Distinct carboxy-termini confer divergent characteristics to the mitogen-activated protein kinase p38α and its splice isoform Mxi2

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Received 1 May 2000

Edited by Shmuel Shaltiel

Abstract The p38 family of mitogen-activated protein kinases is composed of several isoforms. Mxi2 is a splicing variant of p38α that harbors a unique carboxy-terminus. Here we show that this sole divergence results in remarkable differences between Mxi2 and p38α. Mxi2 is distinctively activated by stress stimuli and potently activated by mitogens. Mxi2 affinity for bona fide p38 substrates is remarkably diminished and Mxi2 activity is largely unaffected by the phosphatase CL100. Also, Mxi2 sensitivity to inhibition by SB203580 is greatly reduced. Interestingly, we show that the p38 C-terminus is involved in conferring sensitivity to this compound. Overall, our results point to the p38 carboxy-terminus as a key determinant of the biochemical properties of this family of kinases.

Key words: Mitogen-activated protein kinase; p38; Mxi2; Signal transduction

1. Introduction

Mitogen-activated protein kinases (MAPKs) are cytoplasmic, proline-directed serine/threonine kinases that are activated in response to a wide array of extracellular stimuli, including those that regulate cell proliferation, differentiation, development and inflammation. MAPKs have been shown to be pivotal elements in these cellular processes, acting as essential mediators in the transduction of signals from the cell surface to the nucleus [1]. To date, four groups of mammalian MAPKs have been studied in detail: extracellular signal-regulated kinases (ERKs) [2], stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs), p38 MAPKs [3,4] and the ERK5/BMK1 MAPK [5]. The ERKs are potently activated by growth factors. In contrast, SAPKs/JNKs and p38 are mainly activated by cytokines and environmental stress, although activation of various MAPK pathways by the same stimuli often occurs [1,2].

p38α/RK/CSBP2 MAPK was first identified by virtue of its homology to the yeast HOG MAPK and found to be activated by stress-inducing agents such as osmotic shock, heat shock, UV radiation, lipopolysaccharide, protein synthesis inhibitors and inflammatory cytokines such as IL-1 and TNF-α [6–10]. Once activated, p38 can phosphorylate a number of cytoplasmic proteins like MAPKAPK 2/3 [8,11] and cPLA2 [12]. And nuclear transcription factors including Ets family proteins Elk-1 [13] and Sap-1a [14,15], ATF2 [10,13], MEF2C [16] and CHOP10 [17].

A common feature to all MAPKs is the existence of several isoforms in each family. With respect to the p38 family, in addition to p38α/RK/CSBP2, isoforms p38β [18], p38γ/ERK6/SAPK3 [19,20] and p38δ/SAPK4 [21,22] have been described. Moreover, splicing variants of p38α, namely CSBP1 [7] and Mxi2 [23], and of p38β, termed p38β2 [24] and p38-2 [25], have been reported, but the role of these molecules is still largely unknown.

Mxi2 was isolated in a two-hybrid screen for proteins that bind c-Myc partner protein Max. Mxi2 protein is identical to p38 from amino acids 1 to 280 and harbors a unique C-terminal 17 amino acid sequence. Its relative distribution in human tissues resembles closely that of p38 [23]. However, other than its ability to bind and phosphorylate Max, that is also shared by p38 [23], little is known about its regulation and substrate affinity that could help explain the role of an otherwise almost identical protein to p38. The purpose of this study was to examine Mxi2 biochemical properties, evaluating both upstream and downstream events, which could provide us with an insight into the importance of the C-terminus for p38 MAPKs function. We have found that in spite of sharing common characteristics with p38, Mxi2 activation is stimulated by mitogens and differentially activated by stress-inducing stimuli. Mxi2 phosphorylating activity over bona fide p38 substrates as ATF2, Elk-1, Sap-1 and the MEF2 family is remarkably low. Likewise, Mxi2 is incapable of stimulating the transcriptional activity of the p38-responsive exchange factor MEF2C. Moreover, Mxi2 sensitivity to dual-specificity protein phosphatases capable of inactivating p38 is greatly reduced. Also, Mxi2 sensitivity to the inhibitory properties of pyridinyl imidazole drugs is remarkably lower than that exhibited by p38. Interestingly, we show that the p38 C-terminus can act as an additional determinant of the sensitivity towards these inhibitors. Overall, our results highlight the importance of the p38 carboxy-terminal portion for defining the biochemical properties of these kinases. Moreover, our results are also indicative of Mxi2 having still unveiled regulatory and physiological functions distinct from those of p38.

