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Selective inhibition of oncogenic *ras*-p21 in vivo by agents that block its interaction with *jun*-N-kinase (JNK) and *jun* proteins. Implications for the design of selective chemotherapeutic agents

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Abstract We have obtained evidence that oncogenic and activated normal *ras*-p21 proteins utilize overlapping but distinct signal transduction pathways. Recently, we found that *ras*-p21 binds to both *jun* and its kinase, *jun* kinase (JNK). We now present evidence that suggests that oncogenic but not normal activated p21 depends strongly on early activation of JNK/*jun*. This early activation most likely involves direct interaction between oncogenic p21 and JNK/*jun* because p21 peptides that blocked the binding of p21 to JNK and *jun* strongly inhibited oncogenic p21-induced oocyte maturation while they did not inhibit insulin-activated normal cellular p21-induced maturation. Very similar results were also obtained for a newly characterized specific inhibitor of JNK which blocked oncogenic but not normal activated p21-induced oocyte maturation. We also found that both *jun* and JNK strongly enhanced oncogenic p21-induced oocyte maturation while they inhibited insulin-activated normal p21-induced oocyte maturation. These results suggest that the peptides and JNK inhibitor may be useful agents in selectively blocking the effects of oncogenic but not normal p21 in cells.

Key words Selective inhibition · Oncogenic *ras*-p21 · *jun*-N-kinase · *jun* proteins

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Introduction

The *ras*-oncogene-encoded p21 protein, a membrane-bound G-protein, activated by exchanging GDP for GTP, has been implicated in causing a large number of human tumors [1, 2] and is known to cause transformation of cells in culture and maturation of *Xenopus laevis* oocytes [1, 3]. This protein differs from its normal counterpart protein in that it contains arbitrary amino acid substitutions at critical positions such as Val for Gly 12 and Leu for Gln 61 [1].

Activated p21 interacts with a number of intracellular proteins including: GTPase-activating protein (GAP) that binds to the GTP-bound forms of p21 and enhances GTP hydrolysis of the normal (wildtype) form of the protein [4]; the *raf*-p74 protein that is vital in activating a kinase cascade involving mitogen-activated kinase (MAK) protein which, in turn, activates mitogen-activated protein (MAP) kinase, a critical protein that is involved in nuclear transcriptional events and cytoskeletal rearrangements [5, 6]; phosphoinositide-3-hydroxy kinase (PIK) [7]; and the SOS guanine nucleotide exchange protein that promotes exchange of GDP for GTP [8]. The *ras*-p21 protein has been found to interact with GAP, *raf* and PIK in the same effector domain

involving residues 32–47 [4–7] and with son-of-sevenless (SOS) protein in the region around residues 102–103 [8].

Recently, we have found that p21 interacts directly with the nuclear transcription factor *jun* and its N-terminal kinase, *jun* kinase (JNK) [9], a stress-activated protein (SAP) [10]. The incubation of p21 with JNK isolated from U937 cells and the aminoterminal phosphorylation domain of *jun* (residues 5–89) causes an increase in the rate of JNK-induced phosphorylation of *jun*. The affinity of oncogenic Val 12-p21 for JNK and *jun* is about two to three times that of the normal protein, and the rate of phosphorylation of *jun* by JNK is induced twice as rapidly by oncogenic Val 12-p21 as by the normal protein [9].

The interaction between p21 and JNK and *jun* proteins is blocked by synthetic peptide segments of p21 that have been identified as effector domains from conformational energy calculations of the average structures for oncogenic and non-oncogenic forms of the protein [11–13]. In particular, the p21 peptide corresponding to residues 96–110 blocks the binding of p21 to both JNK and *jun* proteins while the peptide corresponding to residues 115–126 blocks the binding of p21 to JNK [9].

