Independent Human MAP Kinase Signal Transduction Pathways Defined by MEK and MKK Isoforms

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Mammalian mitogen-activated protein (MAP) kinases include extracellular signal-regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 subgroups. These MAP kinase isoforms are activated by dual phosphorylation on threonine and tyrosine. Two human MAP kinase kinases (MKK3 and MKK4) were cloned that phosphorylate and activate p38 MAP kinase. These MKK isoforms did not activate the ERK subgroup of MAP kinases, but MKK4 did activate JNK. These data demonstrate that the activators of p38 (MKK3 and MKK4), JNK (MKK4), and ERK (MEK1 and MEK2) define independent MAP kinase signal transduction pathways.

Several subgroups of MAP kinase exist in mammalian cells (*1*). These include the ERK (*2*, *3*), JNK (*4*, *5*), and p38 (6, 7) MAP kinases. These subgroups are distinguished by the sequence of the tripeptide dual phosphorylation motif that is required for MAP kinase activation: Thr-Glu-Tyr (ERK), Thr-Pro-Tyr (JNK), and Thr-Gly-Tyr (p38). Substantial progress toward understanding the signal transduction pathway that leads to ERK activation has been made (8). However, the signaling pathways that activate the p38 and JNK subgroups of MAP kinase are poorly understood.

The JNK (*4*, *5*) and p38 (6, 7) protein kinases are activated in response to osmotic stress in mammalian cells (6, 9). Furthermore, these kinases can complement mutant strains of the yeast *Saccharomyces cerevisiae* that lack the osmotic stress-activated HOG1 MAP kinase (6, 9). In the case of JNK1, this complementation depends on the expression of the HOG1 MAP kinase kinase PBS2 (9). This observation suggests that the PBS2 protein kinase may phosphorylate and activate p38 and JNK1 in yeast. A homolog of PBS2 may therefore be the physiological MAP kinase kinase that activates p38 and JNK in human cells.

Comparison of the sequence of PBS2 with that of the human MAP kinase kinases MEK1 and MEK2 shows a marked similarity but also differences (Fig. 1). To isolate a human PBS2 homolog, we exploited the distinctive regions of the yeast PBS2 sequence to design polymerase chain reaction (PCR) primers. Amplification of human brain mRNA with these primers resulted in the formation of specific products. These were cloned into a plasmid

