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ORIGINAL ARTICLE

L. Chie · S. Amar · H.-F. Kung · M. C. M. Lin · H. Chen
D. L. Chung · V. Adler · Z. Ronai · F. K. Friedman
R. C. Robinson · C. Kovac · P. W. Brandt-Rauf
Z. Yamaizumi · J. Michl · M. R. Pincus

Induction of oocyte maturation by *jun*-N-terminal kinase (JNK) on the oncogenic *ras*-p21 pathway is dependent on the *raf*-MEK-MAP kinase signal transduction pathway

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Abstract *Purpose*: We have previously found that microinjection of activated MEK (mitogen activated kinase kinase) and ERK (mitogen-activated protein; MAP kinase) fails to induce oocyte maturation, but that maturation, induced by oncogenic *ras*-p21 and insulin-activated cell *ras*-p21, is blocked by peptides from the *ras*-binding domain of *raf*. We also found that *jun* kinase (JNK), on the stress-activated protein (SAP) pathway, which is critical to the oncogenic *ras*-p21 signal transduction pathway, is a strong inducer of oocyte maturation. Our purpose in this study was to determine the role of the *raf*-MEK-MAP kinase pathway in oocyte maturation and how it interacts with JNK from the SAP pathway. *Methods*: We microinjected *raf* dominant negative mutant mRNA (DN-*raf*) and the MAP kinase-specific phosphatase, MKP-T4, either together with oncogenic p21 or c-*raf* mRNA, into oocytes or into oocytes incubated with insulin to determine the effects of these *raf*-MEK-MAP kinase pathway inhibitors. *Results*: We found that oocyte maturation induced by both oncogenic and activated normal p21 is inhibited by both DN-*raf* and by MKP-T4. The latter more strongly blocks the oncogenic pathway. Also an mRNA encoding a constitutively activated MEK strongly induces oocyte maturation that is not inhibited by DN-*raf* or by MKP-T4. Surprisingly, we found that oocyte maturation induced by JNK is blocked both by DN-*raf* and MKP-T4. Furthermore, we discovered that c-*raf* induces oocyte maturation that is inhibited by glutathione-S-transferase (GST), which we have found to be a potent and selective inhibitor of JNK. *Conclusion*: We conclude that there is a strong reciprocal interaction between the SAP pathway involving JNK and the *raf*-MEK-MAP kinase pathway and that oncogenic *ras*-p21 can be preferentially inhibited by MAP kinase inhibitors. The results imply that blockade of both MAP kinase and JNK-oncogenic *ras*-p21 interactions may constitute selective synergistic combination chemotherapy against oncogenic *ras*- induced tumors.

Key words Oncogenic *ras*-p21 · Oocyte maturation · Dominant negative mutant of *raf*

L. Chie · S. Amar · D. L. Chung · C. Kovac
Departments of Biology and Chemistry,
Long Island University, Brooklyn, NY 11201, USA

L. Chie · S. Amar · D. L. Chung · M. R. Pincus (✉)
Department of Pathology and Laboratory Medicine,
VA Medical Center, 800 Poly Place, Brooklyn, NY 11209, USA
e-mail: pincusm@vax.cs.hscsyr.edu

H.-F. Kung · M. C. M. Lin · H. Chen
Laboratory of Biochemical Physiology,
National Cancer Institute (FCRF) Building 567,
Frederick, MD 21702, USA

V. Adler · Z. Ronai
Ruttenberg Cancer Center, Mount Sinai Medical Center,
425 Madison Avenue, New York, NY 10029, USA

F. K. Friedman · R. C. Robinson
Laboratory of Molecular Carcinogenesis, NCI,
National Institutes of Health, Bethesda, MD 20892, USA

P. W. Brandt-Rauf
Division of Environmental Health Sciences,
Columbia School of Public Health, 60 Haven Avenue,
New York, NY 10032, USA

Z. Yamaizumi
National Cancer Institute, Tokyo, Japan

J. Michl · M. R. Pincus
Department of Pathology, SUNY Health Science Center,
450 Clarkson Avenue, Brooklyn, NY 11203, USA

J. Michl
Departments of Microbiology and Anatomy and Cell Biology,
Brooklyn, NY

Address all reprint requests to Dr. Matthew R. Pincus at VA Medical Center (see address above)

Introduction

Oncogenic *ras*-p21 proteins and their activated normal counterpart protein initiate signal transduction cascades that result in cell growth and proliferation [1]. In stage VI metaphase-arrested *Xenopus laevis* oocytes, microinjection of oncogenic, but not normal, p21 induces the

second meiotic division, resulting in oocyte maturation [2]. Insulin induces oocyte maturation by induction of cellular *ras*-p21, since the insulin effect can be completely blocked by microinjection of the inactivating anti-p21 monoclonal antibody, Y13–259 [3].

Both normal and oncogenic *ras*-p21 proteins require membrane insertion via addition of a farnesyl moiety to the SH-group of Cys 186 [4, 5]. Activation of normal p21 is dependent on the binding of GTP in place of GDP promoted by the exchange protein, SOS [6], and is regulated by GTPase activating protein (GAP) [7]. Both proteins bind to and activate the *raf*-p74 protein that activates a mitogen-activated protein (MAP) kinase cascade, in which direct activation of MEK results in downstream activation of MAP kinases ERK1 and ERK2 which are involved in activation of the nuclear transcription factor, *fos* [8, 9]. Microinjecting activated MEK and MAP kinase proteins into oocytes results in no maturation [10], raising the question of the role of the *raf*-MEK-MAP kinase pathway in oocyte maturation.

Subsequent to membrane insertion, oncogenic and normal proteins induce signal transduction pathways that overlap but are distinct from one another [11], as evidenced in part by the selective inhibition of oncogenic p21 versus insulin-activated normal p21 in induction of oocyte maturation [11]. We have found that small molecule inhibitors of p21, such as the agent azatyrosine, selectively block oocyte maturation induced by microinjected oncogenic p21 [11]. Microinjection of the selective protein kinase C (PKC) inhibitor, CGP 41 251, a staurosporine derivative, but not its inactive homolog, CGP 42 700, strongly inhibits oncogenic p21, but only weakly inhibits insulin-induced oocyte maturation [12, 13]. Since microinjection of PKC induces oocyte maturation that cannot be inhibited by Y13–259, but oncogenic p21 induces oocyte maturation that is inhibited by CGP 41 251, we concluded that PKC is a required downstream element on the oncogenic *ras*-p21 signal transduction pathway [11, 12].

In molecular modeling studies [14, 15, 16], we found that there are regions of the average structures of oncogenic forms of p21 that differ significantly in tertiary structure from that of the corresponding regions of the average structure of the normal protein. Peptides corresponding to these regions, in particular p21 peptides 35–47 (corresponding to an effector domain involved in binding to GAP and *raf* [7, 8]), 96–110, and 115–126, block oncogenic p21 protein-induced oocyte maturation but only minimally inhibit insulin-induced oocyte maturation [11]. Thus these peptides selectively block oncogenic p21, but not activated normal p21-induced oocyte maturation.

