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Genetics

Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein

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ABSTRACT **We describe Mxi2, a human protein that interacts with Max protein, the heterodimeric partner of the Myc oncoprotein. Mxi2 encodes a 297-residue protein whose sequence indicates that it is related to extracellular signal-regulated kinases (ERK protein kinases). Mxi2 in yeast interacts with Max and with the C terminus of c-Myc. Mxi2 phosphorylates Max both *in vitro* and *in vivo*. The Mxi2 putative substrate recognition region has sequence similarity to the helix–loop–helix region in Max and c-Myc, suggesting that substrate recognition might be mediated via this motif. Phosphorylation by Mxi2 may affect the ability of Max to oligomerize with itself and its partners, bind DNA, or regulate gene expression.**

Proteins of the Myc family function in the proliferation, differentiation, and oncogenic transformation of higher cells (1, 2). c-Myc, the prototype, requires for its action Max, a protein with which it forms heterodimers via a helix–loop–helix (HLH)–leucine zipper motif (3). Myc/Max heterodimers bind specific sites on DNA and activate transcription, whereas Max homodimers are transcriptionally inactive (4–6). We and others (7, 8) have described two proteins, Mxi1 (for Max-interactor 1) and Mad (for Max-dimerization), that bind specifically to Max protein. Transcription of Mxi1 is induced during differentiation of monocytes into macrophages (7, 9). Both Mxi1 and Mad proteins can suppress the transformation activity of Myc protein (10). They may achieve this either by competing with Myc for Max binding or by forming complexes with Max that bind the same DNA sites as Myc/Max complexes but repress transcription (7, 9). The *MXI1* gene maps to human chromosome 10 band q25 (11), a segment associated with translocations and deletions in a variety of naturally occurring human tumors, including prostate cancers (12), (A.S.Z., L.F., and P. Bao, unpublished data). Here we describe a second Max interacting protein, Mxi2, an ERK (for extracellular signal-regulated kinase)-related protein kinase, which can phosphorylate Max protein and may modulate its activity.‖

MATERIALS AND METHODS

Isolation of Mxi2. An interactor hunt from a HeLa library with strain EGY48/pJK103 that contained a LexA–Max bait was conducted as described (7). One transformant, as judged by transcription phenotypes, contained a fused protein that interacted with Max more weakly than fusion proteins that contained Mxi1 and c-Myc. Plasmid pJG-29 was rescued from these cells into *Escherichia coli* K12 strain KC8 by electroporation (Massachusetts General Hospital Molecular Biology Gopher Server, http://xanadu.mgh.harvard.edu). Both strands of the entire *Eco*RI–*Xho* I insert were sequenced.

Mxi2 Expression in Mammalian Cells and Kinase Activity. The following primers were used to PCR amplify the Mxi2 coding sequence from pJG-29: 5′-AGCTAGTCTAGAGATGTCTCAGGAGAGGCCCAC-3′ and 5′-AAGGAAAAAAGCGGCCGCAATTTATATCATAACCAATTC-3′. The PCR product was cut with *Not* I and *Xba* I and introduced into the pMT3 expression vector (13) to create pMT3–Mxi2, which directs the synthesis of an Mxi2 protein that contains a hemagglutinin (HA) epitope tag at its N terminus. pMT3–Mxi2 was transfected into 70–80% subconfluent COS cells with Lipofectamine (GIBCO/BRL) according to the manufacturer's instructions. Sixteen micrograms of plasmid was used per 100-mm dish. Two days after transfection, the cells were split 1:3 and placed into low serum (0.1% bovine calf serum/Dulbecco's modified Eagle's medium). After 48 hr, cells were placed into Dulbecco's modified Eagle's medium that contained either 20% bovine calf serum (serum stimulated) or 0.1% bovine calf serum (serum deprived). Protein extracts from transfected COS cells were prepared essentially as described (13), and Mxi2–HA protein was purified by using the 12CA5 anti-HA antibody and Sepharose coupled to recombinant *Staphylococcus aureus* protein G (Pharmacia). The kinase activity of Mxi2 was monitored by mixing this protein with either bacterially produced histidine-tagged Max protein (7) or myelin basic protein and [γ-^{32}P]ATP as described (13).

