Functional Interactions of Raf and MEK with Jun-N-Terminal Kinase (JNK) Result in a Positive Feedback Loop on the Oncogenic Ras Signaling Pathway†

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ABSTRACT: In previous studies we have found that oncogenic (Val 12)-ras-p21 induces *Xenopus laevis* oocyte maturation that is selectively blocked by two ras-p21 peptides, 35−47, also called PNC-7, that blocks its interaction with raf, and 96−110, also called PNC-2, that blocks its interaction with jun-N-terminal kinase (JNK). Each peptide blocks activation of *both* JNK and MAP kinase (MAPK or ERK) suggesting interaction between the raf−MEK−ERK and JNK−jun pathways. We further found that dominant negative raf blocks JNK induction of oocyte maturation, again suggesting cross-talk between pathways. In this study, we have undertaken to determine where these points of cross-talk occur. First, we have immunoprecipitated injected Val 12-Ha-ras-p21 from oocytes and found that a complex forms between ras-p21 raf, MEK, MAPK, and JNK. Co-injection of either peptide, but not a control peptide, causes diminished binding of ras-p21, raf, and JNK. Thus, one site of interaction is cooperative binding of Val 12-ras-p21 to raf and JNK. Second, we have injected JNK, c-raf, and MEK into oocytes alone and in the presence of raf and MEK inhibitors and found that JNK activation is independent of the raf−MEK−MAPK pathway but that activated JNK activates raf, allowing for activation of ERK. Furthermore, we have found that constitutively activated MEK activates JNK. We have corroborated these findings in studies with isolated protein components from a human astrocyte (U-251) cell line; that is, JNK phosphorylates raf but not the reverse; MEK phosphorylates JNK but not the reverse. We further have found that JNK does not phosphorylate MAPK and that MAPK does not phosphorylate JNK. The stress-inducing agent, anisomycin, causes activation of JNK, raf, MEK, and ERK in this cell line; activation of JNK is not inhibitable by the MEK inhibitor, U0126, while activation of raf, MEK, and ERK are blocked by this agent. These results suggest that activated JNK can, in turn, activate not only jun but also raf that, in turn, activates MEK that can then cross-activate JNK in a positive feedback loop.

The ras-p21 protein becomes oncogenic when arbitrary amino acid substitutions occur at critical positions, such as at Gly 12, Gly 13, Ala 59, and Gln 61, in the polypeptide chain (*1*). Most commonly, the G12V substitution is found in a variety of common human neoplasms (*2*, *3*). Microinjection of oncogenic, but not wild-type, ras-p21 protein into NIH 3T3 cells induces cell transformation (*1*, *4*). Similarly, microinjection of oncogenic, but not wild-type, ras-p21 protein into *Xenopus laevis* oocytes, which are metaphase-arrested in the second meiotic division, induces them to complete meiosis, or oocyte maturation, as measured by germinal vesicle breakdown (GVBD) (*5*). In addition, insulin induces oocyte maturation in a process that requires activation of cellular wild-type ras-p21 (*6*).

In prior investigations using conformational energy analysis, we have found that oncogenic amino acid substitutions induce stereotypical changes in the conformation of specific domains of ras-p21 including residues 35−47 of the switch 1 effector domain, 55−71 of the switch 2 effector domain, 83−91, 96−110, and 115−126 (*7*, *8*). We have also computed the effects of oncogenic amino acid substitutions

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in p21 on the conformations of its complexes with a number of target proteins, that is, raf, the son-of sevenless (SOS) guanine nucleotide exchange-promoting protein, and GTPase activating protein (GAP), and found specific domains of these proteins that likewise undergo well-defined conformational changes (*8*). We have synthesized peptides corresponding to these domains and tested them for their abilities to inhibit ras signaling in cells (*8*).

In one set of experiments, we have co-injected these peptides together with Val 12-p21 into oocytes and have injected them into oocytes that were then incubated with insulin. We found that three p21 peptides, 35−47, also called PNC-7,[1] 96−110, also called PNC-2, and 115−126, also called PNC-1, raf peptide 97−110, and SOS peptide 994−1004 strongly block Val 12-p21-induced oocyte maturation but have only a minimal effect on insulin-induced maturation (*7*, *8*). In a separate set of experiments, we have linked the p21 35−47 and 96−110 peptides and the raf 97−110 and SOS 994−1004 peptides to an antennapedia transmembrane-penetrating peptide and have incubated these peptides with ras-transformed pancreatic cancer cells and found that the two p21 peptides induce complete reversion of the cancer cells to the untransformed phenotype, while the other two peptides induce tumor cell necrosis (*8*, *9*).

Since these peptides block oncogenic- but only minimally inhibit insulin-activated wild-type p21 protein-induced oocyte maturation, we surmised that oncogenic and activated wild-type p21 utilize overlapping but different signal transduction pathways (*8, 10*).

In investigating possible pathway differences, we have found that Val 12-p21 interacts in vitro with jun-N-terminal kinase (JNK) and with its substrate, the transcriptional activation factor, jun, and induces phosphorylation of both proteins (*7*, *8*, *11*−*13*). Normally, activation of JNK occurs by upstream elements, including JNK kinase (JNKK), of the stress-activated protein pathway (SAP) that is distinct from the raf-kinase pathway (*14*−*16*). Raf, a known direct target of ras-p21, directly activates extracellular mitogen-activated kinase (MEK) that, in turn, activates mitogen-activated protein kinase (MAPK or ERK) that ultimately activates elk and ultimately fos, that forms the active fos−jun transcriptional activation (AP1) complex (*7*, *8*, *14*, *17*).

In our studies on the Val 12-p21−JNK/jun interactions, we further found that the binding of Val 12-p21 to JNK and jun was blocked by both the p21 96−110 and 115−126 peptides (*7*, *8*). Since dose−response curves for the inhibition, by the p21 96−110 peptide, of Val 12-p21-induced oocyte maturation and Val 12-p21 binding to JNK are superimposable, we concluded that one important site of Val 12-p21 inhibition by this peptide is on the Val 12-p21−JNK interaction (*7*). That JNK/jun is vital to oncogenic but not wild-type p21 is suggested by our finding that the protein, glutathione-*S*-transferase-pi (GST-pi), which is a specific inhibitor of JNK-induced jun phosphorylation (*18*), strongly blocks Val 12-p21 but not insulin-induced oocyte maturation (*13*, *19*).

In addition, lysates of oocytes that were induced to mature either by injection of Val 12-p21 or incubation with insulin for activated JNK and MAPK (ERK) exhibited strong phosphorylation signals for both JNK and MAPK that increased with time in the Val 12-p21-injected oocytes, while there were much smaller phosphorylation signals for these two proteins in oocytes induced to mature with insulin (*20*). These and other experiments suggested that oncogenic p21, but not its wild-type counterpart protein, may interact directly with the JNK/jun system. In these experiments, we found that, in lysates from oocytes co-injected with Val 12-p21 and either the p21 96−110 or the 35−47 inhibitory peptide, that were blotted with anti-phospho-JNK and MAPK, the levels of both phospho-proteins were greatly reduced (*20*). While reduction in phospho-JNK was expected, since the p21 96−110 peptide appeared to be selective for the Val 12-p21−JNK interaction, reduction in MAPK phosphorylation was not expected. This result was all the more surprising in view of our prior finding that the p21 96−110 peptide does not inhibit c-raf induction of oocyte maturation (*21*, *22*); raf induces activation of MAPK via activation of MEK.

Conversely, in other studies, we demonstrated that the p21 35−47 peptide strongly inhibits c-raf- but not JNK-induced oocyte maturation (*21*, *22*). Nonetheless, this peptide, which would be expected therefore to block MAPK phosphorylation only, blocks phosphorylation of *both* MAPK *and* JNK. Results with both peptides therefore suggested the presence of interactions between the raf−MEK−MAPK pathway and the JNK/jun system.

Interactions between these two pathways may occur initially where both raf and JNK/jun bind to Val 12-p21 and possibly between specific proteins of the raf-kinase pathway with JNK. That the latter may be important is suggested by further results of studies in which we found that kinase-dead dominant negative raf (dn-raf) not only blocks c-raf-induced oocyte maturation but also blocks JNK-induced oocyte maturation (*21*). Conversely GST-pi, which blocks JNK-induced maturation also inhibits c-raf-induced maturation (*21*).

In this paper, we have studied first the effects of the two ras-p21 peptides 96−110 and 35−47 on the interaction of Val 12-p21 with endogenous raf and JNK when injected with Val 12-p21 into oocytes. In these experiments, we have blotted for raf, MEK, MAPK, and JNK in immunoprecipitated Val 12-p21 from lysates of oocytes injected with Val 12-p21 alone and together with either peptide to determine whether blockade of the binding of one of these proteins to p21 results in diminished binding of the other to p21.

Second, we have investigated the possible points of interaction ("cross-talk") between the raf−MEK−MAPK and JNK/jun pathways in two ways. First, we have injected JNK and proteins on the raf kinase pathway to determine if each one activates both JNK and MAPK. We have then explored the effects of inhibitors of the raf kinase pathway, that is, dn-raf and a MEK inhibitor, on JNK and MAPK phosphorylation to determine whether raf kinase pathway inhibitors block JNK activation in addition to MAPK activation. Second, we have further investigated interactions between isolated proteins on the two pathways. We have prepared activated raf, MEK, MAPK, and JNK from mammalian cell

[1] Abbreviations: JNK, jun-N-terminal kinase that activates jun transcriptionally active protein; MEK, extracellular mitogen-induced kinase; MAPK, mitogen-activated protein kinase also called ERK; dn-raf, kinase-dead dominant negative raf; NPP, nitrophenyl phosphate phosphatase inhibitor; PNC-2 and PNC-7, ras-p21 peptides containing residues 96−110 and 35−47, respectively; GST-pi, glutathione-*S*-transferase, pi isozyme.

extracts and the corresponding nonactivated proteins on antibody-bound beads and have determined the abilities of each of the proteins on the two pathways to activate these bead-bound proteins, for example, raf bound to anti-raf beads incubated with JNK and vice versa. We find a pattern of activation in which both raf and MEK interact in a specific manner with JNK in a positive feedback loop.