2. Materials and methods

2.1. Materials

Hemagglutinin (HA) epitope-tagged p38α and Mxi2 [23] were subcloned in pCDNA3. MKK6 was provided by Dr. R.J. Davis (University of Massachusetts). CL100 was from Dr. J.S. Gutkind (NIDR, NIH). TNF-α was from Genzyme Corp., SB203580 and TPA were from Calbiochem, anisomycin, sorbitol and cycloheximide were from Sigma.

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PII: S0014-5793(00)01598-2

2.2. Cell culture and transfection

Human 293T cells were regularly grown in DMEM–10% fetal calf serum. Subconfluent cells were transfected by the calcium phosphate precipitation technique [26]. The total amount of plasmid DNA was adjusted to 3–4 μg per plate with vector DNA when necessary.

2.3. Kinase assays

Two days after transfection, cells were cultured overnight in serum-free medium. Cells were then left untreated or stimulated with various agents. Kinase assays were performed essentially as described [26] by an in vitro immunocomplex assay in anti-HA immunoprecipitates using myelin basic protein (MBP) (Sigma) as substrate.

2.4. Recombinant proteins

Glutathione *S*-transferase (GST) fusion proteins of the N-terminal portion of ATF2 (1–109), c-Jun (1–93) [27], Elk-1 (310–428) and Sap-1 (287–431) [15] and MEF2A, B and C [28] were purified by standard procedures [15] and used as substrates as described above.

2.5. Immunoblotting

Total lysates were fractionated in SDS–PAGE gels and transferred onto nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham), using horseradish peroxidase-conjugated secondary antibody (Cappel). Mouse monoclonal anti-HA antibody was from Babco, mouse monoclonal anti-GST antibody was from Santa Cruz. Anti-phospho-p38 and anti-p38 antibodies were from New England Biolabs.

2.6. Reporter gene assays

Performed in NIH3T3 cells transfected using the calcium phosphate precipitation technique with a β-galactosidase reporter vector, the reporter plasmid pGE51-luc and vectors encoding either GAL4-MEF2C fusion protein or GAL4 (1–147) [16]. The total amount of DNA for each transfection was kept constant (5 μg) using pCDNA3. The luciferase activities were determined using a commercial kit (Promega) following the manufacturer's instructions and normalized by dividing by the β-galactosidase activity.

3. Results

As a first approach to understand Mxi2 regulation, we investigated its activation kinetics. It is now well-established that MAPKs stimulation is time-dependent, ERKs respond to stimuli with an almost immediate peak of activity, while SAPKs/JNKs and p38 MAPKs maximum activation occurs at later stages [27,10]. Activation time course assays in 293T cells transfected with HA epitope-tagged Mxi2 or p38α (referred to as p38 hereafter) and stimulated with anisomycin revealed that Mxi2 activity curve mirrors that of p38, reaching its maximum after 10–15 min stimulation and decreasing thereafter (Fig. 1). However, Mxi2 phosphorylating activity, even at the maximum levels, was almost 3-fold lower than that exhibited by p38 for the same substrate, MBP, utilized in this assay. Due to this, in subsequent experiments, film exposition times for Mxi2 kinase assays were 3–4 times longer than those for p38.