We found that oncogenic *ras*-p21 binds in vitro to the nuclear transcriptional activation protein, *jun*, and its activating kinase, *jun* N-terminal kinase (JNK) with higher affinities than its normal counterpart protein [17, 18]. JNK is a stress-activated protein (SAP) normally activated by the upstream kinases, MEKK, MEK4, and MEK7 in response to such stimuli as UV light, oxidative stress, and osmotic shock [19]. Microinjection of either JNK or *jun* into oocytes induces oocyte maturation [20, 21], in contrast to MEK and MAP kinase, neither of which produces this effect [10].

In the in vitro assays, we found that the two inhibitory *ras*-p21 peptides, 96–110 and 115–126, strongly block the binding of p21 to JNK in vitro [17, 18]. The dose-response curve for inhibition of the 96–110 peptide of oncogenic p21-induced oocyte maturation superimposes on the inhibition curve for the binding of oncogenic p21 to JNK [22], suggesting that this peptide blocks oncogenic p21-induced maturation by inhibiting the binding of p21 to JNK. Since neither peptide blocks insulin-induced oocyte maturation and since both completely block oncogenic p21-induced maturation, we concluded that oncogenic but not activated normal p21 requires a direct interaction with JNK/*jun*.

In further support of this conclusion, we have identified a specific protein inhibitor of JNK, that binds with high affinity to the JNK-*jun* complex and whose sequence is identical to that for the π isozyme of glutathione-S-transferase (GST-π) [23]. This protein blocks JNK-induced activating phosphorylation of *jun* but has no effect on a number of different kinases including MAP kinase, PKA, *src*, PKC, and CKII [23]. We have found that GST blocks oncogenic p21-induced maturation but has no effect on insulin-induced oocyte maturation [21, 24], further suggesting that oncogenic p21 induces maturation by a JNK/*jun*-dependent pathway not required by activated normal p21 [21, 24].

Since PKC and JNK each induce rapid oocyte maturation and are required for oncogenic p21-induced oocyte maturation, we investigated the interrelationship of these two proteins on the *ras*-p21 signal transduction pathway [25]. CGP 41 251, which blocks PKC-induced oocyte maturation, also blocks JNK-induced maturation; GST, which blocks JNK-induced oocyte maturation, also blocks PKC-induced maturation [25]. These proteins therefore appear to be interdependent in the induction of maturation [25]. Based on molecular dynamics modeling studies of p21 bound to the *ras*-binding domain (RBD) of *raf*, we identified three regions of the RBD (residues 62–76, 97–110, and 111–121), which are candidates for being effector loops of the protein [26]. We have synthesized peptides corresponding to these regions and found that all three strongly block both oncogenic p21- and insulin-induced oocyte maturation [27], suggesting that *raf*-p74 is a common target for both oncogenic and activated normal p21 proteins. Since JNK, *jun*, and PKC are all essential proteins on the oncogenic p21 signal transduction pathway, we now explore the relationship of these proteins to *raf* and its target kinase, MEK. In view of our previous findings that neither activated MEK nor MAP kinase induces oocyte maturation, we also re-examine the role of the MAP kinase pathway in oncogenic *ras*-induced oocyte maturation.

Materials and methods

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 [28]. Protein kinase C, beta isoform, was purchased from Upstate Biotechnology (Lake Placid, N.Y.) and used directly. *Jun*-N-terminal kinase (JNK1) was prepared by overexpression of the pGEX-JNK vector in *E. coli* as described previously [17]. The GST-JNK fusion protein was further purified on glutathione affinity columns as described previously. Insulin and glutathione-S-transferase, purified on a glutathione affinity column, were both purchased from Sigma (St. Louis, Mo., USA).

Peptides, protein kinase C inhibitors, and mRNA constructs

The *ras* peptide (96–110) and the three *raf* peptides (62–76), (97–110), and (111–121) from the *ras* binding domain, as well as the control peptide from cytochrome P-450 called X13, were prepared using solid-phase synthesis. All peptides were purified using HPLC, such that the purity was >99% [27]. A specific PKC inhibitor, CGP 41 251 and its inactive analog, CGP 42 700, were both obtained as gifts from Novartis Pharma (Basel, Switzerland). Dominant negative (DN) *ras*-p21 (encoding [Asn^{17}]Ha-*ras*) was prepared as described in [29]. Construction of both c-*raf* and DN-*raf* cDNAs was performed using pBluescript II SK(+) vector [29]. Each construct was then linearized and transcribed using an in vitro transcription kit (Ambion, Austin, Tex., USA). Constitutively active MEK and MAP kinase-specific phosphatase (MKP-T4) were prepared as described previously [30]. The cDNAs for both were sub-cloned into transcription vectors and were then transcribed with SP6 or T7 RNA polymerase [30]. The control mRNA for β-galactosidase was obtained as described previously [25].

Oocyte microinjection

Oocytes were obtained from *X. laevis* frogs from Connecticut Valley Biological (Southhampton, Mass., USA) as described previously [11]. All microinjection experiments were performed at least six times on 30 oocytes, 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 (GVBD). The proteins [$^{Val\ 12}$]Ha-*ras*-p21, JNK, PKC-β, and the mRNA for c-*raf*, MEK, and MAP kinase phosphatase were all injected alone or co-injected with another protein, peptide or mRNA encoding a protein. Negative controls included β-Gal mRNA and the X-13 peptide, which corresponds to a segment of cytochrome P-450 [21].

Results

As shown in Fig. 1A, dominant negative (DN-) *raf* strongly inhibited maturation induced by both oncogenic p21 and insulin. Injection of the specific inactivating MAP kinase phosphatase, MKP-T4, also blocked maturation induced by oncogenic p21 and insulin but, as shown in Fig. 1A, caused a twofold increase in inhibition of oncogenic p21, compared with that found for insulin, suggesting that insulin can induce pathways that are independent of MEK and MAP kinase. That the inhibition caused by the mRNA for the dominant negative mutant of *raf* and for MKP-T4 was a specific effect of mRNA on Val 12-p21 and insulin is supported by the finding that microinjection of the mRNA for β-gal protein had no inhibitory effect on the ability of either agent to induce maturation (Fig. 1A). The control X13 peptide (Fig. 2A) and the p21 96–110 peptide (not shown) had no effect on c-*raf*-induced oocyte maturation. Typical time courses for oncogenic p21- and insulin-induced oocyte maturation and their inhibition by DN *raf* and MKP-T4 are shown in Fig. 1B.