MATERIALS AND METHODS

1. Materials. 1.1. Ras-Val 12-p21 Protein. Val 12-Ha-*ras*-p21 protein was overexpressed in *Escherichia coli* using the pGH-L9 expression vector containing the chemically synthesized Ha-*ras* gene, as previously described (*23*).

Insulin was purchased from Sigma (St. Louis, MO). Jun-N-terminal kinase was obtained from either U-251 or NIH 3T3 cells as described below. For some of our in vitro phosphorylation experiments, we employed activated JNK purchased from Biomol (Plymouth, PA). Active (phosphorylated) MEK, raf, and MAP kinase were purchased from Upstate Biotechnology (Lake Placid, NY). Myelin-basic protein (MBP) as the p-Gex fusion protein (the MAP kinase substrate) and pGex-jun fusion protein, the jun-kinase substrate peptide, were both purchased from Biosource (Camarillo, CA).

1.2. Peptides. In this study, we employed two ras-p21 peptides, 35−47 (TIEDSYRKQVVID) and 96−110 (YREQIKRVKDSDDVP), designated PNC-7 and PNC-2, respectively, each of which selectively inhibits oncogenic, but not insulin-activated wild-type, ras-p21-induced oocyte maturation. The first blocks the interaction of Val 12-p21 with raf, while the second blocks its interaction with JNK (*7*, *8*). In addition, we employed the negative control peptide, X13 sequence from mammalian cytochrome P450 (MPFSTGKRIMLGE), which has no effect on either Val 12-p21- or insulin-induced maturation (*7*, *8*). All of these peptides were synthesized by solid-phase methods and purified by HPLC to >95% purity as revealed by mass spectroscopy (Macromolecular Resources, Colorado State University, Fort Collins, CO).

1.3. MEK Inhibitor, U0126. This is a kinase inhibitor specific for both isoforms of MEK (MEK-1 and -2) and was obtained from Cell Signaling (Beverly, Mass).

1.4. Constructs for c-Raf, Dominant Negative Raf (dn-raf), JNK, and Constitutively Activated MEK. These are described in a prior set of publications (*11*, *21*, *22*, *24*, *25*). Briefly, construction of both c-*raf* and dn-*raf* cDNAs was performed using pBluescript II SK(+) vector (*24*, *25*). Each construct was then linearized and transcribed using an in vitro transcription kit (Ambion, Austin, TX). Constitutively active MEK (ca-MEK) was prepared as described previously (*21*, *24*, *25*). Its cDNA was subcloned into a transcription vector and was transcribed with SP6 or T7 RNA polymerase (*21*, *24*).

1.5. Cells. The following two cell lines were employed: NIH 3T3 cells obtained from the ATCC (Bethesda, MD) and a human astrocytoma cell line (U-251), a gift from Dr. D. Weinstin (GliaMed) (*27*).

1.6. Buffers. Oocyte (Group VI) lysis buffer consisted of 80 mM beta-glycerophosphate, 20 mM EGTA, 20 mM HEPES, pH 7.5, 1 mM PMSF, 2 μg/μL pepstatin, 1 mM leupeptin, 2 μg/μL aprotinin, 1 mM Na_3VO_4, and 1% Triton X-100 (*20*).

Lysis buffer for cells used to lyse U-251 and NIH 3T3 cells consisted of 0.35 M LiCl, 50 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol (DDT), 2 mM $MgCl_2$, 50 mM NPP (nitrophenyl phosphate phosphatase inhibitor), 1 mM sodium vanadate, and an inhibitor "cocktail" consisting of 1 μg/mL each of the protease inhibitors, pepstatin, leupeptin, and aprotinin, and the phosphatase inhibitors, 1 mM sodium orthovanadate and 5 mM sodium fluoride (*27*, *28*).

Kinase buffer used for all of the pathway proteins consisted of 50 mM Tris-HCI, pH 7.5, 50 mM LiCI, 10% glycerol, 1 mM EGTA, 1 mM DTT, 0.5% NP-40 (nonionic detergent), and 0.5% Triton X-100.

2. Methods. 2.1. Oocyte Microinjection. Oocytes were obtained from *X. laevis* frogs from Connecticut Valley Biological (Southhampton, MA) as described previously (*5*, *6*, *8*). All microinjection experiments were performed at least three times on a minimum of 200 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 for a minimum of 24 h at 25 °C. Oocyte maturation was determined by observing germinal vesicle breakdown (GVBD). All matured oocytes were separated at the end of the 24 h period and subjected to lysis.

The following agents were microinjected into oocytes in a volume of 50 nL per oocyte: [^{Val} 12]Ha-*ras*-p21 was either injected into oocytes (100 μg/mL) alone or co-injected with either the p21 35−47 or 96−110 peptide present at a concentration of 400 μg/mL). Constructs for c-*raf*, c-*raf* + dn-*raf*, c-raf, JNK, JNK + dn-*raf*, and ca-MEK were each injected at a concentration of 10 μg/mL as described previously (*22*). C-*raf* (10 μg/mL) was also co-injected with U0126 MEK inhibitor at a concentration of 10 μM.

3. Oocyte Studies. 3.1. Lysis of Oocytes. The matured oocytes were subjected to lysis in Group VI lysing buffer and mechanical disruption using a tissue homogenizer followed by centrifugation (*20*). The supernatants were stored at −70 °C until employed in Western blotting experiments.

3.2. Immunoblotting of Oocyte Lysates. 3.2.1. Assays for JNK and MAPK Activation. Samples of lysates containing constant amounts of protein (either 50 or 75 μg) were subjected to SDS−PAGE on a 12% resolving gel, and the proteins were then electrophoretically transferred onto nitrocellulose membranes overnight at 4 °C as described previously (*20*). The membranes were then blocked with nonfat dry milk in Tris-buffered saline with 1% Tween-20 (TBS-T) (pH 7.6) and were then incubated with the appropriate anti-kinase antibodies as follows: (1) anti-JNK polyclonal antibody (Sigma, St. Louis, MO), which recognizes both JNK-1 and JNK-2, diluted 1:1 000; (2) anti-di-phospho-JNK (JNK-P) (Promega, Madison, WI), diluted 1:800; (3) anti-MAPK (New England Biolabs, Beverly, MA), diluted 1:500; and (4) anti-di-phospho-MAPK (MAPK-P) (Promega), diluted 1:2 500. All incubations were performed for 12 h at 4 °C, after which the membranes were washed three times with TBS-T and incubated with anti-rabbit secondary antibody (Amersham, Piscataway, NJ) at 1:4 000 dilution. Detection was accomplished using the ECL chemiluminescence detection kit (Amersham) (*20*).

3.3. Isolation of Val 12-ras-p21 Complexes in Oocytes Injected with Val 12-p21 and Val 12-p21 + p21 Inhibitory Peptide. We injected the Ha-ras form of Val 12-p21 into oocytes either alone or together with inhibitory peptide as described above. For oocytes injected with Val 12-p21, we separated the mature oocytes after 24 h (about 50% maturation, approximately 100 oocytes) and subjected them to lysis. For oocytes co-injected with Val 12-p21 and either inhibitory peptide, we lysed 100 mostly nonmatured oocytes (both peptides strongly inhibit Val 12-p21-induced maturation) after 24 h of incubation. Lysis was achieved using a modified oocyte lysis buffer consisting of 0.35 M LiCl, 50 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol (DDT), 2 mM MgCl, 50 mM NPP, 1 mM sodium vanadate, and an inhibitor "cocktail" consisting of 1 μg/mL each of the protease inhibitors, pepstatin, leupeptin and aprotinin, and the phosphatase inhibitors, 1 mM sodium orthovanadate and 5 mM sodium fluoride. The lysate was centrifuged for 15 min at 17 000*g* at 4 °C, and the supernatant was either used directly or was stored at −80 °C.

Since we injected Ha-ras-Val 12-p21 and endogenous ras-p21 protein in oocytes is mainly of the K- isoform (*29*, *30*), we subjected the lysate to immunoprecipitation using an anti-Ha-ras antibody (Calbiochem). In this procedure, cell lysate was first precleared by incubation with 50 μL of protein A beads for 1 h at room temperature, followed by centrifugation. The anti-Ha-ras antibody was added to the lysate such that 0.1 μg of the antibody was added per 250 μg of precleared lysate protein. A volume of 25 μL of protein A agarose beads (Sigma) was then added to the incubation mixture, and the resulting mixture was incubated overnight at 4 °C, after which the mixture was centrifuged, and the immunoprecipitate was washed three times with 0.5 mL of kinase buffer as described above. Immunoprecipitates were subjected to SDS−PAGE as described above in the preceding paragraph and blotted with anti-Ha-ras (1:2 000 with 0.25% BSA), anti-raf (Calbiochem, San Diego, CA) diluted 1:2 000 with 0.25% BSA, anti-JNK polyclonal antibody (1:2 000), and anti-MEK (Calbiochem) diluted 1:2 000 with 0.25% BSA. All the incubations were performed as described in the preceding paragraph, that is, for 12 h at 4 °C, after which the membranes were washed three times with tris-buffered saline with Triton (TBS-T) and incubated with anti-rabbit secondary antibody (Pierce, Rockford, IL) at 1:20 000 dilution. Detection was accomplished using the ECL chemiluminescence detection kit (Pierce).

