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15. For the induction of strabismus and for tracer injections, anesthesia was induced with ketamine (10 mg per kilogram of body weight) and xylazine (2.5 mg/kg) intramuscularly, and maintained with ketamine intraveneously. In six kittens, we induced divergent strabismus at the age of 2 to 3 weeks by cutting the medial rectus muscle of the left eye. At age 2 to 3 months, pressure injections of red and green fluorescent latex microspheres ("beads") [L. C. Katz, A. Burkhalter, W. J. Dreyer, *Nature* **310**, 498 (1984); L. C. Katz and D. M. Iarovici, *Neuroscience* **34**, 511 (1990)] were made in the visual cortex of four squinting cats and of two normally reared control animals. Injection sites were typically 80 to 350 μm in diameter and confined to area 17 as evidenced by the distribution of retrogradely labeled cells in the LGN. One to 4 weeks later, one eye was occluded and a venous catheter implanted under halothane anesthesia. After full recovery from anesthesia, [^{14}C]2-DG (100 μCi/kg) was injected and the cats were allowed to freely move around in the laboratory for effective monocular stimulation. After 45 min, the animals were killed with pentobarbitone (180 mg/kg, intravenously). Flat-mounts of the occipital cortex were prepared and rapidly frozen. To provide landmarks for later superposition, three holes were made in the flat-mounts with warm needles. Subsequently, serial tangential sections were cut at −15°C and exposed to x-ray film for 3 to 4 weeks [S. Löwel, B. Freeman, W. Singer, *J. Comp. Neurol.* **225**, 401 (1987)]. The distributions of retrogradely labeled cells were mapped with a Zeiss fluorescence microscope (objective ×16) with the stage coupled to an analog *X*/*Y*-plotter. Thereafter cell plots and 2-DG autoradiographs from the same, representative supragranular sections were superimposed with the aid of the needle holes. For data presentation, we contrast-enhanced the 2-DG autoradiographs with an image-processing system (Imago II, Compulog) by expanding the gray values over the full modulation range: regions of lowest 2-DG uptake (unstimulated eye) are displayed in white, regions of highest 2-DG uptake in black. For transneuronal labeling of ocular dominance columns in layer IV, 2.5 mCi of [^{3}H]proline (in 50 μl of saline) were injected into one eye of the two remaining squinting cats 2 weeks before the 2-DG experiment. For the visualization of the [^{3}H]proline distributions, sections were postfixed in 4% paraformaldehyde, washed to remove all [^{14}C]2-DG, and then exposed to Ultrofilm (LKB) for 8 to 12 weeks [S. Löwel, H.-J. Bischof, B. Leutenecker, W. Singer, *Exp. Brain Res.* **71**, 33 (1988)].
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21. For statistical analysis of cell distributions, retrogradely labeled cells were classified into three groups depending on whether they were located within columns of the same (group a) or opposite ocularity (group b) as the injection sites or in the transition zone (group c) between bright and dark areas of the autoradiographs (Table 1). Subsequently, the relative areas of both ocular dominance territories were determined and cells in group c were assigned to groups a and b according to this ratio. The resulting distributions were compared with those expected if labeled cells distributed independently of ocular dominance territories according to the χ^2 test.
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Requirements for Phosphorylation of MAP Kinase During Meiosis in *Xenopus* Oocytes

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Mitogen-activated protein (MAP) kinases are activated in response to a variety of extracellular stimuli by phosphorylation on tyrosine and threonine residues. Xp42 is a *Xenopus laevis* MAP kinase that is activated during oocyte maturation. Modified forms of Xp42 that lacked enzymatic activity or either of the phosphorylation sites were expressed in *Xenopus* oocytes. When meiotic maturation was induced with progesterone, each mutant Xp42 was phosphorylated, indicating that at least one kinase was activated that can phosphorylate Xp42 on tyrosine and threonine. Phosphorylation of one residue is not strictly dependent on phosphorylation of the other.