Our results suggest that both oncogenic and activated normal *ras*-p21 require *raf*-p74 in induction of maturation and that MAP kinase appears to be more critical on the signaling pathway for oncogenic p21 than for insulin-activated cellular p21. Since the DN mutant of c-*raf* blocked both oncogenic p21- and insulin-induced oocyte maturation, we investigated whether c-*raf*, as a critical target for both oncogenic and activated normal p21, itself could induce oocyte maturation. As shown in Fig. 2A, c-*raf* mRNA strongly induces oocyte maturation, an effect which is almost completely abolished by co-injection of the mRNA for its DN mutant. Similar inhibition of c-*raf*-induced oocyte maturation was achieved by co-injection of mRNA for MKP-T4 (Fig. 2A). As expected, the [Asn^{17}]Ha-*ras*-p21 DN p21 mutant completely blocked oncogenic p21-induced maturation but failed to inhibit c-*raf*-induced maturation (not shown). Typical time courses for c-*raf*-induced oocyte maturation and its inhibition by DN-*raf*, MKP-T4, and *raf* peptide 97–110 (as discussed below) are shown in Fig. 2B.

In earlier studies, we identified three peptide domains from the RBD of *raf* that were likely to be effector domains and synthesized three peptides corresponding to these domains [26]. Microinjection of each of these peptides into oocytes strongly inhibited both Val 12-p21- and insulin-induced oocyte maturation [27]. Since these peptides were derived from *raf*, we have investigated whether they can also inhibit c-*raf*-induced oocyte maturation. As shown in Fig. 2A, each of these peptides was found to induce a four-to-five-fold inhibition of c-*raf*-induced maturation, suggesting that all three RBD peptides block *ras*-p21 at the level of its interaction with c-*raf*. The most pronounced inhibition was achieved with the 97–110 peptide, a finding that correlates with prior results for its inhibition of oncogenic 21 [27].

Since c-*raf* induces maturation which is inhibited by MKP-T4 (Fig. 2), we investigated whether MEK could itself induce maturation since it is the direct target of activated *raf* [9]. We previously found that microinjection of activated and cloned purified forms of MEK fails to induce oocyte maturation [10]. A construct gene encoding a constitutively active form of MEK [31] has been transfected into NIH 3T3 cells, which were found to undergo transformation [31]. We have obtained the mRNA for this construct and microinjected it into oocytes.

As shown in Fig. 3, constitutively active MEK, in contrast to the wild-type protein, strongly induces

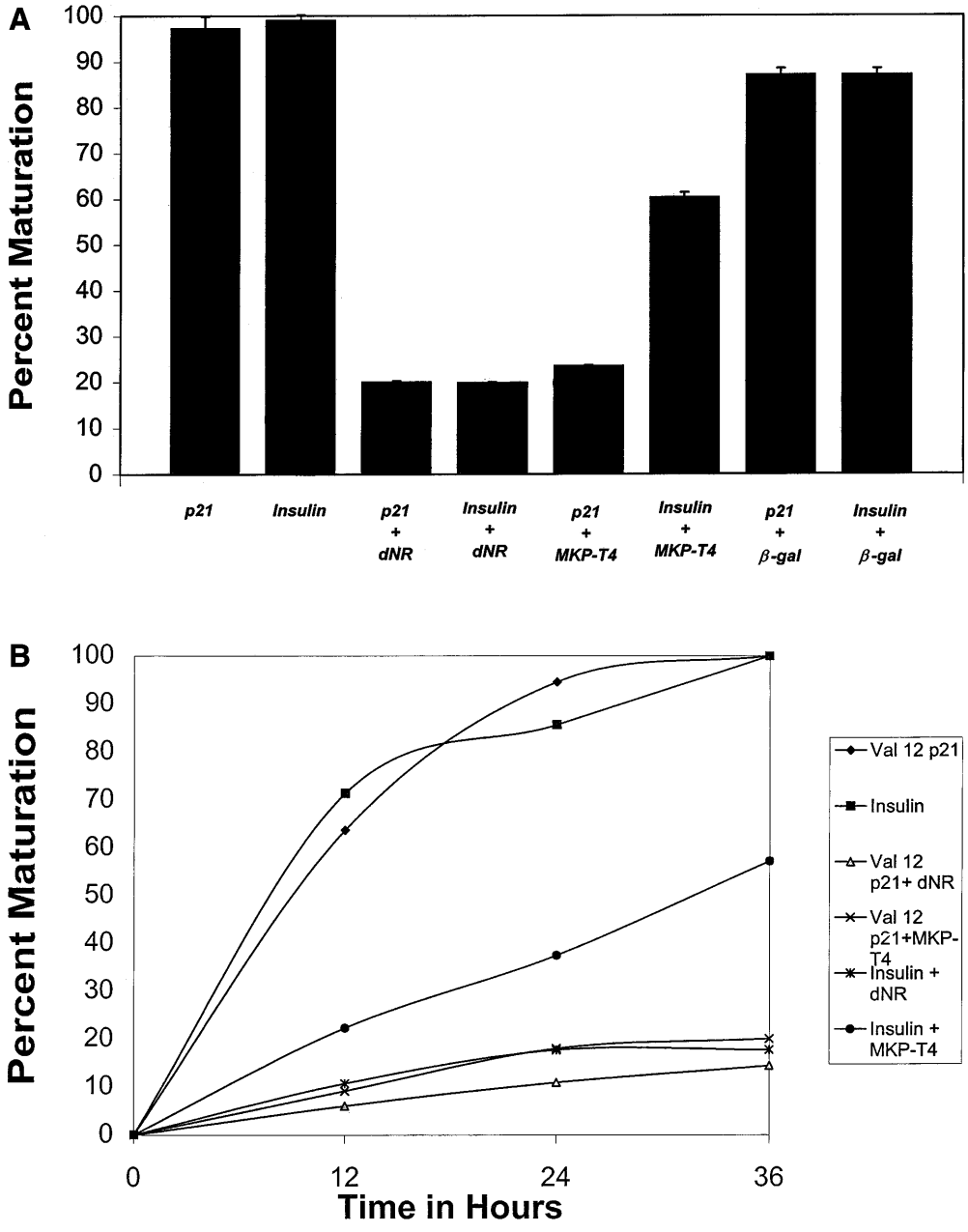

Fig. 1 **A** Effects of dominant negative-*raf* (*dNR* in figure) and the MAP kinase-specific phosphatase, MKP-T4, on oncogenic *ras*-p21 (p21 in figure) and insulin-induced oocyte maturation. β-Gal controls are also shown; *ras*-p21 was injected at a concentration of 100 μg/ml; DN-*raf*, MKP-T4, and β-gal were all microinjected at a concentration of 200 μg/ml; insulin concentration in the medium was 10 μg/ml. Standard errors are shown as horizontal bars. **B** Time course for induction of oocyte maturation induced by Val 12-p21 and insulin and the effects of dominant negative *raf* (dNR) and MKP-T4 on Val 12-p21- and insulin-induced maturation

oocyte maturation, an effect which is not inhibited by DN-*raf* or by MKP-T4. Lack of inhibition of constitutively activated MEK by DN-*raf* is consistent with MEK's being downstream of *raf*. Since MEK is known to activate MAP kinase, it is surprising that MEK-induced maturation is not blocked by MKP-T4. This result suggests that constitutively activated MEK may induce MAP kinase-independent signal transduction pathways.