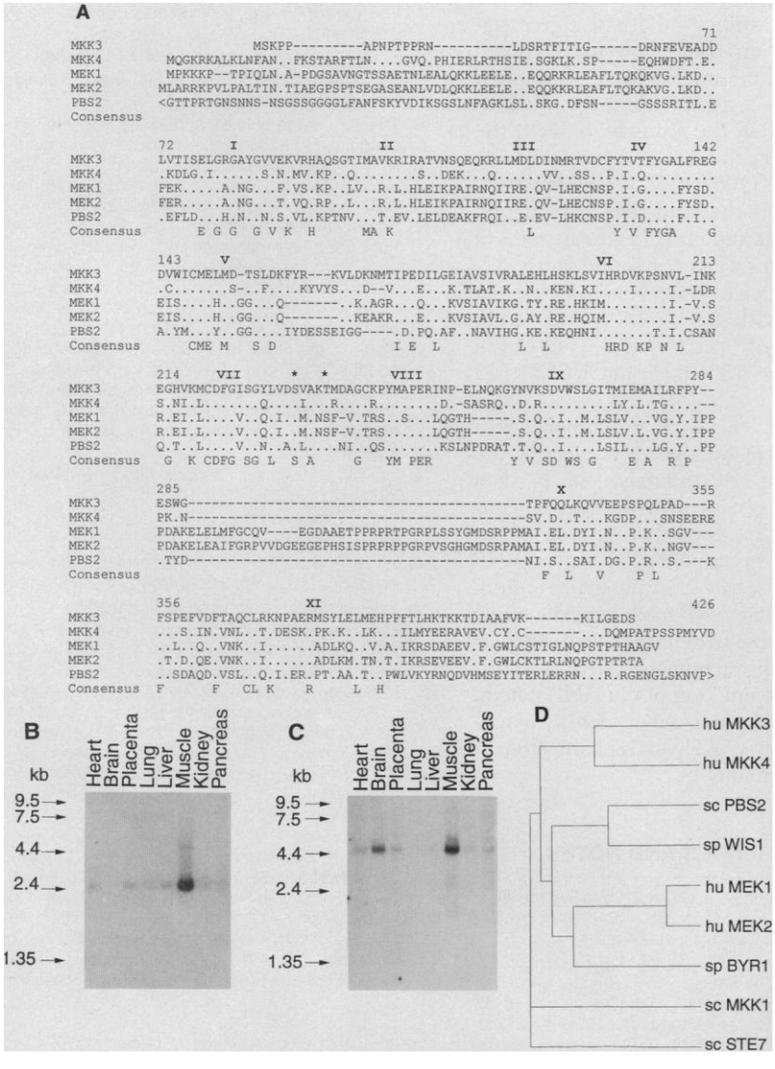

Fig. 1. Primary structure of MKK protein kinases. (**A**) The primary sequences of MKK3 and MKK4 were deduced from the sequence of cDNA clones isolated from a human fetal brain library (*22*). An in-frame termination codon is located in the 5′ untranslated region of the MKK3 cDNA but not in the 5′ region of the MKK4 cDNA. The MKK4 protein sequence presented starts at the second in-frame initiation codon. Usage of the first initiation codon fuses the sequence MAAPSPSGGGGSGGGSGSGTPGPVGSPAPGHPAVSS (*18*) to the NH_2-terminus of the MKK4 sequence shown. These sequences were compared to those of the human MAP kinase kinases MEK1 and MEK2 (*23*) and of the yeast MAP kinase kinase PBS2 (*24*) with the PILE-UP program (version 7.2; Wisconsin Genetics Computer Group). The protein sequences are presented in single-letter code (*18*). The PBS2 sequence is truncated at both the NH_2- (<) and COOH- (>) termini. Gaps introduced into the sequences to optimize the alignment are illustrated by a dash. Identical residues are indicated by a period. The sites of activating phosphorylation in MEK (*10*,*11*) are indicated by asterisks. The identity and similarity of the kinases with human MKK3 (between subdomains I and XI) were calculated with the BESTFIT program (version 7.2; Wisconsin Genetics Computer Group) (percent of identity to percent of similarity: MEK1, 41%/63%; MEK2, 41%/62%; MKK4, 52%/73%; and PBS2, 40%/59%). The identity and similarity with human MKK4 were calculated to be as follows: MEK1, 44%/63%; MEK2, 45%/61%; MKK3, 52%/73%; and PBS2, 44%/58%. The cDNA sequences of MKK3 and MKK4 have been deposited in GenBank with accession numbers L36719 and L36870, respectively. The expression of MKK3 (**B**) and MKK4 (**C**) were examined by Northern blot analysis of poly(A)$^+$ mRNA isolated from various human tissues (*25*). (**D**) The relation between members of the human and yeast MAP kinase kinase group is presented as a dendrogram created by the unweighted pair-group method with the use of arithmetic averages (PILE-UP program). The human (hu) MAP kinase kinases MEK1, MEK2, MKK3, and MKK4; the *Saccharomyces cerevisiae* (sc) MAP kinase kinases PBS2, MKK1, and STE7; and the *S. pombe* (sp) MAP kinase kinases WIS1 and BYR1 are presented.

vector and sequenced. Two different complementary DNAs (cDNAs) that encoded human protein kinases were identified. These kinases are 52% identical and are distantly related to the MAP kinase kinase group (MKK3 and MKK4) (Fig. 1). One region of sequence similarity includes the dual phosphorylation sites that are required for stimulation of the ERK activator MEK (MAP or ERK kinase) (*10*, *11*). Comparison by progressive pairwise alignment to create a dendrogram indicated that MKK3 and MKK4 form a distinct subgroup of MAP kinase kinases (Fig. 1). This subgroup shares sequence similarity with PBS2, including the presence of a large deletion located between subdomains IX and X that is absent in MEK1 and MEK2 (Fig. 1). The MKK3 and MKK4 protein kinases therefore represent putative human homologs of the yeast PBS2 protein kinase.