MAP KINASES (*1*) ARE ACTIVATED in many cell types in response to mitogenic stimuli (*2, 3*). However, MAP kinases are also activated in specialized cells that are not mitogenically responsive, such as *Xenopus* and starfish oocytes undergoing meiosis (*4–6*), PC12 cells differentiating in response to nerve growth factor (*7–9*), adrenal cortical cells secreting catecholamines in response to nicotine or carbachol (*7*), and hippocampus undergoing a seizure response (*10*). The signaling pathways that trigger MAP kinase activation under these different conditions are initiated variously by tyrosine kinases, protein kinase C, or G proteins, and each pathway may activate MAP kinases by a common mechanism or by a distinct one.

Activated MAP kinase from mouse 3T3 cells is phosphorylated on threonine and tyrosine residues (*1*), and dephosphorylation of either or both residues reduces activity (*11*). Therefore, MAP kinases may be activated by the combined actions of tyrosine and serine-threonine kinases, by a single kinase able to phosphorylate threonine and tyrosine residues (*12*), by autophosphorylation, or by a combination of these possibilities. Both the phosphorylated residues are in kinase subdomain VIII (*13*), a region that contains autophosphorylated residues in other protein kinases (*14*). Either autophosphorylation or phosphorylation by other kinases could be induced by extracellular signals.

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Activities that induce serine, threonine, and tyrosine phosphorylation of MAP kinase have been detected in epidermal growth factor–stimulated mouse 3T3 cells and in nerve growth factor–stimulated PC12 cells (*15, 16*). These activators have no apparent protein kinase activity when assayed on other substrates. Studies of the MAP kinase ERK2 made in *Escherichia coli*, which lack tyrosine kinases, indicate that MAP kinases can autophosphorylate on tyrosine (*8, 17, 18*). Furthermore, phosphorylation of bacterially synthesized ERK2 is stimulated by the activator from 3T3 cells (*19*). The activators have been variously assumed to be activators of autophosphorylation (*19*) or specific "MAP kinase kinases" (*16*), but it is not clear which interpretation is correct. The activation of MAP kinase in *Xenopus* oocytes is also correlated with increased phosphorylation of the enzyme (*4, 5*). We now present evidence that this phosphorylation is catalyzed by one or more other kinases that are stimulated during oocyte maturation.

A cDNA for *Xenopus* MAP kinase Xp42 that is phosphorylated and activated during oocyte maturation has been cloned (*4, 20*), and antiserum has been raised to a COOH-terminal peptide (*21*). Xp42 was immunoprecipitated from oocytes and egg extracts and incubated with the substrates myelin basic protein (MBP) and γ-^{32}P–labeled adenosine triphosphate (ATP). Xp42 from eggs was enzymatically active and Xp42 from oocytes was inactive (Fig. 1A). Immunoblotting of the immunoprecipitates revealed that egg Xp42 migrated more slowly than oocyte Xp42 on gel electrophoresis (Fig. 1A). Xp42 from eggs, but not from

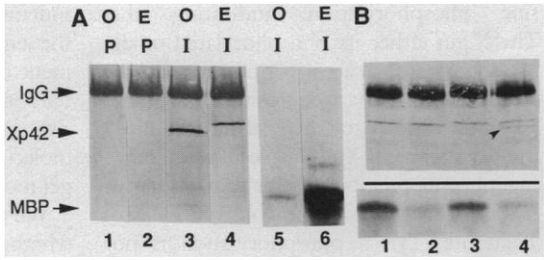

Fig. 1. Characterization of *Xenopus* MAP kinase Xp42. (**A**) *Xenopus* oocyte (O) or egg (E) extract was immunoprecipitated with preimmune (P) rabbit serum (lanes 1 and 2) or antiserum (I) to Xp42 (1913.2) (lanes 3 through 6). In lanes 1 through 4, proteins in the immunoprecipitates were immunoblotted with antiserum 1913.2. In lanes 5 and 6, oocyte or egg immunoprecipitates were incubated with MBP in the presence of [γ-^{32}P]ATP to measure kinase activity. IgG, a nonspecific band present because of the large amounts of immunoglobulin G in the extracts. (**B**) Phosphatase treatment of immunoprecipitated Xp42. Equal amounts of *Xenopus* egg extract were immunoprecipitated with 1913.2. The immunoprecipitates were treated with buffer alone (lane 1), PP2A (lane 2), CD45 (lane 3), or both PP2A and CD45 (lane 4) (top). The arrowhead points to the unphosphorylated Xp42. Kinase activity was assayed with MBP as substrate (bottom).

