utilizing oocytes from four different frogs, microinjection of H-*ras*(Val12) resulted in GVBD in 75 ± 19% of the oocytes. The percentage of oocytes maturing following coinjection of *ras* and *rap*-1B was only 1.25 ± 2.5% GVBD.

As is the case in human platelets and HEL cells (9-13), *rap*-1B is phosphorylated in oocytes. ^{32}P-Labelled proteins obtained from uninjected or *rap*-1B-injected oocytes were analyzed by SDS-PAGE and autoradiography. As shown in Figure 3, a distinct radiolabelled band appears at the precise location as the immunoreactive peptide identified by the M90 antibody. No such band is present in the uninjected oocytes.

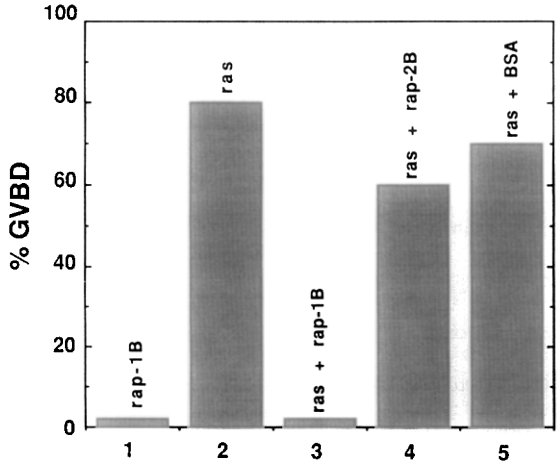
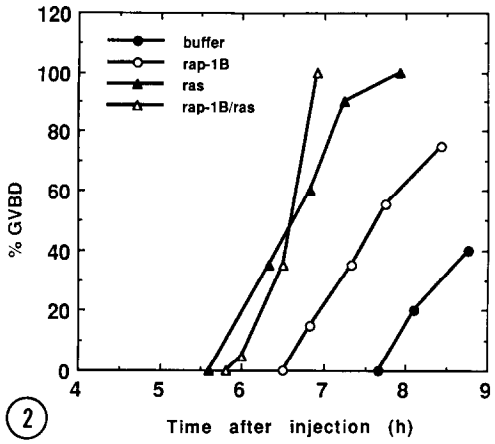


Figure 1. Inhibition of H-*ras*(Val12)-induced GVBD by *rap*-1B.
Primed oocytes were collected and microinjected as described in "Experimental Procedures". All individual proteins and mixtures were microinjected in buffer consisting of 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The protein concentrations at the time of microinjection were as follows: *rap*-1B, 2 mg/ml; *rap*-2B, 2 mg/ml; H-*ras*(Val12), 1.5 mg/ml; bovine serum albumin, 2 mg/ml. Approximately 50 nl were injected into each oocyte. GVBD was scored as described in "Experimental Procedures".



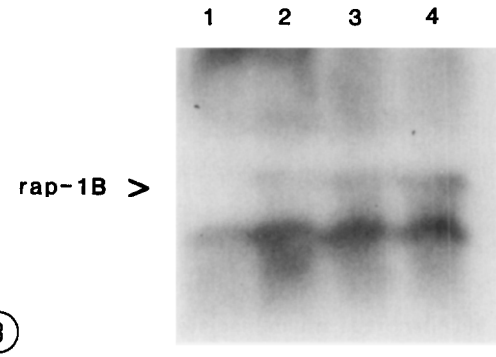


Figure 2. Effect of IGF-1 on the action of *rap*-1B and H-*ras*(Val12) in oocytes.
Microinjection and scoring of GVBD were carried out as described in the legend to Figure 1. All microinjections were carried out in buffer consisting of 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 1.25 mM dithiothreitol, and 10% (v/v) glycerol. Oocytes were incubated in modified Barth's medium supplemented with 0.1% (w/v) bovine serum albumin (radioimmunoassay-grade, Sigma) and containing 2 nM IGF-1.

Figure 3. Phosphorylation of *rap*-1B in oocytes.
^{32}P-Labelled oocytes were microinjected as described in "Experimental Procedures" and subjected to SDS-polyacrylamide gel electrophoresis. Microinjected *rap*-1B was detected by immunoblotting with M90 and ^{32}P-labelled proteins were detected by fluorography. Lane 1, uninjected; lanes 2, 3, and 4, oocytes injected with 50, 100, or 150 ng *rap*-1B each, respectively. Each lane contains the protein from 2 oocytes.

Effect of IGF-1 on the inhibition of *ras*-induced GVBD by *rap*-1B: *Ras*-induced GVBD has been shown to be accelerated by the inclusion of IGF-1 in the incubation medium (19). In our system, not only did IGF-1 accelerate *ras*-induced GVBD, but it also blocked the ability of *rap*-1B to inhibit the process (Figure 2). In the presence of 2 nM IGF-1, the rate of H-*ras*(Val12)-induced GVBD was unchanged whether or not *rap*-1B was microinjected along with the *ras* protein (Figure 2). Furthermore, not only was rap-1B unable to inhibit *ras*/IGF-1-induced GVBD, but it actually accelerated the rate of GVBD brought about by IGF-1 alone.

CONCLUSIONS

Rap-1B, a protein that differs from *rap*-1A in only 8 out of 184 amino acid residues, is able to block *ras*-induced GVBD in oocytes (Figure 1). Because GVBD can be induced by *ras* proteins harboring mutations of the same type as those that lead to cellular transformation, it is conceivable that *rap*-1B has anti-oncogenic activity *in vivo*. Similar to the model proposed for *rap*-1A action (6), *rap*-1B could modulate *ras* activity by controlling its access to GAP. In addition, the ability of IGF-1 to block *rap*-1B inhibition of *ras*-induced GVBD (Figure 2) suggests that the anti-oncogenic activity of *rap*-1B is subject to regulation.

In order to decipher the mechanism by which *rap*-1B inhibits *ras*-induced GVBD, it will be necessary to determine with what endogenous proteins *rap*-1B interacts in the oocyte. In addition,

the elucidation of the modifications (such as phosphorylation) *rap*-1B undergoes once inside the oocyte and the effects these modifications have on protein-protein interaction must be determined.

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