Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580

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Abstract Stress-activated protein kinase 2a, also called p38, is inhibited by SB 203580 and this drug has been used widely to implicate this enzyme in the regulation of many physiological processes. Here, we introduce a novel method of general application, which can be used to establish whether the effects of SB 203580 are mediated via inhibition of stress-activated protein kinase 2a/p38 or whether they result from 'non-specific' effects. Four events thought to occur upon activation of stress-activated protein kinase 2a/p38 have been established unequivocally. These are the activation of mitogen-activated protein kinase-activated protein kinase-2 and mitogen- and stress-activated protein kinase-1 and the phosphorylation of their presumed substrates, heat shock protein 27 and the transcription factor cyclic AMP response element binding protein, respectively. In contrast, the SB 203580-induced activation of c-Raf is independent of stress-activated protein kinase 2a/p38 inhibition.

Key words: SAPK2; p38; SB203580; MAPKAP-K2; MSK1; CREB

1. Introduction

Nearly all aspects of cell life are controlled by the reversible phosphorylation of proteins. Approximately one third of mammalian proteins contain covalently bound phosphate and protein kinases represent the largest single family of enzymes in the human genome, with perhaps 2000 members. Assuming that 100 000 proteins are encoded by the human genome, an 'average' protein kinase should phosphorylate about 15 substrates in vivo. A major challenge is therefore to identify unambiguously all the physiological substrates of each protein kinase. One strategy to address this problem would be to develop small cell-permeant compounds that are specific inhibitors of protein kinases and to examine their effects on intact cells and tissues. The recent discovery of compounds with the requisite specificity establishes this as a valid approach with an enormous potential [1,2].

One such compound is SB 203580, a member of a class of pyridinyl imidazoles that were originally shown to suppress the bacterial lipopolysaccharide (LPS)-induced production of tumour necrosis factorα (TNFα) and to be effective in animal models of chronic inflammatory disease [3,4]. The presumed target of SB 203580, initially identified as a binding protein [3], is a mitogen-activated protein kinase (MAPK) family member termed stress-activated protein kinase 2a (SAPK2a) or p38. Subsequently, SB 203580 was shown to be highly specific for SAPK2a/p38 [5], as other MAPK family members (as well as many other protein kinases) were insensitive to this drug [5,6]. For this reason, SB 203580 has been used in hundreds of publications to implicate SAPK2a/p38 in a wide range of physiological processes with the implicit assumption that the drug was extremely specific for SAPK2/p38 (reviewed in [1]). However, it has emerged that there are other SB 203580-sensitive enzymes, which include cyclo-oxygenases 1 and 2 [7] and the protein kinase Raf [8,9]. These findings now question which of the reported effects of SB 203580 can be attributed to inhibition of SAPK2a/p38 and which are due to the 'non-specific' inhibition of other enzymes.

The co-crystallisation of SAPK2a/p38 in complex with pyridinyl imidazoles related to SB 203580 [10,11] pinpointed Thr-106 as being critical for inhibition by SB 203580. As predicted, mutation of this residue to amino acids with larger side chains was shown to render SAPK2a/p38 insensitive to SB 203580 [10,12,13]. By implication, the insensitivity of other MAPK family members to SB 203580 was due to amino acid residues with larger side chains at this position. Indeed, mutation of these to smaller residues (e.g. threonine) then rendered these kinases sensitive to the drug [12–14]. In this paper, we inducibly express a drug-resistant mutant of SAPK2a/p38 and exploit it to investigate whether some of its putative targets are bona fide substrates in intact cells.