oocytes, is phosphorylated on threonine and tyrosine (*4, 22*). Incubation of an egg Xp42 immunoprecipitate with the serine-threonine–specific protein phosphatase 2A (PP2A) (*23*) abolished the enzymatic activity but did not alter the electrophoretic mobility of Xp42 (Fig. 1B). Incubation of immunoprecipitated Xp42 with the tyrosine-specific protein phosphatase CD45 (*24*) reduced the enzymatic activity of Xp42 by 50% and had no effect on electrophoretic mobility. Incubation with both phosphatases increased the electrophoretic mobility of about 50% of Xp42 molecules, to comigrate with nonphosphorylated oocyte Xp42 (Fig. 1B). These results are consistent with dephosphorylation studies on murine MAP kinase, in which phosphorylation at both tyrosine and threonine was found to be required for activity (*11*), although in our experiments CD45 dephosphorylated Xp42 inefficiently. Xp42 molecules that are phosphorylated at either or both sites migrate slower than nonphosphorylated Xp42 on SDS gels. Thus, gel mobility can be used as an assay for phosphorylation.

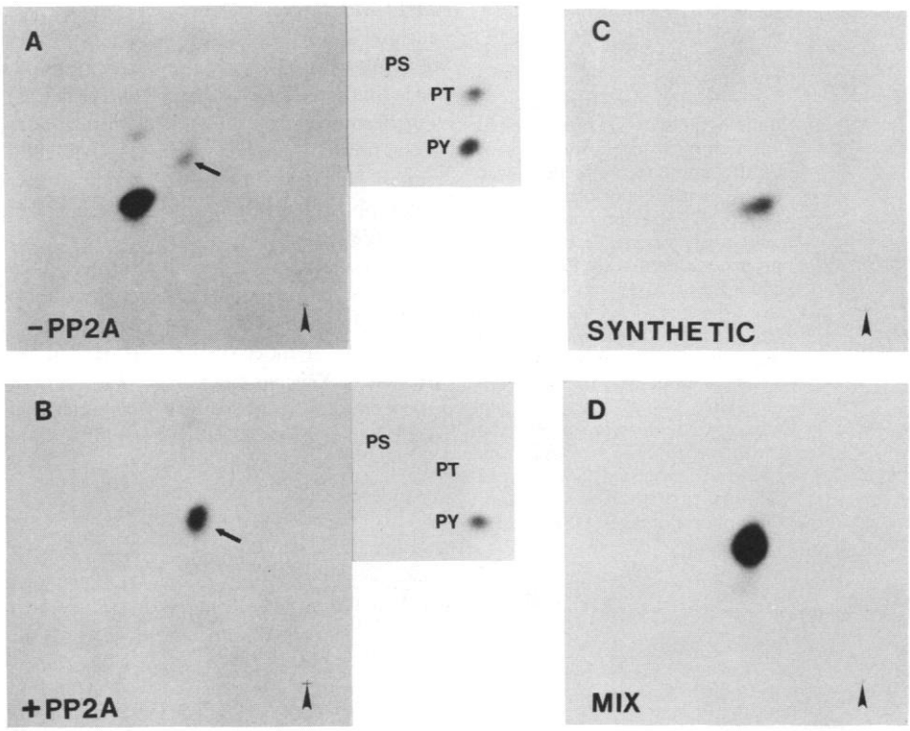

Fig. 2. Identification of the phosphorylation sites in Xp42. (**A**) Phosphopeptide map of Xp42 labeled with $^{32}P_i$ in *Xenopus* egg extract. Phosphoamino acid analysis of the major spot is shown to the right. PS, phosphoserine; PT, phosphothreonine; and PY, phosphotyrosine. (**B**) The same sample as in (A) after PP2A treatment. Phosphoamino acid analysis is shown to the right. Comparison of the PP2A-treated peptide from Xp42 in (B) with a tyrosine-phosphorylated synthetic peptide VADPDHDHTGFLTEYVATR (**C**) (*29*), or a mixture (**D**) of the samples in (B) and (C). The arrowhead indicates the origin.

