Acquisition of Sensitivity of Stress-activated Protein Kinases to the p38 Inhibitor, SB 203580, by Alteration of One or More Amino Acids within the ATP Binding Pocket*

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Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase compete with ATP for binding. Mutation of 23 residues in the ATP pocket indicated that several residues which affected binding of pyridinyl imidazole photoaffinity cross-linker 125I-SB 206718 did not affect kinase activity, and vice versa, suggesting that pyridinyl imidazoles bind p38 differently than ATP. Two close homologues of p38, SAPK3 and SAPK4, are not inhibited by SB 203580 and differ from p38 by three amino acids near the hinge of the ATP pocket. Substitution of the three amino acids in p38 by those in SAPK3/4 (Thr-106, His-107, and Leu-108 to Met, Pro, and Phe) resulted in decreased 125I-SB 206718 cross-linking and loss of inhibition by SB 203580. Substitution of just Thr-106 or Met resulted in incomplete loss of inhibition. Conversely, substitution of the three amino acids of p38 into SAPK3, SAPK4, or the more distantly related JNK1 resulted in inhibition by SB 203580, whereas mutation of just Met-106 to Thr resulted in weaker inhibition. These results indicate that these three amino acids can confer specificity and sensitivity to SB 203580 for at least two different classes of MAPKs.

Mitogen-activated protein kinases (MAPKs)1 have been shown in recent years to be key components of signal transduction pathways regulating both mitogenic and stress-induced stimuli (1). At least three families of MAPKs have been identified in mammalian cells (2). The first family, the extracellular regulated kinases (ERKs), is activated primarily by mitogenic stimuli, growth factors and tumor promoters (2). The other two families, the Jun amino-terminal kinases (JNKs) (JNK1 is also known as stress-activated protein kinase 1 or SAPK1) and the p38 MAPKs (p38 is also known as CSBP2, RK, SAPK2 and, in yeast, HOG1) are activated primarily by environmental stress and inflammatory agents (3–5). All of these MAPKs are serine-threonine protein kinases, which are activated by different upstream MAPK kinases through phosphorylation of a Thr-Xaa-Tyr motif in an activation loop near the ATP and substrate binding sites (2, 6). The length of the activation loop and the identity of the central (Xaa) amino acid (Glu for the ERKs, Pro for the JNKs, and Gly for p38 MAPKs) differ between the three MAPK families as do their in vivo substrate specificities (2, 7–9).

A series of pyridinyl imidazoles, exemplified by SB 203580, which were originally identified by their ability to suppress inflammatory cytokine synthesis, can specifically inhibit p38 MAPK by binding in the ATP pocket (5, 10, 11). Neither JNKs nor ERKs are inhibited by SB 203580 at the same concentrations (12, 13). Recently, three homologues of p38 have been identified: p38β/β2 (14, 15), SAPK3 (16) (also called p38γ (17), and ERK8), and SAPK4 (15, 19, 20). These homologues are between 60% and 74% identical to p38 but show some differences in activation stimuli and substrate specificities (15). Interestingly, p38 and p38β/β2 are inhibited by SB 203580 whereas SAPK3 and SAPK4 are not, indicating the high degree of selectivity of these compounds (15). Recent studies have determined that SB 203580 functions by competitive binding in the ATP pocket (11), and this has been further illustrated by the recent co-crystal structures of p38 with pyridinyl imidazoles2 (21, 22). In the present study, we have determined the contribution of various amino acids to inhibitor binding and find that three amino acids within the ATP pocket play a major role in determining the selectivity of p38 and p38β/β2 for SB 203580. Substitution of these three residues for the corresponding amino acids in SAPK3, SAPK4, or JNK1 is sufficient to confer sensitivity to SB 203580.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—Yeast expression construct p138NBU-CSBP2 (wild-type p38) was engineered as described for p137NBU-CSBP2 (23), except that the copper-inducible CUP1 promoter plasmid, p138NBU, was used instead of the constitutive TDH3 promoter in the p137NBU plasmid. Cloning and construction of mammalian expression constructs for FLAG-tagged p38, SAPK3, and SAPK4 were described previously (9, 15), as were cloning and construction of FLAG-tagged JNK1 (3, 24). Mutagenesis of p38 in yeast expression vectors was performed as described (23). Mutants of p38, SAPK3, SAPK4, and JNK1 were generated using a QuikChange site-directed mutagenesis kit as directed by the manufacturer (Stratagene) using 40-base pair oligonucleotides containing the indicated amino acid
changes in the middle and confirmed using a DNA sequencer (Applied Biosystems, Inc.).