2. Materials and methods

2.1. Antibodies

Antibodies that recognise human HSP27 only when phosphorylated at Ser-15 and Ser-78 were raised in sheep at the Scottish Antibody Production Unit (Carluke, UK). The peptides LLRGPS*[15]WDPFRC and YSRALS*[78]RQLSSC (phosphorylated at the serines equivalent to Ser-15 and Ser-78, respectively) were coupled via their C-terminal cysteines to keyhole limpet haemocyanin using the cross linking agent sulphosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Calbiochem, Nottingham, UK) according to the manufacturer's instructions. The antisera were purified by affinity chromatography on the relevant phosphopeptide-agarose columns followed by chromatography on the relevant dephosphopeptide-agarose. The specificity of these antibodies was established by the finding that they did not recognise bacterially expressed HSP27 and only recognised HSP27 in lysates from U373 cells that had been exposed to chemical stress (sodium arsenite). A SAPK2a/p38-specific antibody raised in sheep against the C-terminal sequence (ISFVPPPLDQEEMES) of the human enzyme was affinity-purified in a similar manner. Polyclonal antisera that recognise phosphorylated and dephosphorylated HSP27 equally well and an antibody that recognises the Ser-133 phosphorylated form of the cyclic AMP response element binding protein (CREB) were purchased from UBI (Lake Placid, NY, USA). A monoclonal antibody 12CA5 that recognises the haemagglutinin epitope was obtained from Boehringer (Lewes, UK). Affinity-purified antisera to rabbit, mouse and sheep IgG were purchased from Pierce and used

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at dilutions of 1:10 000. Immunoreactive protein was detected by the enhanced chemiluminescence technique (Amersham International, UK).

2.2. Construction of stable 293 cell lines inducibly expressing wild-type SAPK2a/p38 or an SB 203580-insensitive mutant

The procedure was carried out using reagents from Invitrogen (Leek, The Netherlands) according to the procedures recommended by the manufacturer. The human SAPK2a/p38 sequence (CSBP2 variant [3]) including a haemagglutinin tag at the N-terminus was subcloned into the *Bam*HI site of the pIND vector [15]. This construct expresses SAPK2a/p38 only in the presence of ponasterone A, an analogue of the insect hormone ecdysone. A drug-resistant mutant (DR-SAPK2a/p38), insensitive to SB 203580, was made by changing the residues Thr-106, His-107 and Leu-108 to Met, Pro and Phe, respectively, using the quick change PCR mutagenesis kit (Stratagene, Cambridge, UK). Constructs were sequenced to verify that the mutagenesis was successful. Human embryonic kidney 293 cells stably expressing the ecdysone receptor subunits were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% (by volume) foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.4 mg/ml zeocin. Wild-type (WT) SAPK2a/p38 or DR-SAPK2a/p38 were transiently transfected into the cells and then selected for, using 1 mg/ml of the antibiotic G418 (Gibco BRL, Paisley, UK) for 14 days to generate cells that inducibly express these enzymes only after stimulation with ponasterone A.

2.3. Cell stimulation and analysis of downstream targets of SAPK2a/p38

Cells were stimulated with 10 μM ponasterone A for 23 h followed by a 1 h treatment with SB 203580. They were then exposed to either anisomycin (10 μg/ml) for 30 min or to UV-C radiation (200 J/m^2) and incubated for 30 min before lysis as in [16]. The lysates were centrifuged (13 000×*g*, 10 min), the supernatants removed and the nuclear pellets extracted in 1% (w/v) SDS. MAP kinase-activated protein kinase-2 (MAPKAP-K2) [17] or mitogen- and stress-activated protein kinase-1 (MSK1) [18] were immunoprecipitated from the supernatants and assayed as described [18]. The SDS-extracted pellets were electrophoresed on 10% SDS/polyacrylamide gels, transferred to nitrocellulose and immunoblotted with the anti-phospho-CREB antibody. A plasmid encoding human HSP27 (pcDNA3.1) was transiently transfected into the 293 cells 24 h before induction with ponasterone A and analysed by immunoblotting the supernatants using anti-HSP27 antibodies (Section 2.1).

3. Results

3.1. SB 203580-insensitive forms of SAPK2a/p38

The mutation of Thr-106, His-107 and Leu-108 to Met, Pro and Phe, respectively, (i.e. to the residues present in the related, but SB 203580-insensitive SAPK3 and SAPK4) is reported to make SAPK2a/p38 even more resistant to SB 203580 than the single mutation of Thr-106 to Met [13]. The triple mutant, hereafter called DR-SAPK2a/p38, was used in the present study but, in our hands, it was found to be no more resistant to SB 203580 than the Thr-106-Met mutant (Fig. 1A).