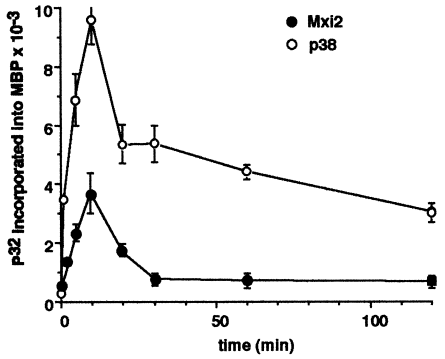

Fig. 1. Time-dependent activation of Mxi2. 293T cells transfected with 1 μg of constructs encoding HA-tagged Mxi2 and p38 were serum-starved for 12 h and then stimulated with 10 μg/ml anisomycin for different times. At the indicated time points, the kinase activity of immunoprecipitated Mxi2 and p38 was determined by an immunocomplex in vitro kinase assay as described in Section 2, using MBP as a substrate. The rate of phosphorylation was quantified by phosphorimager analysis. Results represent the average ± S.E.M. of three independent experiments.

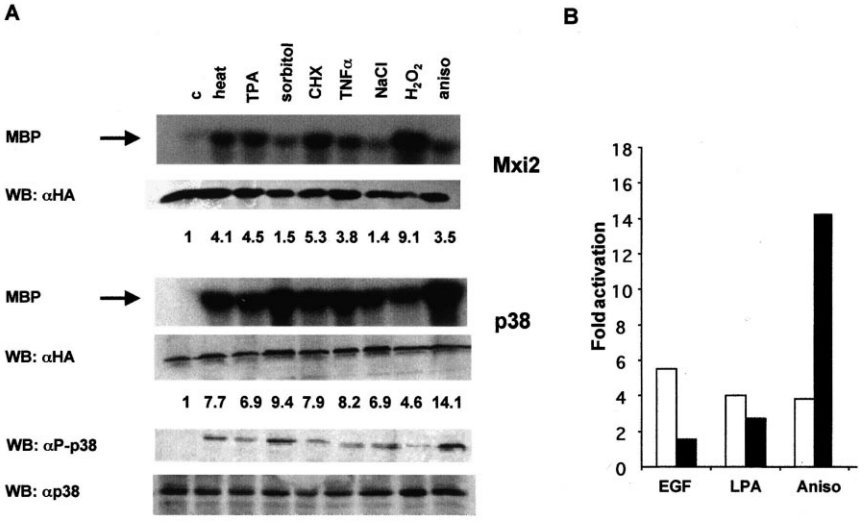

Fig. 2. Mxi2 activation by diverse stimuli. (A) Serum-starved 293T cells previously transfected with HA-tagged Mxi2 and p38 were stimulated for 20 min by heating at 42°C in a water bath, or addition of: 100 nM TPA, 300 mM sorbitol, 60 mg/ml cycloheximide, 10 ng/ml TNF-α, 200 mM NaCl, 4 mM H_2O_2 and 10 μg/ml anisomycin. Lower panels: expression levels of HA-Mxi2 and HA-p38 as determined in total lysates by immunoblotting using an anti-HA mouse monoclonal antibody (Babco). Bottom: phospho-p38 and p38 levels detected in total lysates of cells treated as indicated, determined by Western blotting using the corresponding antibodies. (B) Cells were stimulated by the addition of 10 μM LPA, 100 ng/ml EGF and 10 μg/ml anisomycin for 20 min. Kinase activity of immunoprecipitated Mxi2 (open bars) and p38 (solid bars) was determined by an immunocomplex in vitro kinase assay as described in Section 2, using MBP as a substrate. Data show the average of three independent experiments.

