c-Myc potentiates the mitochondrial pathway of apoptosis by acting upstream of apoptosis signal-regulating kinase 1 (Ask1) in the p38 signalling cascade

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Cell transformation by growth-promoting oncoproteins renders cells extremely sensitive to apoptosis through an unknown mechanism affecting the mitochondrial pathway of apoptosis. We have shown previously that sensitization to apoptosis also correlated with the activation of the stress-activated protein kinase p38. In the present study, we investigated the role of p38 in c-Myc-dependent apoptosis induced by the anticancer agent cisplatin. Cisplatin treatment of Rat1 cells with deregulated expression of c-Myc resulted in nuclear fragmentation that was accompanied in all cells by the activation of Bax and the translocation of cytochrome *c* from the mitochondria to the cytoplasm. None of these features of apoptosis was induced in control Rat-1 cells. p38 was also activated by cisplatin only in cells with deregulated expression of c-Myc, but, in contrast with all features of apoptosis, this activation was not affected by Bcl-2. Remarkably, overexpression of an interfering mutant of the p38 α

isoform, but not p38 β , blocked cisplatin-induced Bax activation or cytochrome *c* release and nuclear fragmentation. Analysis of the kinase cascade upstream of p38 revealed a c-Myc-dependent activation by cisplatin of mitogen-activated protein kinase kinase (MKK) 3/6 and apoptosis signal-regulating kinase 1 (Ask1). Inhibition of Ask1 blocked p38 activation by cisplatin and all features of apoptosis. Several of these data were confirmed using other DNA-damaging agents. The findings indicated that c-Myc potentiation of the mitochondrial pathway of apoptosis results, at least in part, from a sensitization of Ask1 activation, allowing DNA-damaging agents to induce in cascade Ask1, p38 α and Bax.

Key words: Bax, Bcl-2, cytochrome *c*, mitogen-activated protein kinase kinase 3/6 (MKK3/6).

INTRODUCTION

As cell growth and apoptosis are tightly co-regulated processes, oncogenic transformation is often associated with the joint dysregulation of proteins involved in growth promotion and death regulation [1,2]. The dual growth- and apoptosis-promoting activities of the c-Myc oncoprotein are well documented [3,4]. Cell proliferation in the presence of deregulated c-Myc expression requires the presence of growth factors that provide apoptosis-inhibiting surviving signals [5]. In the absence of growth factors or under *in vivo* conditions, c-Myc-dependent tumour development does not occur unless additional molecular modifications are acquired to prevent apoptosis triggering or execution [4,6,7].

One of the most generally recognized features of apoptosis is the activation of caspases, which cause cell death by selectively cleaving several intracellular key substrates, leading to cellular disintegration. Caspases can be activated via the death receptor pathways involving the CD95/tumour-necrosis-factor-related apoptosis-inducing ligand ('TRAIL')/tumour-necrosis-factor-receptor family which, upon activation, trigger the activation in the cascade of caspase 8 and caspase 3 [8]. Caspase 3 can also be activated through the mitochondrial pathway [9]. In that case, caspases are activated following the release of cytochrome *c* from the mitochondria into the cytoplasm, where it catalyses the formation of the apoptotic-protease-activating

factor-1 ('APAF-1') apoptosome, thereby leading to the activation in cascade of caspase 9 and caspase 3 [10]. Anti-apoptotic members of the Bcl-2 protein family, such as Bcl-2 and Bcl-X_L, inhibit the release of cytochrome *c* from the mitochondria [11]. Conversely, pro-apoptotic members of this family, such as Bax or Bak, promote the release of cytochrome *c* [12,13]. In unstimulated cells, Bax is generally found distributed diffusely throughout the cytoplasm, whereas upon activation it changes conformation and associates with mitochondria, a process which can be inhibited by Bcl-2 family members [13–16].

There is mounting evidence that c-Myc promotes apoptosis by sensitizing the activation of the mitochondrial pathway [17]. In the absence of serum or pro-survival factors, such as insulin-like growth factor ('IGF')-1, that can neutralize endogenous death signals, or in the presence of pro-apoptotic factors such as X-rays, Fas ligand or diverse toxic agents, including etoposide, taxol or doxorubicin, c-Myc allows apoptosis induction by promoting the release of cytochrome *c* from the mitochondria, an effect that can be inhibited by Bcl-2 [6,17,18]. However, the nature of the signalling pathway affected by c-Myc transformation and which mediates or amplifies the death signal at the mitochondrial level is unclear.

The stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 are activated in many cell types by virtually all stressing stimuli, including cancer chemotherapeutic drugs,

Abbreviations used: Ask1, apoptosis signal-regulating kinase 1; BH, Bcl-2 homology; DAPI, 4-6-diamidino-2-phenylindole; D-JNK11, JNK peptide inhibitor 1D-stereoisomer; DTT, dithiothreitol; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; MKK, MAP kinase kinase; OHT, 4-hydroxytamoxifen; TBS-T, Tris-buffered saline containing 0.1% Tween 20.

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osmotic shock, heat shock or UV irradiation, and are thought to play an important role in signalling apoptosis [19,20]. We reported recently [21] that activation of p38 by many of these toxic agents was also dependent on the deregulated expression of c-Myc and played an important role in the pro-apoptotic effect of c-Myc. In the present study, we examined further the interplay between c-Myc-dependent apoptosis and p38 activation using c-Myc-transformed Rat-1 cells exposed to cisplatin and other anticancer agents. The results indicated that c-Myc transformation mediates an essential step required for p38 activation upstream of the p38 activator, the mitogen-activated protein (MAP) kinase kinase ('MAPKKK') apoptosis signal-regulating kinase 1 (Ask1), and that this activation of p38 is, in turn, required for the efficient activation of Bax and the initiation of mitochondrial apoptosis.

MATERIALS AND METHODS

Reagents

[γ - 32 P]ATP (3000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.). Cisplatin, etoposide, doxorubicin, daunorubicin, SB203580, 4,6-diamidino-2-phenylindole (DAPI), 4-hydroxytamoxifen (OHT), H₂O₂ and streptolysin O were from Sigma Chemicals (St Louis, MO, U.S.A.). JNK peptide inhibitor 1 D-stereoisomer (D-JNKI1) was from Alexis Biochemicals (San Diego, CA, U.S.A.). The electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, U.S.A.) and Fisher Scientific (Pittsburgh, PA, U.S.A.).

Antibodies

The anti-Bax (clone N-20) and anti-Bcl-2 (clone C-2) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-Bax antibody was raised against sequences in the N-terminus of Bax, which are located in a domain that is accessible only when Bax is activated [22]. The mouse monoclonal anti-cytochrome *c* antibody 6H2.B4 was used for immunofluorescence and clone 7H8.2C12 for Western-blot analysis. Both antibodies were from BD PharMingen (Mississauga, ON, Canada). The rabbit antibodies against the phosphorylated form of p38, phosphorylated c-jun, phosphorylated MAP kinase kinase (MKK) 3/6 and cleaved caspase-3 were all obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). The anti-haemagglutinin (HA) antibody HA.11 was from Babco (Richmond, CA, U.S.A.). The anti-(mouse Alexa 594) and the anti-(rabbit Alexa 488) were from Molecular Probes (Eugene, OR, U.S.A.). The horseradish peroxidase-conjugated anti-(mouse IgG) and anti-(rabbit IgG) antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). The anti-p38 α antibody is a rabbit polyclonal antibody raised against the C-terminal sequence PPLQEEMES (where single-letter amino-acid notation has been used) of murine p38 α [23]. The rabbit anti-p38 β antibody was developed for this study against the C-terminal sequence EPPKPPGSLEIEQ of human p38 β . Anti-Ask1 antibody is a rabbit polyclonal antibody and was raised against the C-terminal sequence KALLDFRNKQT of human Ask1 [24].

Plasmids

The plasmid used to express green fluorescent protein (GFP) (pEGFP) was from Clontech (Palo Alto, CA, U.S.A.). Bcl-2-GFP and Bcl-2-HA were expressed using the plasmids

pEGFP-C3-hBcl2 and pcDNA3-hBcl2-Ha respectively [15]. p38 α -glutathione S-transferase (GST) was expressed using pEBG-p38 [25]. pcDNA3-Ha-Ask1 and pcDNA3-Ha-ASK-KM were used to express wild-type Ask1 and a kinase-inactive variant of Ask1 respectively [26]. A dominant-negative form of p38 β (also called p38 β 2 [27,28]) was expressed using pcDNA3-p38 β -AGF (where AGF refers to the changed amino acid composition of the p38 phosphorylation site motif TGY) [27]. A Flag-tagged (where Flag is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) version of the dominant negative form of p38 α (pCMV-Flag-p38AGF) [27] was recloned by PCR to obtain a HA-tagged version of p38 α -AGF (pCMV-HA-p38 α AGF).