We tested whether phosphorylation of Xp42 occurred at the same sites as murine MAP kinase (*13*). Xp42, immunoprecipitated from ^{32}P-labeled egg extracts, contained one predominant tryptic phosphopeptide (*25*) (Fig. 2A). Removal of phosphothreonine with PP2A altered the migration of the peptide to a position indicative of less acidic, less hydrophilic properties (Fig. 2B), corresponding to the position of migration of a minor phosphopeptide in the original digest (Fig. 2A, arrow). This mobility shift is consistent with removal of one phosphate from a peptide containing two phosphates. A synthetic peptide containing the Xp42 phosphorylation sites (residues 176 to 194) comigrated with the tyrosine-phosphorylated Xp42 tryptic peptide (Fig. 2, C and D) when phosphorylated in vitro at Tyr^{190} by an Src immunoprecipitate. Therefore, the tyrosine phosphorylated in Xp42 is Tyr^{190}, and the threonine residue also lies within the synthetic peptide at either Thr^{184} or Thr^{188}. This suggests that Xp42 molecules that are active are phosphorylated at both Thr^{188} and Tyr^{190}, as is the murine MAP kinase activated by mitogenic stimulation (*13*).

We fused a cDNA encoding a repeated Myc protein epitope to the 5′ end of the Xp42 cDNA (Xp42-MT). This allowed the separation of the cDNA product from endogenous Xp42 by immunoprecipitation with the monoclonal antibody 9E10 (*26*). We expressed Xp42-MT in *Xenopus* oocytes by injecting mRNA transcribed in vitro (*27*). After labeling the oocytes with [^{35}S]methionine for 2 days, we incubated them with progesterone to induce meiotic maturation. The oocytes were then lysed, and Xp42-MT immunoprecipitated (Fig. 3). The Xp42-MT from progesterone-treated wild-type (WT) oocytes had a reduced mobility in SDS gels, indicative of phosphorylation (Fig. 3A, WT lanes). Phosphorylation of Xp42-MT at tyrosine was also detected by immunoblotting the immunoprecipitates with antibody to phosphotyrosine (Fig. 3B). These phosphorylated molecules were enzymatically active (Fig. 3D). To test whether phosphorylation required intrinsic protein kinase activity (and was therefore intramolecular), we made a mutation in the Xp42-MT cDNA so that it encoded arginine in place of Lys^{57} (K57R mutant). A corresponding mutation has been made in many other protein kinases, as well as in murine MAP kinase (*17*), and in all cases it inhibits enzymatic activity. Another highly conserved residue in the phosphotransferase region, Ile^{86}, was changed to tyrosine (I86Y). The COOH-terminal 34–amino acid residues were also deleted (−COOH). Xp42-MT made from each of

these cDNAs was also phosphorylated during meiotic maturation (Fig. 3, A and B), but these molecules were enzymatically inactive (Fig. 3D). Because kinase-inactive mutant Xp42-MT was phosphorylated in vivo during meiosis, this phosphorylation must have been carried out by one or more maturation-activated tyrosine and threonine kinases.

To investigate the specificity of the protein kinase or kinases that phosphorylate Xp42 in meiotic oocytes, we tested four phosphorylation site mutants of Xp42-MT as substrates (Fig. 3). These mutants have Thr^{188} changed to valine or aspartate (T188V and T188D), Tyr^{190} changed to phenylalanine (Y190F), or both Thr^{188} changed to valine and Tyr^{190} changed to phenylalanine (TY/VF). Mutation of Thr^{188} to valine or aspartate had no effect on tyrosine phosphorylation, indicating that Thr^{188}, in either its phosphorylated or dephosphorylated state, is not part of the recognition site for the tyrosine kinase. Mutation of Tyr^{190} to phenylalanine reduced the percentage of Xp42 molecules undergoing phosphorylation at Thr^{188}, but about half the molecules were still phosphorylated. Therefore, Tyr^{190} phosphorylation is not essential for, but may increase the efficiency of, Thr^{188} phosphorylation. No phosphorylation of the double mutant was detected, confirming that the phosphorylations causing the mobility shifts are occurring at the sites identified by peptide mapping. Expression of WT or mutant Xp42-MT had no apparent effect on oocyte maturation or on phosphorylation of endogenous Xp42 (Fig. 3C). To verify that the Myc epitope did not alter the regulation of Xp42, we expressed the various mutants without the Myc tag (*28*). Although the endogenous Xp42 prevented measurement of the activity of the exogenous proteins, the phosphorylation of the kinase-inactive and phosphorylation site mutant molecules could be detected by their altered gel mobilities.

