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ORIGINAL ARTICLE

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# Plasmid expression of a peptide that selectively blocks oncogenic *ras*-p21-induced oocyte maturation

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**Abstract** *Purpose*: We have previously found that a synthetic peptide corresponding to *ras*-p21 residues 96–110 (PNC2) selectively blocks oncogenic (Val 12-containing) *ras*-p21 protein-induced oocyte maturation. With a view to introducing this peptide into *ras*-transformed human cells to inhibit their proliferation, we synthesized an inducible plasmid that expressed this peptide sequence. Our purpose was to test this expression system in oocytes to determine if it was capable of causing selective inhibition of oncogenic *ras*-p21. *Methods*: We injected this plasmid and a plasmid expressing a control peptide into oocytes either together with oncogenic p21 or in the presence of insulin (that induces maturation that is dependent on normal cellular *ras*-p21) in the presence and absence of the inducer isopropylthioglucose (IPTG). *Results*: Microinjection of this plasmid into oocytes together with Val 12-p21 resulted in complete inhibition of maturation in the presence of inducer. Another plasmid encoding the sequence for the unrelated control peptide, X13, was unable to inhibit Val 12-p21-induced maturation. In contrast, PNC2 plasmid had no effect on the ability of insulin-activated normal cellular or wild-type *ras*-p21 to induce oocyte maturation, suggesting that it is selective for blocking the mitogenic effects of oncogenic (Val 12) *ras* p21. *Conclusion*: We conclude that the PNC2 plasmid selectively inhibits oncogenic *ras*-p21 and may therefore be highly effective in blocking proliferation of *ras*-induced cancer cells. Also, from the patterns of inhibition, by PNC2 and other *ras*- and *raf*-related peptides, of *raf*- and constitutively activated MEK-induced maturation, we conclude that PNC2 peptide inhibits oncogenic *ras* p21 downstream of *raf*.

**Keywords** Oncogenic *ras*-p21 · PNC2 plasmid · Selective inhibition of oncogenic *ras*-p21 · Oocyte maturation

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## Introduction

Oncogenic *ras*-p21 protein containing Val for the normally occurring Gly at position 12 in its amino acid sequence induces malignant transformation of cells and is a strong inducer of oocyte maturation [1]. Insulin also induces oocyte maturation through activation of normal cellular wild-type *ras*-p21 [2]. From conformational energy analysis of the low-energy structures of oncogenic and wild-type p21 alone and bound to different known intracellular target proteins, we have identified domains of oncogenic p21 that differ in conformation from those of the wild-type protein [3]. Similarly, we have identified regions of target proteins that differ in conformation when bound either to oncogenic or to wild-type p21 [4, 5].

We have synthesized peptides corresponding to these domains of p21 and target proteins and have microinjected them into oocytes that either were coinjected with oncogenic *ras*-p21 or were incubated subsequently with insulin [6, 7]. Several peptides selectively inhibited oncogenic p21 but were found to have only a minimal effect on insulin-induced wild-type p21 [6, 7]. Among these peptides from *ras*-p21 are the sequences 35–47, 96–110 and 115–126. The first peptide segment is from a known effector domain of p21 involved in its binding to such target proteins as the SOS-guanine nucleotide exchange protein, GTPase activating protein (GAP), the *ras*-binding domain (RBD) of *raf*-p74 protein and phosphoinositide-3-hydroxy kinase (PI3K) [8]. The second and third sequences have been implicated in the binding of *ras*-p21 to *jun*-N-terminal kinase and/or to *jun* protein [9].

We have also synthesized peptides from the RBD of *raf* and SOS that correspond to segments of each of these target proteins that differ in structure depending on whether the target protein is bound to activated normal or oncogenic p21. These include residues 62–72 of the p21-RBD interaction domain, 97–110 and 111–121 of the RBD and residues 631–641, 676–691, 718–729 and 994–1004 of SOS [6, 7]. All of these peptides, but not control peptides, block Val 12-p21-induced oocyte maturation. However, the peptides corresponding to residues 97–110 of *raf* and 994–1004 of the SOS protein have been found to block oncogenic but not insulin-activated normal (wild-type) p21 in oocytes [6, 7]. The other peptides inhibit both oncogenic and insulin-activated normal (wild-type) p21 protein-induced oocyte maturation.