Oncogenic p21 microinjected into metaphase-arrested stage VI oocytes in the second meiotic division causes completion of the second division, called oocyte maturation [3]. Insulin has also been found to induce oocyte maturation by uniquely activating normal cellular *ras*-p21 protein [14]. Three peptides corresponding to three regions identified as effector domains from molecular modeling studies, residues 35–47, 96–110 and 115–126, all inhibit oncogenic p21 protein-induced oocyte maturation [15, 16], and the 35–47 peptide inhibits Val 12-p21-induced transformation of NIH 3T3 cells [17]. The 35–47 peptide also inhibits insulin-induced maturation but achieves a 70% maximal level of inhibition [15]. The agent azatyrosine, which completely inhibits oncogenic p21 protein-induced oocyte maturation, has also been found to block insulin-induced maturation at the same maximal level [15, 16].

Additionally, we have found that the selective anti-protein kinase C agent, CGP 41 251, blocks oncogenic p21 induction of oocyte maturation [18] but inhibits activated normal p21 to a maximum of only 40% in inducing oocyte maturation [19, 20]. Since protein kinase C (PKC) lies downstream of p21, we have proposed that mitogenic signaling by the activated normal protein occurs by increasingly branched pathways as the signal proceeds downstream, so that signal transduction can occur by any of a number of different, presumably highly regulated, pathways, only one of which overlaps with the oncogenic pathway [19, 20].

Similarly, we have identified essential targets of activated normal p21 that are nonessential for oncogenic p21. In induction of oocyte maturation, oncogenic p21 is known to cause early activation of MAP kinase and, subsequently, of cyclins A and B which form active complexes with the p34 protein [21]. Normally, in the signal transduction pathway leading to maturation, phosphorylation and activation of cyclins occurs prior to phosphorylation of MAP kinase [21]. The activity of p34 is blocked by phosphatase 2A, the active component of the inhibitory protein, INH [22]. We have microinjected purified phosphatase 2A into oocytes together with oncogenic p21 or into oocytes incubated in insulin and have found that it selectively blocks insulin- but not oncogenic p21-induced oocyte maturation [23]. This result suggests that activation of p34 is critical to the activated normal, but not the oncogenic, signal transduction pathway.

We have recently found that a peptide from the aminoterminal *jun* regulatory domain (residues 5–89) blocks oncogenic p21- but not insulin-induced oocyte maturation, suggesting that JNK/*jun* are critical to the oncogenic but not to the normal p21 signal transduction pathway [24]. In the study reported here, we demonstrated that the *ras*-p21 96–110 and 115–126 peptides strongly blocked oncogenic p21 but did not affect insulin-activated normal p21, that a selective protein inhibitor of JNK blocked oncogenic but not normal p21, and that both JNK and *jun* proteins act synergistically with oncogenic p21 but not with activated normal p21. Our results suggest that the oncogenic *ras* pathway involves direct interaction of oncogenic p21 with JNK and *jun*, allowing for direct activation of nuclear transcription, bypassing normal cellular control mechanisms.

Methods

Materials

Oncogenic and normal p21 proteins were overexpressed in *E. coli* employing an expression vector (pGH-L9) containing the chemically synthesized c-Ha-*ras* gene and purified as described previously [25]. The 46-kDa protein isoform of JNK and its substrate, *jun* protein, were overexpressed as the p-GEX and hexa-histidinyl-construct proteins in *E. coli*, respectively, and were isolated on glutathione and nickel-affinity columns, respectively, as described previously [9]. In experiments involving inhibition by p21 peptides of *jun*-induced oocyte maturation, the pGEX construct protein was used. All peptides used in the experiments described were synthesized using solid-phase synthesis and purified by HPLC to 99% purity. Their compositions were confirmed from amino acid analysis of hydrolysates. The control tridecapeptide from cytochrome p450, called X13, has the sequence MPFSTGKRIMLGE. Activated MAP kinase was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.) and was employed directly in the microinjection experiments described below. Insulin and bovine serum albumin were purchased from Sigma (St. Louis, Mo.) and progesterone was purchased from Calbiochem (La Jolla, Calif.).