We designed in vitro experiments to test whether phosphorylation of kinase-inactive Xp42-MT in progesterone-treated oocytes was catalyzed by endogenous Xp42 or another kinase. Egg extract was separated by ion-exchange chromatography into a 0.1 M NaCl fraction depleted of Xp42 and a 0.3 M NaCl fraction that contained Xp42 (Fig. 4B). WT and K57R Xp42-MT were synthesized in reticulocyte lysate and immunoprecipitated. The immunoprecipitates were incubated with ATP and either the egg fractions or the column buffer (Fig. 4A). The egg fraction depleted of Xp42 catalyzed phosphorylation of K57R Xp42-MT, causing the characteristic mobility shift (Fig. 4A, lane 6), and activated WT Xp42-MT (lane 5), suggesting phosphorylation at both sites. In contrast, the fraction containing Xp42 neither phosphorylated K57R mutant Xp42-MT (lane 8) nor activated WT Xp42-MT (lane 7). In the control incubations with column buffer, a small but reproducible fraction of WT Xp42-MT molecules was phosphorylated (lane 3), but kinase-inactive Xp42-MT molecules were not (lane 4). The autophosphorylation of Xp42-MT appeared to result in singly phosphorylated molecules because there was no kinase activity (lane 3). Furthermore, the phosphorylation appears to be limited to Tyr^{190} because the T188V mutant, but not the Y190F mutant, underwent phosphorylation under these conditions (*28*).

These data suggest that although autophosphorylation results in a small amount of Xp42 phosphorylation, full activation requires one or more distinct protein kinases present in *Xenopus* egg extract. Our data do not exclude the possibility that autophosphorylation of MAP kinases may also be regulated in maturing oocytes and other stimulated cells.