We have recently found that oncogenic *ras*-p21-induced oocyte maturation requires at least two critical signal transduction pathways, one involving the *raf*-MEK-MAP kinase pathway and the other the *jun*-kinase (JNK)-*jun* pathway [9]. Activation of *raf* is also required for insulin-induced oocyte maturation. Both pathways appear to be interdependent because inhibition of either *raf* or MAP kinase results in inhibition of JNK-induced maturation while inhibition of JNK results in blockade of *raf*-induced maturation. Both pathways require activation by protein kinase C (PKC) [9]. Surprisingly, inhibition of MAP kinase in oocytes only minimally affects insulin-induced maturation, suggesting that *raf* activated by normal cellular *ras*-p21 induces elements of pathways other than the MEK-MAP kinase pathway [9].

We have found that the p21 96–110 peptide, which blocks oncogenic p21- but not activated wild-type p21-induced maturation, inhibits the interaction of oncogenic p21 with JNK and *jun* proteins [10]. The dose-response curve for this inhibition coincides with that for its inhibition of oncogenic *ras*-p21-induced oocyte maturation, suggesting that the site of action of this peptide is at the interaction of p21 with JNK/*jun* [8]. Microinjection of JNK into oocytes results in maturation that is not, however, inhibited by this peptide, presumably because JNK does not interact efficiently with normal cellular *ras*-p21. We concluded that activation of JNK occurs by alternate pathways [9], in agreement with previous findings [10].

One such pathway may involve the *raf*-MEK-MAP kinase pathway since blockade of *raf* by its dominant negative mutant protein and of MAP kinase by the MAP kinase-specific MKP-T4 phosphatase also block maturation induced by JNK. Maturation induced by *raf* is not inhibited by the p21 96–110 peptide but is strongly inhibited by the 35–47 peptide, suggesting that *raf* is the site of action of this peptide. Since the 35–47 peptide does not block insulin-induced oocyte maturation, but insulin-induced maturation requires activation of *raf*, we concluded that this peptide selectively inhibits *raf*-activated oncogenic pathways such as those involved with oncogenic p21. One pathway that this peptide may inhibit is the MEK-MAP kinase pathway that is required by oncogenic p21 but apparently not by insulin-activated normal p21.

Many of the signaling proteins that are activated by oncogenic *ras*-p21 in oocytes are likewise activated in *ras*-transformed mammalian cell lines [8]. For example, in 3T3/4A cells transfected with an oncogenic form of *ras*-p21, JNK and *jun* expression is significantly increased [11], and in a *ras*-transformed rat pancreatic carcinoma cell line, called TUC-3 [12], MAP kinase and JNK expression is markedly increased as compared with the counterpart untransformed cells in growth phase (Kanofsky et al., manuscript in preparation). Therefore, because specific p21 peptides selectively inhibit oncogenic p21-induced oocyte maturation by blocking activation of these proteins, they may also be effective in reversing oncogenic p21-induced cell transformation. Introduction of these peptides into large numbers of such cells can be effected either by attaching penetratin sequences to the peptides allowing them to traverse the cell membranes or by preparing plasmids that encode the peptide sequences and by transfecting these plasmids into *ras*-transformed cells.

We describe here the preparation of a plasmid encoding the *ras*-p21 96–110 sequence and the induced expression by this plasmid of the 96–110 peptide that has been found to be highly selective in blocking oncogenic *ras*-p21-induced maturation. This plasmid was microinjected into oocytes together with oncogenic p21 protein or into oocytes that were subsequently incubated in insulin in the presence of a promoter to determine whether it was capable of inhibiting oncogenic p21 selectively.

In addition, we also explored whether these anti-oncogenic *ras*-p21 peptides act on the MEK-MAP kinase pathway that appears to be preferentially activated by oncogenic *ras*-p21.