***In Vivo* Labeling.** COS cells were transfected with pMT3–Max or pMT3–Mxi2, which encode HA-tagged constructs of the cognate proteins. Cells transfected with Max construct were labeled with ^{32}P-labeled H_3PO_4 (1 mCi/ml; 1 Ci = 37 GBq) for 2 hr. Half of the cells were treated with arsenite as described (14). HA–Max or HA–Mxi2 were immunoprecipitated with anti-HA antibody, and *in vitro* phosphorylation of purified, bacterially expressed hexahistidine–Max was done by using described techniques (15).

Northern Blot Analysis. The following Mxi2-specific primers were used to amplify a DNA probe: 5′-^{881}TGGGTAAGTTGACCATATATC^{902}-3′ and 5′-^{992}CAACTAATGGTACTTTATTT^{973}-3′. The 111-bp DNA fragment was ^{32}P-labeled by using the same PCR primers in a modified Klenow reaction. For the p38 the following primers were used to PCR a p38-specific fragment (22): 5′-^{1200}TGGCTGTCGACTTGCTGGAGAAGAT^{1225}-3′ and 5′-^{1529}GGCACTTGAATAATATTTGGAGAGT^{1504}-3′. The 329-bp fragment was ^{32}P-labeled by using random primers (Prime-It II, Stratagene). A human multiple-tissue Northern blot (Clontech) was probed for Mxi2 and p38 kinase expression as described (7).

RESULTS

We used the interaction trap (17), a yeast two-hybrid system (18), to isolate Mxi2, a protein that interacted specifically with

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Abbreviations: HA, hemagglutinin; HLH, helix–loop–helix; ERK, extracellular signal-regulated kinase.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U19775).

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GAATTCGGCACGAGGCGCCTTCTTGCCCGGCGGCTGCTGGAAAATGTCTCAGGAGAGGCC
                                              M  S  Q  E  R  P
CACGTTCTACCGGCAGGAGCTGAACAAGACAATCTGGGAGGTGCCCGAGCGTTACCAGAA
  T  F  Y  R  Q  E  L  N  K  T  I  W  E  V  P  E  R  Y  Q  N

CCTGTCTCCAGTGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAAC
  L  S  P  V  G  S  G  A  Y  G  S  V  C  A  A  F  D  T  K  T

GGGGTTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTCAGTCCATCATTCATGCGAA
  G  L  R  V  A  V  K  K  L  S  R  P  F  Q  S  I  I  H  A  K

AAGAACCTACAGAGAACTGCGGTTACTTAAACATATGAAACATGAAAATGTGATTGGTCT
  R  T  Y  R  E  L  R  L  L  K  H  M  K  H  E  N  V  I  G  L

GTTGGACGTTTTTACACCTGCAAGGTCTCTGGAGGAATTCAATGATGTGTATCTGGTGAC
  L  D  V  F  T  P  A  R  S  L  E  E  F  N  D  V  Y  L  V  T

CCATCTCATGGGGGCAGATCTGAACAACATTGTGAAATGTCAGAAGCTTACAGATGACCA
  H  L  M  G  A  D  L  N  N  I  V  K  C  Q  K  L  T  D  D  H

TGTTCAGTTCCTTATCTACCAAATTCTCCGAGGTCTAAAGTATATACATTCAGCTGACAT
  V  Q  F  L  I  Y  Q  I  L  R  G  L  K  Y  I  H  S  A  D  I

AATTCACAGGGACCTAAAACCTAGTAATCTAGCTGTGAATGAAGACTGTGAGCTGAAGAT
  I  H  R  D  L  K  P  S  N  L  A  V  N  E  D  C  E  L  K  I

TCTGGATTTTGGACTGGCTCGGCACACAGATGATGAAATGACAGGCTACGTGGCCACTAG
  L  D  F  G  L  A  R  H  T  D  D  E  M  T  G  Y  V  A  T  R