Expression of Constructs in Saccharomyces cerevisiae, Immunoprecipitations, and Kinase Assays—p38 wild-type and mutant yeast expression constructs were introduced into the JBY10 (hog1 Δ) strain of S. cerevisiae. Induction for expression with copper, stimulation for phosphorylation with 0.9 M potassium chloride and lystate preparation were performed as described previously (23). Proteins were immunoprecipitated from yeast cell extracts using anti-CSBP polyclonal antibody and protein G-agarose (Life Technologies, Inc.). Immunocomplex kinase assays were performed on five of the extracts as described previously (9) using anti-FLAG M2 antibody conjugated to agarose (Santa Cruz) or anti-CSBP antibody were performed as described previously (9, 23). Experiments were performed at least three times with similar results.

Mammalian Transfections and Preparation of Lysates—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a humidified 5% CO2 incubator at 37 °C. Transient transfections were performed using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). 24 h after transfection, cells were split into several plates. 48 h later, cells were treated with 0.4% sorbitol (Sigma) for 20 min and then harvested as described (9).

Immunoprecipitations, Kinase Assays, and Western Blotting—Epitope-tagged proteins were immunoprecipitated from HeLa cell extracts as described previously (9) using anti-FLAG M2 antibody conjugated to agarose (Eastman Kodak Co.) and then divided into six equal portions. Immune complex kinase assays were performed on five of the six fractions. For drug inhibition experiments, samples were preincubated for 10 min at room temperature with different concentrations of SB 203580 in 15 μl of kinase buffer and then another 15 μl of kinase buffer was added containing 50 μM cold ATP, 2 μCi of [γ-32P]ATP (specific activity 4500 Ci/mmole) and 10 μg of MBP or 2 μg of glutathione S-transferase–c-Jun (1–79) (Santa Cruz Biotechnology, Inc.) as a substrate for Western blotting using anti-phosphotyrosine PY69 monoclonal antibody (Santa Cruz) or anti-CSBP antibody were performed as described previously (9, 23). Experiments were performed at least three times with similar results.

Preparation of Inhibitors and Photoaffinity Cross-linking Experiments—Expression of Inhibitors and Photoaffinity Cross-linking Experiments—Synthesis of SB 203580 and 125I-SB 206718 was described previously (25, 26), as was photoaffinity cross-linking of yeast extracts to 125I-SB 206718 (5, 11). Epitope-tagged proteins from HeLa cell extracts were first immunoprecipitated as described above, then cross-linked to 125I-SB 206718 as described for yeast extracts (5) in the presence or absence of cold competitor, SB 203580. Photoaffinity-labeled proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

Mutation of Thr-106 to Met, in Yeast-expressed p38, Disrupts Cross-linking and Results in Loss of Sensitivity to Inhibition by SB 203580—Two closely related homologues of p38 (SAPK3 and SAPK4) are insensitive to p38 inhibitors at concentrations up to 10 μM (Ref. 15 and Fig. 4A). There are three amino acid residues within the ATP pocket, which differ between p38 and SAPK3 or SAPK4. In p38, they are Thr-106, His-107, and Leu-108, whereas in SAPK3 they are Met-109, Pro-110, and Phe-111, and in SAPK4, Met-107, Pro-108, and Phe-109. A mutant p38 in which these three residues were changed to the corresponding residues in SAPK3 or SAPK4 (mutant p38(MPF)), was expressed in yeast and shown to have significantly decreased cross-linking to [125I]-SB 206718 (Fig. 2A). A mutant p38 with a single substitution of Thr-106 to Met (mutant p38(106M)) showed a similar decrease in cross-linking to [125I]-SB 206718 (Fig. 2A), indicating that only Thr-106 was necessary for cross-linking. Western blotting of equal portions of extracts showed that the differences in cross-linking were not due to differences in expression levels of the constructs (Fig. 2B).