The expression of these human MKK isoforms was examined by Northern (RNA) blot analysis of mRNA isolated from different adult tissues. Both protein kinases are widely expressed in human tissues (Fig. 1). The most expression of both isoforms was observed in skeletal muscle (Fig. 1).

We investigated the substrate specificity of MKK3 in an in vitro assay with recombinant MAP kinases (JNK1, p38, and ERK2) as substrates (Fig. 2). Autophosphorylation of MKK3 was observed. However, no phosphorylation of JNK1 or ERK2 was detected. In contrast, p38 was a substrate (Fig. 2). Phosphoaminoacid analysis of p38 demonstrated the presence of phosphothreonine and phosphotyrosine (*12*). Mutational analysis demonstrated that the sites of phosphorylation were Thr^{180} and Tyr^{182}, because the replacement of these residues with Ala and Phe, respectively, blocked p38 phosphorylation (Fig. 2). These data establish that MKK3 functions in vitro as a p38 MAP kinase kinase.

In vitro protein kinase assays demonstrated that MKK4, like MKK3, is a p38 MAP kinase kinase (Fig. 3). However, three differences between MKK3 and MKK4 were observed. First, the amount of autophosphorylation of MKK3 was greater than that of MKK4 (Fig. 3). Second, MKK4 (but not MKK3) was a substrate for activated MAP kinase (Fig. 3), like MEK1 and MEK2 (*13*). Third, MKK4 (but not MKK3) activated JNK. The rate of p38 phosphorylation by MKK4 was greater than the rate of phosphorylation of JNK (Fig. 3). MKK4 is therefore a p38 MAP kinase kinase that also phosphorylates the JNK subgroup of MAP kinases.

The activation of p38 is mediated by dual phosphorylation on Thr^{180} and Tyr^{182} (*14*). The MKK isoforms phosphorylated p38 at these sites in vitro (Figs. 2 and 3). We therefore tested the hypothesis that MKK activation causes increased activity of p38 MAP kinase. Epitope-tagged MKK3 expressed in cultured COS cells and then immunoprecipitated from cell lysates caused a small amount of phosphorylation of p38 MAP kinase (Fig. 4). This phosphorylation resulted from the basal activity of MKK3. We therefore investigated the activity of MKK3 isolated from activated cells. MKK3 from ultraviolet (UV)-irradiated cells caused increased phosphorylation of p38 MAP kinase but not of JNK (Fig. 4). An increase in p38 MAP kinase activity was detected in assays in which the transcription factor ATF2 was a substrate (Fig. 4). MKK3 is therefore activated by UV radiation. MKK3 activation was also observed in cells exposed to inflammatory cytokines (interleukin-1 and tumor necrosis factor) and osmotic stress but was not observed in cells treated with epidermal growth factor (EGF) or phorbol ester (*12*). Together, these data establish that MKK3 is activated by cytokines and by environmental stress in vivo.

MKK3 and MKK4 may be relevant to the activation of p38 MAP kinase in vivo. To test this hypothesis, we examined the effect of overexpression of these MKK isoforms on p38 MAP kinase activity. The expression of the ERK activator MEK1 did not alter p38 protein kinase activity (Fig. 5). In contrast, expression of MKK3 or MKK4 caused increased activity of p38 MAP kinase (Fig. 5). The activation of p38 caused by MKK3 and MEK4 was similar to that observed in UV-irradiated cells and

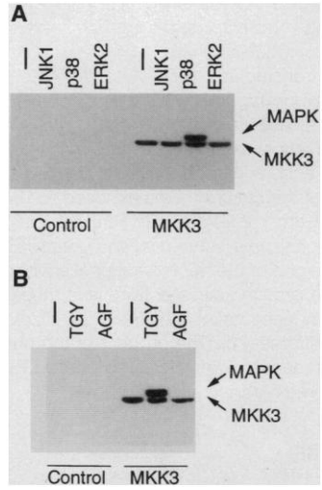

Fig. 2. Phosphorylation of p38 MAP kinase by MKK3. (**A**) Recombinant GST-MKK3 was incubated with [γ-^{32}P]ATP and buffer (dashed lanes), GST-JNK1 (JNK1), GST-p38 MAP kinase (p38), or GST-ERK2 (ERK2) (*26*). The phosphorylation reaction was terminated by addition of Laemmli sample buffer, and the phosphorylated proteins were detected after SDS-PAGE by autoradiography (*20*). (**B**) GST-MKK3 was incubated with [γ-^{32}P]ATP and buffer (dashed lanes), wild-type GST-p38 MAP kinase (TGY), or mutated GST-p38 MAP kinase (AGF). In the mutated p38 MAP kinase, Thr^{180} and Tyr^{182} were replaced with Ala and Phe, respectively. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

