RESEARCH COMMUNICATION

Effect of synthetic peptides representing the hypervariable region of p21^{ras} on *Xenopus laevis* oocyte maturation

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The carboxy-terminal hypervariable regions of p21^{ras} proteins have been highly conserved throughout evolution but no function has been assigned to them yet. This region has been suggested as a possible candidate for receptor recognition. We have tested the possibility of this region being involved in p21^{ras} biological function. Synthetic peptides corresponding to the hypervariable domains of p21^{N-ras} and p21^{K(B)-ras} were microinjected into *Xenopus* oocytes to assess their effect on oocyte maturation. The K(B)-ras peptide inhibited insulin-dependent but not pro-

gesterone-dependent maturation, in contrast with the N-ras peptide which did not inhibit maturation significantly. A control peptide, with the same amino acid composition as the K(B)-ras peptide but with a scrambled sequence, and poly(D,L-lysine) were inactive. Pentalysine had partial activity which may be due to its mimicking the lysine-rich stretch of the K(B)-ras sequence. The data support the hypothesis that the K(B)-ras gene product specifically is involved in transducing the insulin and/or insulin-like growth factor 1 signal.

INTRODUCTION

The ubiquitous mammalian *ras* family of proto-oncogenes, H-, K- and N-*ras*, encode highly conserved proteins with $M_r = 21\,000$ (p21) which bind GDP and GTP and possess low intrinsic GTPase activity (Barbacid, 1987). Dramatic progress has recently been made in elucidating the mechanism of action of *ras* proteins. GTP-bound *ras* proteins activate the mitogen-activated protein kinase pathway via Raf, leading to multiple phosphorylation events and the regulation of cell growth and differentiation (Maruta and Burgess, 1994). Regulation of the guanine nucleotide binding of *ras* proteins is performed by at least four exchange factors in mammals, CDC25Mm, mSos1, mSos2 and smgGDS, whose activities are regulated by tyrosine kinase receptors (Feig, 1993). Plasma membrane localization of *ras* proteins is dependent on the CXXX sequence at the C-terminus (Willumsen et al., 1984) and three common post-translational modification steps have been identified: farnesylation of cysteine 186, cleavage of XXX and carboxyl-methylation (Willumsen et al., 1984; Fujiyama and Tamanoi, 1986; Clarke et al., 1988; Gutierrez et al., 1989; Hancock et al., 1989). In addition, the H-, N- and K(A)-*ras* proteins can be palmitoylated on nearby cysteine residues while in contrast the K(B)-*ras* protein contains a polybasic stretch (Willumsen et al., 1984; Hancock et al., 1989). Turnover of p21^{ras}-bound fatty acid is rapid compared with the lifetime of the protein, suggesting that an acylation-deacylation cycle may play a role in modulating the dynamic association of *ras* proteins with the plasma membrane and subsequent signal transduction (Magee et al., 1987).

Exogenous mutationally activated p21^{ras} proteins induce meiotic maturation of *Xenopus laevis* oocytes (Stacey and Kung, 1984; Birchmeier et al., 1985). This effect is different from the

maturation induced by progesterone, since it is independent of the regulation of the levels of cyclic AMP (Birchmeier et al., 1985). Induction of oocyte maturation by insulin is dependent on endogenous p21^{ras} since it is blocked by injection of either anti-p21^{ras} monoclonal antibody Y13-259, which reacts with all known *ras* gene products (Furth et al., 1982), or 6B7, a monoclonal antibody directed against a synthetic peptide corresponding to a highly conserved region of p21^{ras} that is required for oncogenic function (Korn et al., 1987). These results suggest that p21^{ras} proteins take part in a cascade of events which mediate insulin-induced oocyte maturation and that the insulin receptor tyrosine kinase somehow interacts with this family of proteins to initiate the maturation process. However, the biochemical pathway influenced by *ras* proteins has not been fully elucidated.

Despite considerable knowledge about the structural and functional aspects of the *ras* proteins, their role in physiological and pathological processes still remains obscure. The three mammalian p21^{ras} proteins share an identical effector domain that is essential for mitogenic activity (Sigal et al., 1986; Stein et al., 1986), therefore the protein(s) required for downstream signalling may be the same. On the other hand, the hypervariable regions near the C-termini of the *ras* proteins differ considerably from each other and it has been proposed that this region may be necessary for interaction with different upstream proteins (Milburn et al., 1990) in the form of 'detector' or receptor molecules for each of the *ras* proteins. However, the hypervariable domains of the different *ras* proteins are well conserved between species, indicating selective pressure to maintain function. We have tested whether the hypervariable region is required for p21^{ras} biological action involved in insulin-dependent maturation in *Xenopus* oocytes. In this study, the effect of microinjecting

Abbreviations used: Fmoc, fluorenyl-methoxycarbonyl; TFA, trifluoroacetic acid; NAM, normal amphibian medium; GVBD, germinal vesicle breakdown.