In contrast to the results we obtained with wild-type MEK protein, microinjection of wild-type JNK protein into oocytes strongly induces maturation [20, 21]. Since oncogenic p21 interacts with JNK but also requires *raf* and MEK (Figs. 1 and 2), we explored whether JNK may require activation of the *raf*-MEK-MAP kinase pathway. Surprisingly, as shown in Fig. 4, co-injection of DN-*raf* with JNK or co-injection of MKP-T4, strongly inhibits JNK-induced oocyte maturation. In contrast to Fig. 4, the *ras*-p21 96–110 peptide that blocks the interaction of p21 with JNK does not block JNK-induced maturation, as found previously [20, 21].

These results suggest that JNK, an element of the SAPs pathway [19], depends for its effect on the *raf*-MEK-MAP kinase pathway. Other studies have suggested that there is minimal cross-talk between these pathways [32]. The results also suggest the possible activation of JNK by *raf* and/or by MEK/MAP kinase in oocytes.

Since JNK is dependent upon *raf*/MEK in induction of maturation, we have further explored whether inhibition of JNK has an effect on *raf*-induced oocyte maturation. We have found that GST protein is a strong

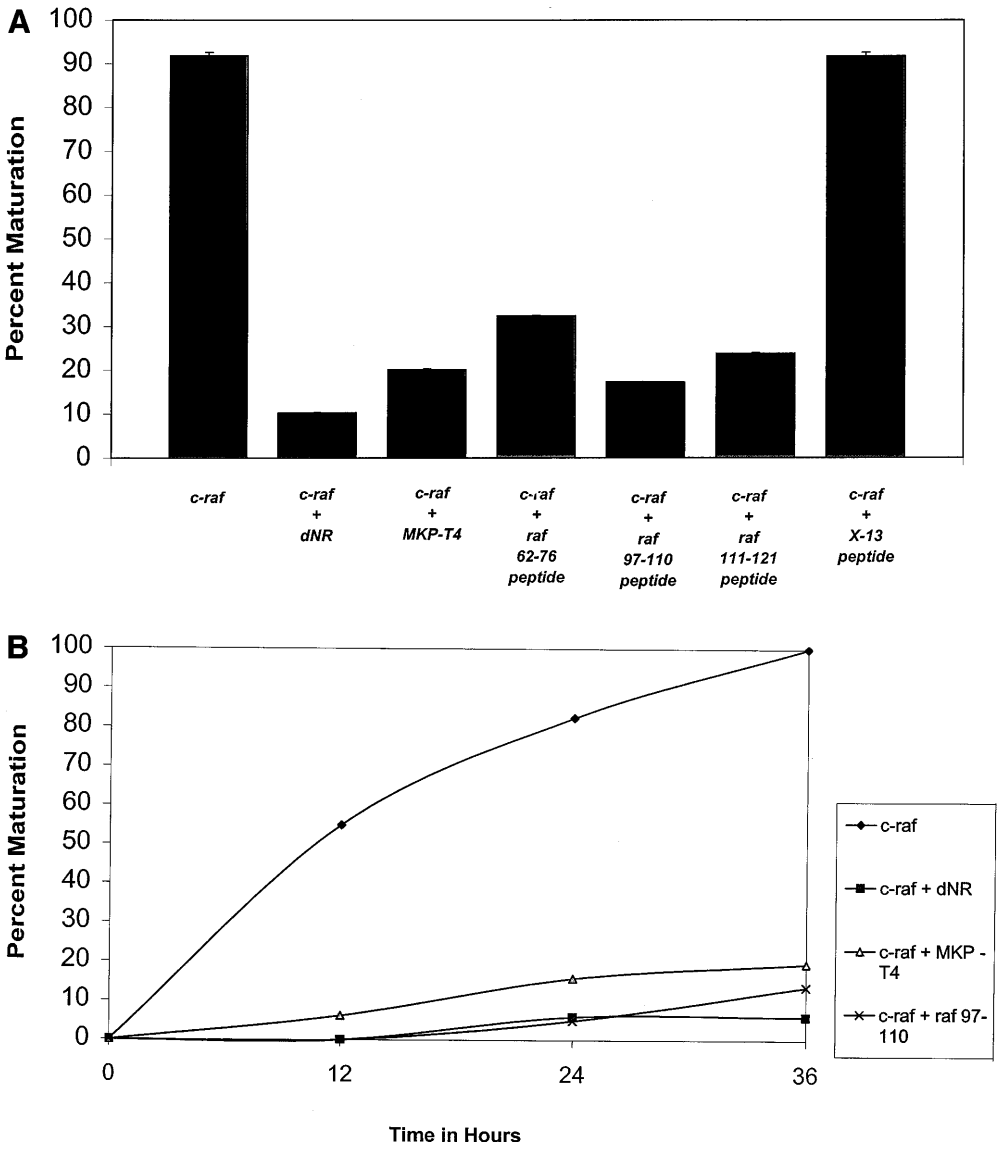

Fig. 2 **A** c-*raf* induces oocyte maturation, which is inhibited by dominant negative-*raf* (*dNR*), MKP-T4, and three peptides from the *ras*-binding domain of *raf*, corresponding to residues 62–76, 97–110, and 111–121. DN*raf* and MKP-T4 were injected at a concentration of 200 μg/ml; c-*raf* and all peptides were injected at a concentration of 100 μg/ml. **B** Time course of c-*raf*-induced oocyte maturation alone and with dominant negative *raf* (*dNR*), MKP-T4, and *raf* 97–110 peptide