JNK inhibitor protein

We have recently isolated an inhibitor protein that specifically blocks the phosphorylation of *jun* protein by JNK (Adler V et al., submitted for publication) from whole cell lysates of 3T3/4A cells and have purified it to homogeneity using gel filtration and anion exchange chromatography. UV light activates JNK, a SAP, in this cell line [26]. The protein preparation from resting (non-UV-treated) cells completely blocks JNK-induced phosphorylation of *jun*. It does not inhibit the phosphorylation activities of *src*, PKA,

MAP kinase or casein kinase. We have found that this protein cannot be isolated from the same cell line immediately after treatment of the cells with UV light which causes the dissociation of the JNK-inhibitor complex. We have found that this protein blocks JNK-catalyzed phosphorylation of *jun* only when the two protein are bound to one another. Solutions of this protein purified to homogeneity were microinjected into oocytes at a concentration of 100 μg/ml.

Methods

Oocytes were obtained from *X. laevis* frogs from the Xenopus-1 Corp (Ann Arbor, Mich.). All microinjection experiments were performed in triplicate on 15–25 oocytes, prepared from collagenase-digested ovarian follicles. Microinjected oocytes were incubated for 12–46 h at 20 °C in Barth's medium [3, 14, 15]. Oocyte maturation was determined by observing germinal vesicle membrane breakdown (GVBD) [3, 14, 15]. Harvey-*ras* Val 12-p21 protein was microinjected at concentrations ranging from 50 to 200 μg/ml. For experiments involving incubation of oocytes with insulin or progesterone, these agents were present at concentrations of 20 μg/ml and 10 μg/ml, respectively. JNK and *jun* proteins were microinjected at a concentration of 500 μg/ml; activated MAP was microinjected at a concentration of 100 μg/ml.

Results

Effects of microinjection of MAP kinase, JNK and *jun* proteins on oocytes

We have found previously that activated and purified, cloned MEK and MAP kinase fail to induce oocyte maturation over a 24-h period [23]. Since we recently identified JNK and *jun* as being proteins to which p21 binds directly [9], we also microinjected these proteins into oocytes to determine whether they induced oocyte maturation. The results are shown in Fig. 1, which shows the effects of microinjected MAP kinase, JNK and *jun* proteins over a 46-h period. Both JNK and *jun* induced 100% maturation while activated MAP kinase caused no induction of maturation.

These results have added significance because neither JNK nor *jun* was injected in its activated phosphorylated form, but MAP kinase was injected in its activated form [23]. Thus MAP kinase is not sufficient to induce oocyte maturation while JNK and *jun* proteins are sufficient for induction of maturation. Presumably, these proteins become phosphorylated in the oocytes subsequent to microinjection.