3.2. Identification of stable cell lines that inducibly express SAPK2a/p38

Cells that stably express transfected HA-tagged pIND SAPK2a/p38 contain a gene that confers resistance to the antibiotic G418, whereas untransfected cells are killed [16]. After 14 days growth in 1.0 mg/ml G418, 20 colonies were picked, further cultured in the presence of 0.5 mg/ml G418 and tested for ponasterone A-dependent induction of SAPK2a/p38. About half of these colonies showed an inducible SAPK2a/p38 protein. One cell line expressing WT-SAPK2a/p38 and one expressing the drug-resistant mutant DR-SAPK2a/p38 (Fig. 1B) were chosen for further study. After stimulation with 10 μM ponasterone A, both cell lines overexpressed the

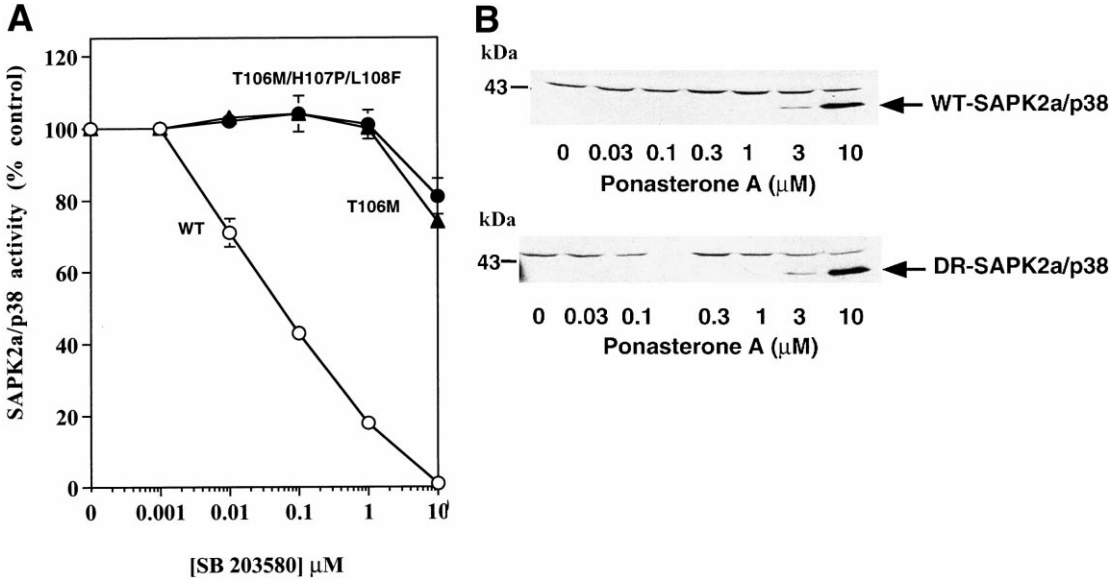

Fig. 1. Characterisation of the SB 203580-sensitivity of WT-SAPK2a/p38 and DR-SAPK2a/p38 and their inducible expression in 293 cells. A: Inhibition by SB 203580 of WT-SAPK2a/p38 (open circles), the triple mutant DR-SAPK2a/p38 in which Thr-106, His-107 and Leu-108 have been mutated to Met, Pro and Phe, respectively (closed circles), and the single mutant Thr-106-Met SAPK2a/p38 (closed triangles). Each enzyme was expressed as a GST fusion protein in *Escherichia coli* and assayed using myelin basic protein as substrate [12]. B: 293 cells stably expressing DNA encoding HA-tagged WT-SAPK2a/p38 or DR-SAPK2a/p38 were stimulated for 24 h with the indicated concentrations of ponasterone A. Aliquots of the cell lysates were denatured in SDS and subjected to polyacrylamide gel electrophoresis. After transfer to nitrocellulose, the membranes were immunoblotted with an anti-HA antibody. The positions of WT-SAPK2a/p38 and DR-SAPK2a/p38, which migrated slightly faster than the ovalbumin marker (43 kDa), are indicated. No HA-SAPK2a/p38 was detected in the absence of ponasterone A. The cross-reactive band present in all lanes, and which migrates slightly slower than ovalbumin, is an unknown protein recognised non-specifically by the HA antibody which demonstrates equal loading of the samples.