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Table 1 Peptides representing the hypervariable region at the C-termini of N-ras and K(B)-ras

The single-letter amino acid code is used. Bio = biotin.

N-ras	Bio-Y	R	M	K	K	L	N	S	S	D	D	G	T	N	G	K	(P1; M_r = 2038)
K(B)-ras	Bio-Y	K	E	K	M	S	K	D	G	K	K	K	K	K	K	K	(P2; M_r = 2077)
K(B)-ras	Acetyl	K	K	D	K	G	K	Y	K	S	K	M	K	E	K	K	(P2SCR; M_r = 1896)
scrambled													K	K	K	K	(Lys ₅ ; M_r = 658)

Table 2 Amino acid analysis of purified peptides

Values were normalized to D for P1 and E for P2.

Residue	P1	P2
D	4.0	1.23
T	1.0	—
S	1.75	0.84
E	—	1.0
G	2.09	1.24
M	0.70	0.14
L	1.01	—
Y	0.62	0.56
K	2.51	9.53
R	0.85	—

peptides representing the hypervariable regions of N-ras and K(B)-ras proteins on oocyte maturation was tested. The results support the possibility that the hypervariable region is required for p21^{ras} biological action in *Xenopus* oocytes and that this protein is involved in insulin-dependent maturation.

MATERIALS AND METHODS

Peptide synthesis

Solid-phase synthesis was performed using fluorenyl-methoxycarbonyl (Fmoc) amino acid active esters (LKB) in a glass shaker on 0.5 g of *p*-benzyloxybenzyl alcohol resin (Nova biochem) (Merrifield, 1963). Peptides were biotinylated at their N-termini using NHS-biotin (Sigma). Peptide 2SCR was synthesized by the peptide synthesis service at the National Institute for Medical Research on an Applied Biosystems 430A synthesizer using Fmoc chemistry. Peptide resins were cleaved in 95% trifluoroacetic acid (TFA) and 5% of appropriate scavengers to de-protect side chains. The resulting crude peptide was extracted in 20% acetic acid and chromatographed on a Sephadex G10 column in the same solvent. Peptides were purified by HPLC (Beckman System Gold) on a Brownlee Prep10 C18 reverse-phase cartridge using a gradient from 0.1% TFA (solvent A) to 0.05% TFA in acetonitrile (solvent B). A linear gradient of 0–60% B in 65 min at 2 ml/min was used. For the basic K(B)-ras peptide a Mono S column (Pharmacia) was used with the solvent system A = 10 mM Na-phosphate, pH 6.8, B = 1 M NaCl in A. A linear gradient of 0–100% B in 30 min at 0.5 ml/min was used. The authenticity of the purified peptides was confirmed by amino acid analysis and/or by m.s. Poly(D,L-lysine) (P-0171, M_r 1000–4000) and pentyllysine (L-9151) were obtained from Sigma (Poole, Dorset, U.K.).

Oocyte microinjection

Ovarian fragments were surgically removed from *Xenopus laevis* frogs that were anaesthetized by hypothermia. To remove follicle

cells, the ovarian fragments were incubated with 2 mg/ml of collagenase (Sigma) for 2 h in normal amphibian medium (NAM: 88 mM NaCl/0.33 mM Ca(NO₃)₂/1 mM KCl/0.41 mM CaCl₂/0.82 mM MgSO₄/10 mM Hepes, pH 7.6) containing 100 µg/ml streptomycin (Sigma) and 100 i.u./ml penicillin (Sigma) at room temperature. The reaction was stopped by washing the fragments five times in fresh NAM. The enzyme-treated oocytes were allowed to recover overnight before injection. Stage VI oocytes (Dumont, 1972) to be injected were selected and transferred to plastic dishes containing NAM made 4% with Ficoll (type 400-D). Microelectrodes were pulled with a vertical electrode puller (Scientific Research Instruments Ltd.). The electrodes were calibrated by injection into liquid paraffin and measurement of droplet diameter. Oocytes were injected at the equator level into the animal pole with antibodies, peptides or buffer alone using a pressurized injector (Micro Instruments Ltd., Oxford, U.K.). Injected oocytes were incubated for 3 h to allow healing of the injection wound and equilibration of the injected solution within the oocyte cytoplasm. After 3 h groups of 20–30 oocytes were stimulated with 1 µM progesterone (Sigma) or 10 µM insulin (Sigma) plus 0.01 µM progesterone for 18–20 h at 15 °C in an incubator. Germinal vesicle breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole. In some cases nuclear breakdown was confirmed by dissection of oocytes fixed in 10% trichloroacetic acid.