3.4. Control Experiment with Insulin. As a control experiment for the above study with the oncogenic form (Val 12-containing), we performed duplicate experiments identical to the above-described experiment, except that we incubated 200 oocytes in each experiment with insulin (10 μg/mL). At this concentration, the time course for maturation is the same as for oocytes injected with 100 μg/mL of Val 12-p21 (*20*). After 24 h, about 100 oocytes were found to have matured. These oocytes were lysed and subjected to the identical protocol described in the preceding paragraph.

4. Studies on Mammalian Cell Lines. 4.1 Lysis of U-251 Astrocytes and NIH 3T3 Cells. U-251 cells were used as the source of each pathway protein as described below. NIH 3T3 cells were used as another source of activated JNK (*27*). For cells activated with anisomycin, a total of 2×10^6 U-251 or NIH 3T3 cells was incubated in DMEM containing 5% bovine calf serum in the presence of anisomycin (anandamide, Calbiochem, San Diego, CA) (12.5 μg/mL) for 20 min at 37° C. This agent is known to induce the stress-activated protein kinase system (SAP) resulting in JNK activation (*18*, *27*). After incubation, the cells were washed twice with cold PBS and lysed by adding the lysis buffer described in the preceding paragraph. The same number of untreated (resting) cells serving as a source of inactivated proteins was subjected to the same lysis procedure directly with no incubation with anisomycin.

4.2. In Vitro Studies with Component Proteins Isolated from U-251 Cells. 4.2.1. Immunoaffinity Beads. Each of the component proteins on the two signal transduction pathways was isolated on immunoaffinity beads either as potential substrates for another component protein or as potential activating proteins for another bead-bound component protein. In the latter case, the protein was eluted from the immuno-affinity bead with 2-fold concentrated SDS sample buffer and then used in a phosphorylation assay.

In all cases, resting or anisomycin-treated (see above) confluent U-251 cells (1×10^6) were lysed with the cell lysis buffer described in the Materials section. In experiments in which total protein concentration in lysates was found to be 1 μg/μL, 0.5 mL of lysate was incubated with 1 μL of primary antibody (concentration 0.1 μg/μL) (the exact volume of primary antibody was always such that 0.1 μg of antibody was present for every 500 μg of total protein), followed by addition of 1 μL of 1 μg/μL biotinylated anti-mouse or anti-rabbit (as appropriate) IgG (Pierce, Rockford, IL). To this mixture, 25 μL of streptavidin beads (Pierce) was then added, and the resulting mixture was incubated overnight at 4 °C, after which it was centrifuged at 10 000*g* for 2 min and then washed three times, each time with 0.5 mL kinase buffer (see Materials section). These immunoaffinity bead-bound proteins were used directly in the experiments described below.

4.3. Phosphorylation Assays. These were performed using as substrates immunoaffinity bead-bound proteins from untreated cells incubated either with affinity-purified proteins eluted from the immuno-affinity beads described in the preceding section or obtained as the active proteins from Upstate Biotechnology Corp. (see Materials section) as follows.

4.3.1. JNK + Raf Immunoaffinity Beads, Raf + JNK Immunoaffinity Beads, JNK + MEK Affinity Beads, and MEK + JNK Immunoaffinity Beads. A volume of 10 μL of immunoaffinity bead-bound protein (e.g., raf) in 50 μL kinase buffer was incubated in the presence of activated kinase protein (e.g., JNK) (2 μL of 0.05 μg/μL kinase protein). As a control, the same concentration of immunopurified non-activated kinase (e.g., JNK) from untreated cells was also used in this assay. The reaction was commenced either by addition of 10 μL of 10 uM ATP for blotting experiments or by addition of 10 μL of 10 μCi of $\gamma[^{32}P]$-ATP (3 000 Ci/mM, Perkin-Elmer Life and Analytical Science, Boston, MA) in the presence of 10 μM cold ATP for autoradiography experiments. The mixture was incubated for 45 min at room temperature. The samples were then centrifuged and washed three times, each time with 0.5 mL kinase buffer, as described above, boiled with 50 μL of SDS sample buffer, and electrophoresed on 4−20% polyacrylamide gel (Novex, Invitrogen, Carlsbad, CA). After transfer to nitrocellulose

membranes, phosphorylation was either directly analyzed by autoradiography using a computerized radio-imaging blot analyzer (Molecular Dynamics, Boston, MA) or analyzed using Western blotting with anti-kinase and anti-phosphokinase antibodies. For blotting, after transfer, the membrane was blocked with a solution containing 50 mL 5% skimmed milk and 5% BSA (Sigma) and incubated for 30 min at room temperature. The membrane was then washed four times for 10 min each with 50 mL TBS-T and was then incubated with primary antibody solution containing 15 mL of antibody solution that was diluted 1:2 000 in TBS-T, containing 0.25% BSA, with shaking overnight at 4°C. The membrane was then washed four times with TBS-T (50 mL) for 10 min each time. The membrane was then incubated for 1 h at room temperature with 50 mL of either goat anti-mouse or goat anti-rabbit secondary antibody (Pierce Supersignal West Pico Chemiluminescent kit) that was diluted 1:25 000 in TBS-T buffer. The membrane was again washed four times for 10 min each with 50 mL of TBS-T, and the signals were developed with enhancer, as per manufacturer's instructions, on X-ray film.

4.3.2. Activated JNK + MAPK Immunoaffinity Beads and Activated MAPK + JNK Immunoaffinity Beads. Incubations, autoradiography, and Western blotting were the same as described in the preceding paragraph. In addition, to ascertain whether MAPK-treated JNK beads and JNK-treated MAPK beads had any kinase activity, we performed kinase assays for each protein using its appropriate substrate (*11*, *27*). Using the procedures described above, on immuno-affinity beads, after the third wash, the pellet containing the immunoaffinity beads was mixed with 40 μL kinase buffer (Materials section) containing 5 μg of the specific substrate (pGex-jun N-terminal peptide for JNK or pGex-myelin basic protein for MAPK), and phosphorylation was initiated by addition of 10 μL of a mixture of 10 μM ATP with 10 μCi [^{32}P]-ATP as described in the preceding paragraph. The reaction mixture was incubated at room temperature for 45 min, after which the beads were centrifuged, and the supernatant was mixed with an equal volume of 2-fold concentrated SDS protein sample buffer and electrophoresed on 4−20% PAAG gel (Novex), followed by transfer to a nitrocellulose membrane that was subjected to autoradiography as described in the preceding paragraph.

As controls for these experiments, we also prepared immunoaffinity beads of activated JNK and MAPK immunoprecipitated from anisomycin-treated U-251 cells. These beads were incubated directly with the appropriate substrates (pGex-jun or pGex MBP) in a manner that was identical to the procedure described in the preceding paragraph.

RESULTS

Effects of Inhibitory Ras-p21 Peptides on Val 12-p21 Complexes with Raf and JNK. We injected 100 μg/mL Val 12-p21 into 200 oocytes. This purified protein is the Harvey (Ha) form of oncogenic Val 12-p21. After 24 h, all oocytes (over 100, around 50%) that matured in this time period were lysed and subjected to immunoprecipitation with anti-Ha-ras-p21. Counterblotting of the immunoprecipitate with anti-Ha-ras antibody showed a large band at 21 kDa (not shown).

As shown in Figure 1A, when this Ha-ras immunoprecipitate was blotted with anti-raf (lane 2), anti-MEK (lane 4), anti-JNK (lane 6), and anti-MAPK (lane 8), all four proteins were present, indicating that each is present in complexes with Val 12-p21. On the other hand, when oocytes that were co-injected with Val 12-p21 and the p21 96−110 peptide, that we found blocks the interaction of Val 12-p21 with JNK and jun, there was a marked decrease in the amount of each of these proteins as can be seen in lanes 1, 3, 5, and 7 for raf, MEK, JNK, and MAPK, respectively. Similar amounts of p21 were immunoprecipitated in these injection experiments from blots with anti-Ha-ras antibody. Identical results (not shown) were found using the p21 35−47 peptide.

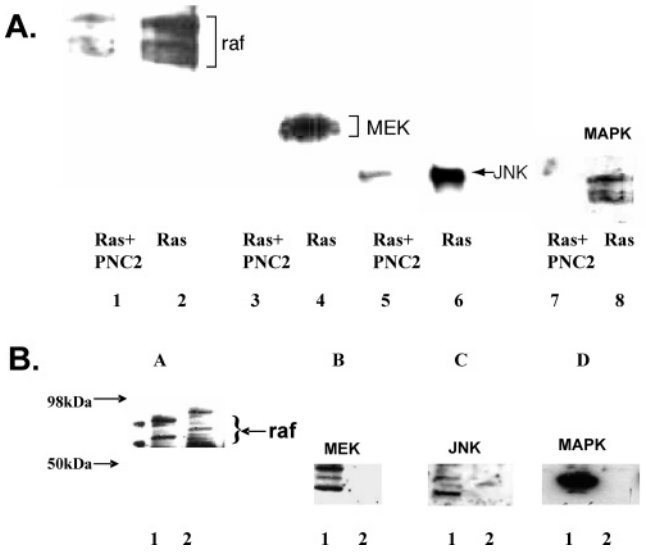

FIGURE 1: (A) Injected Val 12-p21 forms a complex with raf, MEK, JNK, and MAPK (ERK). Oocytes that matured after being injected with Val 12-Ha-ras-p21 were lysed and immunoprecipitated with anti-Ha-ras antibody. The immunoprecipitate was blotted with anti-raf (lane 2), anti-MEK (lane 4), anti-JNK (lane 6), and anti-MAPK (lane 8). Oocytes were also injected with Val 12-p21 and ras-p21 inhibitory peptide 96−110, labeled as PNC-2, lysed, and subjected to immunoprecipitation with anti-Ha-ras. These immunoprecipitates were then blotted with anti-raf (lane 1), anti-MEK (lane 3), anti-JNK (lane 5), and anti-MAPK (lane 7). (B) The same experiment as in panel A was performed on oocytes that were induced to mature with insulin. This panel shows blots for raf (A), MEK (B), JNK (C), and MAPK (D). The first lane for each of these four sets presents the results for the blots of whole cell lysate to demonstrate the presence of each protein. The second lane in each set of blots shows the results of blotting for each protein in the anti-Ha-*ras*-p21 immunoprecipitate.