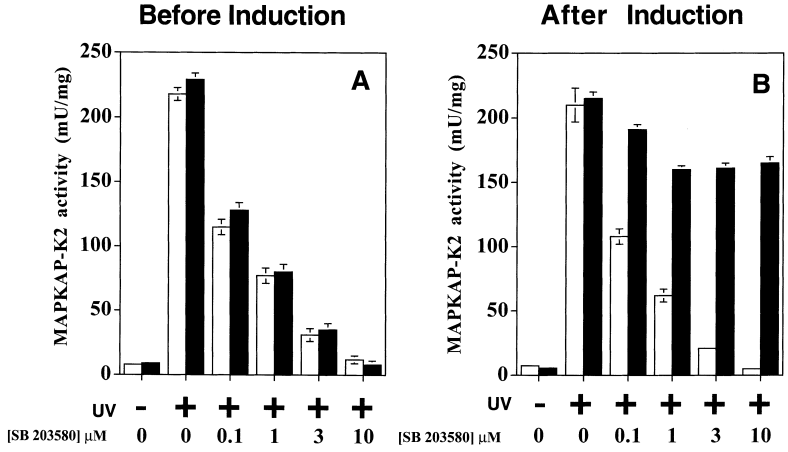

Fig. 2. SB 203580 no longer blocks the activation of MAPKAP-K2 in 293 cells expressing DR-SAPK2a/p38. A: Cells not stimulated with ponasterone A were pre-incubated for 1 h with the indicated concentrations of SB 203580 and then exposed to UV-C radiation. After cell lysis, MAPKAP-K2 was immunoprecipitated from 50 μg of lysate and assayed [16]. Units of MAPKAP-K2 are defined in [16]. The open bars show cells stably transfected with WT-SAPK2a/p38 and the closed bars cells stably transfected with DR-SAPK2a/p38. B: The same as A, except that the cells were first stimulated for 23 h with 10 μM ponasterone A prior to incubation with SB 203580 and exposure to UV-C radiation. The data shown are the mean ± S.E.M. for three separate ponasterone A inductions. All assays were carried out in triplicate.

HA-tagged enzymes approximately 5-fold as compared to the endogenous WT enzyme (data not shown).

3.3. The activation of MAPKAP-K2 and the phosphorylation of HSP27 are no longer sensitive to SB 203580 in cells expressing DR-SAPK2a/p38

The availability of a cell line capable of overexpressing DR-SAPK2a/p38 provided an opportunity to establish whether putative downstream targets identified in earlier studies were indeed physiological substrates. One such enzyme is MAPKAP-K2, which is activated by SAPK2a/p38 in vitro [19] and whose activation by cell damaging agents and other stimuli is prevented by SB 203580 ([5], reviewed in [1]). Before induction with ponasterone A, cells stably transfected with WT-SAPK2a/p38 or DR-SAPK2a/p38 behaved like untransfected 293 cells, in that the endogenous MAPKAP-K2 was essentially inactive until exposed to UV-C radiation. As expected, the UV-induced activation of the endogenous MAPKAP-K2 was suppressed by SB 203580 with an IC_{50} of 100 nM (Fig. 2A). After induction by ponasterone A, the basal level of MAPKAP-K2 was not elevated nor was the activity attained after exposure to UV-C radiation, despite the 5-fold overexpression of SAPK2a/p38 (Fig. 2B). In contrast, the activation of MAPKAP-K2 in cells overexpressing DR-SAPK2a/p38 was no longer suppressed significantly by SB 203580, whereas the sensitivity of the cells overexpressing WT-SAPK2a/p38 (Fig. 2B) was the same as before induction (Fig. 2A).

