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BRIEF COMMUNICATION

Astrocytes express Mxi2, a splice isoform of p38MAPK

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Abstract Mitogen-activated protein kinases (MAPKs) are a superfamily of cytoplasmic serine/threonine kinases that transduce many types of extracellular stimuli into cellular responses. p38MAPK is a member of this family with its active form in a diphosphorylated state (p38MAPKdiP). Two strong anti-p38MAPKdiP immunoreactive bands (apparent molecular weight 38 and 34 kDa) were detected by Western blotting in cultured astrocytes. Using a specific antibody and employing immunoprecipitation procedures and SELDI-TOF analysis, the 34 kDa band was found to correspond to Mxi2, a splice variant of p38MAPK; cultured astrocytes therefore express Mxi2. Separate protein extractions of different subcellular fractions, and fluorescent immunovisualisation employing confocal microscopy, showed Mxi2 to have a non-nuclear, cytosolic distribution in the studied cells. ERK1/2, protein whose intracellular distribution is influenced by Mxi2, showed the same cytoplasmic pattern than Mxi2.

Keywords p38MAPK · Mxi2 · ERK1/2 · Astrocyte · SELDI-TOF · Immunoprecipitation · Confocal microscopy · RT-PCR

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Mitogen-activated protein kinases (MAPKs) are a superfamily of ubiquitous cytoplasmic serine/threonine kinases that play an important role in transducing many types of extracellular stimuli into cellular responses. At least four groups of MAPKs have been identified in mammalian cells: extracellular signal-regulated kinases (ERKs, p42/p44 MAPK), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK1), ERK/big MAP kinase (ERK/BMK), and the p38 group of protein kinases. To date, four members of the p38 group of MAPKs have been characterized: p38α (or CSBP2, RK), p38β, p38δ (or SAPK4) and p38γ (or ERK6, SAPK3) (New and Han 1998; Roux and Blenis 2004; Sudo et al. 2002; Zarubin and Han 2005). In addition to p38α itself, CSBP1 (Lee et al. 1994), Mxi2 (Zervos et al. 1995) and Exip (Sudo et al. 2002) have been identified as splice variants.

In our previous studies (Bodega et al. 2006, 2007a, b) on the involvement of p38MAPK in the astroglial response to ammonia (the main neurotoxic agent in hepatic encephalopathy), we had observed two different p38MAPKdiP (the active diphosphorylated form of p38MAPK) immunoreactive bands; the heavier band with a molecular weight of 38–40 kDa, the other 32–34 kDa. To ascertain the nature of this last band was the goal of the present work.

Primary cultures of astroglial cells were obtained using cells from the cerebral hemispheres of P0-P1 Wistar rats.

Cells were cultured in 25 cm^2 flasks containing DMEM medium (Gibco), 12% fetal bovine serum (FBS) (Gibco) and an antibiotic/antimycotic solution (Gibco), at 37°C in a 5% CO_2 atmosphere. Five days after confluence, total proteins were extracted using CelLytic buffer (Sigma), including 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktails I and II (Sigma), and following the instructions provided by the manufacturer. The total protein concentration was determined using the Bradford microassay (Bio-Rad). After electrophoresis (12% polyacrylamide gels, 8 μg of total protein per lane), the proteins were transferred to Immobilon-P membranes (Millipore); these were then washed in Tris buffer (0.05 M, 7.6 pH) for 5 min and incubated (30 min, room temperature) with agitation in Tris buffer with 5% (w/v) non-fat powdered milk (blotto buffer). Without further washing, the membranes were incubated overnight at 4°C with the primary antibody (anti-p38MAPdiP or anti-Mxi2). After three 5 min washes, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody for 2 h at room temperature. The peroxidase reaction was performed in a solution of 0.05 M Tris buffer containing 0.1% 3,3-diaminobenzidine (Sigma) and 0.05% H_2O_2. The monoclonal anti-p38MAPdiP (clone p38-TY) used was purchased from Sigma; the rabbit polyclonal anti-Mxi2 antibody (generated by injecting rabbits with a peptide from the Mxi2-specific 17-amino-acid C-terminus (Esparís-Ogando et al. 2002)) used was kindly provided by Dr. Piero Crespo.