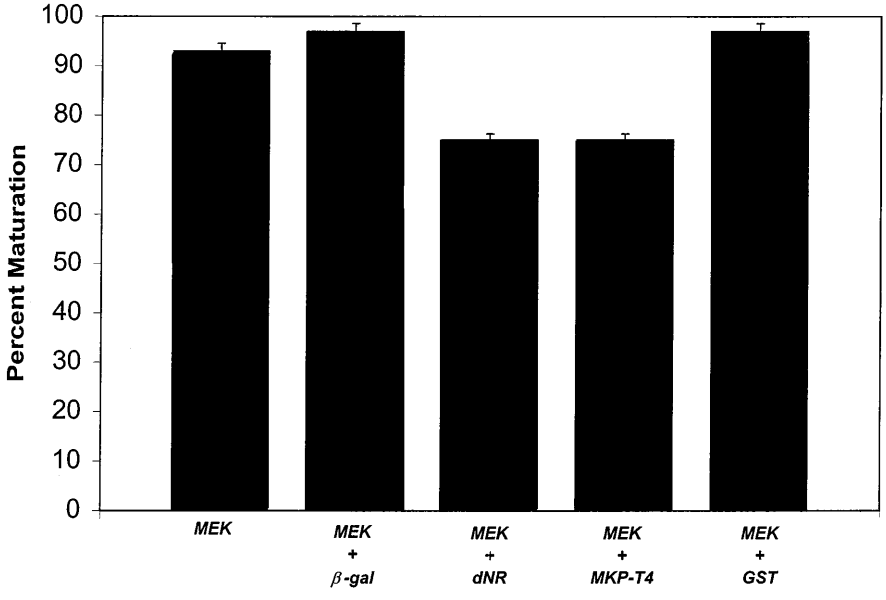

Fig. 3 Constitutively activated MEK induces oocyte maturation which is not inhibited by dominant negative-*raf* (*dNR*; 200 μg/ml), MKP-T4 (200 μg/ml), glutathione-S-transferase (100 μg/ml), a *jun*-N-terminal kinase inhibitor or the β-Gal control mRNA (200 μg/ml)

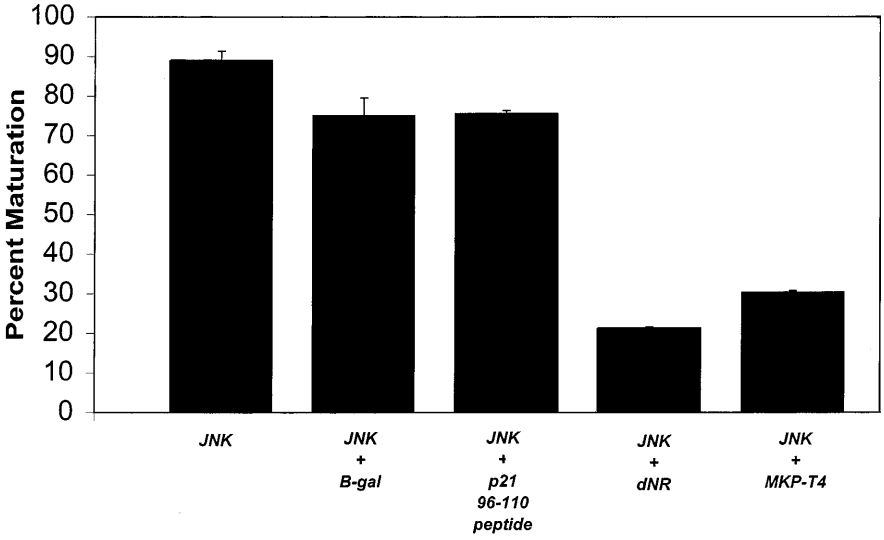

Fig. 4 *jun*-N-terminal kinase induces oocyte maturation that is not inhibited by the β-Gal control (200 μg/ml) or by the *ras*-p21 peptide 96–110 (425 μg/ml), but is inhibited by dominant negative-*raf* (*dNR*) and MKP-T4 (each at a concentration of 200 μg/ml)

inhibitor of JNK-induced activation of *jun* but has no effect on activation of MAP kinase [23]. Therefore, we co-injected c-*raf* with GST into oocytes. As shown in Fig. 5, GST causes about a twofold inhibition of c-*raf*-induced oocyte maturation. On the other hand GST fails to inhibit oocyte maturation when co-injected with constitutively activated MEK (Fig. 3).

These results suggest that JNK and c-*raf* require one another on the maturation induction pathway and that the GST inhibition of oocyte maturation induced by c-*raf* occurs at the level of this protein rather than at MEK or MAP kinase. This conclusion is further based on our previous observation that GST does not inhibit MAP kinase in vitro [23]. If JNK is a target of *raf*, GST inhibition of JNK could also explain its inhibition of *raf*-induced maturation.

We have found that induction of maturation by JNK depends on activation of PKC [25]. Reciprocally, to induce maturation, PKC requires activation of JNK [25]. These conclusions are based on the findings that the PKC inhibitor, CGP 41 251, which selectively blocks PKC, also blocks JNK-induced oocyte maturation and that GST, which selectively blocks JNK-induced activation of *jun*, reciprocally blocks PKC-induced oocyte maturation [25].

Since PKC is a known activator of *raf* [33], we explored the effects of CGP 41 251 on *raf*-induced oocyte maturation and the effect of DN mutant *raf* on PKC-induced oocyte maturation. As shown in Fig. 5, the PKC inhibitor, CGP 41 251, caused almost a twofold inhibition of c-*raf*-induced oocyte maturation. Conversely, both DN-*raf* and MKP-T4, strongly blocked PKC-induced oocyte maturation. None of these agents (DN-*raf*, MKP-T4, GST, and CGP 41 251) inhibited constitutively activated MEK as shown in Fig. 3 (results for CGP 41 251 not shown), a result that is consistent with previous findings that MEK is a downstream element on the *raf* pathway [9].

Discussion

Our results establish that c-*raf* is a vital target of both oncogenic and activated cellular *ras*-p21 in oocytes, as

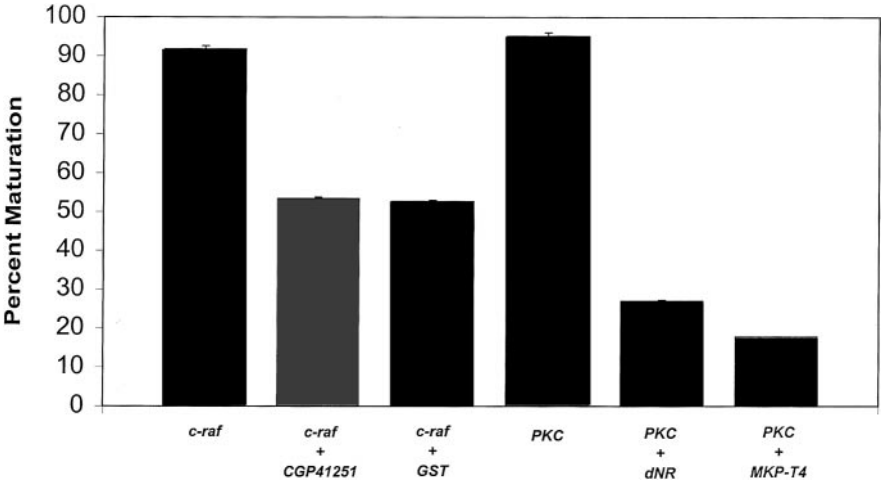

Fig. 5 Effects of protein kinase C- (PKC) specific inhibitor, CGP 41 251 (100 μg/ml) and the *jun*-N-terminal kinase (JNK) specific inhibitor, glutathione-S-transferase (100 μg/ml), on c-*raf* (100 μg/ml) induction of oocyte maturation. Also shown are the effects of dominant negative *raf* (*dNR*) and MKP-T4 (both at 200 μg/ml) on PKC-induced oocyte maturation

evidenced by complete inhibition of Val 12-p21- and insulin-induced oocyte maturation by DN-*raf*. This agent has been found to block erythroid differentiation of the ventral mesoderm in *Xenopus* embryonic development [29], establishing that the *ras*-*raf* signal transduction pathway is central to this stage of embryogenesis. Thus both oncogenic and normal proteins require intact *raf* for expression of their cellular effects. That *raf* lies downstream of *ras* in oocyte maturation is supported by the findings that neither DN-*ras* nor the p21 96–110 inhibitory peptide blocks c-*raf* induction of oocyte maturation.