A putative physiological substrate for MAPKAP-K2 is HSP27. It is phosphorylated by MAPKAP-K2 in vitro at the same residues that become phosphorylated in response to signals that activate SAPK2a/p38 ([20], reviewed in [1]) and these phosphorylations are suppressed by SB 203580 [5]. The results obtained in the present study establish that HSP27 is indeed a 'downstream component' of the SAPK2a/p38 pathway, because its phosphorylation at Ser-15 (Fig. 3) and Ser-78 (data not shown), induced by either UV-C radiation or the protein synthesis inhibitor anisomycin, is no longer sup-

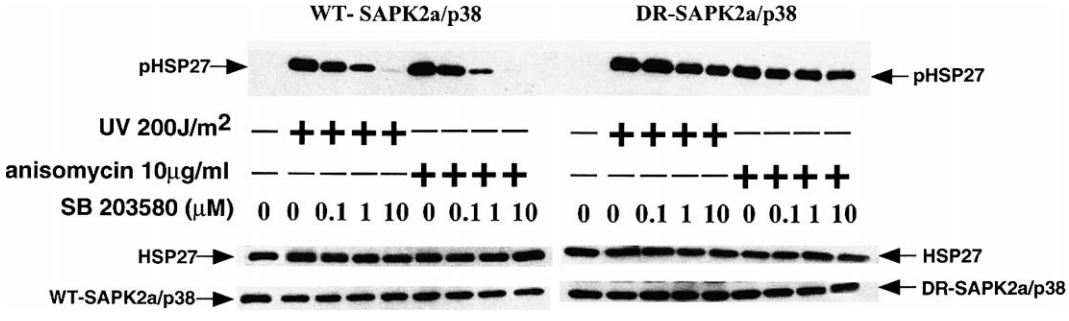

Fig. 3. SB 203580 no longer blocks the phosphorylation of HSP27 in 293 cells expressing DR-SAPK2a/p38. Cells stably expressing DNA encoding HA-tagged WT-SAPK2a/p38 or DR-SAPK2a/p38 were transiently transfected with a plasmid encoding human HSP27. After 24 h, the cells were stimulated for 23 h with 10 μM ponasterone A, then incubated for 1 h with the indicated concentrations of SB 203580. After exposure to either UV-C radiation or anisomycin (Section 2.3), the cells were lysed and the extent of phosphorylation of HSP27 was measured by immunoblotting with a phospho-specific antibody that only recognises HSP27 phosphorylated at Ser-15. Similar results were obtained with a second phospho-specific antibody that only recognises the Ser-78-phosphorylated form of HSP27 (not shown). The lysates were also blotted with an anti-HSP27 antibody that recognises both phosphorylated and dephosphorylated HSP27, further establishing the specificity of the phospho-specific antibodies. An HA antibody (that recognises the induced SAPK2a/p38) was used to show equivalent expression in all lysates. Similar results were obtained in two separate experiments.

pressed significantly by SB 203580 in cells overexpressing DR-SAPK2a/p38.

3.4. The activation of MSK1 and the phosphorylation of CREB are no longer sensitive to SB 203580 in cells expressing DR-SAPK2a/p38

MSK1 is a recently identified protein kinase that can be activated in response to either growth factors/phorbol esters or cell damaging agents/pro-inflammatory cytokines. The activation of MSK1 by growth factors and phorbol esters appears to be mediated via the classical MAP kinase cascade because it is prevented by a specific inhibitor of this pathway (the drug PD 98059). Activation of MSK1 by cell damaging stimuli and pro-inflammatory cytokines may be mediated by SAPK2a/p38, because it is prevented by SB 203580 [18]. Consistent with this hypothesis, MSK1 can be activated in vitro by either MAPK2/ERK2 or SAPK2a/p38 [18]. The results presented in Fig. 4 establish that the activation of MSK1 in response to UV-C radiation is mediated by SAPK2a/p38, because it is hardly suppressed by SB 203580 in cells overexpressing DR-SAPK2a/p38. In contrast, SB 203580 blocks UV-induced MSK1 activation in cells overexpressing WT-SAPK2a/p38 (Fig. 4).