GTGGTACAGGGCTCCTGAGATCATGCTGAACTGGATGCATTACAACCAGACAGTTGATAT
  W  Y  R  A  P  E  I  M  L  N  W  M  H  Y  N  Q  T  V  D  I

TTGGTCAGTGGGATGCATAATGGCCGAGCTGTTGACTGGAAGAACATTGTTTCCTGGTAC
  W  S  V  G  C  I  M  A  E  L  L  T  G  R  T  L  F  P  G  T

AGACCATATTGATCAGTTGAAGCTCATTTTAAGACTCGTTGGAACCCCAGGGGCTGAGCT
  D  H  I  D  Q  L  K  L  I  L  R  L  V  G  T  P  G  A  E  L

TTTGAAGAAAATCTCCTCAGAGTCTGCAAGAAACTATATTCAGTCTTTGACTCAGATGCC
  L  K  K  I  S  S  E  S  A  R  N  Y  I  Q  S  L  T  Q  M  P

GAAGATGAACTTTGCGAATGTATTTATTGGTGCCAATCCCCTGGGTAAGTTGACCATATA
  K  M  N  F  A  N  V  F  I  G  A  N  P  L  G  K  L  T  I  Y

TCCTCACCTCATGGATATTGAATTGGTTATGATATAAATTGGGGATTTGAAGAAGAGTTT
  P  H  L  M  D  I  E  L  V  M  I  *

CTCCTTTTGACCAAATAAAGTACCATTAGTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAA
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FIG. 1. Sequence of Mxi2 cDNA and protein. The sequences of the 1023-bp Mxi2 cDNA isolated in the interactor hunt and the predicted 297-residue protein are shown.

Max protein from a HeLa cDNA library (7). We isolated 7.5×10^5 colonies that contained individual members of a HeLa cDNA interaction library (17) and plated cells from these colonies on selective medium plates at a multiplicity of 10. From these cells we isolated 80 plasmids that interacted with a LexA–Max bait (7). cDNA inserts from these plasmids fell into three restriction map classes, one of which, composed of a single plasmid, contained the Mxi2 coding sequence. As judged by growth on Leu-galactose plates and blue color on 5-bromo-4-chloro-3-indolyl β-D-galactoside (dependent on the medium-strong pJK103 LexAop–LacZ reporter), Mxi2 was the third strongest interactor (after c-Myc and Mxi1), consistent with its low frequency of isolation. Mxi2 was specific for Max protein; in the interaction trap, it did not interact with LexA–Cdc2, LexA–Fus3, or LexA–Bicoid baits but interacted with a bait containing the C terminus of Myc (data not shown).

Fig. 1 shows the sequence of Mxi2. The 1023-bp cDNA encodes a polypeptide of 297 amino acids with significant sequence similarity to the yeast *HOG1* gene product (see Fig. 2), an ERK involved in osmoregulation (20). The sequence suggests that Mxi2 is an alternatively spliced form of the recently described human p38 protein kinase (19). In human cells, p38 kinase is activated by tyrosine and threonine phosphorylation in response to lipopolysaccharide, inflammatory cytokines (tumor necrosis factor, interleukin 1), and environmental stress (19, 20). Recently another protein kinase, CSPB1, that is involved in lipopolysaccharide-induced cytokine biosynthesis and is the target of several antiinflammatory drugs, has been isolated; an alternative spliced form, CSBP2, was shown to be p38 kinase (22).

Mxi2 protein is identical in sequence to p38 kinase (19, 22) from amino acids 1–280; Mxi2 contains 17 C-terminal amino acids not found in p38 kinase, and p38 kinase contains 80 residues not found in Mxi2 protein (see Fig. 2). Northern analysis of mRNA from different human tissues using probes specific for Mxi2 and p38 kinase revealed that Mxi2 protein is expressed at much lower levels than p38 kinase in all tissues tested (Fig. 3). The size of the mRNA is 4.2–4.4 kb, and the relative distribution of Mxi2 in different human tissues mirrors closely the distribution of p38 mRNA, except in heart, where the relative expression of Mxi2 is higher.