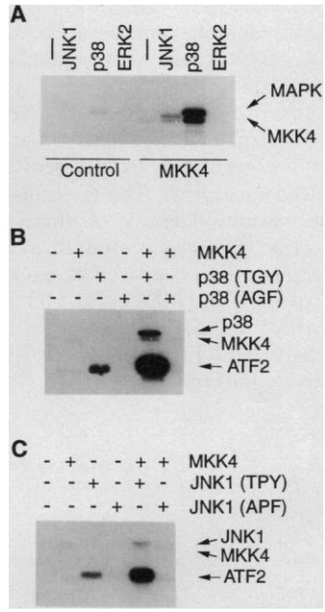

Fig. 3. Phosphorylation and activation of JNK and p38 MAP kinase by MKK4. (**A**) Recombinant GST-MKK4 was incubated with [γ-^{32}P]ATP and buffer (dashed lanes), GST-JNK1 (JNK1), GST-p38 MAP kinase (p38), or GST-ERK2 (ERK2) (*26*). The phosphorylation reaction was terminated by addition of Laemmli sample buffer, and the phosphorylated proteins were detected after SDS-PAGE by autoradiography (*21*). (**B**) GST-MKK4 was incubated with [γ-^{32}P]ATP and buffer (dashed lanes), wild-type GST-p38 MAP kinase (TGY), or mutated GST-p38 MAP kinase (AGF). The p38 substrate ATF2 (*14*) was included in each incubation. In the mutated p38 MAP kinase, Thr^{180} and Tyr^{182} were replaced with Ala and Phe, respectively. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. (**C**) GST-MKK4 was incubated with [γ-^{32}P]ATP and buffer (dashed lanes), wild-type GST-JNK1 (TPY), or mutated GST- JNK1 (APF). The JNK1 substrate ATF2 (*19*) was included in each incubation. The mutated JNK1 was constructed by replacement of the phosphorylation sites Thr^{183} and Tyr^{185} with Ala and Phe, respectively. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

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was much greater than that detected in EGF-treated cells (Fig. 5). Overexpression of MKK3 and MKK4 did not alter ERK2 protein kinase activity (*12*). MKK3 also caused no change in JNK activity (*12*). In contrast, expression of MKK4 increased the activity of JNK (*12*). The lack of effect of MKK3 on JNK activity and the increased JNK activity caused by MKK4 are consistent with the substrate specificity of these MKK isoforms (Figs. 2 and 3). These data demonstrate that MKK3 and MKK4 represent physiologically relevant activators of p38 MAP kinase. In addition, these data establish a role for MKK4 in the activation of JNK.

ERKs are potently activated by treatment of cells with EGF or phorbol ester (*15, 16*). In contrast, p38 MAP kinase is only slightly activated under these conditions (*14*). However, UV radiation, osmotic stress, and inflammatory cytokines cause a marked increase in p38 MAP kinase activity (*14*). This difference in the pattern of activation of ERK and p38 suggests that these MAP kinases are regulated by different signal transduction pathways. The molecular basis for the separate identity of these signal transduction pathways is established by the demonstration that the MAP kinase kinases that activate ERK (MEK1 and MEK2) and p38 (MKK3 and MKK4) are distinct (Fig. 6). Because MEK1, MEK2, and MKK3 do not activate JNK in vitro (*4*) (Figs. 2 and 4)