## Materials and methods

### Oligonucleotide sequences

To construct plasmids that express either the Ha-*ras* Val 12-p21 peptide sequence 96–110, called PNC2 (YREQIKRVKDSDDVP)

or the control peptide from mammalian cytochrome p450, called X13 (MPFSTGKRIMLGE), four oligonucleotides (plus and minus strands) encoding each sequence and including Not1 (5′) and Kpn1 (3′) restriction sites were synthesized by solid phase methods. Sequential degradation of each oligonucleotide confirmed its sequence. The oligonucleotide sequences used were, for PNC2:

- *Upper:* 5′-CGCCGCCATGGGTACAGGGAGCAGATCAAGAGGGTGAAGGACAGCGACGACGTGCCCTA
- *Lower:* 3′-GGCCGCTTAGGGCACGTCGTCGCTGTCCTTCACCCTCTTGATCTGCTCCCTGTAGCCCATand for X13:
- *Upper:* 5′-CGCCGCCATGGGCCCTTCAGCACCGGCAAGAGGATCATGCTGGGCGAGTAAGC
- *Lower:* 3′-GGCCGCTTACTCGCCCAGCATGATCCTCTTGCCGGTGCTGAAGGGGCCCATGGCGGCGGT.

In a total volume of 20 μl, 4 μl each of 0.5 m*M* upper and lower oligonucleotides including the restriction sites were annealed at 80°C, 60°C, 37°C and room temperature for 5 min each. After annealing, the 3′ ends were phosphorylated in the following reaction mixture: 2.5 μl of 10 m*M* ATP, 19 μl of annealed oligonucleotide (2 n*M*) with 2.5 μl tenfold concentrated Tris-EDTA (TE) buffer, pH 7.5, and 1 μl of T4 polynucleotide kinase (Invitrogen, Carlsbad, Calif.). The annealed, phosphorylated oligonucleotides were run on a 4% agarose TAE gel and purified using a Qiagen (Piscataway, N.J.) Gel Pure kit.

### Vector digestion and purification

We used the pOPRSVI/MCS vector from the Lac switch II mammalian expression system inducible by isopropylthioglucose (IPTG) from Stratagene (La Jolla, Calif.). This vector was cut with Kpn1 and Not1 and then incubated with 0.2 μl of calf intestinal phosphatase at 37°C and 50°C for 60 min at each temperature to remove 3′ phosphates that prevent self-ligation. The cut vector was then purified by phenol/chloroform extraction, ethanol precipitation and then resuspension in 10 μl TE buffer.

### Ligation of oligonucleotides and vector

The annealed oligonucleotides were then separately ligated into the purified pOPRSVI/MCS vector at a 3:1 ratio of target to vector using T4 ligase overnight at 4°C. The vectors containing the cloned oligonucleotides were transfected into DH5α-competent cells (Gibco-BRL, Grand Island, N.Y.) by a standard heat-shock protocol and spread on LBamp plates for overnight incubation.

From each plate, 20 colonies were selected and grown overnight at 37°C in 5 ml of LBamp liquid medium. DNA was prepared by the Qiagen Miniprep procedure, cut with Kpn1/Not1, and run on 2% agarose/TAE to estimate the size of the inserts. Clones with the correct size DNA inserts were regrown in 500 ml LBamp overnight at 37°C, and plasmids were then purified by the Qiagen Maxiprep method. An aliquot of each positive DNA was sequenced using T3 or T7 primers. Bacterial clones containing DNA plasmids with the inserted oligonucleotide sequences were frozen at –70°C in LBamp medium containing 50% glycerol and used in the microinjection experiments.

### Proteins

Val 12-Ha-*ras*-p21 and the normal Gly 12-p21 proteins were overexpressed in *E. coli* using the pGH-L9 expression vector containing the chemically synthesized Ha-*ras* gene, as previously described [13]. Insulin was purchased from Sigma (St. Louis, Mo.).

### Peptides

The ras peptide (96–110) (PNC2) and the three *raf* peptides (62–76) (PNC12), (97–110) (PNC13) and (111–121) (PNC14) from the RBD, as well as the control peptide from cytochrome p450 called X13 were prepared using solid phase synthesis. All peptides were purified using HPLC to a purity of >99%.

### Constitutively active MEK

Constitutively active MEK (caMEK) mRNA was prepared as described previously [9, 14].

### Oocyte microinjection

Oocytes were obtained from *Xenopus laevis* frogs from Connecticut Valley Biological (Southampton, Mass.) as described previously [15]. All microinjection experiments were performed at least six times on 30 oocytes (each injected with 50 nl of the appropriate solution), prepared from collagenase-digested ovarian follicles which were then incubated at 19°C for 12–24 h. Microinjected oocytes were incubated in Barth's medium or Barth's medium containing insulin, present at a concentration of 10 μg/ml, for 36 h at 19°C. Oocyte maturation was determined by observing germinal vesicle breakdown. Val 12-*ras*-p21 (100 μg/ml) (6–9) was injected alone or coinjected with either the PNC2-expressing plasmid or the negative control X13-expressing plasmid at different concentrations ranging from 50 to 500 μg/ml. For experiments involving microinjection of plasmids, injected oocytes were incubated in either the presence (50 m*M*) or the absence of the plasmid inducer, IPTG.