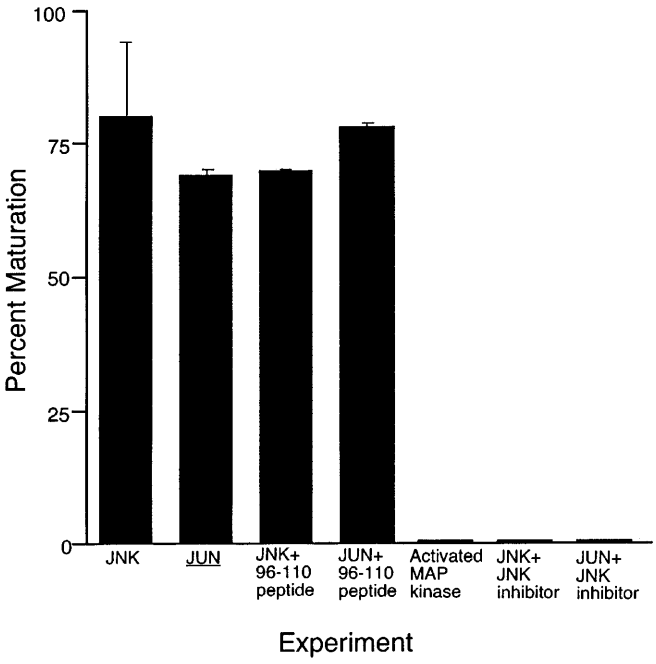

Fig. 1 Induction of oocyte maturation (observed for a maximum of 48 h) by microinjection of 500 μg/ml JNK or *jun* protein into oocytes. This contrasts with the absence of maturation induced by activated MAP kinase. Comicroinjection of the p21 peptide 96–110 (425 μg/ml), that blocks oncogenic p21-induced oocyte maturation, with JNK or *jun* at the same concentrations, is shown not to inhibit oocyte maturation induced by either of these proteins. A specific JNK inhibitory protein is seen to block both JNK and *jun*-induced oocyte maturation

Effects of JNK inhibitory protein on oocyte maturation

In resting 3T3/4A cells, a high concentration of phosphorylated JNK is present, but *jun* transcriptional activity is low [26]. Lysates of these resting cells, when incubated with activated JNK bound to GST-*jun* beads, block the phosphorylation of *jun* by JNK. We have found that this inhibitory activity results from the presence of a 21-kDa protein which does not bind to JNK or to *jun* alone but binds only to the JNK-*jun* complex. Stress, such as exposure of the cells to UV light, results in the inactivation of this inhibitor. Lysates of cells exposed to UV light, when incubated with JNK-*jun* complex, fail to inhibit phosphorylation of *jun* by JNK. This inhibitor protein has been purified to homogeneity as described in the Methods section. It selectively blocks phosphorylation of *jun* by JNK but does not affect the activity of a wide variety of other kinases as described in the Methods section.

As shown in Fig. 1, the JNK inhibitor completely blocked JNK- and *jun*-induced oocyte maturation. This result is compatible with the in vitro finding that the inhibitor protein binds and inactivates the JNK-*jun* complex. In contrast, the inhibitor protein did not affect progesterone-induced maturation (not shown), a finding that supports the conclusion that it selectively affects its target, JNK/*jun*, in vivo.

We also comicroinjected this inhibitor with oncogenic p21 into oocytes and microinjected it into oocytes incubated with insulin. The results are shown in Fig. 2A,B. The inhibitor protein completely blocked oncogenic p21-induced oocyte maturation (Fig. 2A) but did not affect the ability of insulin to induce maturation (Fig. 2B). These results clearly point to a selective inhibition of the oncogenic p21 pathway by the specific JNK inhibitory protein. Since insulin induces oocyte maturation through activation of normal cellular *ras*-p21 [14],