Western blotting detected two p38MAPKdiP immunoreactive bands, one with a molecular weight of 38–40 kDa, the other 32–34 kDa. The position of the anti-Mxi2 immunoreactive band in the Western blot was the same as that shown by the lower molecular weight immunoreactive band detected using anti-p38MAPKdiP (Fig. 1a). This suggests that this latter band might represent Mxi2. Moreover, MDCK cells express Mxi2 at high levels (Casar et al. 2007), and the anti-Mxi2 immunoreactive band was observed in a similar position in astroglial cells and in MDCK cell lysates used as positive controls (Fig. 1b).

To confirm the identity of this band, double immunoprecipitation with magnetic beads was performed. Dynabeads Protein G (Invitrogen) and the DynaMag-2 apparatus (Invitrogen) were used for immunoprecipitation experiments. The monoclonal anti-p38MAPKdiP or the rabbit polyclonal anti-Mxi2 antibodies- were coupled to Dynabeads Protein G to form a Dynabeads Protein G-antibody complex. Washing, IgG capture, immunoprecipitation and elution procedures were performed following the Dynabead protein G manufacturer's instructions. Samples were first subjected to immunoprecipitation using anti-p38MAPKdiP antibody. The proteins of the eluted material (that should contain activated p38MAPK and Mxi2) were precipitated and solubilized in PBS (0.1 M, pH 7.2). The resulting sample was then subjected to a second immunoprecipitation process using anti-Mxi2 as the primary antibody. In this case both the supernatant (which should contain only p38MAPK) and the material remaining adhered to the beads (which should contain only Mxi2) were analysed. SELDI-TOF mass spectrometry recorded a band of 27.8 kDa for the supernatant (Fig. 2a) and a band of 22.8 kDa for the eluted material (Fig. 2b, c). Clearly, the molecular weight of these proteins is 10–11 kDa less than those expected for p38MAPK and Mxi2. However, a band with a molecular weight of 10.8 kDa (Fig. 2c) was also found; this might represent a fragment in the case of both proteins. If the molecular weight of the latter is included, the final molecular weight of the eluted proteins is 38.6 and 33.6 kDa, respectively. Therefore, the possibility that some plasma protease might cleave Mxi2 to give rise to a fragment of approximately 10.7 kDa should be contemplated, especially when the antibody to Mxi2 is not pure and might contain plasma proteins (as confirmed in the present work by the presence of albumin; see Fig. 2 caption). A sequence of 20 amino acids (from positions 84 to 104) was analysed using PeptideCutter software (ExPASy Proteomics Server of Swiss Institute of Bioinformatics), a tool that predicts the potential protease cleavage sites in a protein sequence; this sequence was found cleavable by at least 14 proteolytic enzymes.

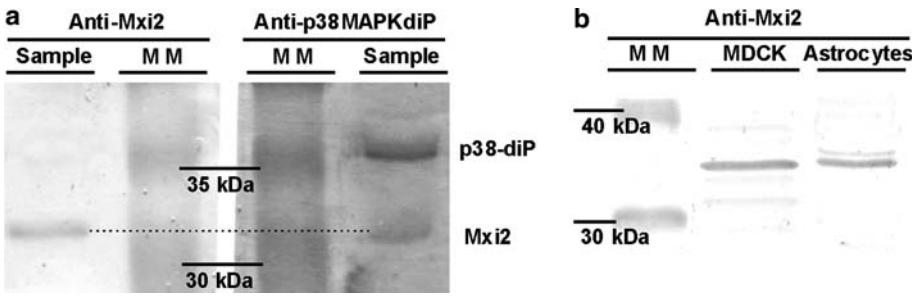

Fig. 1 **a** Western blots immunostained with anti-Mxi2 and anti-p38MAPKdiP. Note that the lower immunoreactive band detected using anti-p38MAPKdiP is located in the same position as the anti-Mxi2 immunoreactive band. **b** Western blots of MDCK cell lysate and astroglial cells; note the similar position of the anti-Mxi2 immunoreactive bands. *MM* molecular markers