We have previously found that both oncogenic and activated normal p21 proteins require membrane insertion prior to induction of cell signaling [11]. We postulate that, subsequent to membrane insertion, both proteins activate c-*raf*. Since each protein induces oocyte maturation by divergent pathways, we further infer that pathway divergences must therefore occur concurrently with or after the interaction of each protein with *raf*.

Microinjection of c-*raf* into oocytes induces maturation, an effect which is blocked both by DN-*raf* and by MAP kinase phosphatase. The latter result supports the finding that MAP kinase is a critical target of *raf* [34] on the oocyte maturation pathway. That MEK is downstream of *raf* on the *raf* signal transduction pathway in oocytes is further supported by the findings that constitutively activated MEK induces oocyte maturation which is not inhibited by DN-*raf* (Fig. 3).

From molecular modeling studies [26], we identified three regions of the RBD of *raf* (i.e., RBD residues 62–76, 97–110, and 111–121) as putative effector domains involved in *raf* activation. Peptides corresponding to these three domains were synthesized and were found to block both oncogenic p21- and insulin-activated c-*ras*-p21-induced oocyte maturation. From these results, we postulated that both oncogenic and activated normal p21 require activation of *raf* on their respective pathways [27]. We now find that these three RBD peptides block oocyte maturation induced by c-*raf* (Fig. 2). Since these peptides inhibit *ras*-p21 and *raf*, a downstream target of *ras*, we conclude that the three RBD peptides inhibit *raf* directly. These peptides may (1) block the interaction of *ras*-p21 with the RBD, (2) block the activation by the RBD of the kinase domain of *raf*; or (3) interfere with the interaction of *raf* with MEK, its immediate downstream target.

Since MAP kinase is a known direct target of *raf*-activated blockade of MAP kinase using MKP-T4, would be expected to block both oncogenic p21- and insulin-induced oocyte maturation. However, as shown in Fig. 1, MKP-T4 exerts a significantly stronger effect on oncogenic p21 than on its normal counterpart protein, suggesting that induction of oocyte maturation by activated normal p21 is partially independent of MEK activation. This finding points to the possibility that MAP kinase inhibitors may effect the selective inhibition of oncogenic p21 leaving the normal pathway intact and may therefore be effective as anti-*ras*-induced tumor agents.

A unique pathway induced by oncogenic p21, but *not* by normal activated p21, is direct activation of JNK/*jun* [17, 21]. Since microinjection of JNK (or *jun*) into oocytes results in maturation (Fig. 5; [20, 21]), activation of JNK is not likely to be due to its interaction with cellular p21. Consistent with this conclusion is the finding that the *ras*-p21 96–110 peptide, which blocks the interaction between JNK and *ras*-p21, does not interfere with activation of microinjected JNK (Fig. 1A). JNK is a downstream target on the SAP pathway, which has been found to be almost completely independent of the *raf*-MEK-MAP kinase pathway [32].

It is therefore surprising that JNK-induced maturation is blocked by both *raf*- and MAP kinase-specific inhibitors (Fig. 4). These results imply that an intact *raf*-MEK-MAP kinase axis is required for proper expression of JNK. There are several possible interactions among these pathway elements that can explain these results. One possibility is that activation of microinjected JNK occurs as a consequence of a direct interaction between JNK and cellular *raf* between JNK and *raf*-activated MEK or between JNK and MAP kinase. Any of these two possible events would imply that microinjected JNK can be directly activated by any of these proteins. Inhibition of JNK-induced maturation by MKP-T4 may be brought about either if activated cellular MAP kinase is involved in the activation of JNK or if MKP-T4 has a broader specificity such that activated JNK is directly dephosphorylated by this phosphatase. Either event would result in a decreased level of activated JNK in oocytes.

Alternatively, activation of microinjected JNK may be independent of the *raf*-MEK pathway, but its expression in inducing maturation may require an intact but independent *raf*-MEK-MAP kinase pathway. The latter results in activation of the nuclear transcription factor, *fos*, which is required for the formation of the heterodimeric AP-1 complex with *jun*, the direct target of JNK, although JNK can activate *fos* [35]. This latter possibility still leaves open the question as to the mechanism by which, in oocytes, the *raf*-MEK-MAP kinase pathway becomes activated after microinjection of JNK. We have recently found that MAP kinase (ERK-1) levels in resting oocytes are high, but the active, phosphorylated levels are not expressed in these oocytes (J. Michl and M.R. Pincus, unpublished observations).

Regardless of the nature of the interaction between JNK and *raf*-MEK, it is clear that a strong reciprocal interaction exists between these proteins in the induction of oocyte maturation. Not only do *raf* and MAP kinase inhibitors cause about a fivefold inhibition of JNK-induced maturation (Fig. 4), but GST, a specific inhibitor of JNK [23], causes about a twofold inhibition of *raf*-induced oocyte maturation (Fig. 5). The latter implies that the *raf*-MEK pathway is partially dependent on an intact JNK-*jun* pathway.

This particular interaction between the pathways appears to be unique to the oncogenic *ras* pathway because GST inhibits oncogenic p21-induced maturation but does not inhibit insulin-induced oocyte maturation [24]. However, insulin-induced maturation depends on *raf* (Fig. 1), which itself is partially inhibited by GST. We therefore conclude that GST selectively blocks a *raf*-induced pathway utilized selectively by oncogenic *ras*-p21.