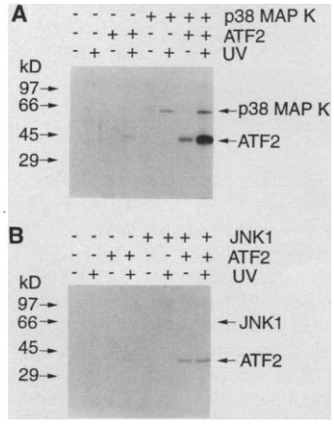

Fig. 4. Phosphorylation and activation of p38 MAP kinase by UV-stimulated MKK3. Epitope-tagged MKK3 was expressed in COS-1 cells (*27*). The cells were exposed in the absence and presence of UV-C (40 J/m^2). MKK3 was isolated by immunoprecipitation and used for protein kinase assays (*21*) with the substrates GST-p38 MAP kinase (**A**) or GST-JNK1 (**B**). In some assays, the transcription factor ATF2 was included in the incubation as a substrate for p38 and JNK (*14, 19*). The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP and was terminated by the addition of Laemmli sample buffer. The phosphorylated proteins were visualized after SDS-PAGE by autoradiography.

and MKK4 activates both p38 and JNK (Fig. 3), we predict the possible existence of an additional class of MAP kinase kinases that are specific for JNK (Fig. 6).

The mammalian p38 MAP kinase and the yeast HOG1 MAP kinase share the dual phosphorylation motif Thr-Gly-Tyr. This structural relation is reflected by a functional similarity between these protein kinases. Both p38 and HOG1 are activated by osmotic stress (*6, 14, 17*). Furthermore, the mammalian p38 MAP kinase can complement yeast strains that are defective in HOG1 expression (*6*). The signal transduction pathways that lead to activation of p38 in mammals and HOG1 in yeast may therefore be similar. Indeed, the MAP kinase kinases that activate p38 (MKK3 and MKK4) and HOG1 (PBS2) are structurally related (Fig. 1). A two-component histidine kinase response regulator is an upstream component of the HOG1 signal transduction pathway (*17*). By analogy, it is possible that similar proteins may function within the signal transduction pathway that leads to activation of the mammalian p38 MAP kinase.

Note added in proof: MKK3 is a specific activator of p38 MAP kinase. In contrast, MKK4 activates both p38 and JNK in vitro.

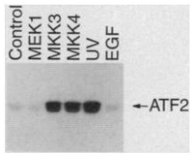

Fig. 5. Activation of p38 MAP kinase in cells expressing MKK3 and MKK4. COS-1 cells were transfected (*27*) with epitope-tagged p38 MAP kinase together with an empty expression vector or an expression vector encoding MEK1, MKK3, or MKK4 (*26*). Some of the cultures were exposed to UV radiation (40 J/m^2) or treated with 10 nM EGF. The p38 MAP kinase was isolated by immunoprecipitation with the use of the M2 monoclonal antibody (*27*), and the protein kinase activity was measured in the immunecomplex with [γ-^{32}P]ATP and ATF2 as substrates (*19*). The product of the phosphorylation reaction was visualized after SDS-PAGE by autoradiography.

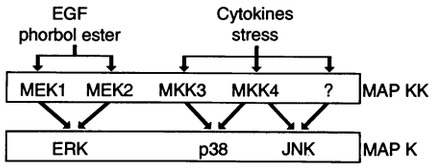

Fig. 6. MEK and MKK isoforms define independent MAP kinase signal transduction pathways. The ERK, p38, and JNK signal transduction pathways are illustrated schematically. MEK1 and MEK2 are activators of the ERK subgroup of MAP kinase. MKK3 and MKK4 are activators of p38 MAP kinase. MKK4 is identified as an activator of both the p38 and JNK subgroups of MAP kinase. A distinct MAP kinase kinase isoform (question mark) that is specific for the JNK group of MAP kinases remains to be established.

However, MKK4 may be a specific activator of JNK when expressed at physiological levels in vivo because cotransfection assays demonstrate that MEKK1 (*28*) causes marked activation of JNK but not of p38 (*12*). The molecular cloning of a murine homolog of human MKK4 (SEK1) was recently reported by Sanchez *et al.* (*29*).