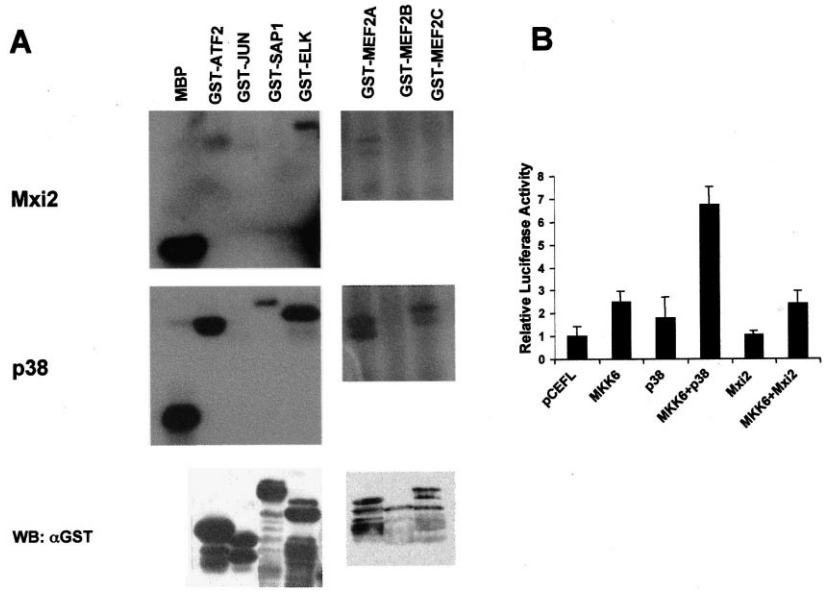

Fig. 3. Substrate specificity of Mxi2. In vitro kinase assays were performed with HA-tagged Mxi2 and p38 immunoprecipitated from transiently transfected 293T cells stimulated with 10 μg/ml anisomycin for 20 min. The kinase reactions were performed utilizing equal amounts (lower panel) of MBP and bacterially produced GST-ATF2 (1–109), GST-Jun (1–93), GST-Elk-1 (310–428) and GST-Sap-1 (287–431), GST-MEF2A, B and C as substrates. Substrate phosphorylation was quantified by phosphorimager analysis. (B) MEF2C-dependent gene expression was determined in NIH3T3 cells cotransfected with a β-galactosidase reporter vector, the reporter plasmid pGE51-luc and an expression vector encoding the GAL4-DNA binding domain fused to MEF2C transactivation domain, in addition to plasmids encoding MKK6, Mxi2 and p38 as activators. The luciferase activities were normalized by the β-galactosidase activity. Values are expressed relative to the luciferase activity detected in vector-transfected cells. Results represent the average ± S.E.M. of three independent experiments.

We next examined Mxi2 sensitivity to activation by external agents. For this purpose, 293T cells were subjected to a panel of stimuli that included: environmental stress stimuli as: heat, osmotic shock induced by high concentrations of sorbitol and NaCl, oxidative stress produced by H_2O_2, inhibitors of protein synthesis like cycloheximide and anisomycin; and TNF-α, an inflammatory cytokine. All of them well-known activators of the p38 family [1,10]. All the stimuli tested activated Mxi2 to different extents, however, maximum activation was induced by H_2O_2, while hyperosmolarity caused by sorbitol and NaCl had minimal effects on Mxi2 activation (Fig. 2A). In sharp contrast to p38, which was potently activated by osmotic shock and to a lesser extent by oxidative stress. Differences in activation were not due to variations in the expression of Mxi2 and p38 proteins as demonstrated by anti-HA Western blot analysis of the corresponding lysates (lower panels). As an additional control, we assayed whether the activation elicited on the transfected HA-tagged versions of Mxi2 and p38 mimicked that one induced over the endogenous proteins. For that purpose, we utilized an anti-phospho-p38 antibody that specifically recognizes activated p38. As shown in Fig. 2A (lower panels), activation of the endogenous p38, as determined by anti-phospho-p38 Western blotting, resembled the one assayed on the transfected HA-p38. However, the anti-phosoho-p38 antibody utilized did not recognize Mxi2. Neither the endogenous in the total lysates nor the HA-tagged version in anti-HA immunoprecipitates (data not shown). It is likely that conformational changes masking the epitope account for this lack of reactivity in Mxi2.

In a similar fashion, we tested how Mxi2 reacted to stimulation by mitogenic stimuli. Interestingly, we found that mitogens such as lysophosphatidic acid (LPA) and epidermal growth factor (EGF) that barely affected p38 catalytic activity, when compared to bona fide p38 stimulators as anisomycin, markedly induced Mxi2 activation (Fig. 2B). Overall, the observed differences on the activation patterns of Mxi2 and p38 by various stimuli suggest that these two isoforms may be differentially regulated.