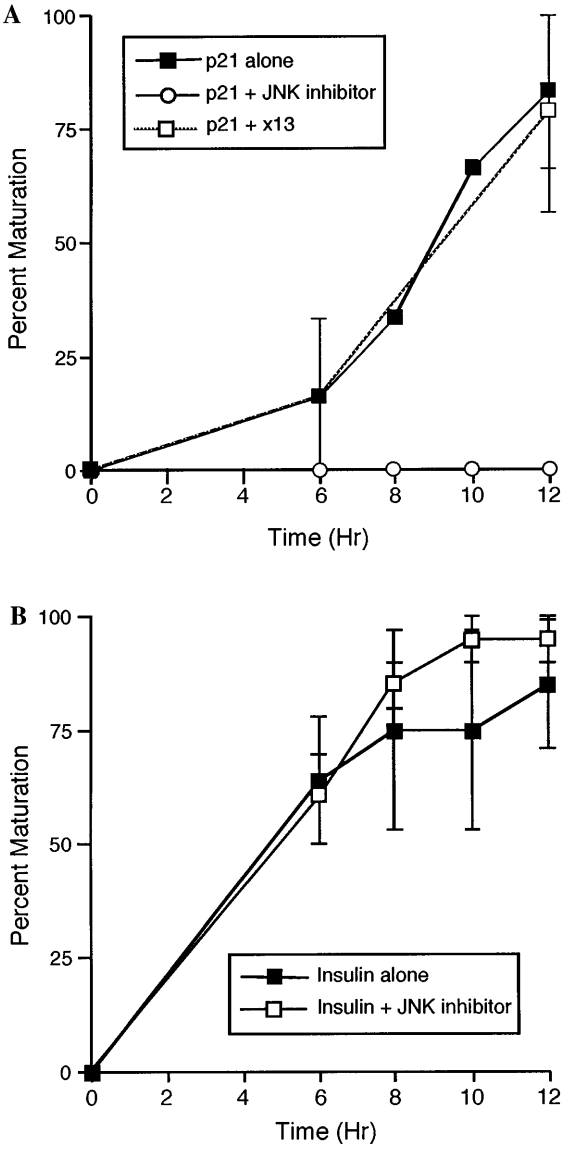

Fig. 2A Time course for the effects of comicroinjection of the 21 kDa JNK inhibitor on oncogenic p21-induced oocyte maturation (*filled squares* p21 alone, *open circles* p21 + JNK inhibitor, *open squares* p21 + control peptide X13 from cytochrome P450). No error bars have been placed on points for which the standard deviation was < 5%. **B** The effects of the same inhibitor on insulin-induced oocyte maturation (*filled squares* insulin alone, *open squares* insulin + JNK inhibitor)

the lack of inhibition by the JNK inhibitor of insulin-induced oocyte maturation implies that the normal *ras* signal transduction pathway does not require JNK/*jun*.

Selective inhibition of oncogenic p21 peptides from effector domains of p21

By comparing the average three dimensional structures for the oncogenic and normal forms of p21 using computer modeling [11–13], we have previously shown that three p21 peptides, corresponding to residues 35–47, 96–

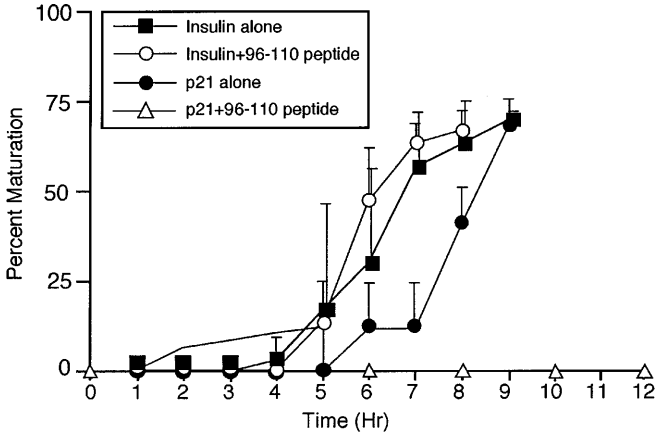

Fig. 3 The effects of comicroinjection into oocytes of the 96–110 peptide (425 μg/ml) with oncogenic p21 on oocyte maturation (*open triangles*) and of injection of the same concentration of this peptide on insulin-induced oocyte maturation (*open circles*). The effects of microinjection of 100 μg/ml oncogenic p21 alone (*filled circles*) and of incubation of the oocytes with 20 μg/ml insulin (*filled squares*) are also shown

10 and 115–126, all inhibit oncogenic p21-induced oocyte maturation [15, 16]. The 35–47 peptide has been found to block insulin-induced oocyte maturation up to a maximum of 70% [19, 20]. As shown in Fig. 3, the 96–110 peptide completely blocked oncogenic p21, as found previously, but did not affect insulin-induced oocyte maturation. Identical results (not shown) were obtained for the 115–126 peptide. Both of these peptides have been found to block directly the interaction of p21 with JNK and *jun* proteins in vitro. Combining these in vitro results with the present findings, we suggest that oncogenic p21 interacts directly with JNK/*jun* proteins, but this direct interaction is not required for the activated normal protein.