Blots of anti-Ha-ras immunoprecipitates from lysates of oocytes that were injected with Val 12-p21 and the negative control X13 peptide from cytochrome P450 with anti-raf, -MEK, and -JNK were identical to those shown in lanes 2, 4, 6, and 8. This peptide has no effect on either Val 12-p21- or insulin-induced oocyte maturation (*7*).

These effects are specific since the control X13 peptide has no effect on the amount of complex formed. The 35−47 peptide specifically blocks raf and does not inhibit JNK-induced oocyte maturation, while the 96−110 peptide blocks the Val 12-p21−JNK complex formation and does not inhibit c-raf-induced maturation (*7*, *8*). Yet, each peptide blocks the association of Val 12-p21 with both raf and JNK, suggesting that the interaction of Val 12-p21 with these proteins is cooperative and that blockade of its binding to one of these proteins reduces its affinity for the other protein.

These results help to explain why co-injection of either peptide with Val 12-p21 into oocytes results in a reduction

of both phosphorylated JNK and MAPK. If the binding of Val 12-p21 to JNK is blocked by the p21 96−110 peptide and this reduces its affinity for raf, then not only would a reduction in phosphorylated JNK occur but, because of diminished raf activation due to decreased raf−Val 12-p21 interactions, a reduction in activation of MAPK would also occur.

Formation of the Complex Is Unique to Oncogenic p21. To test whether the formation of the p21−ras−MEK−JNK complex is unique to oncogenic (Val 12-containing) p21, we performed identical experiments on oocytes induced to mature with insulin. This agent induces maturation by activating intracellular wild-type p21 (*6*). Figure 1B shows the results of these experiments. In this figure, four sets of blots are shown. In each set, blots were performed with an antibody to one of the pathway proteins, that is, raf, MEK, MAPK, and JNK, against the protein in whole cell lysate (first lane) and against the protein in the anti-Ha-ras immunoprecipitate (second lane). In set A of Figure 1B, raf is seen to be present in whole cell lysate (lane A-1) and is also present in substantial amount in the anti-Ha-*ras*-p21 immunoprecipitate (lane A-2), although the isoforms of this protein appear to migrate somewhat differently, possibly due to differing phosphorylation states. Blots of total Ha-ras protein from the anti-ras-p21 immunoprecipitates from lysates from oocytes that were either treated with Val 12-p21 or with insulin revealed similar amount of Ha-ras-p21 protein (not shown). Thus, differences in our blotting results cannot be attributed to significantly reduced Ha-ras-p21 protein in the insulin-treated oocytes.

Importantly, blots for MEK, JNK, and MAPK in whole cell lysate are seen to be positive in lanes B-1, C-1, and D-1, respectively, but are negative for MEK, JNK, and MAPK in the anti-Ha-*ras*-p21 immunoprecipitates (lanes B-2, C-2, and D-2, respectively). (In lane C-2, the blot for JNK in the immunoprecipitate reveals only a trace of this protein that migrates somewhat differently.)

These latter results are in contrast to those in Figure 1A, which shows that MEK and JNK are present in substantial quantity in the immunoprecipitate. However, they are consistent with our prior findings that suggest that insulin-induced maturation, which activates cellular p21, requires raf but not MEK, MAPK, or JNK (*8*). Thus the formation of a p21−ras−MEK−JNK−MAPK complex appears to be specific to oncogenic p21.

Effects of Pathway Component Proteins on JNK and MAPK Activation. While the results in Figure 1 suggest interaction of raf and JNK through interactions of both proteins with ras-p21, other pathway interactions between the raf and JNK pathways may also exist. In a prior study (*21*), we found that dn-raf blocks JNK-induced oocyte maturation, suggesting interactions between JNK and elements of the raf−MEK−MAPK pathway. To attempt to define where these interactions occur, we injected each pathway component into oocytes to determine its effects on the phosphorylation of JNK (Figure 2A) and MAPK (Figure 2B). Each of these figures contains an upper panel that shows the blot results with antibody to total JNK (Figure 2A) and MAPK (Figure 2B) and a lower panel that shows the blot results with antibody to phosphorylated JNK (labeled as JNK-P) (Figure 2A) and MAPK (labeled as MAPK-P (Figure 2B).

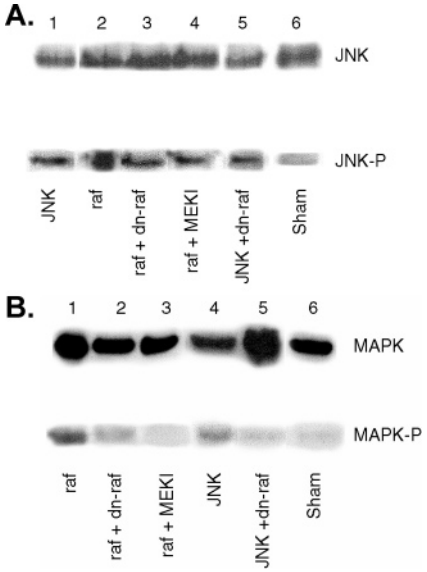

FIGURE 2: Effects of injection of different ras pathway proteins and ras pathway proteins plus inhibitors on phosphorylation of JNK (A) and of MAPK (B). In A, each protein or protein + inhibitor is listed below its lane. Upper panel, blots for total JNK; lower panel, blots for phospho-JNK. In B, as in A, each protein or protein + inhibitor is listed below its lane. Upper panel, blots for total MAPK; lower panel, blots for phospho-MAPK. Abbreviations: MEKI is the MEK inhibitor, U0126; dn-raf is a kinase-dead dominant negative mutant form of raf; and sham refers to injection of oocytes with Barth's saline (*19*).

As shown in Figure 2A, microinjection of both JNK and raf (lanes 1 and 2, respectively) induces phosphorylation of JNK. (The amount of total JNK in lane 1 was somewhat reduced compared with that in lane 2; in both lanes, quantitative scans revealed a ratio of JNK-P (lower lanes) to JNK (upper lanes) of about 0.5.) Surprisingly, co-injection of dn-raf with JNK does not inhibit phosphorylation of JNK (lane 5). Since dn-raf inhibits JNK-induced oocyte maturation, we anticipated that raf or one of its downstream targets (like MEK and/or MAPK) might activate JNK, an activity that would be blocked by kinase-dead dn-raf. This result suggests that raf does not activate JNK directly unless dn-raf and raf itself can effect activation of JNK either in a different phosphorylation domain than the one involved in MEK activation or in a process that does not involve direct phosphorylation of JNK by raf.

Also shown in Figure 2A are the effects of dn-raf and the MEK inhibitor, U0126, on raf-induced phosphorylation of JNK, lanes 3 and 4, respectively. Neither of these inhibitors induces a decrease in phosphorylated JNK, suggesting that JNK activation appears to be independent of the kinase activities of raf and MEK. However, activation of JNK either by injection of exogenous JNK or raf is a specific phenomenon since a sham injection of oocytes with incubation buffer (Barth's saline) gives no phosphorylation signal for either JNK or MAPK (both lanes 6 in Figure 2A,B) (*20*).

Figure 2B shows that raf induces strong phosphorylation of MAPK (lane 1), an effect that is strongly blocked both by dn-raf (lane 2) and MEK U0126 inhibitor (lane 3). As can be seen in the upper panel, lanes 1−3 of Figure 2B, the total protein in these lanes is about the same. Importantly, as shown in lane 4, JNK induces phosphorylation of MAPK, an effect that is blocked by dn-raf as shown in lane 5. (Although total MAPK is higher in lane 5 than in lane 4,

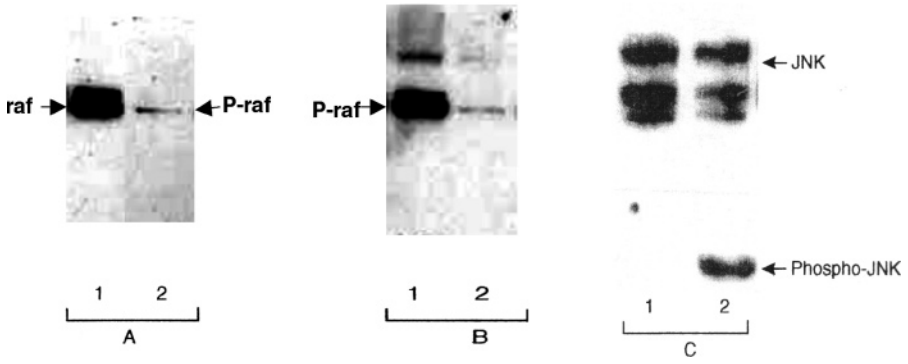

FIGURE 3: JNK activates raf. (A) Lane 1, Anti-JNK immunoprecipitate from oocytes blotted with anti-raf; lane 2, blotted with anti-phospho-raf. (B) Lane 1, incubation of streptavidin bead-bound raf with anisomycin-activated JNK and [^{32}P]-ATP from U-251 cells results in phosphorylation of raf (P-raf); lane 2, incubation of raf beads with [^{32}P]-ATP in the absence of activated JNK gives no band. (C) Lane 1, lower blot, streptavidin bead-bound JNK from untreated U-251 cells was incubated with raf immunoprecipitated from anisomycin-treated U-251 cells and blotted with anti-phospho-JNK, showing no phosphorylation of JNK. Lane 2, lower blot, streptavidin bead-bound JNK from U-251 cells treated with anisomycin and blotted with anti-phospho-JNK as a control, showing a prominent phospho-JNK band. Upper lanes blotted with nonphospho-specific anti-JNK.

the level of phosphorylated MAPK in lane 5 is not above background, as in lane 6.) These results suggest that JNK can effect activation of MAPK by inducing activation of raf since dn-raf blocks phosphorylation of MAPK induced by JNK but does not block JNK activation.