We then compared the substrate specificity of Mxi2 and p38. To do so, we immunopurified HA-tagged Mxi2 and p38 from anisomycin-stimulated cells. The expression levels of each construct were verified by anti-HA immunoblot (data not shown) and equal amounts were utilized in an in vitro kinase assay using bacterially produced ATF2 (1–109), Jun (1–93), Elk-1 (310–428), Sap-1 (287–431), MEF2A, MEF2B and MEF2C GST fusion proteins as substrates. As shown in Fig. 3A, p38 strongly phosphorylated ATF2 and Elk-1 and to a lesser extent Sap-1, MEF2A and MEF2C. In contrast, Mxi2 activated to a level at which it efficiently phosphorylated the MAPK unspecific substrate MBP, failed to recognize Sap-1 and MEF2C as substrates. Likewise, the degree of ATF2, Elk-1 and MEF2A phosphorylation by Mxi2 was also markedly diminished, about 7.5- and 4-fold, respectively. Similar results were observed when H_2O_2 and sorbitol were used as activators (data not shown).

As a parallel approach, we studied the response of a MEF2C-dependent reporter, known to be activated by p38 [16], to stimulation by Mxi2. It was found that expression of the upstream p38-specific MAPKK MKK6 or p38 induced a moderate increase on MEF2C-dependent luciferase activity (Fig. 3B). But when co-expressed, these two proteins acted synergistically to induce a potent MEF2C response. On the other hand, Mxi2 failed to elicit any substantial increase on MEF2C transcriptional activity, alone or in synergy with MKK6. This lack of effect was not due to Mxi2-reduced expression as verified by Western blotting (data not shown).

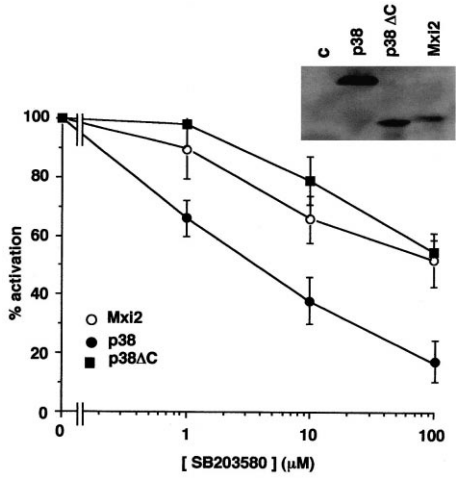

Fig. 4. Effects of pyridinyl imidazole on Mxi2 activity. 293T cells transiently transfected with HA-tagged Mxi2, p38 or p38ΔC were serum-starved for 12 h and treated with increasing concentrations of SB203580 for 30 min, prior to 20 min stimulation with 10 μg/ml anisomycin. After which kinase activities were determined as previously described in Section 2. Substrate phosphorylation was quantified by phosphorimager analysis. Results represent the average ± S.E.M. of three independent experiments. Inset: protein expression levels as determined by anti-HA immunoblot.

Together, these results, showing reduced affinity and a low catalytic activity exhibited by Mxi2 towards well-characterized p38 substrates and effectors, strongly argue in favor of Mxi2 and p38 having different biochemical roles.

A characteristic of most members of the p38 family is its sensitivity to pyridinyl imidazoles that, in contrast, have no inhibitory effects on the activity of ERKs and JNKs [7]. Therefore, we proceeded to determine if the pyridinyl imidazole derivative SB203580 could efficiently block the kinase activity of Mxi2. As shown in Fig. 4, SB203580 could potently block anisomycin-induced p38 activation in vivo with an IC_{50} of around 2 μM. In contrast, Mxi2 was much less sensible to inhibition by this compound, showing an IC_{50} almost two orders of magnitude higher, of around 100 μM.