Mxi2 protein is the smallest ERK family member identified thus far. It lacks a C-terminal stretch (domain XI) (21), present in other protein kinases (Fig. 2). Interestingly, the extreme C terminus of Mxi2 (residues 283–297) shows some sequence similarity to residues 292–304 of ERK2 (**LTIYPHLM-DIELVMI** vs. **LTFNPHKR.IE.VEQ**). In ERK2 these residues lie immediately N-terminal to helices αI and αL16, which fold

```
                                       I                      II                III
Mxi2  MSQERPTFYRQELNKTIWEVPERYQNLSPVGSGAYGSVCAAFDTKTGLRVAVKKLSRPFQSIIHAKRTYRELRLLK
p38   ----------------------------------------------------------------------------
Hog1   MTTNEE-I-TQIFGTVF-ITN--ND-N---M--F-L--S-T--L-SQP--I--IMK--STAVL--------K---

              IV                    V                   VIA                VIB
      HMKHENVIGLLDVFTPARSLEEFNDVYLVTHLMGADLNNIVKCQKLTDDHVQFLIYQILRGLKYIHSADIIHRDLK
      ----------------------------------------------------------------------------
      -LR---L-C-Q-I-.....-SPLE-I-F--E-Q-T--HRLLQTRP-EKQF--YFL---------V---GV------

                 VII               VIII            IX
      PSNLAVNEDCELKILDFGLARHTDDEMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHI
      -----------------------------------------------------------------------------
      ---ILI--N-D---C------IQDPQ-----S--Y--------T-QK-DVE-----A---F--MIE-KP----K--V

         X                                                            XI
      DQLKLILRLVGTPGAELLKKISSESARNYIQSLTQMPKMNFANVFIGANPLGKLTIYPHLMDIELVMI*.........
      ---------------------------------------------------AVDLLEKMLVLDSDKRITAAQALAHAY
      H-FSI-TD-L-S-PKDVINTIC--NTLKFVTS-PHRDPIP-SER-KTVE-D----------F-PK------D----P-

      ..............................................................................
      FAQYHDPDDEPVADP.YDQSFESRDLLIDEWKSLTYDEVISFVPPPLDQEEMES*.......................
      S-P----T------AKF-WH-NDA--PV-T-RVMM-S-ILD-HKIGGSDGQIDISATFDDQVAAATAAAAQAQAQAQAQ

      .....................................
      .....................................
      VQLNMAAHSHNGAGTTGNDHSDIAGGNKGQRSCSCK*
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FIG. 2. Comparison of Mxi2 protein with human p38 kinase (19) and *Saccharomyces cerevisiae* Hogl protein (20). Gaps introduced to optimize the alignment are illustrated by periods, identical residues are illustrated by dashes, and stop codons are illustrated by asterisks. Conserved subdomains in protein kinases, described in refs. 16 and 21, are indicated by Roman numerals. Protein sequences were aligned by the Genetics Computer Group (Madison, WI) PILEUP program and visual inspection.

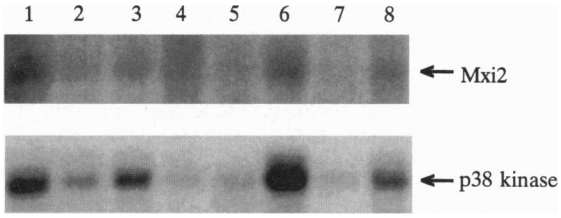

FIG. 3. Mxi2 and p38 mRNA expression in different human tissues. The Northern blot contains 2 μg of poly(A)$^+$ RNA per lane from eight different human tissues (Clontech). It was probed with an Mxi2- or a p38-specific probe, as described. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; and 8, pancreas.

along the back of the molecule (23). This portion of Mxi2 and ERK2 (but not of p38 kinase) may thus mediate interaction with a common modulatory protein.