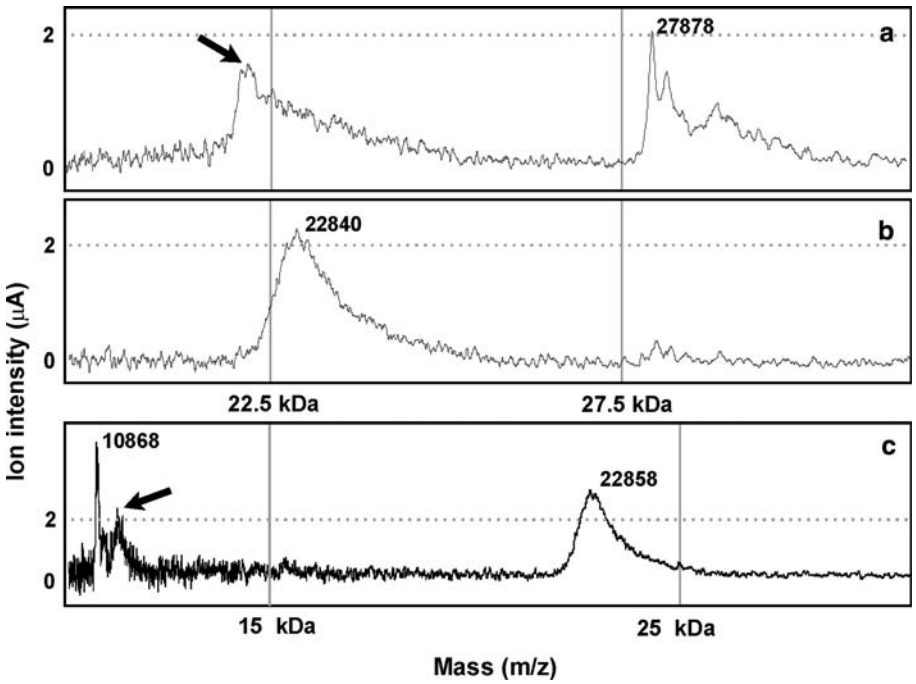

Fig. 2 Results of SELDI-TOF mass spectrometry following immunoprecipitation. **a** Plot obtained from the analysis of the supernatant after the second immunoprecipitation process. The *arrow* indicates a 22.2 kDa band identified by the software provided with the SELDI-TOF mass spectrometer as derived from serum albumin in a triple protonated state. **b**, **c** Plots obtained from the analysis of the eluted material after the second immunoprecipitation process. A fragment with a molecular weight of 10.8 kDa is clearly observed in plot "**c**"; the *arrow* indicates an 11.3 kDa band identified by the software as a quadruple protonated form of serum albumin. The proteins eluted in the immunoprecipitation procedures were deposited on a H50 protein chip and allowed to air-dry. The energy-absorbing matrix of 1 μl of 50% SPA matrix solution (saturated sinapinic acid in 50% v/v aqueous acetonitrile containing 0.5% v/v trifluoroacetic acid) was added to each spot. Calibration was performed in parallel using protein standards for M_r from 8,564 (ubiquitin) to 14,7300 (bovine IgG). Mass spectra were acquired in the positive-ion mode using a Bio-Rad PCS-4000 linear laser desorption/ionization-time-of-flight mass spectrometer equipped with time-lag focusing (SELDI-TOF) capability. TOF mass spectra were generated in the 1–100 kDa range by averaging 260 laser shots (laser intensity 4000, focused at m/z 30 kDa). Following mass calibration, total ion current normalization and baseline subtraction, p38MPK and Mxi-2 peaks were manually labelled and intensities (peak heights) extracted using Bio-Rad Express-Datamanager 3.0.6 software (Bio-Rad Laboratories)

In order to incorporate data that support the expression of Mxi2 in rat cultured astrocytes we have also tried to detect Mxi2 mRNA. Total RNA of confluent cultured astrocytes was isolated, and its integrity analysed by electrophoresis (2100 Bioanalyzer, Agilent Technologies). cDNA was obtained from 2 μg of RNA using 500 ng of oligo dT, 1× first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 40U RNaseOut, and Superscript III (Invitrogen) as reverse transcriptase, incubating at 46°C for 2 h and inactivating reverse transcripase at 70°C for 10 min. MAPK14 was amplified from rat astrocytes by PCR using the following oligonucleotides: MAPK14_F (GAA CTT CGC AAA TGT ATT TAT TGG T) and MAPK14_R (CGA GTC CAA AAC CAG CAT CT). Given that there is not a known sequence for rat Mxi2 we tried to amplify it from rat astrocytes using the two primers utilized by Zervos et al. (1995) to amplify human Mxi2. The primers were MXI2_F (TGG GTA AGT TGA CCA TAT ATC) and MXI2_R (CAA CTA ATG GTA CTT TAT TT) and both correspond to the Mxi2 specific region of 17 aminoacids. PCR was developed using a StepOnePlus real-time and Fast SYBR® Green master Mix (Applied Biosystems) following manufacturer's instructions for presence/absence experiments. An initial incubation at 60°C for 30 s and 95°C for 10 min was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. StepOne Software v2.1 (Applied Biosystems) was used to analyze the presence/absence of Mapk14 and Mxi2. Mapk14 was detected by real-time PCR, but Mxi2 failed to be detected by this method. In order to explain the negative result on Mxi2 mRNA expression it is necessary take into account that primers were obtained from the Mxi2 human sequence; at the moment rat sequence is not known. Nevertheless, we decide to use the human sequence because of the high homology of the human and rat protein. In this sense, we are now trying to ascertain the sequence of rat Mxi2.