The mutants were also tested for kinase activity and sensitivity to inhibition by SB 203580. Little change was observed in kinase activity of the mutants relative to wild-type p38; however, significant changes in sensitivity to the inhibitor were observed. Mutants p38(MPF) and p38(106M) were no longer sensitive to inhibition by SB 203580 (Fig. 2C). Both activation
To determine if Thr-106 or the triple mutation (Thr, His, and Phe) resulted in decreased cross-linking to $^{125}$I-SB 206718 (Fig. 3), mutations were engineered into the homologues corresponding to 106, 107, and 108 of p38. The mutants were transfected with these mutants were immunoprecipitated with anti-FLAG as the probe (Fig. 3A). Again, differences in kinase activity without drug inhibition to $^{125}$I-SB 206718 (Fig. 3A, top panel). However, in contrast to the inhibition results with yeast-expressed p38(106M), mammalian-expressed p38(106M) remained partially inhibitable by SB 203580, with approximately a 10-fold higher IC$_{50}$ (Figs. 3A, top panel, and 5A) than wild-type p38. In further experiments, the IC$_{50}$ for mutant p38(MPF) was determined to be between 80 and 100 $\mu$m (data not shown).

Similarly, the reverse mutations in SAPK3 and SAPK4 resulted in increased sensitivity to inhibition by SB 203580. Mammalian-expressed mutants SAPK3(THL) and SAPK4(THL) both exhibited equivalent sensitivity to SB 203580 inhibition as wild-type p38, whereas SAPK3(106T) and SAPK4(106T) were approximately 10-fold less sensitive, similar to p38(106M) (Figs. 4A and 5, B and C).

**DISCUSSION**

Mutagenesis of the ATP pocket of p38 and its homologues has revealed distinctions between the residues required to bind pyridinyl imidazoles and those required for kinase activity. This is illustrated in Fig. 7; Panel A shows residues that result in loss of kinase activity together with a model of ATP bound in the ATP pocket (Fig. 7B), suggesting no significant intrinsic changes in kinase activity. However, significant changes in sensitivity to the inhibitor were observed. Like yeast-expressed mutant p38(MPF), mammalian-expressed mutant p38(MPF) was no longer sensitive to inhibition by SB 203580 (Fig. 4A, top panel). In contrast to the inhibition results with yeast-expressed p38(106M), mammalian-expressed p38(106M) remained partially inhibitable by SB 203580, with approximately a 10-fold higher IC$_{50}$ (Figs. 3A, top panel, and 5A) than wild-type p38. In further experiments, the IC$_{50}$ for mutant p38(MPF) was determined to be between 80 and 100 $\mu$m (data not shown).

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the ATP pocket of p38. Panel B highlights residues found to increase or decrease cross-linking to SB 206718; these are shown in the context of the x-ray co-crystal structure of SB 203580 bound to p38. The residues that affect kinase activity are consistent with what is known from related crystal structures. For example, Lys-53, Lys-152, and Asp-168 are known catalytic residues conserved in all serine-threonine protein kinases. Val-38, Ile-84, and Leu-171 are highly conserved nonpolar residues in the pocket. Comparison to the activated ERK2 structure suggests that Arg-67 may form an ion-pair interaction with the phosphorylated Thr-180 in the activation loop, which is critical for the domain rotation and closure which occurs upon MAPK activation (27). On the other hand, Ala-157, Asn-114, and Asn-115 are not conserved among kinases. The particularly strong effect of mutating Ala-157 to Leu (the equivalent residue in ERK) may be due to its effect on Met-109 packing, which is different in p38 than in ERK (27).

Among the residues involved in photoaffinity cross-linking to 125I-SB 206718, several overlap with those required for kinase activity (Val-38, Lys-53, Arg-67, Ile-84, Lys-152, Ala-157, Leu-171, and Asp-168). As indicated above, many of these are highly conserved in all serine-threonine protein kinases (Lys-53, Lys-152, and Asp-168) or MAPK (Arg-67 and Ile-84). Those residues that affect only 125I-SB 206718 cross-linking include Val-30, Tyr-35, and Thr-106. Although Tyr-35 is fairly well conserved, being either a Phe or Tyr in most protein kinases, Val-30 and Thr-106 are not conserved with other MAPKs. All of the mutations that affect cross-linking of 125I-SB 206718 are also those that are in intimate contact with its analogue, SB 203580, in the co-crystal structure (2) with p38 (Fig. 7B) (see also Refs. 21 and 22), suggesting that the mutations alter binding of the cross-linking agent, not the chemical reaction at the cross-linking site itself, which is known to be close to residue Thr-175 (11). The overall picture that emerges is that the inhibitors interact with a combination of both conserved and non-conserved residues, some of which are unique for binding of the inhibitors and some of which are also used for binding ATP.