Overall, the results shown in Figure 2A,B suggest an explanation for our prior observation (*21*) that dn-raf blocks JNK-induced oocyte maturation: when JNK is injected into oocytes, it activates raf that then activates MEK and MAPK, an effect that is blocked by dn-raf. If oocyte maturation requires activation of both JNK and MAPK, dn-raf inhibits JNK-induced maturation by blocking MEK activation and, ultimately, MAPK activation.

Componential Analysis of Interprotein Interactions. The above results suggest that JNK is activated by mechanisms that do not depend on the raf-MEK-MAPK pathway and that activated JNK activates raf resulting ultimately in MAPK activation. To test whether these interactions take place, we have isolated each of the potentially interacting component proteins on immuno-affinity beads and tested each of the other component proteins for their abilities to induce phosphorylation of the bead-bound proteins.

In previous studies, we have found that the U-251 human astrocyte cell line contains abundant amounts of each of the proteins on the raf-kinase pathway and also of JNK and jun (*27*). JNK is rapidly activated in these cells by the antibiotic anisomycin (*27*), known to induce chemical stress on cells, resulting in activation of the stress-activated protein pathway (SAP) and, ultimately, in activation of JNK and jun (*14*−*16*). Therefore, we utilized these cells in our studies on potential interprotein interactions.

JNK Interacts with Raf and Phosphorylates It. To determine whether JNK interacts with raf, we immunoprecipitated JNK from lysates of resting U-251 cells that were then blotted with pan-anti-raf antibody. As shown in Figure 3 (lane A-1), intense bands corresponding to known isoforms of raf are obtained. Though the JNK in these cells was not activated with aniosmycin, a small band was obtained for phospho-raf at 70 kDa (P-raf, lane A-2). Thus, JNK interacts with raf in these cells. We then immunoprecipitated raf from resting U-251 cells; the resulting immunoprecipitate was conjugated to biotin-labeled anti-IgG and bound to sepharose-bound streptavidin, giving immuno-bead-bound raf as revealed in a blot for raf with pan-anti-raf antibody. These beads were incubated with activated JNK obtained from anisomycin-treated U-251 cells in the presence of [^{32}P]-ATP, washed, and subjected to SDS−PAGE and autoradiography. As shown in lane B-1 of Figure 3, a strong band for phosphorylated raf was obtained. In a control, incubation of bead-bound raf with [^{32}P]-ATP in the absence of JNK gave only a faint phosphorylation signal as shown in lane B-2. These results therefore suggest that activated JNK induces phosphorylation of raf.

Raf Does Not Phosphorylate JNK. We then performed the converse experiment in which we immunoprecipitated JNK from resting U251 cell lysate and attached the immunoprecipitated JNK to streptavidin beads which were subsequently incubated, in the presence of ATP, with raf immunoprecipitated from anisomycin-treated U-251 cells. Raf obtained from these cells induced phosphorylation of immuno-affinity bead-bound MEK (described in the next section) suggesting that it was activated (not shown). We probed blots of bead-bound JNK with antibody to total JNK (upper bands in panel C in Figure 3) and with anti-phospho-JNK (lower bands in panel C in Figure 3). Lane C-1 of Figure 3, the blot for anti-phospho-JNK, shows that no phospho-JNK could be detected. As a control, we obtained immunoprecipitated JNK from cells treated with anisomycin that induces JNK phosphorylation. As shown by the lower band in lane C-2 of Figure 3, blots of bead-bound anisomycin-activated JNK with anti-phospho-JNK resulted in a strong signal. These results suggest that activated raf does not phosphorylate JNK. Overall, the results of both sets of experiments suggest that JNK and raf interact, that JNK phosphorylates raf, but that raf does not induce phosphorylation of JNK and are consistent with our findings from the microinjection experiments in oocytes in Figure 2 above.

MEK Activates JNK. We have likewise incubated bead-bound JNK, from resting U-251 cells, with MEK that we immunoprecipitated from anisomycin-treated cells. As shown in Figure 4, blots for phospho-JNK of JNK beads incubated with ATP alone show little or no phospho-JNK (panel A, lane 1). On the other hand, blots of these beads incubated with activated MEK and ATP show a strong phospho-JNK signal (panel A, lane 2). Lane 3 in Figure 4A shows blots for total JNK.

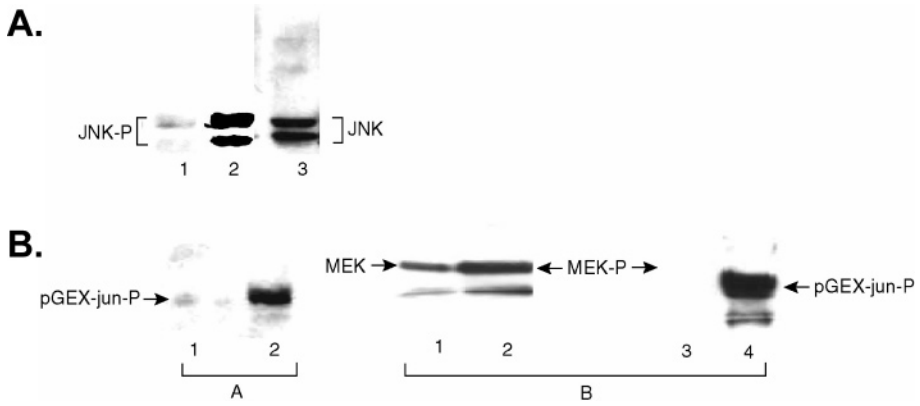

FIGURE 4: MEK activates JNK, but JNK does not activate MEK. Streptavidin-bound JNK immunoprecipitated from untreated U-251 cells incubated with ATP and blotted with anti-phospho-JNK (lane 1) or with activated MEK + ATP and blotted with anti-phospho-JNK (lane 2). Lane 3, blot of beads in lane 2 with anti-JNK. (B) Lane A-1, autoradiogram showing that JNK from upper lane 1 does not phosphorylate p-Gex-jun peptide (37 kD), while lane A-2, MEK-phosphorylated JNK from upper lane 2 does phosphorylate this JNK substrate. (B lanes) Streptavidin bead-bound immunoprecipitated MEK from resting U-251 cells, blotted with anti-MEK (lane B-1) and from anisomycin-treated U-251 cells blotted with anti-phospho-MEK (lane B-2). Lane B-3, Autoradiogram showing that phospho-JNK from anisomycin-treated U-251 cells does not activate bead-bound MEK, but as shown in lane B-4, does phosphorylate p-Gex-jun peptide substrate.

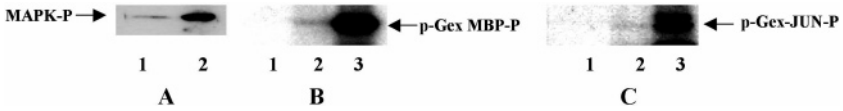

FIGURE 5: JNK does not activate MAPK nor does MAPK activate JNK. (A) Lane 1, blot for phospho-MAPK of immuno-affinity-purified MAPK from resting U-251 cells incubated with activated JNK. Lane 2, blot with anti-phospho-MAPK for immuno-affinity-purified MAPK (from resting U-251 cells) incubated with activated MEK. (B) Functional assay for MAPK by testing its ability to induce phosphorylation of myelin basic protein (MBP), in the presence of [^{32}P]-ATP. Lane 1, MBP + [^{32}P]-ATP alone, negative control. Lane 2, MAPK incubated with activated JNK + [^{32}P]-ATP does not phosphorylate p-Gex myelin basic protein (p-Gex MBP-P, phosphorylated myelin basic protein in the figure). Lane 3, MAPK incubated with activated MEK + [^{32}P]-ATP does phosphorylate MBP. (C) Functional assay for JNK by testing its ability to induce phosphorylation of p-Gex c-jun 5−89 peptide. Lane 1, p-GEX jun + [^{32}P]-ATP alone, negative control. Lane 2, p-Gex-jun + immunopurified JNK from resting U-251 cells incubated with activated MAPK + [^{32}P]-ATP. Lane 3, p-Gex-jun + activated JNK + [^{32}P]-ATP.