The function of Mxi2 is not clear: it has been shown that it modulates Max activity, that it interacts with the C-terminus of Myc (Zervos et al. 1995), and it has also been

implicated in the nucleocytoplasmic distribution of ERK1/2 (Casar et al. 2007). Mxi2 has not been detected in HeLa or other cell lineages (Faccio et al. 2000); however, Mxi2 was reported predominantly nuclear in the COS7 and MDCK cell lines, especially when the cells were growing or under acute mitogenic stimulation (Casar et al. 2007). To analyse the Mix2 subcellular distribution, the soluble cytosolic proteins, membrane/organelle protein and the nuclear protein fractions were extracted separately using buffers I, II and III, (respectively) of the ProteoExtract kit (Calbiochem); moreover, the subcellular distribution of Mxi2 was also studied by means of confocal microscopy. The subcellular fractionation of protein extraction showed that Mxi2 was mainly located in the soluble cytosolic fraction and, to a lesser extent, in the membrane/organelle fraction. Mxi2 was not detected in the nuclear fraction (Fig. 3). These results were confirmed by the confocal microscopy analysis (Fig. 4). Neither Mxi2 nor p38MAPKdiP showed a nuclear location; the immunofluorescent signal always showed a diffuse cytosolic distribution. However a strong signal with a vesicular distribution was also observed in a few cells. The distribution of p38MAPKdiP and Mxi2 was similar, although p38MAPKdiP showed a more intense vesicular pattern. We have also analyzed the nucleocytoplasmic distribution of extracellular regulated kinases 1/2 (ERK1/2) by confocal microscopy. ERK1/2 showed a cytoplasmic location in confluent cultured astrocytes (Fig. 4). The identical pattern of nucleocytoplasmic distribution showed by Mxi2 and ERK1/2 supports our data on Mxi2 expression in cultured astrocytes because it has been demonstrated that Mxi2 influences ERK1/2 intracellular distribution (Casar et al. 2007).

Differential expression has been described for the different p38MAPK subfamilies in different human tissues and cell lines (Jiang et al. 1997), and differential expression and activation has been observed in inflammatory cell

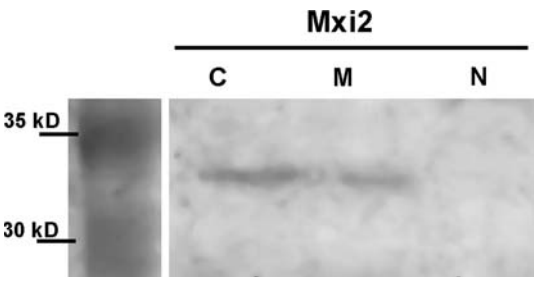

Fig. 3 Subcellular localization of Mxi-2. Note that the strongest immunoreactivity appears in the cytosolic fraction (*C*) and is absent from the nuclear fraction (*N*). *M* membrane/organelle fraction