MSK1 is predominantly localised to the nucleus and one of its putative physiological substrates is the transcription factor CREB, which it phosphorylates at Ser-133 with an extremely low K_m value in vitro. Ser-133 becomes phosphorylated in response to signals that activate SAPK2a/p38 [18] and phosphorylation is suppressed by SB 203580 [21]. Here, we establish that CREB and the closely related activating transcription factor 1 (ATF1) do indeed lie 'downstream' of SAPK2a/p38. This is because their phosphorylation can be induced by either UV-C radiation (Fig. 5) or the protein synthesis inhibitor anisomycin (data not shown). Their phosphorylation is also not suppressed significantly by SB 203580 in cells overexpressing DR-SAPK2a/p38. In contrast, CREB and ATF1 phosphorylation remain sensitive to the drug in cells overexpressing WT-SAPK2a/p38 (Fig. 5).

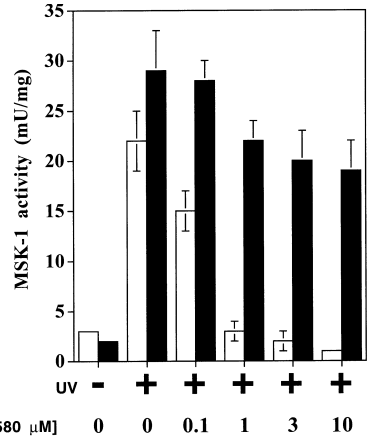

Fig. 4. SB 203580 no longer blocks the activation of MSK1 in 293 cells expressing DR-SAPK2a/p38. Experimental conditions were as in Fig. 2B, except that MSK1 was immunoprecipitated from 500 μg of cell lysate and assayed as in [18]. Units of MSK1 activity are defined in [18]. The open and closed bars show cells stably transfected with WT-SAPK2a/p38 and DR-SAPK2a/p38, respectively. The data shown are the mean ± S.E.M. for three separate ponasterone A inductions. All assays were carried out in triplicate.

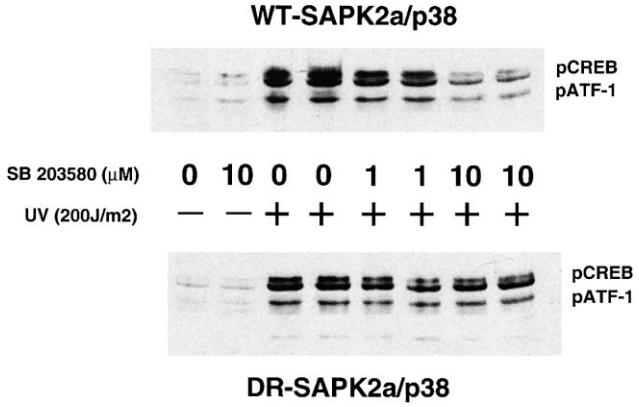

Fig. 5. SB 203580 no longer blocks the phosphorylation of CREB at Ser-133 or ATF1 at Ser-63 in 293 cells expressing DR-SAPK2a/p38. The experiment was carried out as in Fig. 3, except that the phosphorylation of CREB and ATF1 was visualised by immunoblotting using SDS-extracted nuclear extracts and a phospho-specific antibody that recognises Ser-133-phosphorylated CREB and Ser-63-phosphorylated ATF1. 10 μg of nuclear lysate protein was loaded per gel lane and the data shown are from one experiment performed in duplicate. Similar results were obtained in several other experiments.