In addition to JNK, oncogenic p21 requires PKC on the oocyte maturation pathway [23]. Both JNK and PKC require reciprocal activation on the oncogenic p21 pathway [25]. Since PKC is known to activate *raf* [33], we have studied the effects of PKC inhibitors on *raf*-induced oocyte maturation. The specific PKC inhibitor, CGP 41 251 [13], induces about a twofold inhibition of *raf*-induced oocyte maturation (Fig. 5). Reciprocally, both DN-*raf* and MKP-T4 induce a fivefold inhibition of PKC-induced oocyte maturation (Fig. 5). Since the concentration of CGP 41 251 (1 μM) completely blocks maturation induced by PKC but partially blocks *raf*-induced maturation, it is likely that *raf* can be activated by PKC-independent pathways. Inhibition of PKC-induced oocyte maturation by DN-*raf* and MKP-T4 may imply that PKC acts directly through the *raf*-MEK pathway; or that PKC requires JNK which, in turn, requires the *raf*-MEK pathway; or that mutually activated PKC and JNK both require *raf*-MEK-MAP kinase.

The possible relationships among the different elements of the pathway involved in oncogenic p21- and insulin-activated cellular p21-induced oocyte maturation are summarized in Fig. 6. Both oncogenic and activated normal p21 require direct interaction with *raf*. Oncogenic p21 requires *raf* activation of MEK (*solid arrow*), while activated normal p21 does not completely depend on activation of MEK (*broken arrow*). Since activated normal p21 requires *raf*, the possibility exists that *raf* may activate another pathway (*question mark in the box*).

Previously, we found that the cyclin inhibitor, INH, the active component of which is phosphatase-2A, blocks insulin-induced oocyte maturation but only minimally affects oncogenic p21-induced maturation [10]. This result suggested that activated cellular but not oncogenic p21 required early activation of the cyclins. Thus in Fig. 6, we show an "alternative" *raf* pathway for activated cellular p21 involving cyclins. Oncogenic p21 activates JNK, which, for induction of maturation, depends on *raf* and MEK (*arrow 1*) and on PKC (*arrow 2*). PKC, in turn, depends on both JNK and on the *raf*-MEK pathway (*arrow 3*). Activation of JNK and MAP kinase results in activation of *jun* and *fos*, respectively, and in the formation of the AP1 transcription complex. Fig. 6 summarizes discrete steps at which oncogenic p21 can be selectively inhibited: at the p21-JNK level (p21 peptide 96–110), at the JNK-PKC level (GST and CGP 41 251), at the PKC-*raf*-MEK level (GST and CGP 41 251), and at the MEK-MAP kinase level (MKP-T4). Inhibition at more than one of these steps should result in a synergistic block of oncogenic *ras*-p21.

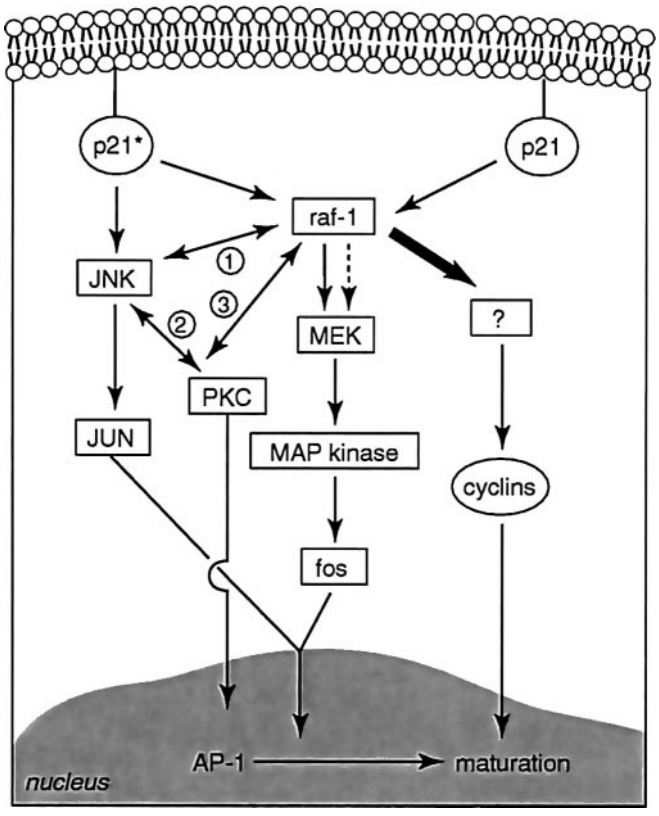

Fig. 6 Summary of proposed pathway elements of the oncogenic (p21*, *left side* of figure) and activated normal *ras*-p21 (*right side* of figure) proteins. Both oncogenic and normal proteins depend on *raf*. Since MKP-T4, the MAP kinase phosphatase, inhibits oncogenic more than activated normal p21, two arrows from *raf* have been drawn, one dashed to MEK, indicating that this is one of a number of possible targets for c-*ras*-activated *raf* and the rightmost (*large*) arrow from *raf* indicating other pathways that may be activated by *raf* (*box with question mark*). The leftmost arrow from *raf* shows that activation of MEK by *raf* is the unique pathway induced by oncogenic p21. Since activated normal p21 has been found to require activation of cyclins, the latter proteins are shown to be essential to the signal transduction pathway induced by this protein [10]. Oncogenic p21 requires *jun*-N-terminal kinase (JNK) [21] which, reciprocally, requires activation of *raf* (*circled 1* in figure). Similarly, JNK reciprocally requires protein kinase C (PKC; *circled 2* in figure) [25]. Finally PKC and *raf* interact in a mutually dependent manner (*circled 3* in figure). Activation of *jun* and *fos* by JNK and mitogen-activated protein (MAP) kinase on the stress-activated and *raf*-MEK-MAP kinase pathways, respectively, results in the formation of the heterodimeric AP-1 complex

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References

1. Barbacid M (1987) *ras* Genes. Ann Rev Biochem 56: 779
2. Birchmeier C, Broek D, Wigler M (1985) *ras* Proteins can induce meiosis in *Xenopus* oocytes. Cell 43: 615