RESULTS

In this study two different peptides representing the hypervariable region at the C-termini of N- and K(B)-ras p21 proteins were synthesized; they do not include the CAAX box (Table 1). The peptides were N-terminally biotinylated to protect them from aminopeptidase degradation and to aid in their analysis and use. The peptides were designed in such a manner as to be detected via an iodinated tyrosine or the biotin at the N-terminus. Therefore it was possible to use peptides as antigens for production of monoclonal antibodies and to attach them to solid supports for ELISA assays and radioassays. Quantitative amino acid analysis of resin-bound peptide and free peptide in the different purification steps was performed. The amino acid composition was as expected (Table 2). A scrambled version of peptide 2 (peptide 2SCR; Table 1) was also synthesised as a control, and its relative molecular mass confirmed by m.s.

Effect of synthetic peptides on oocyte maturation

To determine the effect of synthetic peptides representing the hypervariable regions of N-ras and K(B)-ras on *Xenopus* oocyte maturation, P1 (N-ras) and P2 [K(B)-ras] were microinjected. The oocytes were microinjected with 50 nl of PBS containing increasing concentrations of each peptide (60, 120 and 240 ng/oocyte; 30, 60 and 120 pmol). After microinjection, groups of 30 oocytes were incubated in 3 ml of NAM buffer containing 1 µM progesterone alone or 10 µM insulin plus 0.01 µM progesterone to potentiate insulin action (Sadler and

Figure 1 Effect of synthetic ras peptides on *Xenopus* oocyte maturation

After microinjection of indicated amounts of peptides P2 (a), P1 (b) or antibody Y13-259 (c), oocytes were incubated in the presence of 1 μ M progesterone (●) or 10 μ M insulin plus 0.1 μ M progesterone (○). As a control oocytes were injected with buffer only followed by 10 μ M insulin plus 0.1 μ M progesterone (○). After 18 h, oocytes were analysed for GVBD. For each point 30 oocytes were microinjected. Data are representative of three independent experiments. Standard deviations are not shown for clarity but variation was less than $\pm 5\%$.

Maller, 1989). Eighteen hours after the addition of the hormones, the cells were scored for GVBD, which was indicated by the appearance of a white spot on the pigmented animal pole. As shown in Figure 1(a), P2 [K(B)-ras] inhibited GVBD with high efficiency since the lowest concentration of peptide ($\sim 25 \mu$ M final cytoplasmic concentration, taking the volume of a stage VI oocyte as 1.325 μ l) inhibited about 50% of insulin-induced maturation. Conversely, P1 (N-ras) was virtually inactive at the same concentration; only the highest concentration of the peptide (240 ng/oocyte) inhibited $\sim 30\%$ of insulin-induced maturation (Figure 1b). Oocytes were microinjected with the same concentrations of Y13-259 antibody as a positive control for inhibition of insulin-induced maturation (Figure 1c). Antibody Y13-259 inhibited insulin-induced maturation but not progesterone-induced maturation. This pattern of inhibition is similar to the one observed previously (Desphande and Kung, 1987). Untreated or PBS-injected oocytes which gave high levels of GVBD were used as negative controls.

The scrambled version of P2, poly(D,L-lysine) and pentyllysine were used as controls to compare their effects with that of P2 (Figure 2). After microinjection, groups of 20 oocytes were incubated in 3 ml of NAM buffer containing either progesterone or insulin as indicated above. Neither P2SCR (Figure 2b) nor poly(D,L-lysine) (Figure 2c) had any effect on insulin-induced maturation. However, pentyllysine had an inhibitory effect (Figure 2d) similar to the one induced by P2 (Figures 1a and 2a) although its potency was 4–5-fold lower. None of these peptides had a significant effect on the progesterone-induced maturation.