3.5. The activation of c-Raf by SB 203580 is not mediated by inhibition of SAPK2a/p38

The proto-oncogene c-Raf possesses a threonine residue at the position equivalent to Thr-106 of SAPK2a/p38 and, for this reason, is inhibited by SB 203580. The IC_{50} in vitro (2 μM when assayed at 0.1 mM ATP) is 40-fold higher than for human SAPK2a/p38 assayed under the same conditions [8]. Remarkably, however, incubation of mammalian cells with

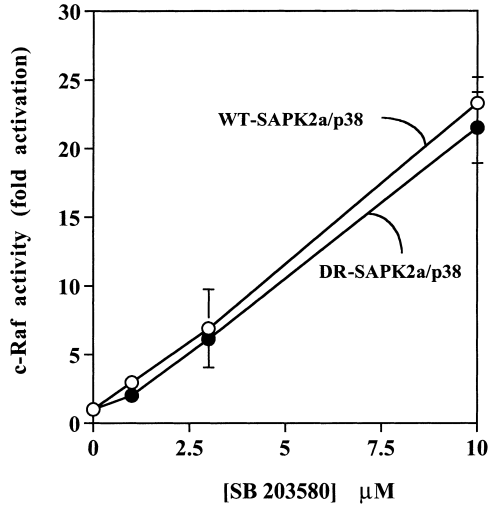

Fig. 6. SB 203580 activates c-Raf in cells expressing either WT-SAPK2a/p38 or DR-SAPK2a/p38. Cells were serum-starved for 23 h in the presence of 10 μM ponasterone A, then incubated for 30 min with the indicated concentrations of SB 203580 followed by a 30 min incubation with 10 μg/ml anisomycin to activate MAPKAP-K2. Anisomycin does not induce any activation of c-Raf. The cells were lysed, c-Raf immunoprecipitated from 0.1 mg lysate protein and assayed as in [25]. The open circles show c-Raf activation in cells overexpressing WT-SAPK2a/p38 and the closed circles show Raf activation in cells overexpressing DR-SAPK2a/p38. The results are presented as fold activation relative to control cells incubated in the absence of SB 203580 and are shown ± S.E.M. for two dishes of cells each assayed in triplicate. Similar results were obtained in two separate experiments.

SB 203580 triggers a 25-fold activation of c-Raf when it is assayed after immunoprecipitation from cell lysates in the absence of SB 203580 [8,9]. The results presented in Fig. 6 demonstrate that the activation of c-Raf triggered by SB 203580 does not result from inhibition of SAPK2a/p38, because the same activation still occurs in cells overexpressing DR-SAPK2a/p38. In contrast, the anisomycin-induced activation of MAPKAP-K2 in the same cell lysates was not suppressed significantly by any concentration of SB 203580 in the cells overexpressing DR-SAPK2a/p38 (data not shown).

4. Discussion

Although SB 203580 is one of the most specific protein kinase inhibitors to have been developed, it is not quite as specific as previously supposed. Nearly all human protein kinases are insensitive to SB 203580, because they possess a side chain larger than threonine at the position equivalent to Thr-106. A few other protein kinases possess threonine or serine at this position (although none have glycine or alanine). Those that have been tested (the type-I and type-II TGFβ receptors, the tyrosine kinase lck and the proto-oncogene c-Raf) are all inhibited by SB 203580, albeit more weakly than SAPK2a/p38 [8,12]. Inhibition of the TGFβ receptors is caused by the presence of Ser and Thr at the relevant position, because their mutation to Met abolishes sensitivity to SB 203580 [12]. In our standard in vitro assay, conducted at 0.1 mM ATP, the type-I and type-II TGFβ receptors and lck are inhibited by SB 203580 with IC_{50} values of 20, 40 and 25 μM, respectively, 400–800-fold higher than the IC_{50} for human SAPK2a/p38. Since inhibition by SB 203580 is competitive with respect to ATP, it is most unlikely that (at 10 μM) this drug will inhibit these protein kinases in the intracellular milieu, where mM concentrations of ATP are present.