## Results and discussion

### Effect of PNC2 plasmid on Val 12-p21 and insulin induction of maturation

As shown in Fig. 1, co-microinjection of the PNC2 IPTG-inducible plasmid together with oncogenic p21 protein into oocytes resulted in complete inhibition in the presence of the inducer (compare the first two bars in Fig. 1). This was not a nonspecific or toxic effect of plasmid injection because injection of the plasmid for the control X13 peptide, at the same concentration as that for the PNC2 plasmid, gave no inhibition in the presence of the inducer (Fig. 1, third bar). Identical results (not shown) were obtained with microinjection of empty vector in the absence or presence of promoter. As shown in Fig. 1 (fourth bar) injection of the PNC2 plasmid alone caused about a 50% inhibition of oocyte maturation, suggesting baseline expression of PNC2 by noninduced plasmid. On the other hand, microinjection, in the presence of IPTG, of the PNC2 plasmid into oocytes did not inhibit insulin-induced maturation (Fig. 1, fifth bar). This corresponds to our prior observation that the PNC2 peptide from *ras*-p21 selectively blocks oncogenic *ras*-p21 but not insulin-activated wild-type *ras*-p21 [16].

Inhibition of oncogenic p21-induced maturation by plasmid-expressed PNC2 exhibited a dose-dependence as illustrated in Fig. 2. At 500 μg/ml of plasmid, inhibition approached 100%, at 250 μg/ml the level of inhibition fell below 50%, and at lower doses of plasmid the inhibition fell to lower levels. The dose dependence for the PNC2 plasmid parallels that found for the synthetic peptide which shows a narrow range for its dose-