DISCUSSION

In amino acid positions 171–185, the N-, H- and K-ras proteins show little similarity, therefore it has been proposed that this hypervariable region may determine the physiological specificity of the ras proteins by interacting with different regulatory proteins. In this study we have investigated the effect of synthetic peptides representing this region on *Xenopus* oocyte maturation, to test whether the hypervariable region may indeed confer specific properties on each of the members of the ras gene family. Oocyte maturation *in vitro* can be stimulated by treatment of isolated oocytes with insulin or insulin-like growth factor 1 (El-Etr et al., 1979; Maller and Koontz, 1981). Insulin-induced oocyte maturation is apparently ras-dependent, since microinjection of anti-p21^{ras} antibodies (Y13-259) inhibits oocyte maturation induced by insulin but not by progesterone (Desphande and Kung, 1987; Korn et al., 1987). The synthetic peptide representing the hypervariable region of N-ras (P1) had a very low inhibitory effect in comparison with that of P2 representing the hypervariable region of K(B)-ras. This might be due to the fact that the major, if not the only, ras protein which is expressed in *Xenopus* oocytes is of the K(B)-ras type.

Recently it was shown that the first characterized *Xenopus* ras cDNA is an analogue of the human K(B)-ras gene (Andeol et al., 1990; Baum and Bebernitz, 1990). The high level of homology between the human K(B)-ras protein and the *Xenopus* protein suggests a strong selective pressure for the conservation of this specific gene in the vertebrate phylum. K(B)-ras is present throughout *Xenopus* oogenesis, and in slightly higher amounts in stage II–III oocytes compared with older oocytes (Baum and Bebernitz, 1990). To date, no H- or N-ras has been shown to be expressed in *Xenopus* oocytes, however, the possibility of N-ras or H-ras also being present in *Xenopus* cannot be ruled out. This could be the reason why some inhibition was observed with a high concentration of the N-ras P1.

A functional linkage between insulin and normal p21^{K(B)-ras} is supported by the finding that the insulin-induced GVBD is specifically inhibited by P2. This peptide showed the same effect as Y13-259 antibody, inhibiting insulin- but not progesterone-induced maturation. It is likely that P2 is competing with p21^{ras} for a 'detector' or regulatory protein, whereas Y13-259 antibody is neutralizing p21^{ras} protein. Our data suggest that the polybasic region of six lysines at the C-terminal end of P2 is involved in this effect. The fact that the scrambled version of P2 did not have any effect indicates that the sequence plays an important role in this effect as well as the overall charge of the peptide; this peptide was designed to maximize the separation of the lysine residues. This interpretation is supported by the finding that pentyllysine, which may mimic the lysine stretch of K(B)-ras, had an inhibitory effect, albeit 4–5-fold less potent than P2. On the other hand, poly(D,L-lysine) of relative molecular mass 1000–4000 did not have any effect on the insulin-induced maturation, indicating that non-specific ionic interactions of a highly basic peptide cannot explain the observations. This also suggests that the

Figure 2 Effect of synthetic peptides on *Xenopus* oocyte maturation

The experiments were performed as in Figure 1 except that 20 oocytes were microinjected per point. Oocytes were injected with the indicated amounts of P2 (a), scrambled P2 (b), poly-(D,L-lysine) (c) or Lys₅ (d). Oocytes were exposed to progesterone (■) or insulin plus progesterone (●) as before. As a control, oocytes were injected with buffer alone followed by 10 μ M insulin plus 0.1 μ M progesterone (○). Data are representative of at least two experiments. Standard deviations are not shown for clarity but variation was less than $\pm 5\%$.

inhibitory effect of the peptides is not due to competition with binding of the polybasic region of K(B)-ras to anionic phospholipid headgroups, thus interfering with correct targeting of the protein (Hancock et al., 1990). Other studies have reported evidence for a direct interaction between p21^{K(B)-ras} and insulin receptor and for activation of the receptor kinase activity by polybasic peptides (O'Brien et al., 1987; Fujita-Yamaguchi et al., 1989a,b; Morrison et al., 1989; Sacks et al., 1989; Xu et al., 1991). Moreover, the fact that P2 inhibited insulin-induced maturation and P1 did not, suggests that the hypervariable region may confer specific properties on each of the members of the ras family by interacting with different proteins, candidates for which could be the exchange factors that are known to interact directly with ras proteins or other unknown components of the pathway. Each p21^{ras} protein has a unique hypervariable domain that has been maintained throughout evolution and that may confer the specificity to interact with different proteins and to participate in different functions.

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