As shown in Fig. 1, the 96–110 peptide was found not to inhibit either JNK- or *jun*-induced oocyte maturation. Similar results were obtained with the 115–126 peptide (not shown). This result is compatible with our previous finding that JNK and *jun* proteins lie downstream of p21 on the signal transduction pathway [24].

Synergy between p21 and JNK and *jun* proteins

If direct interaction between oncogenic p21 and JNK/*jun* is required for the promotion of oocyte maturation, then coinjection of either of these proteins with oncogenic p21 should result in a heightened maturation response. As shown in Fig. 4, microinjection of 500 μg/ml of *jun* protein caused 100% maturation in a 3-h period whereas, without the presence of *jun*, 100% maturation occurred in a minimum of 12 h. Identical results (not shown) were obtained with coinjection of JNK (100 μg/ml) with oncogenic p21.

Also shown in Fig. 4 are the results of experiments in which JNK was microinjected into oocytes that

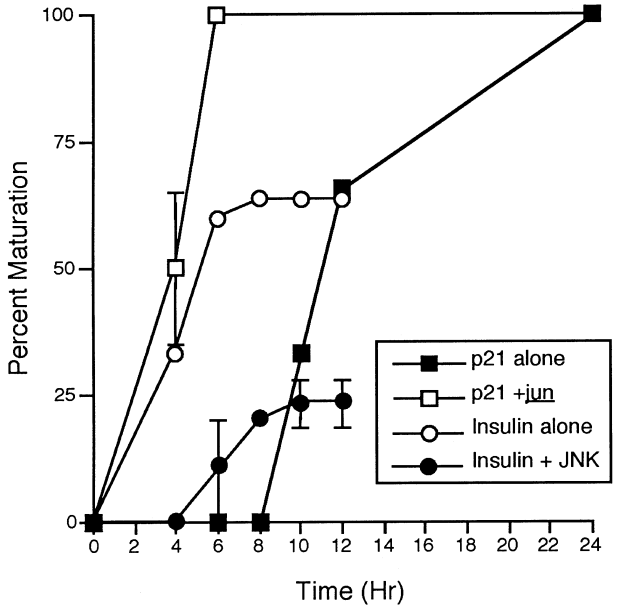

Fig. 4 Synergy between oncogenic p21 and *jun* protein. Comicroinjection of 100 mg/ml of oncogenic p21 and 500 mg/ml *jun* protein (*open squares*) caused rapid oocyte maturation. Injection of the same concentration of JNK into oocytes incubated with 20 mg/ml insulin (*filled circles*) resulted in inhibition of insulin-induced maturation. Controls: 100 mg/ml oncogenic p21 (*filled squares*) and 20 mg/ml insulin (*open circles*). No error bars have been placed on points for which the standard deviation was $< 5\%$

were then incubated in insulin. Not only was there no enhancement of maturation, but there was significant *inhibition* of insulin-induced oocyte maturation. Identical results were obtained with microinjected *jun* protein (not shown). Insulin caused a slight enhancement of oocyte maturation induced by these two proteins (not shown). The augmentation of maturation by combined JNK/*jun* with oncogenic but not with insulin-activated normal p21 further confirms the conclusion that JNK and *jun* proteins are essential direct targets for oncogenic p21 but not for normal, activated p21.

Discussion

We have previously presented results that suggest that the oncogenic and normal p21 proteins induce overlapping but distinct mitogenic signaling pathways [19, 20]. We have now identified a major early branch-point between oncogenic and activated normal p21. Oncogenic but not normal p21 protein appears to interact directly with JNK and *jun* proteins. That direct interaction between oncogenic p21 and JNK/*jun* is required on the oncogenic pathway is strongly supported by the finding that the p21 96–110 and 115–126 peptides, which were found to block the interaction of p21 with these two proteins in vitro, selectively block only oncogenic but not insulin-activated normal p21-induced oocyte maturation.