To ascertain whether the JNK, that was phosphorylated by activated MEK, was active, we washed the MEK-phosphorylated JNK beads, incubated them with p-Gex-jun 5−89 substrate peptide (*11*) in the presence of [^{32}P]-ATP, and then subjected them to SDS−PAGE and autoradiography. As a control, we also incubated untreated bead-bound JNK with the c-jun beads. As shown in panel B, lane A-2, MEK-phosphorylated JNK strongly induces c-jun phosphorylation while, as shown in panel B, lane A-1, nonactivated JNK induces only a minimal level of phosphorylation of c-jun. These results suggest that activated MEK can induce phosphorylation of JNK resulting in its activation.

JNK Does Not Induce Phosphorylation of MEK. We prepared immuno-beads of MEK immunoprecipitated from resting cells and incubated these with activated JNK. As a control, we also prepared immuno-beads of MEK immunoprecipitated from anisomycin-treated U-251 cells. Results of blots of nonactivated MEK beads with anti-MEK and of activated MEK beads with anti-phospho-MEK are shown in Figure 4B, lanes B-1 and B-2, respectively. Blots of nonactivated MEK beads incubated with anisomycin-activated JNK using anti-phospho-MEK antibody result in little or no phosphorylation signal, as shown in lane B-3 of Figure 4B. That this JNK was active is shown in lane B-4 of panel B, in which the same activated JNK was incubated with p-Gex-jun peptide and [^{32}P]-ATP, and the p-Gex-jun peptide was subjected to autoradiography. There is a large band (37 kDa) for the radiolabeled phospho-jun peptide, showing that the JNK used for the incubation with MEK immuno-beads was active.

These results suggest that activated MEK can phosphorylate JNK but that JNK does not phosphorylate MEK. In the results shown in Figure 2A, dn-raf does not block phosphorylation of JNK when co-injected with JNK into oocytes. This suggests that JNK phosphorylation does not depend on raf or MEK activation. In other experiments, however, we have found that constitutively activated MEK (*26*), strongly activates JNK in oocytes (results not shown). Thus, while it is not required for JNK activation, activated MEK may induce further activation of JNK.

JNK and MAPK Do Not Interact Directly. In previous studies, we have found that cloned, purified MAPK and activated MAPK bound to non-hydrolyzable ATP injected into oocytes both fail to induce maturation (*31*). This result suggested that MAPK alone, unlike JNK (*7*, *8*, *13*, *21*), is not sufficient to induce oocyte maturation and cannot activate JNK. From our results in Figure 2, it further appears that JNK does not activate MAPK directly but rather via activation of raf.

To test whether JNK can directly activate MAPK, we incubated bead-bound MAPK with JNK from anisomycin-treated cells in the presence of ATP and blotted for phosphorylated MAPK (labeled as MAPK-P) as shown in lane 1 of Figure 5A. This lane shows that no phosphorylation occurs in contrast to the results obtained when activated MEK is incubated with the bead-bound MAPK, wherein high levels of phosphorylation of MAPK are seen to occur (lane 2 of Figure 5A).

To test the functionality of MAPK incubated with activated JNK and activated MEK, we have assayed MAPK for its

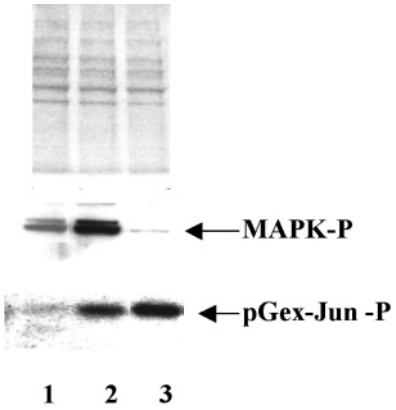

FIGURE 6: Anisomycin-induced MAPK phosphorylation depends on activation of MEK, while phosphorylation of JNK has no such dependence. U-251 cells were treated with anisomycin in the absence or presence or U0126 MEK inhibitor and then subjected to lysis followed by immunoprecipitation with either anti-MAPK or anti-JNK, and the immunoprecipitates were blotted with anti-phospho-MAPK or anti-phospho-JNK. JNK isolated from the cells was further incubated with p-Gex-jun in the presence of [^{32}P]-ATP. Lane 1, resting cells; lane 2, cells treated with anisomycin; lane 3, cells treated with anisomycin + U0126. The total protein in each lane was the same as shown in the Ponceau red stain for total protein shown above the blots.

ability to induce phosphorylation of myelin basic protein (MBP) as the p-Gex construct protein. MAPK incubated with activated JNK is seen in lane 2 of Figure 5B not to phosphorylate MBP. On the other hand, MAPK incubated with activated MEK strongly induces phosphorylation of MBP as shown in lane 3 of Figure 5B. Thus, activated JNK does not induce activation of MAPK.

We also performed the converse experiment in which we incubated JNK immunoaffinity beads with activated MAPK. As shown in lane 2 of Figure 5C, incubation of JNK beads with activated MAPK, followed by incubation of the bead-bound JNK with its substrate, p-Gex-jun peptide, does not result in its ability to induce phosphorylation of this substrate. On the other hand, as shown in lane 3 of Figure 5C, bead-bound JNK from anisomycin-treated cells strongly induces p-Gex-jun peptide phosphorylation.

These results suggest that MAPK does not induce JNK phosphorylation directly, while JNK likewise does not induce direct MAPK phosphorylation and are consistent with the results shown in Figure 2B. JNK induces phosphorylation of MAPK which is blocked by both dn-raf and U0126. Neither of these inhibitors would block JNK activation of MAPK if JNK directly induced MAPK phosphorylation.

Anisomycin Activates JNK, but Not MAPK, Independently of the Raf−MEK−MAPK Pathway. Since anisomycin induces activation of both JNK and MAPK in U-251 cells, we further explored the sequence of events that lead to phosphorylation of both of these critical proteins. We incubated U-251 cells with anisomycin in the presence of U0126, which blocks MEK-induced phosphorylation of MAPK and crosses the cell membrane (*32*). We then blotted lysates of these cells for activated JNK and MAPK. As shown in lane 1 of Figure 6, there is some background phosphorylation of MAPK and some ability of the JNK to induce phosphorylation of the p-Gex-jun substrate. As shown in lane 2 of this figure, anisomycin strongly induces phosphorylation of MAPK and activation of JNK (as revealed by the latter's ability to induce phosphorylation of the p-Gex-jun peptide). On the other hand, as shown in lane 3, treatment of the U-251 cells with the MEK inhibitor U0126 results in significant reduction of phospho-MAPK but has no effect on the activation of JNK. These results support our conclusion that JNK activation is largely independent of the raf−MEK−MAPK pathway but that activation of MAPK depends on activation of raf and MEK that may be stimulated by activated JNK.

DISCUSSION

Oncogenic Ras-p21-Induced Maturation Requires Phosphorylation of JNK and MAPK. In our previous studies in which we followed activation of JNK and MAPK in oocytes injected with oncogenic (Val 12-containing) p21 over a 24 h period, we found that there was early phosphorylation of both proteins that increased markedly with time and that correlated with the extent of oocyte maturation (*20*). This finding suggested that activation of both proteins is required for Val 12-p21-induced maturation.

This conclusion is further supported by our results in this study. As can be seen in Figure 2, injection of any agent that induces oocyte maturation, such as JNK, raf, and MEK (not shown), induces phosphorylation of both JNK and MAPK. Furthermore, agents that block MAPK phosphorylation, but do not affect JNK phosphorylation, such as dn-raf and U0126 MEK inhibitor, nonetheless block oocyte maturation.

We also showed that a peptide from the enzyme GST-pi, encompassing residues 34−50 of this protein, which completely blocks Val 12-p21-induced oocyte maturation (*33*), diminishes JNK phosphorylation and its phosphorylation of jun but does not affect MAPK phosphorylation or its phosphorylation of a specific MAPK substrate peptide (*27*). Thus, oocytes injected with Val 12-p21 and the GST-pi 34−50 peptide, that contain activated MAPK but not activated JNK, likewise do not undergo maturation. These results suggest that both JNK and MAPK must become phosphorylated for theoncogenic ras-p21 to induce oocyte maturation.

Phosphorylations of JNK and MAPK Are Linked. In our prior studies we found that the two p21 inhibitory peptides, 35−47 (PNC-7), that blocks Val 12-p21−raf interactions (*7*, *8*), and 96−110 (PNC-2), that blocks Val 12-p21−JNK interactions (*7*, *8*), inhibit Val 12-p21-induced oocyte maturation and both JNK and MAPK phosphorylation. We also found that dn-raf blocks JNK-induced oocyte maturation (*21*). Both sets of results suggest that JNK and MAPK phosphorylations are linked. Our current results (Figure 2) confirm this conclusion. Injection of JNK and of raf into oocytes induces maturation and, as shown in Figure 2, each induces the phosphorylation of both JNK and MAPK.

One Point of Linkage (Cross-Talk) between Pathways Is at Val 12-p21 Complex Formation with Raf and JNK. Our results in this study suggest that two points of linkage between the ras−MEK−MAPK and JNK/jun pathways are in the initial complex formed between Val 12-p21 and raf and JNK and in a loop in which JNK induces raf phosphorylation and MEK induces JNK phosphorylation. For the first point of interaction (Figure 1A), immunoprecipitates of Val 12-p21 from lysates of matured oocytes injected with this

protein blot positively for raf, JNK, MEK, and MAPK, indicating that a multiple component protein complex forms. This complex is specific for oncogenic p21 since, as shown in Figure 1B, immunoprecipitates of p21 in oocytes induced to mature with insulin, that activates endogenous p21, do not contain MEK, JNK, or MAPK. However, when Val 12-p21 is injected with either inhibitory p21 peptide, these immunoprecipitates contain greatly diminished levels of raf, MEK, JNK, and MAPK, suggesting that interaction of raf and JNK with Val 12-p21 may be interdependent.