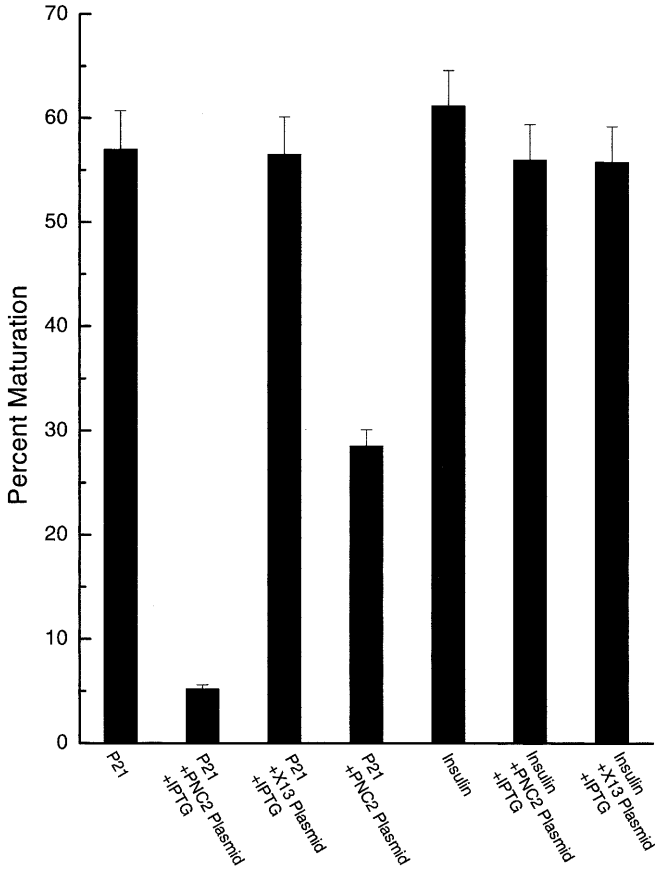


**Fig. 1** Effects of microinjection into oocytes of plasmids encoding PNC2 or X13 peptides on Val 12-p21- or insulin-induced maturation. *Bar 1* shows maturation induced by Val 12-p21 alone; *bar 2* shows the effect of coinjection of PNC2 plasmid and p21 in the presence of the inducer, IPTG; *bar 3* shows that the plasmid expressing the unrelated control X13 peptide had no effect on Val 12-p21-induced maturation; *bar 4* shows that the PNC2 plasmid expressed PNC2 peptide even in the absence of inducer, resulting in inhibition; *bars 5–7* show that insulin induced maturation (*bar 5*) which was not inhibited either by PNC2 (*bar 6*) or by X13 (*bar 7*) plasmids in the presence of IPTG. Injected p21 protein was present at a concentration of 100 μg/ml, plasmids at a concentration of 500 μg/ml, insulin at a concentration of 10 μg/ml, and IPTG at a concentration of 50 m*M*. All experiments were performed over a 36-h period

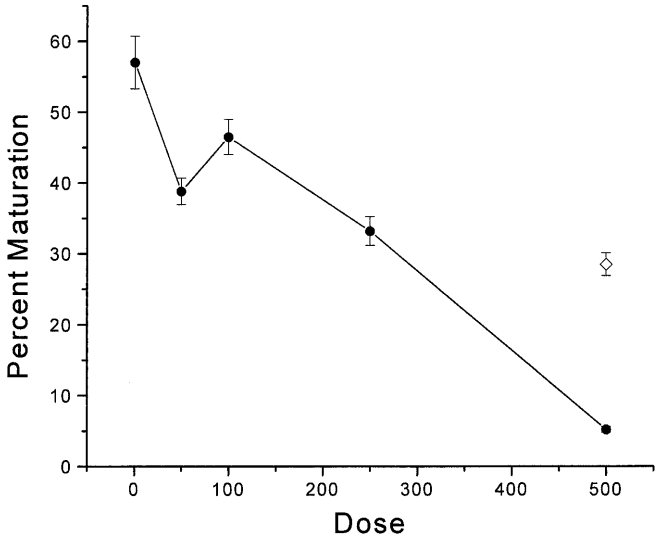


**Fig. 2** Dose-response curve for the inhibition of Val 12-p21-induced oocyte maturation by PNC2 plasmid in the presence of IPTG (*filled circles*). For reference, the level of inhibition of PNC2 plasmid in the absence of inducer at the highest concentration used (500 μg/ml) is also shown (*open diamond*)

dependence [8] that varies between low values of inhibition at concentrations below 100 μg/ml and 100% inhibition at concentrations at or above 500 μg/ml. This finding is compatible with expression of PNC2 peptide at similar concentrations by the plasmid.

Since expression of PNC2 plasmid in oocytes selectively blocked oncogenic p21 but did not affect cell viability even at high concentration (500 μg/ml), and it did not affect the ability of oocytes to respond to insulin, this vector may be effective in inducing phenotypic reversion of *ras*-transformed cells in *ras*-induced human tumors. In employing this plasmid as a therapeutic agent, it would be desirable to determine the precise steps on the *ras* signal on which it exerts an inhibitory effect.

## Sites of action of PNC2

In previous studies we have found that PNC2 peptide itself strongly blocks oncogenic p21- but not insulin-induced oocyte maturation [8, 16]. This level of inhibition correlates closely with its ability to block the binding of Val 12-p21 to JNK, suggesting that it functions to interrupt this unique interaction between oncogenic *ras*-p21 and JNK [8]. Because oncogenic p21 also induces other pathway elements that appear to be unique to it, we sought to determine whether this peptide could also inhibit these elements.

Recently, we found that both activated normal and oncogenic p21 utilize the *raf*-MEK-MAP kinase pathway since both are blocked by dominant negative *raf* [9]. Significantly, however, MPK-T4, a specific MAP kinase phosphatase, strongly blocks oncogenic p21-induced maturation but only weakly inhibits insulin-induced maturation [9]. We therefore sought to determine whether PNC2 could inhibit elements downstream of *raf* such as MEK. Since caMEK strongly induces oocyte maturation, we investigated whether PNC2 could inhibit this protein.

Figure 3 shows the effects of co-microinjecting purified mRNA encoding caMEK with PNC2 (Fig. 3, fifth bar). As can be seen in Fig. 3, caMEK induced oocyte maturation and this was strongly inhibited by PNC2. In contrast, the unrelated X13 peptide had no effect on caMEK-induced maturation (Fig. 3, seventh bar). This result stands in contrast to our previous finding [17] that PNC2 does not inhibit c-*raf*-induced oocyte maturation. This latter result can be explained if c-*raf* induces other signal transduction proteins that can either induce MAP kinase independently of MEK or can induce other MAP kinase-independent pathways. One such pathway

involves the *raf*-activated G-proteins including rac, rho and CDC 42. Both rac and CDC 42 activate JNK [18].