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18. Single-letter 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.
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21. Protein kinase assays were done in kinase buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (pH 7.4), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM orthovanadate]. The assays were initiated by the addition of 1 μg of substrate proteins and 50 μM [γ-^{32}P]ATP (adenosine triphosphate) (10 Ci/mmol) in a final volume of 25 μl. The reactions were terminated after 30 min at 25°C by addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after SDS–polyacrylamide gel electrophoresis (PAGE) by autoradiography. Phosphoaminoacid analysis was done by partial acid hydrolysis and thin-layer electrophoresis (*4*).
22. We designed the primers TTYTAYGGNGCNTTYTTYATHGA and ATBCTYTCNGGNGCCATKTA (*18*) on the basis of the sequence of PBS2 (*17*). These primers were used in a PCR reaction with human brain mRNA as a template. Two sequences that encoded fragments of PBS2-related protein kinases were identified. Similar sequences have been identified in *Xenopus laevis* [B. M. Yashar, C. Kelly, K. Lee, B. Errede, L. I. Zon, *Mol. Cell. Biol.* **13**, 5738 (1993)]. Full-length human cDNA clones were isolated by screening of a human fetal brain library (*4*). The cDNA clones were examined by sequencing with an Applied Biosystems model 373A machine. The largest clones obtained for MKK3 [2030 base pairs (bp)] and MKK4 (3576 bp) contained the entire coding region of these protein kinases.
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25. Northern blot analysis was done with polyadenylated [poly(A)$^+$] mRNA (2 μg) isolated from different human tissues. The mRNA was fractionated by denaturing agarose gel electrophoresis and was transferred to a nylon membrane. The blot was probed with the

MKK3 and MKK4 cDNAs labeled by random priming with [α-^{32}P]dATP (deoxyadenosine triphosphate) (Amersham International PLC).
26. GST-ATF2, GST–c-Jun, GST-JNK1, and GST-ERK2 have been described (*4*, *19*, *20*). GST-p38 MAP kinase was prepared from the expression vector pGSTag [H. Dressler *et al.*, *Biotechniques* **13**, 866 (1992)] and a PCR fragment containing the coding region of the p38 MAP kinase cDNA. GST-MKK3 and GST-MKK4 were prepared with pGEX-3X (Pharmacia–LKB Biotechnology) and PCR fragments containing the coding region of the MKK3 and MKK4 cDNAs. The GST fusion proteins were purified by affinity chromatography with the use of GSH-agarose [S. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. The expression vectors pCMV-Flag-JNK1 and pCMV-MEK1 have been described (*4, 20*). The plasmid pCMV-Flag-p38 MAP kinase was prepared with the expression vector pCMV5 [S. Andersson, D. L. Davis, H. Dahlbäck, H. Jörnvall, D. W. Russell, *J. Biol. Chem.* **264**, 8222 (1989)] and the p38 MAP kinase cDNA. The expression vectors for MKK3 and MKK4 were prepared by subcloning of the cDNAs into the polylinker of pCDNA3 (Invitrogen). The Flag epitope (-Asp-Tyr-Lys-Asp–Asp-Asp-Aps-Lys-; Immunex, Seattle, WA) was inserted between codons 1 and 2 of the kinases by insertional overlapping PCR [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)]. A similar PCR procedure was used to replace Thr^{180} and Tyr^{182} of p38 MAP kinase, and Thr^{183} and Tyr^{185} of JNK1, with Ala and Phe, respectively. The sequence of all plasmids was confirmed by automated sequencing on an Applied Biosystems model 373A machine.
27. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (5%) (Gibco-BRL). The cells were transfected with the lipofectamine reagent according to the manufacturer's recommendations (Gibco-BRL) and treated with UV radiation or EGF as described (*4*). The cells were solubilized with lysis buffer [20 mM tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml)] and centrifuged at 100,000*g* for 15 min at 4°C. The epitope-tagged protein kinases were immunoprecipitated by incubation for 1 hour at 4°C with the M2 antibody (IBI-Kodak) bound to protein G–Sepharose (Pharmacia–LKB Biotechnology). The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (*21*).
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30. We acknowledge the excellent secretarial assistance of M. Shepard. R.J.D. is an Investigator at the Howard Hughes Medical Institute. Supported by NIH grants CA58396, GM37696, and AI15136.