It is also possible that Val 12-p21 forms two separate complexes with raf and JNK. This scenario, however, would not explain why each peptide that inhibits either Val 12-p21−raf (35−47 or PNC-7 peptide) or Val 12-p21−JNK (96−110 or PNC-2 peptide) complex formation blocks phosphorylation of both JNK and MAPK unless these peptides act downstream of the complex formation at some point in common to both pathways. Yet, while the 35−47 peptide blocks c-raf-induced oocyte maturation (*34*), it does not block oocyte maturation induced by constitutively active raf lacking the ras-binding domain, suggesting that this peptide blocks ras−raf interactions specifically. Also, this peptide does not block JNK-induced oocyte maturation (*21*, 22). If it were to inhibit both pathways downstream of complex formation, it would be expected to block both of these maturation-inducing agents. Similarly, the p21 96−110 peptide does not block c-raf-induced oocyte maturation (*21*, *22*), which would be expected for a peptide that blocked at a common point downstream of complex formation.

Downstream Points of Cross-Talk between Raf−MEK−MAPK and JNK/Jun. JNK Induces Raf Phosphorylation. Since we found that dn-raf, which has no kinase activity to MEK, blocks JNK-induced oocyte maturation, we investigated possible downstream points of cross-talk between the two pathways. One reasonable conclusion from this finding is that raf and/or one or more of its downstream targets is involved in JNK phosphorylation. Surprisingly, the results shown in Figure 2A show that injection of JNK into oocytes results in JNK phosphorylation whether or not dn-raf is present. Thus, unless raf can induce JNK phosphorylation in ways that are independent of the activity of its MEK activation domain, it is necessary to conclude that raf does not induce JNK phosphorylation and that JNK phosphorylation occurs by another pathway, as, for example, by activation by upstream elements, such as JNKK, on the SAP pathway (*14*−*16*).

From the results shown in Figure 2B, dn-raf blocks JNK-induced MAPK phosphorylation. This would explain why dn-raf blocks JNK-induced maturation; JNK stimulates MAPK phosphorylation by activating raf, rather than the reverse; raf, in turn, activates MAPK; dn-raf blocks raf-induced activation of MEK and MAPK. Since oocyte maturation requires activation of both JNK and MAPK and MAPK phosphorylation is inhibited, maturation does not occur.

Our in vitro results with raf and JNK immuno-affinity beads confirm this conclusion. Activated JNK induces raf phosphorylation (Figure 3), but activated raf does not phosphorylate bead-bound JNK. Possible caveats to this finding are that raf-induced phosphorylation of JNK is blocked by anti-JNK antibody and/or that raf is inactive. However, MEK stimulates JNK phosphorylation (Figure 4) with no apparent antibody blockade, and raf induces MEK phosphorylation, making both of these possibilities unlikely.

Possible Routes for Phosphorylation of JNK. Formation of a Raf−JNK Complex. In Figure 2B, injection of raf into oocytes results in phosphorylation of JNK and MAPK. Since the above-described results indicate that raf does not phosphorylate JNK directly, another route for phosphorylation of endogenous JNK must exist. Since JNK is activated by stress, oocyte microinjection by itself might induce JNK phosphorylation. However, injection of buffer into oocytes failed to induce JNK phosphorylation ("sham" lane 6 in Figure 2A) (*20*) indicating that nonspecific activation of JNK does not occur.

Since JNK phosphorylates raf and immunoprecipitated JNK coprecipitates raf, it is likely that these two proteins are associated. In a previous study in which we investigated why JNK is inactive in quiescent cells, we found that the JNK/jun complex is bound to GST-pi (*18*). If injection of raf into cells results in intracellular levels sufficient to enable raf to compete with GST-pi for interacting with JNK, this would enable JNK to become activated by release from the inactivation complex.

MEK Is Sufficient but Not Necessary for JNK Phosphorylation. Another possibility for a route to JNK phosphorylation in response to raf injection is suggested in Figure 4 showing that MEK induces JNK phosphorylation in a one-way activation step, since activated JNK does not induce MEK phosphorylation. Since injected raf activates MEK, the latter could phosphorylate JNK. That MEK can phosphorylate JNK is also suggested by our finding that microinjection of constitutively activated MEK into oocytes induces phosphorylation of JNK. On the other hand, injection of raf and U0126 MEK inhibitor does not significantly diminish JNK phosphorylation (Figure 2A). This may be due to the binding of JNK to injected raf, enabling phosphorylation to occur. Activated MEK may therefore be sufficient but not necessary for JNK phosphorylation.

In contrast to the above results, there appears to be no interaction between JNK and MAPK. In previous studies, we have injected two different forms of MAPK into oocytes: an overexpressed baculoviral MAPK construct in Sf9 cells cotransfected with *ras*, *raf*, MEK, and *src* gene constructs enabling activation of MAPK that was maintained in its activated state bound to 8-thio-ATP and bacterially overexpressed MAPK. Neither of these forms induces oocyte maturation (*31*). In contrast, activated JNK from mammalian cell lines and bacterially overexpressed JNK both induce oocyte maturation when injected into oocytes (*13*). These results suggest that MAPK cannot activate JNK, resulting in its failure to induce maturation. As evidenced in Figure 2, JNK can induce its own activation and that of MAPK and hence induce maturation.

Figure 5 shows that anisomycin-activated JNK does not induce phosphorylation of MAPK and that MAPK from anisomycin-treated cells likewise fails to induce phosphorylation of JNK. These results imply that JNK-induced phosphorylation of MAPK (Figure 2B) must occur via activation of raf since JNK induces phosphorylation of raf but not of MEK (Figure 4) or of MAPK (Figure 5). Activation of raf then induces MEK and ultimately MAPK activation. This activation pathway also exists in the mammalian U-251 cell line. Anisomycin activates JNK and

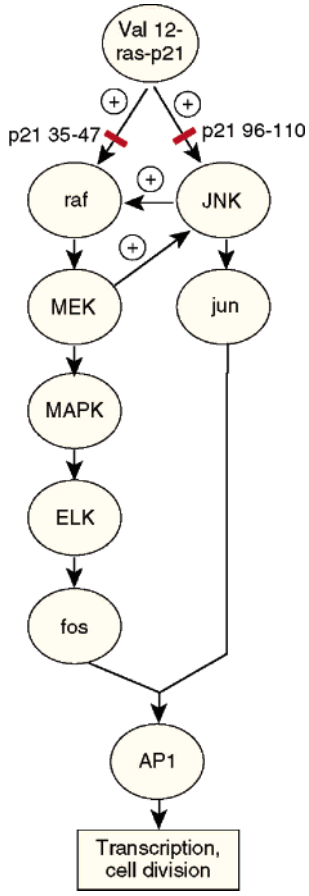

FIGURE 7: Scheme for how proteins on the ras pathway may interact to enhance mitogenic signal transduction. It is based on the findings that Val 12-p21 forms a complex with raf, MEK, and JNK and that JNK can phosphorylate raf and MEK can phosphorylate JNK (+ signs) in a positive feedback loop. Val 12-ras-p21 activates both raf and JNK resulting in concomitant activations of JNK, jun, and MAPK and ultimately fos. This effect is blocked (two solid bars on arrows) by the two p21 35−47 and 96−110 peptides. In addition, raf is also activated by JNK; when activated raf activates MEK, the latter further activates JNK leading to further raf and MEK activation.

MAPK; incubation of the anisomycin-treated cells with U0126 MEK inhibitor blocks MAPK phosphorylation but not JNK activation. Thus, activation of MAPK may depend on JNK activation through activation of raf by activated JNK as described above.

Scheme for Oncogenic Ras-p21-Induced Signal Transduction. A model incorporating the above findings is shown in Figure 7 where oncogenic (Val 12-containing) ras-p21 is shown as interacting directly with raf and JNK leading to the activation of both proteins. Interactions between Val 12-p21 and raf or JNK are blocked by the two p21 peptides 35−47 and 96−110 (Figure 7). Binding of Val 12-p21 to raf and JNK may involve cooperative interactions so that blockade of the binding of one of these two proteins to ras-p21 may diminish the affinity of the other for ras-p21.

Although not shown explicitly in Figure 7, from our immunoprecipitation results in Figure 1A, these interactions occur in a supercomplex of Val 12-p21, raf, JNK, MEK, and MAPK. In the complex, raf becomes activated in its interaction with Val 12-p21 and then induces activation of MEK that induces MAPK activation; the latter can then activate downstream proteins, like elk and fos, ultimately resulting in enhanced transcriptional activity by the fos−jun AP1 complex. From its interaction with Val 12-p21, JNK also becomes activated and induces jun activation enabling the latter to form the activated AP1 complex with MAPK-activated fos.

Our results, from the microinjection experiments shown in Figure 2 and from the in vitro componential studies shown in Figures 3-6, further suggest that activated JNK can phosphorylate raf and that MEK can phosphorylate JNK. We therefore surmise that these same interactions among these pathway proteins also occur in the complex with Val 12−ras-p21.

Thus Val 12-p21 activates both raf and JNK, an event that would initiate concurrent activation of both of these critical proteins; activated JNK, in addition to activating jun, also activates raf. This may result in hyperactivation of MEK that, in addition to superactivating MAPK, can also further activate JNK that can, in turn, further activate raf causing it to send strong activation signals to MAPK. In this manner, a strong positive feedback loop may exist among these proteins resulting in continuous mitogenic signaling.