3. Deshpande AK, Kung H-F (1987) Insulin induction of *Xenopus laevis* oocyte maturation is inhibited by monoclonal antibody against p21 *ras* proteins. Mol Cell Biol 7: 1285
4. Willumsen BM, Christensen A, Humbert NL, Papageorge AG, Lowy DR (1984) The p21 *ras* C-terminus is required for transformation and membrane association. Nature 310: 583
5. Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS (1990) Inhibition of purified p21-*ras* farnesyl-protein transferase by Cys-AAX tetrapeptides. Cell 62: 81
6. Chardin P, Camonis JH, Gale NW, Van Aelst L, Schlessinger J, Wigler MH, Bar-Sagi D (1993) Human SOS1: a guanine nucleotide exchange factor for ras that binds to Grb2. Science 260: 1338
7. Adari H, Lowy DR, Willumsen BF, Der CJ, McCormick F (1988) Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science 240: 518
8. Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993) Complexes of ras-GTP with raf-1 and mitogen-activated protein kinase. Science 260: 1658
9. Zhang X-F, Settleman J, Kryiakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J (1993) Normal and oncogenic $p21^{ras}$ protein bind to the amino terminal regulatory domain of c-raf-1. Nature 364: 308
10. Haspel J, Dent P, Haystead T, Brandt-Rauf PW, Chung D, Weinstein IB, Nishimura S, Yamaizumi Z, Pincus MR (1995) *raf*-Induced kinases may be necessary but are not sufficient for *ras*-p21 protein induction of oocyte maturation. Med Sci Res 23: 455
11. Chung DL, Joran A, Friedman FK, Robinson RR, Brandt-Rauf PW, Weinstein IB, Ronai ZA, Baskin L, Dykes DC, Murphy RB, Nishimura S, Yamaizumi Z, Pincus MR (1992) Evidence that oocyte maturation induced by an oncogenic *ras* p21 protein and insulin is mediated by overlapping yet distinct mechanisms. Exp Cell Res 203: 329
12. Chung DL, Brandt-Rauf PW, Weinstein IB, Nishimura S, Yamaizumi Z, Murphy RB, Pincus MR (1992) Evidence that the *ras* oncogene-encoded p21 protein induces oocyte maturation via activation of protein kinase C. Proc Natl Acad Sci USA 89: 1993
13. Meyer T, Regenass U, Fabbro D, Alteri E, Rosel J, Muller M, Caravatti G, Matter A (1989) A derivative of staurosporine (CPG 41 251) shows selectivity for protein kinase C inhibition and in vivo anti-tumor activity. Int J Cancer 43: 851
14. Liwo A, Gibson KD, Scheraga HA, Brandt-Rauf PW, Monaco R, Pincus MR (1994) Comparison of the low energy conformations of an oncogenic and a non-oncogenic p21 protein, neither of which binds GDP or GTP. J Protein Chem 13: 237
15. Monaco R, Chen JM, Friedman FK, Brandt-Rauf PW, Pincus MR (1995) Structural effects of the binding of GTP to the wild-type and oncogenic forms of the *ras*-gene-encoded p21 proteins. J Protein Chem 14: 721
16. Chen JM, Brandt-Rauf PW, Pincus MR (1996) Oncogenic amino acid substitutions in the inhibitory rap-1 A protein cause it to adopt a *ras*-p21-like conformation as computed using molecular dynamics. J Biomol Struct Dyn 13: 925
17. Adler V, Pincus MR, Brandt-Rauf PW, Ronai Z (1995) Complexes of *ras*-p21 with *jun*-N-kinase and c-*jun* proteins. Proc Natl Acad Sci USA 92: 10585
18. Adler V, Pincus MR, Polotskaya A, Montano X, Friedman F, Ronai Z (1996) Activation of C-*jun* NH_2-terminal kinase by UV-irradiation is dependent on $p21^{ras}$. J Biol Chem 271: 23304
19. Ip YT, Davis, RJ (1998) Signal transduction by the c-*jun* N-terminal kinase (JNK) from inflammation to development. Opin Cell Biol 10: 205
20. Glozman A, Amar S, Chung D, Adler V, Ronai Z, Brandt-Rauf PW, Nishimura S, Yamaizumi Z, Pincus MR (1996) Evidence that signal transduction by oncogenic *ras*-p21 protein depends on its interaction with *jun* kinase and *jun* proteins. Med Sci Res 24: 331
21. Amar S, Glozman A, Chung DL, Alder V, Ronai Z, Friedman FK, Robinson R, Brandt-Rauf PW, Yamaizumi Z, Pincus MR (1997) 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. Cancer Chemother Pharmacol 41: 79
22. Pincus MR, Brandt-Rauf PW, Michl J, Friedman FK (1999) ras-p21-Induced cell transformation: unique signal transduction pathways and implications for the design of new chemotherapeutic agents. Cancer Invest (in press)
23. Adler V, Yin Z, Fuchs S, Benezra M, Rosario L, Tew K, Pincus MR, Sardana M, Henderson C, Wolf CR, Davis R, Ronai Z (1999) GSTπ – a regulator of JNK signaling. EMBO J 18: 1321
24. Villafania A, Anwar K, Amar S, Way D, Chung D, Adler V, Ronai Z, Brandt-Rauf PW, Yamaizumi Z, Kung H-F, Pincus, MR (1999) Glutathione-S-transferase as a selective inhibitor of oncogenic *ras*-p21-induced mitogenic signaling through blockade of activation of *jun* by *jun*-N-terminal kinase. Ann Clin Lab Sci 30: 57
25. Chung D, Villafania A, Anwar K, Amar S, Rijwani K, Kung H-F, Adler V, Ronai Z, Brandt-Rauf PW, Yamaizumi Z, Pincus MR (1998) Mutual dependence of *jun*-N-terminal kinase and protein kinase C on the oncogenic *ras*-p21 protein-induced mitogenic signaling pathway. Med Sci Res 26: 147
26. Chen JM, Monaco R, Manolatos S, Brandt-Rauf PW, Friedman FK, Pincus MR (1997) Molecular dynamics on complexes of *ras*-p21 and its inhibitor protein, rap-1 A, bound to the *ras*-binding domain of the *raf*-p74 protein. Identification of effector domains in the *raf* protein. J Protein Chem 16: 619
27. Chung D, Amar S, Glozman A, Chen JM, Friedman FK, Robinson R, Monaco R, Brandt-Rauf PW, Yamaizumi Z, Pincus MR (1997) Inhibition of oncogenic and activated wild-type *ras*-p21 protein-induced oocyte maturation by peptides from the *ras* binding domain of the *raf*-p74 protein, identified from molecular dynamics calculations. J Protein Chem 16: 631
28. Miura K, Inouye Y, Nakamori H, Iwai S, Ohtsuka E, Ikehara M, Noguchi S, Nishimura S (1986) Synthesis and expression of a synthetic gene for the activated human c-Ha-*ras* protein. Jpn J Cancer Res (Gann) 77: 45
29. Xu R-H, Dong Z, Maeno M, Kim J, Suzuki A, Ueno N, Sredni D, Colburn NH, Kung H-F (1996) Involvement of Ras/Raf/Ap-1 in BMP-4 signaling during *Xenopus* embryonic development. Proc Natl Acad Sci USA 93: 834
30. LaBonne C, Burke B, Whitman M (1995) Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. Development 121: 1475
31. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265: 966
32. Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL, Karin M (1994) Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEK. Science 266: 1719
33. Troppmair J, Bruder JT, App H, Cai H, Liptak L, Szeberenyi J, Cooper G, Rapp UR (1992) *ras* Controls coupling of growth factor receptors and protein kinase C in the membrane to raf-1 and B-raf protein serine kinases in the cytosol. Oncogene 7: 1867
34. Ferrell JE, Machleder EM (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. Science 280: 895
35. Karin M (1995) Regulation of activity by mitogen activated protein kinases. J Biol Chem 270: 16483