It is possible that these two peptides may also inhibit at other sites on the oncogenic p21 signal transduction pathway and this may explain their selective inhibition. However, these two peptides, unlike any of six other p21 peptides and several unrelated control peptides [9], exhibit a high level of in vitro inhibition of the binding of p21 to JNK and *jun* proteins, suggesting their uniqueness in binding to JNK and *jun* [9]. Also, we found that the 96–110 peptide inhibits oocyte maturation with an IC_{50} of 133 μg/ml that is identical to its IC_{50} for inhibition of binding of p21 to JNK [24].

That JNK and *jun* proteins are required for oncogenic but not activated normal p21 is further supported by the selective inhibition of oncogenic p21-induced oocyte maturation by the 21-kDa JNK inhibitory protein and by the synergistic interaction of oncogenic but not normal p21 protein with JNK and *jun* proteins. Our conclusion that insulin-induced oocyte maturation does not lead to a direct activation of JNK and *jun* proteins is further supported by the finding that insulin stimulates the phosphorylation of MEK but not of JNK in Chinese hamster ovary (CHO) cells that contain insulin receptors [27].

A surprising finding in these studies is that both JNK and *jun* proteins appeared to have an inhibitory effect on insulin-induced oocyte maturation. In the study of insulin-induced MEK phosphorylation [27], it was found that activated MEK causes an inactivating phosphorylation of SOS protein that results in negative feedback on the *ras* pathway. Possibly, in an analogous manner, activated JNK may also negatively feed back on the normal p21 activation process.

With respect to this latter possibility, an alternate signal transduction pathway for p21 has been described that is synergistic with but independent of the *raf*/MEK/MAP kinase pathway [28]. In the alternate pathway, the cytoskeletal proteins RAC, RHO and CDC 42 are activated by *ras*-p21. RAC and CDC 42 have been shown to stimulate the phosphorylation of JNK [29, 30]. However, RAC can also stimulate mitogenesis by pathways that are independent of JNK [30].

Since activated normal p21 can result in the phosphorylation of JNK via the RAC/RHO/CDC 42 pathway, we postulate that activated JNK and *jun* may likewise negatively feed back to activation of normal p21 as part of a highly regulated signal transduction pathway. Thus microinjection of either of these proteins into oocytes incubated with insulin may actually cause inhibition of insulin-induced maturation by negative feed back of these injected proteins of the *ras* activation process.

These considerations would not apply to the oncogenic form of the protein which directly interacts with JNK/*jun* to stimulate nuclear transcription directly. Therefore, the addition of JNK and *jun* to oocytes with oncogenic p21 would result in the observed enhancement of productive p21-JNK/*jun* complexes.

In Fig. 5, we propose a model for the differences between the oncogenic and normal *ras*-p21 pathways. Both oncogenic and activated p21 are shown to interact with *raf* p74 protein. Possibly, each of these proteins

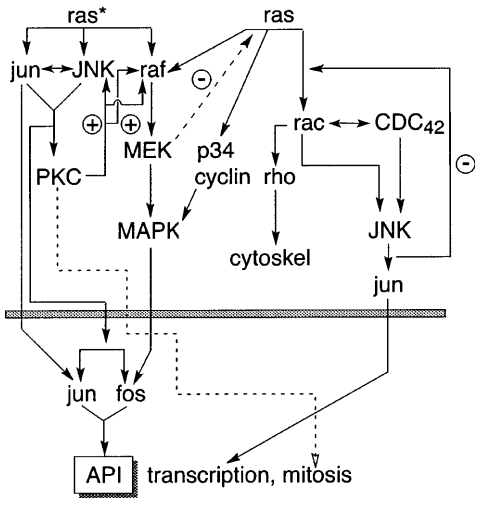

Fig. 5 Model for pathway differences between oncogenic p21 (*ras** on left side of figure) and activated normal p21 (right side of figure). Both oncogenic and normal p21 are shown in interest with *raf*, activating the MAP kinase cascade, resulting in activation of *fos* [10]. Essential elements on the right-sided oncogenic pathway are JNK/*jun* from this study and PKC [18–20]. The activated normal p21 pathway is seen to be more branched and involves activation of the RAC/RHO/CDC 42 pathway [28–30] that can result in activation of JNK [29, 30] and cause cytoskeletal rearrangements. Also p34 and cyclin are essential elements of the normal pathway but not of the oncogenic p21 protein. See text for explanation