Effects of other *ras*-p21-inhibiting peptides on caMEK

Because PNC2 inhibits caMEK-induced oocyte maturation, we also determined the effects on caMEK-induced maturation of a set of peptides all of which block oncogenic p21 and which, unlike PNC2, also inhibit *raf*-induced maturation. These peptides have sequences corresponding to p21 residues 35–47 (PNC7) from the GAP-, *raf*-, SOS- and PI3K-binding domain of *ras*-p21 (reviewed in reference 8), and to residues 62–72 (PNC12), 97–110 (PNC13) and 111–121 (PNC14) of the RBD of *raf* [6].

As shown in Fig. 3 (bars 2–6), each of these peptides blocked MEK-induced oocyte maturation. The most effective ones were PNC2 and PNC13, both of which have the highest selectivity for inhibiting oncogenic p21 [6, 8]. Since these peptides blocked MEK, it might be concluded that their blockade of *raf* was due to their downstream effects on MEK. However, PNC2 blocked MEK but not *raf*, suggesting that downstream blockade of one target of *raf* does not inactivate *raf*-induced signal transduction.

Therefore, it seems more plausible that each of the *raf*-inhibiting peptides blocks *raf* so that it cannot interact with downstream targets such as MEK. Inactivation of caMEK by these peptides would be due to the requirement that it must interact with cellular *raf* to enable it to transmit downstream signals, despite the fact that it does not require phosphorylation by *raf*. This model predicts that caMEK binds to cellular *raf* and that this interaction is disrupted by each of the *raf*-inhibiting peptides. PNC2 would not block this interaction but would interfere in the interaction of MEK with other more downstream targets.

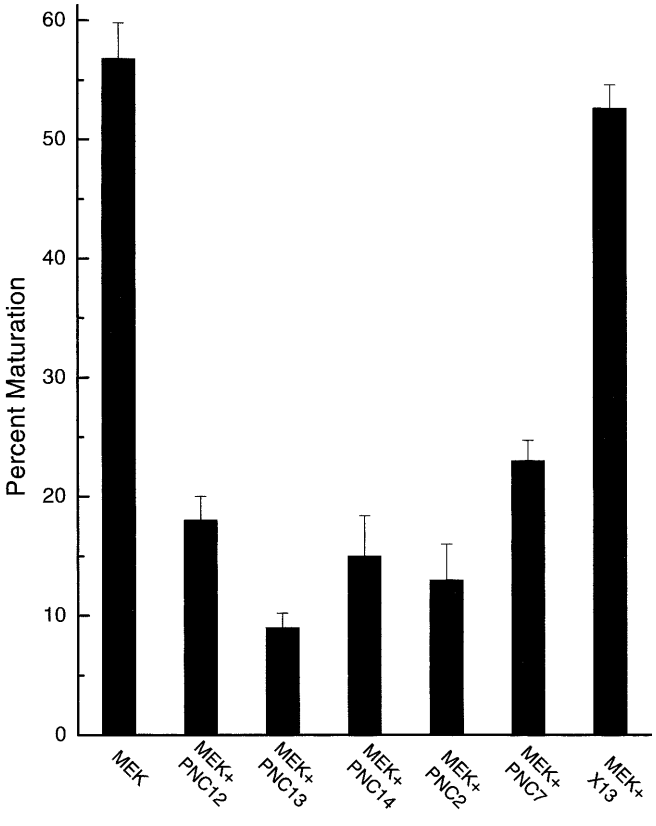


**Fig. 3** Effects of three effector *raf* RBD peptides, PNC12, PNC13 and PNC14 (*bars 2–4*) on caMEK-induced (*bar 1*) oocyte maturation and two *ras*-p21 peptides, PNC2 and PNC7 (*bars 5 and 6*) on caMEK-induced oocyte maturation. MEK mRNA was microinjected at a concentration of 100 μg/ml; all peptides were microinjected at a concentration of 400 μg/ml. All experiments were performed over a 36-h period

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