p38 and Mxi2 differ solely in their respective 80 and 17 C-terminal amino acids, but are identical in the 'canonical' region that confers sensitivity to pyridinyl imidazoles [29]. This raised the possibility that the C-terminus of p38 MAPKs might also determine to some extent the response to these inhibitors. To check this hypothesis, we generated a p38 mutant lacking its C-terminal 80 amino acids (p38ΔC) and assayed its responsiveness to SB203580. It was found that the catalytic activity of this protein was markedly reduced (3-fold) with respect to the wild type (data not shown). On the other hand, its sensitivity to SB203580 was greatly reduced, with an IC_{50} that resembled that of Mxi2 (Fig. 4), suggesting that the p38 carboxy-terminus may act as an additional determinant of the sensitivity towards pyridinyl imidazole inhibitors.

Finally, in mammalian cells, inactivation of MAPKs can be achieved by the action of dual-specificity MAPK phosphatases, which dephosphorylate the critical threonine and tyrosine residues in the activation loop of MAPKs [30]. Of these, CL100/MKP-1 [31] has been shown to potently inhibit p38 activity [10]. Thus, we investigated the inhibitory effects of this phosphatase on Mxi2. Upon cotransfection with increasing concentrations of a plasmid encoding CL100, it was found that the phosphatase almost completely abolished p38 catalytic activity induced by treatment with anisomycin. In contrast, CL100 effects over anisomycin-induced Mxi2 activation were far less dramatic with a barely 20% inhibition at its maximum (Fig. 5). Identical results were obtained upon stimulation with H_2O_2 and sorbitol (data not shown). This lack of response of Mxi2 to pharmacological and biochemical inhibition further supports the idea that Mxi2 and p38 have distinct regulation and biological functions, specified primarily by their different carboxy-termini.

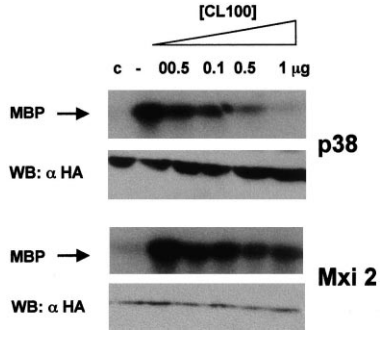

Fig. 5. Effects of CL100 dual-specificity phosphatase on Mxi2 activity. 293T cells were transiently transfected with HA-tagged Mxi2 or p38, with increasing concentrations (50 ng–1 μg) of a vector encoding the phosphatase CL100. Kinase activities were determined as described in Section 2, using MBP as a substrate. Lower panels: expression levels of HA-Mxi2 and HA-p38 as determined in total lysates by anti-HA immunoblotting.

4. Discussion

The existence of multiple isoforms is a general characteristic to all MAPKs [1]. Although the reason for this apparent redundancy is not clearly understood, it could be speculated that the presence of different isoforms provides the basis for the generation of cell-specific and stimulus-specific responses within a MAPK signaling cascade. In this study, we have investigated the biochemical properties of Mxi2, a splicing variant of p38α/CSBP2/RK [23] in comparison to those of p38α, the best-studied member of the p38 family MAPKs. We have found that Mxi2 is activated by stimuli such as hyperosmolarity, environmental and oxidative stress, protein inhibitors and inflammatory cytokines to different extents, being most sensitive to oxidative stress and least to osmotic shock. Distinct responses to stimuli are a common feature to most p38 isoforms. As such, p38β/β2 are only modestly sensible to cytokines [18,24] and a reduced sensitivity towards osmotic changes is also observed in p38δ [21]. However, in sharp contrast to most p38 family members, Mxi2 is potently activated by mitogens as LPA and EGF. How Mxi2 unique C-terminus accounts for this unprecedented behavior is presently not known. A previous study performed in COS cells has found no increment on Mxi2 activity upon serum stimulation, but in these cells Mxi2 exhibits an extremely high basal activity, possibly due to cell-specific variations, that may have masked any stimulation [23].