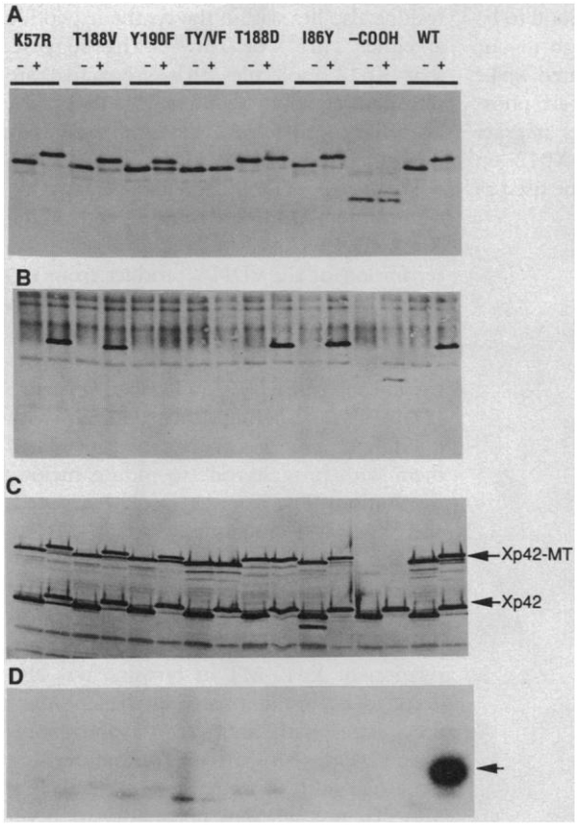

Fig. 3. Expression of Xp42-MT mutants in *Xenopus* oocytes. Stage VI *Xenopus* oocytes were injected with [^{35}S]methionine and RNA transcribed in vitro from various Xp42-MT mutant cDNAs. After 48 hours, oocytes were incubated in the absence or presence of progesterone (as indicated at the top of the lanes) for a further 6 hours. (**A**) Xp42-MT immunoprecipitates. Newly synthesized Xp42-MT immunoprecipitated with monoclonal antibody 9E10 was detected by autoradiography. (**B**) Immunoblot with antibody to phosphotyrosine. 9E10 immunoprecipitates were probed with monoclonal antibody PY-20. (**C**) Xp42 immunoblot. Aliquots of oocyte extract were probed with antibody 1913.2. The endogenous Xp42 migrates faster than the ectopic Xp42-MT. The COOH-terminal deletion is not recognized because it is missing the 1913.2 epitope. (**D**) Immune complex kinase assay. The 9E10 immunoprecipitates were incubated in the presence of [γ-^{32}P]ATP and bacterially expressed GST-Myc fusion protein (*30*). The arrow indicates phosphorylated GST-Myc.

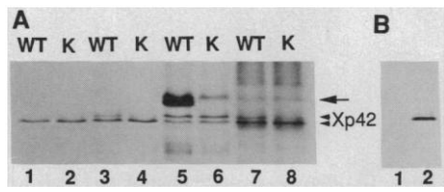

Fig. 4. (**A**) In vitro activation of MAP kinase. WT and kinase-inactive K57R mutant (K) Xp42-MT were immunoprecipitated from reticulocyte lysates after translation of the mRNA. The immunoprecipitates were incubated in the presence of ATP and phosphatase inhibitors with egg extract that had been fractionated by DEAE-cellulose chromatography (*31*). Immunoprecipitates were then washed and assayed for kinase activity with GST-Myc as a substrate. Lanes 1 and 2, incubation in kinase buffer alone; lanes 3 and 4, kinase buffer with ATP (5 mM); lanes 5 and 6, 0.1 M NaCl fraction with ATP; lanes 7 and 8, 0.3 M NaCl fraction with ATP. The upper arrow indicates ^{32}P-labeled GST-Myc substrate. The lower arrowheads indicate the phosphorylated and nonphosphorylated forms of ^{35}S-labeled methionine Xp42-MT. (**B**) Xp42 immunoblot of column fractions. Lane 1, 0.1 M NaCl fraction; and lane 2, 0.3 M NaCl fraction.