Mxi2 isolated from COS cells transfected with a construction that directs the synthesis of epitope-tagged Mxi2 phosphorylates Max protein *in vitro* (Fig. 4). It also phosphorylates myelin basic protein, a common nonspecific substrate of many ERK family kinases (Fig. 3). Mxi2 expressed in transfected cells displays a strikingly high basal activity toward Max and is equally active at phosphorylating Max when isolated from COS cells that are serum-starved, serum-stimulated, or treated with arsenite [which increases p38 activity (19)] (Figs. 4 and 5). We studied Max phosphorylation *in vivo* in COS cells treated with arsenite. There was no detectable difference in Max phosphorylation between control cells, serum-starved cells, serum-stimulated cells, or arsenite-treated cells (Figs. 4 and 5).

Like Mxi2, p38 isolated from COS cells also phosphorylates Max *in vitro* (data not shown); however, it is possible that their relative affinities for their substrates differ; in the yeast two-hybrid system we were unable to detect interaction between a LexA–p38 kinase bait and Max protein (data not shown). Consistent with this idea is the fact that p38 kinase is expressed in the HeLa interaction cDNA library we used (L.F. and A.S.Z., unpublished work), but Mxi2 was the only kinase that we isolated.

In yeast, Mxi2 interacts with LexA–Max bait and also with a LexA–c-Myc bait that lacks the Myc–terminus but contains the C-terminal basic-HLH–leucine zipper region (7). This fact led us to search for a region in Mxi2 protein that could direct its interaction with a common region in these two proteins. The Mxi2 C terminus (residues 227–262) carries a stretch of amino acids with significant homology to the HLH of both Max and Myc proteins (Fig. 6). Alignment of this stretch of Mxi2 with ERK2 reveals that it lies in a region of ERK2 that contains helices αG, α1L14, and α2L14; residues in αG are involved in substrate recognition (23). Inspection of this portion of the Mxi2 sequence suggests that it could form two α-helices separated by a GTGPA linker; and it is thus possible that this stretch helps Mxi2 recognize Max protein by directing oligomerization with the Max HLH. It is interesting to note that CSBP1, an alternatively spliced form of p38 kinase (21), differs from p38 kinase by 25 amino acids in a region that includes the Mxi2 and Max/Myc homology, and it is plausible that this region of CSBP1 directs its interaction with a different set of proteins.

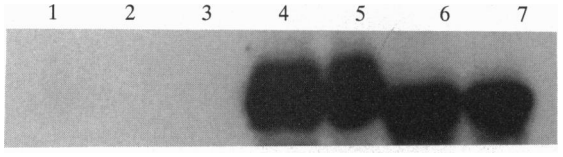

FIG. 4. Kinase activity of Mxi2 protein *in vitro*. Autoradiograph shows transfer of $^{32}PO_4$ to Max protein (lanes 4 and 5) and to myelin basic protein (lanes 6 and 7). Reactions were done as in ref. 13. Lanes: 1, 5 μg of Max and no Mxi2; 2, Mxi2 from the low-serum COS cells and no Max protein; 3, Mxi2 from serum-stimulated COS cells and no Max protein; 4, Mxi2 from the low-serum COS cells and 5 μg of Max protein; 5, Mxi2 from serum-stimulated COS cells and 5 μg of Max protein; 6, Mxi2 from the low-serum COS cells and 5 μg of myelin basic protein; 7, Mxi2 from serum-stimulated COS cells and 5 μg of myelin basic protein.

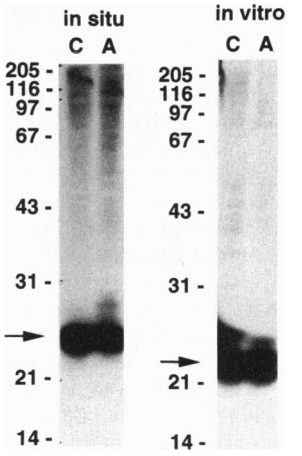

FIG. 5. Max phosphorylation by Mxi2 *in vitro* or *in vivo* with and without arsenite. SDS/PAGE of HA–Max phosphorylated *in situ* (*Left*) or bacterially expressed, purified hexahistidine–Max phosphorylated by immunoprecipitated HA–Mxi2 *in vitro* (*Right*). Arrows show Max protein. C, control cells; A, arsenite-treated cells.

DISCUSSION