Variations in substrate specificity are a common characteristic among the members of the different MAPK families. As such, JNK splicing isoforms exhibit a wide range of affinities towards JNK effectors [32]. Likewise, p38 isoforms α, β and γ

also show differences in their recognition of p38 family substrates [18,19,21,22,24]. In the same fashion, the MEF2C transcription factor is targeted differentially by p38 isoforms [33]. Consequently, our results indicate that Mxi2 exhibits a reduced affinity for p38 substrates like the transcription factors ATF2, Elk-1, Sap-1, MEF2A and MEF2C. Accordingly, Mxi2 is also incapable of stimulating MEF2C transcriptional activity, further emphasizing Mxi2 differential behavior.

Pyridinyl imidazole compounds are known to inhibit the enzymatic activity of the p38 family proteins [7], and the various p38 isoforms show different sensitivities to these drugs. As such, p38α, β, β2 and γ are potently inhibited at low concentrations [18,24,19,22]. In contrast, p38δ harboring a T107M substitution that makes it insensible to pyridinyl imidazoles [29] is largely unaffected [21]. Conversely to its splicing isoform p38α, Mxi2 exhibits only a modest susceptibility to inhibition by SB203580. The fact that Mxi2 and p38 are 100% homologous in the region to which pyridinyl imidazoles bind, the ATP binding pocket [34], implies that some degree of sensitivity to this compounds could be conferred by some motif C-terminal to residue 280, in which Mxi2 and p38 diverge completely. Indeed, we show that a p38 mutant lacking its C-terminal 80 amino acids exhibits a markedly reduced sensitivity to SB203580. These variations on the behavior of p38 isoforms to inhibition by pyridinyl imidazole compounds should be taken into account when interpreting the effects of such inhibitors on potential p38 functions.

Our results also demonstrate that Mxi2 activation is largely unaffected by the over-expression of the dual-specificity protein phosphatase CL100/MKP-1 [31], as opposed to p38 that is efficiently inactivated by the phosphatase. It is noteworthy that the *sevenmaker* mutation (D319N) in ERK2 makes it resistant to inactivation by dual-specificity phosphatases as MPK-3 [35,36], probably due to the loss of its ability to bind the phosphatases [36]. In this respect, the relevance of our findings is bolstered by the recent description of the 'CD domain' [37], a conserved docking motif in MAPKs that serves as a common site for binding to substrates, activators and dual-specificity protein phosphatases. The highly conserved residue homologous to ERK2 D319 maps within this motif. In the case of p38α, the CD domain is located at residues 308–321, included in the 80 C-terminal amino acids that are absent from Mxi2. This strongly suggests that the reduced affinity that Mxi exhibits towards well-established p38 substrates and p38-interacting phosphatases is due to the absence of a docking motif to which p38-interacting proteins could effectively bind. The CD domain contains an acidic cluster crucial for efficiently interacting with a region of basic amino acids present in MAPK-docking sites [37]. Mxi2 lacks the 'high affinity' -DPDD- acidic cluster found in p38. However, Mxi2 unique C-terminus harbors a negatively charged -DIE- cluster. Whether this sequence could represent a 'low affinity' docking motif is presently under study.

Overall, we conclude that Mxi2 particular carboxy-terminus confers unique characteristics to this protein. Differential responses to external stimuli, in particular to mitogens, its low specificity for known p38 substrates and its reduced responsiveness to inhibitors such as pyridinyl imidazoles and dual-specificity phosphatases, clearly point to Mxi2 as an atypical member of the p38 MAPKs, with yet to be unveiled, distinctive, biochemical roles, that in addition to its regulatory characteristics are under current investigation.

Acknowledgements: We are indebted to Drs. R.J. Davis, J.S. Gutkind, J. Han and T. Zervos for sharing constructs and to Drs. J. León, J.C. Rodríguez and J.M. Ortiz for critical discussion. V.S. is a Universidad de Cantabria predoctoral fellow. This work was supported by Grant PM 98-0131 from the Spanish Ministry of Education and a grant from Fundación Marcelino Botín.

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