interacts differently with this target which may partially explain why the 35–47 peptide selectively blocks oncogenic p21-induced oocyte maturation [15, 19, 20] and why oncogenic p21 causes immediate phosphorylation of MAP kinase [21]. Direct interaction of oncogenic p21 with JNK/*jun* would lead to direct stimulation of nuclear transcription and, presumably, to oocyte maturation. It is possible that even with constitutive activation of JNK/*jun* , the *raf*-induced MEK/MAP kinase pathway may be necessary for induction of oocyte maturation [21, 28] even though activation of this pathway alone is not sufficient for induction of oocyte maturation [24] or cell transformation [31].

In Fig. 5, we also show the activated normal pathway as being under tight regulatory control. Activation of MEK on the normal p21 pathway results in the down-regulation of SOS-induced p21 activation [27], represented by the negative arrow from MEK to normal *ras*. We postulate analogously that activated JNK would perform a similar function on the normal signal transduction pathway (negative arrow from JNK to normal *ras* in Fig. 5).

Since we have previously found that PKC is essential to signal transduction by oncogenic but not activated normal p21, it is represented as a target of oncogenic p21 in Fig. 5. The relationship of PKC to JNK/*jun* is not clear. We postulate that activation of JNK/*jun* may, in turn, activate PKC, which may positively feed back further to activate JNK/*jun*. It also may induce maturation by independent pathways (dotted line in Fig. 5).

PKC has also been found to activate *raf*-p74 (see, for example, reference 32), which, in turn, activates the MEK/MAP kinase cascade that may be vital for oocyte maturation. Therefore, PKC activation of *raf* is shown in Fig. 5.

As shown in Fig. 5, the activated normal protein stimulates multiple pathways which themselves are branched as postulated previously [19, 20]. Besides stimulating the tightly controlled *raf*/MEK/MAP kinase pathway, activated normal p21 activates other, branched pathways, including the RAC/RHO/CDC 42 pathway which either can stimulate JNK activation or proceed by a JNK-independent route [30] (not shown), and a pathway that results in activation of the p34 protein that then forms active complexes with cyclins A and B. This pathway may be essential in the activated normal p21 pathway because inhibition of p34 by phosphatase 2A, the active component of INH, the p34-specific inhibitor [22], results in inhibition of insulin- but *not* oncogenic p21-induced oocyte maturation [23]. Phosphatase 2A can also inhibit MEK and MAP kinase [33]. However, neither activated MEK nor MAP kinase has been found to induce oocyte maturation when injected into oocytes [23]. This finding suggests that these proteins may be necessary [28] but are not sufficient for inducing oocyte maturation by oncogenic p21 [23]. Since phosphatase 2A does not block oncogenic p21-induced oocyte maturation [23], which also presumably requires the *raf*/MEK/MAP kinase pathway, its site of action is most likely not on MEK and MAP kinase, but more likely on p34 [23].

This model can explain why overexpression of normal cellular p21 is oncogenic. At elevated concentrations of this protein intracellularly, a sufficient concentration of forms that bind directly to JNK/*jun* are present so that these two proteins become constitutively activated, causing unregulated nuclear transcription and cell division. Agents such as the p21 96–110 and 115–126 peptides and the selective JNK inhibitor protein should therefore prove to be effective in selectively inhibiting tumor growth caused by oncogenic and overexpressed normal p21 protein.

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