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21. *Xenopus* egg or oocyte extracts were prepared as described (*4*) and immunoprecipitated with rabbit polyclonal antiserum (1913.2) to the COOH-terminal Xp42 peptide KELIFEETARFQPGY (*29*). For immune complex kinase assays, immunoprecipitates were incubated in 30 μl of KB [20 mM Hepes (pH 7.5), 2 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM Na_3VO_4] with MBP (1 mg/ml) (Sigma) and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Du Pont Biotechnology Systems) for 15 min at 30°C before electrophoresis. For phosphatase treatment, immunoprecipitates were incubated in phosphatase buffer [25 mM imidizole (pH 7.5), 0.1% 2-mercaptoethanol, and bovine serum albumin (0.1 mg/ml)] with the indicated phosphatase for 20 min at 30°C and washed twice in KB.
22. Phosphothreonine in Xp42 was not detected previously because longer acid hydrolysis times are required to liberate phosphothreonine from partial hydrolysis products. We now hydrolyze for 2 hours at 110°C.
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25. *Xenopus* egg extract was labeled in vitro with [^{32}P]orthophosphate [J. A. Cooper, *Mol. Cell. Biol.* **9**, 3143 (1989)] and immunoprecipitated with antiserum 1913.2. The immunoprecipitates were run on SDS gels, and Xp42 was excised from the gel and digested with trypsin. The tryptic phosphopeptides were separated in two dimensions on thin-layer chromatography plates by electrophoresis at pH 8.9 (anode at left) and ascending chromatography with buffer II [A. Kazlauskas and J. A. Cooper, *Cell* **58**, 1121 (1989)].
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27. Xp42 cDNA was subcloned into plasmid pSP64A (Promega). Site-directed mutagenesis of Xp42 cDNA was done as described [T. A. Kunkel, J. D. Roberts, R. A. Zabour, *Methods Enzymol.* **154**, 367 (1987)]. RNA was transcribed in vitro with SP6 RNA polymerase (Promega). [^{35}S]methionine (1100 Ci/mmol, Tran^{35}S label, ICN Biomedicals) was dried and resuspended in a solution of RNA (0.5 mg/ml) at 38 μCi/μl. This solution (30 to 50 nl) was injected into the cytosol of Stage VI oocytes. The oocytes were incubated in 50% L-15 medium (Gibco) with gentamycin (0.05 μl/ml) at 15°C for 2 days. On day 3, progesterone (2 μg/ml) was added, and incubation was continued for 16 hours. Extracts were prepared by homogenizing groups of 20 to 35 oocytes in 20 mM Hepes (pH 7.5), 2 mM DTT, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 2 mM Na_3VO_4, and 0.05 μM microcystin (Calbiochem), and insoluble debris was removed by centrifugation.
28. J. Posada and J. A. Cooper, unpublished results.
29. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. A bacterial protein containing glutathione transferase (GST) fused to the NH_2-terminus of Myc (GST-Myc) (AMRAD) was prepared according to the manufacturer's instructions. This protein contains a site phosphorylated by MAP kinase [E. Alvarez *et al.*, *J. Biol. Chem.* **266**, 15277 (1991)].
31. Egg extract (2 mg of protein) was diluted in column buffer [20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 1 mM PMSF, 2 mM Na_3VO_4, and 0.5 μM microcystin] containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and was applied to a 1-ml DE-52 (Whatman, Madistone Kent, England) anion exchange column. The column was washed with one bed volume and eluted with 0.1 M and 0.3 M NaCl in column buffer (1 ml of each). The fractions were concentrated in centrifugal concentrators (Amicon), made 5 mM in ATP, and mixed with 9E10 immunoprecipitates containing ^{35}S-labeled methionine Xp42-MT. The mixtures were incubated at 30°C for 30 min and washed, and immune complex kinase assays carried out in the presence of GST-Myc and [γ-^{32}P]ATP.
32. We thank C. Diltz and E. Fischer for CD45, T. Haystead for PP2A, B. Blackwood for GST-Myc, M. Roth for Myc tag reagents, and A. Kashishian for technical assistance. Supported by grants from the NIH (CA-28151 and CA-08860).

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Technical Comment

Localization of Responses to Pain in Human Cerebral Cortex

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J. D. Talbot *et al.* (*1*) found significantly increased cerebral blood flow (CBF) in the contralateral anterior cingulate cortex (area 24) in response to thermal pain in normal volunteers. We have confirmed this result (*2*) using different methods of statistical analysis, but we also found increased CBF in the contralateral thalamus ($z = 4.193$, $P < 0.01$ for the center of both areas). We conclude that these two areas are likely to be the main sites in the brain where the "suffering" component of pain is experienced. The results of cingulumotomy on patients with intractable pain suggest that both attentional and emotional components of pain may be processed in the anterior cingulate cortex (*3*).

In contrast to the findings in (*1*), we did not see any evidence of increased CBF in the primary or secondary somatosensory cortices in response to an increasing intensity of nonpainful heat or to a painful heat stimulus. The explanation for this different result may well be that we stimulated the same area of the back of the hand to exclude the tactile and positional components of the stimulus, whereas Talbot *et al.* carefully moved the site of stimulation around the forearm. It seems likely that, in the latter procedure, the variable tactile components, and the subject's variable attention to the positional components of the stimulus, produced increased CBF in the primary and secondary sensory cortices.

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Response: Although our finding of activation of the anterior cingulate cortex in response to pain has been confirmed by Jones *et al.* (*1*), our interpretation of this activation differs from their "suffering" hypothesis. Psychophysical studies (*2, 3*) have demonstrated that phasic noxious heat stimuli evoke pain sensations with a low affective component. On the basis of anatomical (*4*), neurophysiological (*5*), and human stimulation (*6*) studies, we postulate that (exclusively contralateral) cingulate activation may indicate a direct encoding of stimulus intensity rather than any substantial "suffering" experienced by the subjects. Our views are not completely at odds with those of Jones *et al.*, as we presume that the encoding of pain intensity is a major factor in the regulation of emotional and autonomic responses. Nevertheless, "suffering" is not likely to be