In contrast to the TGFβ receptors and lck, c-Raf is inhibited by SB 203580 with an IC_{50} of 2 μM in vitro [8], only 40-fold higher than SAPK2a/p38 and only 4-fold higher than SAPK2b/p38β2 [12]. However, paradoxically, 10 μM SB 203580 induces a huge (25-fold) activation of c-Raf (measured in the absence of SB 203580) [8,9]. These observations suggest that cells contain a feedback loop by which Raf suppresses its own activity, perhaps explaining why SB 203580 does not affect signalling through the classical growth factor-stimulated MAP kinase cascade in cells. Alternatively, and in contrast to current thinking, c-Raf activity may not be rate limiting for activation of the Ras-MAP kinase cascade. These unexpected findings are fortuitous, otherwise, SB 203580 would have little use as a specific signalling inhibitor!

It has also been reported that two isoforms of SAPK1/JNK, another MAP kinase family member, show some sensitivity to SB 203580, even though they contain a large residue (Met) at the position equivalent to Thr-106 of SAPK2a/p38 [22]. In our standard in vitro assay, SAPK1a/JNK2α2 and SAPK1b/JNK3α1 were inhibited by SB 203580 with IC_{50} values of 20–40 μM and SAPK1b/JNK3α2 with an IC_{50} value of 3 μM. These are 50–500-fold higher than the IC_{50} for SAPK2a/p38 measured under the same conditions. Moreover, in the fibroblast cell lines that we have studied [23,24] and in 293 cells (P.A.E. unpublished work), the phosphorylation of c-Jun and ATF2, two putative SAPK1/JNK substrates, are unaffected by exposure of the cells to 10 μM SB 203580.

It is clear from the studies cited above that it would be a great advantage to know whether a reported effect of SB 203580 is mediated via inhibition of SAPK2a/p38 or another enzyme. After all, other protein kinases and enzymes may exist that are potently inhibited by SB 203580. In this paper, we introduce a simple method for establishing whether the cellular effects of SB 203580 are mediated by inhibition of SAPK2a/p38 and have exploited this to prove that MAPKAP-K2 and MSK1, and their presumed substrates HSP27 and CREB/ATF1, lie 'downstream' of SAPK2a/p38. In contrast, the activation of c-Raf induced by SB 203580 is independent of the inhibition of SAPK2a/p38 and presumably caused by the binding of SB 203580 to c-Raf itself. Our results also imply that none of these four proteins are specific substrates for the closely related SAPK2b/p38β2, the only other MAP kinase family member inhibited by SB 203580. Otherwise, the overexpression of DR-SAPK2a/p38 would not have affected the sensitivity of these four proteins to SB 203580. Although DR-SAPK2a/p38 was overexpressed compared to the endogenous enzyme, no activation occurred until it was exposed to a cell damaging agent and, even then, the activity of a downstream reporter (MAPKAP-K2) was only increased to the same extent as in uninduced cells. To our knowledge, this is the first time that a drug-insensitive protein kinase (or perhaps any enzyme) has been used in this way for the purpose of drug target validation and this approach should be generally applicable to the study of other protein kinases. In the future, it might be possible to improve this method by generating mice in which a drug-insensitive protein kinase replaces the WT enzyme so that possible interference caused by the presence of the endogenous WT enzyme is eliminated. Adenoviral vectors and other methods with the potential for quantitative transfection of mammalian cells could be exploited to express drug-insensitive protein kinases in primary cells and tissues, as opposed to just transformed cell lines.

The methodology described in this paper could also be exploited in a further way to help elucidate the physiological roles of other MAP kinase family members for which suitable inhibitors have not yet been identified. Thus, we and others have been able to confer SB 203580-sensitivity to other MAP kinases by mutation of the relevant residue from Met/Gln to Thr or a smaller residue [10,12–14]. Indeed, mutation to Ala converts SAPK1/JNK1, SAPK3/p38γ and SAPK4/p38δ to forms that are as sensitive or even more sensitive to SB 203580 than human WT-SAPK2a/p38 [12]. In principle, it should be possible, using the system described here, to inducibly express these mutant enzymes in mammalian cells and to identify their physiological substrates by finding which protein phosphorylation events are suppressed by SB 203580 that were previously resistant to this drug. In this way, SB 203580 could be used to elucidate the physiological roles of several MAP kinase family members.

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