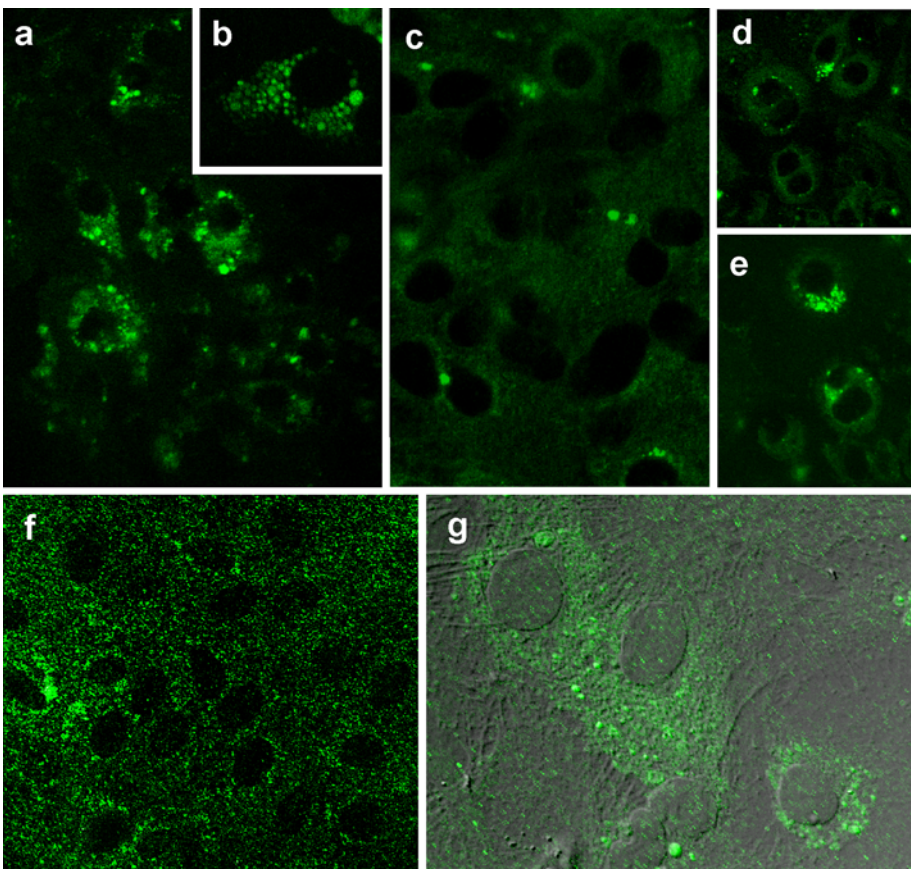

Fig. 4 p38MAPKdiP (**a**, **b**), Mxi2 (**c**, **d**, **e**) and ERK1/2 (**f**, **g**) immunolocalization by confocal microscopy. Nuclei appear as spherical or elliptic holes with no immunofluorescent signal, as is demonstrated in "g" picture, where a single confocal image overlaps with a DIC image of the same field. Cells were fixed in methanol-acetone (50–50%) for 2 min, washed in PBS and blocked in the appropriate serum (1/30 diluted) for 30 min. They were then incubated with primary antibody (anti-p38MAPKdiP, anti-Mxi2 or anti-ERK1/2 (Santa Cruz)) for 24 h at 4°C. After three 5 min washes in PBS the cells were incubated for 2 h at room temperature with the appropriate secondary antibody conjugated to fluorescein isothiocyanate. After washing, coverslips were mounted in VectaShield. A Leica TCS-SL (488 nm excitation wavelength) argon laser confocal microscope was used to detect fluorescent signals

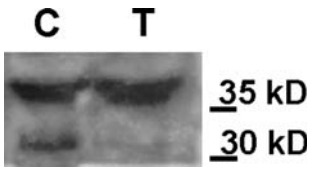

Fig. 5 Western blots immunostained with anti-p38MAPKdiP. The ammonia-treated astrocytes (T) were grown in 2.5% FBS and exposed to 3 mM NH_4Cl for 3 days. *C* control sample. Note that the lower immunoreactive band, corresponding to Mxi2, is not present in treated sample; however, the upper immunoreactive band, corresponding to p38MAPK is bigger. Protein extraction and electrophoresis were carried out as described in the third paragraph

lineages (Hale et al. 1999). Using Western blotting, Faccio et al. (2000) reported Mxi2 expression was exclusive to the kidney in mice (it was not detected in the brain, heart, liver, lung, spleen or skeletal muscle); further immunohistochemical studies localized Mxi2 to the cells of the distal tubules, where it showed a cytoplasmic staining distribution. Given the existence of the two immunopositive bands detected in the present work, and their molecular weights, the present astrocytes only seem to have two active forms of p38: p38MAPK and its splice variant Mxi2. No immunoreactive bands heavier than 38 kDa (the position occupied by p38MPK) were seen, and the β, δ and γ p38 family members all have molecular weights of over 40 kDa (New and Han 1998). All in all, it would seem that Mxi2 expression depends on cell type and cell activation status.

The biological significance of the Mxi2 expression in astrocytes is unknown; however, the expressions of Mxi2 and p38MAPK were modified to different extents in cultured astrocytes subjected to hyperammonemic conditions (Fig. 5). Because of this, we are now analysing the possibility that Mxi2 could have a specific role in osmotic stress and/or ammonia-related disorders.

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