Phosphorylation of Max protein by casein kinase II inhibits the DNA-binding activity of Max homodimers but not of Myc/Max heterodimers (26). What might be the effect of Max protein phosphorylation by Mxi2? Because Myc protein expression in the absence of growth factors leads to p53-dependent apoptosis (27, 28) and because Max protein may well contribute to the function of Myc protein in this process, Mxi2 might activate apoptosis by phosphorylating Max, and it is interesting in this regard that cells transfected with Mxi2 expression constructs show substantial cell death (L.F. and A.S.Z., unpublished work). Max protein does not contain the Pro-Xaa-(Ser/Thr)-Pro or Ser/Thr-Pro motifs recognized by classical ERKs (29), raising the possibility that Mxi2 may have a different substrate-recognition site from the other ERKs. Our data are consistent with the hypothesis that Mxi2 has a distinctive proline requirement: Ser/Thr-Xaa-Pro. Indeed, Max protein has two such sites, S40 (Ser-Val-Pro) and S135 (Ser-Glu-Pro). S40 is located in helix I of the Max HLH motif. Because Max can dimerize with itself as well as form heterodimers with Myc, Mxi1, and Mad proteins, phosphorylation at S40 might change its preference for particular oligomeric partners. Alternatively, because the Max protein basic region and helix I form a continuous α-helix that interacts with DNA

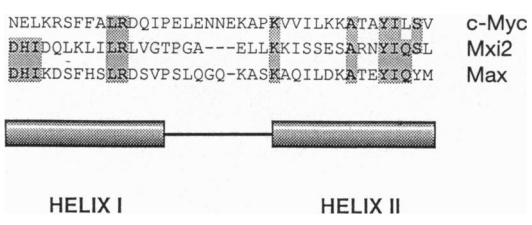

FIG. 6. Myc and Max HLH sequence and the similarity to a stretch of amino acids in Mxi2. Residues 227–262 of Mxi2, 386–406 of human c-Myc (25), and 28–65 of human Max (3) are shown. Gaps introduced to optimize the alignment are illustrated with dashes; identical residues in corresponding positions are shown by stippling.

(30), phosphorylation of this residue could also affect the ability of Max protein to bind its sites.

These experiments define Mxi2 as another member of the burgeoning subfamily of the ERK protein kinases, whose members transduce extracellular signals to the nucleus. These proteins include p44 mitogen-activated protein kinase kinase (ERK1) and p42 mitogen-activated protein kinase kinase (ERK2), which phosphorylate transcription factors Elk1/p62^{TCF} (24, 31), and the stress activated protein (SAP) kinase subfamily of ERKs, which phosphorylate Jun and transcription factor ATF2 (15, 32, 33). By analogy with other ERKs, we view it as likely that Mxi2 will be activated by a mitogen-activated protein kinase/ERK kinase (MEK), in response to some stress signal(s), causing it to phosphorylate Max protein and perhaps other basic-HLH proteins. Recently, our understanding of mammalian stress signaling has progressed rapidly. Two human mitogen-activated protein kinase kinases, MKK3 and SEK1 (MKK4) (34, 35), have been cloned. MKK3 kinase can specifically activate p38 kinase, whereas SEK1 kinase activates the stress-activated protein kinases and possibly p38 kinase as well (34, 35); SEK1 kinase, in turn, is a substrate of MEK kinase 1 (35). Because of the sequence homology, we expect the MEKS to activate Mxi2, supporting the hypothesis that Mxi2 protein is also part of a stress-activated signaling pathway. We do not know, as yet, how Mxi2 protein becomes activated or the kind of signals it mediates. Phosphorylation of Max protein by activated Mxi2 should result in changes in Max activity, which will modulate the transcription of target genes in response to stress.

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