REFERENCES

1. Barbacid, M. (1987) *ras* Genes, *Annu. Rev. Biochem. 56*, 779−827.
2. Pincus, M. R., Brandt-Rauf, P. W., Koslosky, W., and Appruzzese, W. (2001) Cell Biology and Early Tumor Detection, in *Clinical Diagnosis and Management by Laboratory Methods* (Henry, J. B., Ed.) 19th ed., pp 1344−1354, W. B. Saunders, Philadelphia, PA, Chapter 64.
3. Pincus, M. R., and Friedman, F. K. (2003) Oncoproteins in the Detection of Human Malignancies, in *Molecular Diagnostics*, Vol. 1, pp 23−38, Reed Business Information Publishers, New York.
4. Stacey, D. W., and Kung, H.-F. (1984) Transformation of NIH 3T3 Cells by Microinjection of Ha-*ras* p21 Protein, *Nature 310*, 508−511.
5. Birchmeier, C., Broek, D., and Wigler, M. (1985) *ras* Proteins Can Induce Meiosis in *Xenopus* Oocytes, *Cell 43*, 615−621.
6. Deshpande, A. K., and Kung, H.-F. (1987) Insulin Induction of *Xenopus laevis* Oocyte Maturation Is Inhibited by Monoclonal Antibody against p21 *ras* Proteins, *Mol. Cell. Biol. 7*, 1285−1288.
7. Pincus, M. R., Brandt-Rauf, P. W., Michl, J., and Friedman, F. K. (2000) *ras*-p21-Induced Cell Transformation: Unique Signal Transduction Pathways And Implications for the Design of New Chemotherapeutic Agents, *Cancer Invest. 18*, 39−50.
8. Pincus, M. R. (2004) Development of New Anti-Cancer Peptides from Conformational Energy Analysis of the Oncogenic *ras*-p21 Protein and Its Complexes with Target Proteins, *Front. Biosci. 9*, 3486−3509.
9. Kanovsky, M., Michl, J., Botzolaki, G., Morin, J., Kovac, C., Chung, D., Friedman, F. K., and Pincus, M. R. (2003) Peptides, Designed from Molecular Modeling Studies of the *ras*-p21 Protein, Induce Phenotypic Reversion of a Pancreatic Carcinoma Cell Line but Have No Effect on Normal Pancreatic Acinar Cell Growth, *Cancer Chemother. Pharmacol. 52*, 202−208.
10. Chung, D. L., Joran, A., Friedman, F. K., Robinson, R. R., Brandt-Rauf, P. W., Weinstein, I. B., Ronai, Z. A., Baskin, L., Dykes, D. C., Murphy, R. B., Nishimura, S., Yamaizumi, Z., and Pincus, M. R. (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*, 329−335.
11. Adler, V., Pincus, M. R., Brandt-Rauf, P. W., and Pincus, M. R. (1995) Complexes of *ras*-p21 with *jun*-N-Kinase and c-*jun* Proteins, *Proc. Natl. Acad. Sci. U.S.A. 92*, 10585−10589.
12. Adler, V., Pincus, M. R., Polatskaya, A., Montano, X., Friedman, F. K., and Ronai, Z. (1996) Activation of c-*jun* NH_2 Kinase by UV Irradiation Is Dependent on $p21^{ras}$, *J. Biol. Chem. 271*, 23304−23309.
13. Amar, S., Glozman, A., Chung, D. L., Alder, V., Ronai, Z., Friedman, F. K., Robinson, R., Brandt-Rauf, P. W., Yamaizumi, Z., and Pincus, M. R. (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*, 79−85.
14. Karin, M. (1995) Regulation of Activity by Mitogen Activated Protein Kinases, *J. Biol. Chem. 270*, 16483−16486.
15. Dunn, C., Wiltshire, C., MacLaren, A., and Gillespie, D. A. (2002) Molecular Mechanism and Biological Function of c-Jun N-Terminal Kinase Signalling via the c-Jun Transcription Factor, *Cell. Signalling 14*, 585−593.
16. Xia, Y., and Karin, M. (2004) The Control of Cell Motility and Epithelial Morphogenesis by Jun Kinases, *Trends Cell Biol. 14*, 94−101.
17. Troppmair, J., and Rapp, U. R. (2003) Raf and the Road to Cell Survival: A Tale of Bad Spells, Ring Bearers and Detours, *Biochem. Pharmacol. 66*, 1341−1345.
18. Villafania, A., Anwar, K., Amar, S., Chung, D., Adler, V., Ronai, Z., Brandt-Rauf, P. W., Yamaizumi, Z., Kung, H.-F., and Pincus, M. R. (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*, 61−68.
19. Ranginwale, M., Smith, S., Flom, J., Chie, L., Kanovsky, M., Chung, D., Friedman, F. K., Robinson, R. C., Brandt-Rauf, P. W., Yamaizumi, Z., Michl, J., and Pincus, M. R. (2001) Differences in Patterns of Activation of MAP Kinases Induced by Oncogenic *ras*-p21 and Insulin in Oocytes, *Exp. Cell Res. 269*, 162−169.
20. Chie, L., Amar, S., Kung, H.-F., Lin, M. C. M., Chen, H., Chung, D., Adler, V., Ronai, Z., Friedman, F. K., Robinson, R. C., Kovac, C., Brandt-Rauf, P. W., Yamaizumi, Z., Michl, J., and Pincus, M. R. (2000) Induction of Oocyte Maturation by *jun*-N-Terminal Kinase (JNK) on the Oncogenic *ras*-p21 Pathway Is Dependent on the *raf*-MEK Signal Transduction Pathway, *Cancer Chemother. Pharmacol. 45*, 441−449.
21. Kovac, C., Chie, L., Morin, J., Friedman, F. K., Robinson, R., Chung, D. L., Kanovsky. M., Flom, J., Brandt-Rauf, P. W., Yamaizumi, Z., Michl, J., and Pincus, M. R. (2000) Plasmid Expression of a Peptide that Selectively Blocks Oncogenic *ras*-p21-Induced Oocyte Maturation. *Cancer Chemother. Pharmacol. 45*, 441−449.
22. Miura, K., Inouye, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S., and Nishimura, S. (1986) Synthesis and Expression of a Synthetic Gene for the Activated Human c-Ha-*ras* Protein, *Jpn. J. Cancer Res. 77*, 45−51.
23. LaBonne, C., Burke, B., and Whitman, M. (1995) Role of MAP Kinase in Mesoderm Induction and Axial Patterning during *Xenopus* Development, *Development 121*, 1475−1486.
24. Xu, R.-H., Dong, Z., Maeno, M., Kim, J., Suzuki, A., Ueno, N., Sredni, D., Colburn, N. H., and Kung, H.-F. (1996) Involvement of Ras/Raf/Ap-1 in BMP-4 Signaling during *Xenopus* Embryonic Development, *Proc. Natl. Acad. Sci. U.S.A. 93*, 834−838.
25. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) Transformation of Mammalian Cells by Constitutively Active MAP Kinase Kinase, *Science 265*, 966−970.
26. Adler, V., and Pincus, M. R. (2004) Effector Peptides from Glutathione-S-Transferase-pi Affect the Activation of *jun* by *jun*-N-Terminal Kinase, *Ann. Clin. Lab. Sci. 34*, 35−46.
27. Adler, V., Yin, Z., Fuchs, S., Benezra, M., Rosario, L., Tew, K., Pincus, M. R., Sardana, M., Henderson, C., Wolf, C. R., Davis, R., and Ronai, Z. (1999) GSTpi-A Regulator of JNK Signaling, *EMBO J. 18*, 1321−1334.
28. Monaco, R., Friedman, F. K., Hyde, M. J., Chen, J. M., Manolatus, S., Adler, V., Ronai, Z., Koslosky, W., and Pincus, M. R. (2000) Identification of a Glutathione-S-Transferase Effector Domain for Inhibition of *jun* Kinase, by Molecular Dynamics, *J. Protein Chem. 18*, 859−866.
29. Baum, E. Z., and Bebernitz, G. A. (1990) K-ras Expression in *Xenopus laevis*, *Oncogene 5*, 763−767.
30. Soto-Cruz, I., and Magee, A. I. (1995) Effect of Synthetic Peptides Representing the Hypervariable Region of P21 ras on *Xenopus laevis* Oocyte Maturation, *Biochem. J. 306*, 11−14.
31. Haspel, J., Dent, P., Haystead, T., Brandt-Rauf, P. W., Chung, D., Weinstein, I. B., Nishimura, S., Yamaizumi, Z., and Pincus, M. R. (1995) *raf*-Induced Kinases May Be Necessary but Are Not Sufficient for *ras*-p21 Protein Induction of Oocyte Maturation, *Med. Sci. Res. 23*, 455−457.
32. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherel, P. A., and Trzaskos, J. M. (1998) Identification of a Novel Inhibitor of Mitogen-Activated Protein Kinase Kinase, *J. Biol. Chem. 273*, 18623−18632.
33. Chie, L., Friedman, F. K., Chung, D. L., and Pincus, M. R. (2004) An Effector Peptide from Glutathione-S-Transferase-pi Strongly and Selectively Blocks Mitotic Signaling by Oncogenic *ras*-p21, *Protein J. 23*, 235−238.
34. Chie, L., Friedman, F. K., Kung, H.-F., Lim, M. C. M., Chung, D. L., and Pincus, M. R. (2002) Identification of the Site of Inhibition of Mitogenic Signaling by Oncogenic *ras*-p21 by a *ras* Effector Peptide, *J. Protein Chem. 21*, 367−369.

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