Further evaluation of effects of various mutations on the
Acquisition of Sensitivity to the p38 Inhibitor, SB 203580

Fig. 7. Crystal structure of p38 with SB 203580 revealing the location of residues important for binding of pyridinyl imidazole inhibitors and important for kinase activity. A, residues that resulted in a decrease in kinase activity when mutated are shown in magenta. ATP is shown in green with the phosphates in orange. B, residues that, when mutated, showed a decrease in binding to SB 206718 are shown relative to the ATP pocket in orange, and those that increased binding are shown in blue. The three residues that were required for sensitivity to SB 203580 are shown in red. SB 203580 is shown in green. Residues that did not affect kinase activity when mutated are shown in gray in A, and residues that did not affect cross-linking when mutated are shown in gray in B.

ability of SB 203580 to inhibit p38 kinase activity localized the critical region to three residues, Thr-106, His-107, and Leu-108, which lie at the back of the ATP pocket and form the hinge between the two domains flanking the active site. This region is also close to three of the less conserved amino acids important for binding (Val-30, Ala-157, and Leu-167) (Fig. 7B). Although substitution of just Thr-106 with Met in p38 and vice versa in SAPK3, SAPK4, and JNK1 led to kinases with intermediate sensitivity to SB 203580 when these mutants were expressed in mammalian cells, all three residues (Thr-106, His-107, and Leu-108) were required for complete sensitivity. Thus, apparently, one or both of His-107 and Leu-108 are also important for inhibitor sensitivity. The idea that His-107 is also critical for sensitivity to the inhibitor (in addition to Thr-106) is supported by the data with JNK1 and its mutants, which indicate that both Thr-106 and His-107 must be present for complete sensitivity to SB 203580. Neither Leu-108 alone nor the combination of Leu-108 and Thr-106 are sufficient for complete sensitivity. This part of the p38 chain adopts a different conformation from ERK2 (27), and so it is possible that mutations in more than one of the residues in this hinge region alter its conformation and orientation relative to the ATP pocket. The structure of the co-complex of SB 203580 with p38 shows that Thr-106 contacts the inhibitor and that the imidazole ring of His-107, which faces away from the ATP pocket, is involved in hydrogen bonding networks that change on inhibitor binding.2

The role of Thr-106 has been alluded to previously (21, 22). Its small size allows the formation of a pocket into which the fluorophenyl ring of the pyridinyl imidazoles can fit. Substitution with Ala, another small amino acid, had no impact on inhibition by pyridinyl imidazoles (22). Indeed, we saw enhanced cross-linking of 125I-SB 206718 to this mutant p38. In contrast, substitution with a bulky residue such as Met resulted in reduced binding and inhibition consistent with its blocking access to the fluorophenyl pocket. However, our inhibition results do not agree quantitatively with the published work (22), where substitution of Thr-106 by Met resulted in complete loss of inhibition by the related SB 202190 up to 30 μM; in our hands, it was still sensitive to inhibitor at 10 μM. This difference might result from the different source of p38, which in our hands was immunoprecipitated from mammalian cells, but in the previous work was expressed in Escherichia coli and activated in vitro with constitutively active MKK6. The idea that the expression system may influence the inhibition results is supported by kinase inhibition data we obtained with extracts from yeast expressing human p38, p38(106M), and p38(MPF), which revealed that, as in bacteria, the single mutation (p38(106M)) is sufficient to convert p38 to an SB 203580-insensitive phenotype at concentrations up to 10 μM. In contrast, mutation of all three residues (MPF) was required for an insensitive phenotype in the mammalian expression system. These differences may be due to additional proteins, which are associated with mammalian p38 immunoprecipitates and which may interact with His-107 or Leu-108 and alter the conformation of p38. The idea that the hinge region is involved in protein/protein interactions is further supported by the existence of dominant negative mutations near this locus in the yeast MAPK homologue FUS3 (2, 28).

We have demonstrated that alteration of three residues at the back of the ATP binding pocket to Thr-106, His-107, and Leu-108 of p38 is sufficient to confer sensitivity to SB 203580 to p38 homologues such as SAPK3 and SAPK4 and to more distantly related MAPKs such as JNK1. It will be of interest to determine whether introduction of these changes into non-MAPK protein kinases can confer sensitivity to the pyridinyl imidazole inhibitors. Through their altered sensitivity to SB 203580, the mutant p38, SAPK3, SAPK4, and JNK1 now provide tools for further dissecting in vitro pathways in which they have been implicated. In addition, understanding the manner in which pyridinyl imidazoles interact with p38 may now allow synthesis of compounds that specifically interact with other MAPKs but not p38.

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