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Association Between X-Linked Mixed Deafness and Mutations in the POU Domain Gene *POU3F4*

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Deafness with fixation of the stapes (DFN3) is the most frequent X-linked form of hearing impairment. The underlying gene has been localized to a 500-kilobase segment of the Xq21 band. Here, it is reported that a candidate gene for this disorder, *Brain 4* (*POU3F4*), which encodes a transcription factor with a POU domain, maps to the same interval. In five unrelated patients with DFN3 but not in 50 normal controls, small mutations were found that result in truncation of the predicted protein or in nonconservative amino acid substitutions. These findings indicate that *POU3F4* mutations are a molecular cause of DFN3.

Severe, inherited childhood deafness occurs in about 1 out of 1000 births and presents a serious worldwide public health problem (*1*). In 70% of these cases, deafness is not associated with other clinically recognizable features (*2*). To date, genes for nonsyndromic sensorineural deafness have been mapped to five different autosomes (*3*), but none of these has been isolated yet. The most frequent cause of X-linked hearing impairment, X-linked mixed deafness (DFN3) (McKusick catalog number 304400), is characterized by a conductive hearing loss that results from stapes fixation and progressive sensorineural deafness (*4*, *5*). However, a profound sensorineural deafness sometimes masks the conductive element (*6*). Computerized tomography (CT) studies in people with DFN3 demonstrated an abnormal dilatation of the internal acoustic canal (IAC) as well as an abnormally wide communication between the IAC and the inner ear compartment (*7*). As a result, there is an increased perilymphatic pressure that is thought to underlie the observed "gusher" during the opening of the stapes footplate. The gene underlying DFN3 has been mapped to Xq21 by linkage analysis (*8*, *9*) and through molecular characterization of large and submicroscopic deletions (*10*, *11*). Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and an 850-kb cosmid contig was constructed (*12*). This enabled us to identify and characterize two additional microdeletions, as well as a 150-kb duplication in patients with DFN3, and to assign the gene underlying DFN3 to a 500-kb interval of Xq21.1 (*12*, *13*) (Fig. 1B).

Recently, the gene *Brain 4* (*Pou3f4*), which codes for a transcription factor, was mapped between the proteolipid protein locus *Plp* and the *DXMit6* marker near the phosphoglycerate kinase 1 (*Pgk1*) gene on the murine X chromosome (*14*). The chromosomal region between *Pgk1* and *Plp* is evolutionarily conserved between humans and mice, which suggests that the human *POU3F4* gene is located in the Xq13-q22

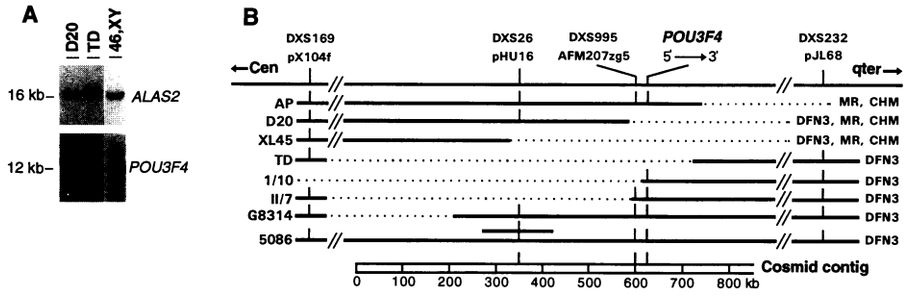

Fig. 1. Localization of the human *POU3F4* gene. (**A**) Southern blot analysis with the use of a mouse *Pou3f4* probe and a control probe, δ-aminolevulinate synthetase (*ALAS2*), with genomic DNAs from a control male, D20, and TD (*16*). Patient D20 has choroideremia (CHM), mental retardation (MR), and X-linked mixed deafness (DFN3); patient TD shows evidence of DFN3 (*12*). (**B**) Physical map of the DFN3 critical region. All patients with deletions have been described elsewhere (*12*). Patient 5086 carries a 150-kb duplication spanning DXS26. The extent of the cosmid contig is given at the bottom. *POU3F4* is located on cosmids IC2 (ICRFc104L0131) and IC3 (ICRFc104B1939), approximately 20 kb distal to DXS995 (*12*). Restriction mapping of the cosmid containing the *POU3F4* gene indicated that the *POU3F4* gene is oriented with its 5′ end toward the centromere.

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