Cell culture and cell line

Rat-1 MycERTM cells express a fusion protein of c-Myc and a modified oestrogen receptor (ERTM), so that c-Myc is activated in the presence of OHT. Cells expressing a non-functional truncated form of c-Myc fused to ERTM (Rat-1 Δ MycERTM) were used as a control [29]. The cells were cultured in α -modified Eagle's medium containing NaHCO₃ (2.2 g/l) and supplemented with 10 % (v/v) fetal bovine serum. For stock maintenance, the selection was maintained with puromycin (5 μ g/ml), and the selection was removed at least 24 h before the experiment. c-Myc was activated by adding OHT to the medium 16 h before the experiment to obtain a final concentration of 100 nM. HeLa/p38 α (AGF) cells, expressing a non-activatable/non-phosphorylatable mutant form of p38 α , and their parental cell line HeLa/HIVcat [30] were cultured in Dulbecco's modified Eagle's medium containing NaHCO₃ (2.2 g/l) and glucose (4.5 g/l) and supplemented with 10 % fetal bovine serum. Selection was maintained in stock cultures by adding G418 (200 μ g/ml) and hygromycin (200 μ g/ml). All cell lines were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂.

Immunocytochemistry

After the treatments, the cells were washed twice with PBS and fixed in 3.7 % (v/v) formaldehyde in PBS for 15 min. The cells were incubated for 1 h at room temperature in blocking buffer [0.1 % saponin and 3 % (w/v) BSA in PBS] and incubated for 1 h at 37 °C either with anti-Bax (1:150), anti-cytochrome *c* (1:100), anti-p38 β (1:300) or anti-HA (1:300) antibodies in blocking buffer. The cells were washed three times in blocking buffer and incubated for 1 h at 37 °C in blocking buffer containing DAPI (2.5 μ g/ml) to stain nuclei and anti-(mouse IgG Alexa 594) (1:2500) and/or anti-(rabbit IgG Alexa 488) (1:2000) antibodies. The observations were made with a Nikon Eclipse E600 upright microscope (Tokyo, Japan) equipped with a 60 $\times$ 0.85 numerical aperture objective by counting at least 300 different cells in 15 random microscopic fields/dish. Experiments were carried out in triplicate and were repeated at least three times. In many cases, independent counts were obtained from two investigators. Images were captured as 16 bit TIFF files with a Micromax 130 YHS cooled (−30 °C) charged-coupled device camera (Princeton Instruments, Trenton, NJ, U.S.A.) driven by Metamorph software version 4.5 (Universal Imaging Corp., Downingtown, PA, U.S.A.). Images were subsequently analysed with Metamorph. For staining mitochondria, cells were seeded on to glass slides. After the appropriate treatment, they were incubated with MitoTracker Red CMXRos (10^{−1} M) for 10 min and then fixed and stained as described above. Confocal images were taken with a MRC-1024 confocal system (Bio-Rad) on a Diaphot inverted microscope (Nikon) and captured with a Laser Sharp software (Bio-Rad).

Cellular fractionation

Cells were seeded at a density of 1.2×10^6 cells in 10-cm dishes. OHT was added at least 4 h later for 16 h. After treatments, the floating cells were collected by centrifugation at 250 *g*, resuspended in 100 μ l of ice-cold PBS and combined with the attached cells after they were washed twice with ice-cold PBS and scraped off the culture dishes into 200 μ l of PBS. The cells were centrifuged at 250 *g* for 5 min at 4 °C, resuspended in 50 μ l of stabilization buffer [20 mM Hepes (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT) and the Complete™ protease inhibitor cocktail at the concentration recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany)] and incubated with 200 units of streptolysin O at 37 °C for 30 min. Cytosolic and particulate fractions were obtained by centrifugation at 1500 *g* for 30 min at 4 °C, diluted with sample buffer and boiled for 5 min before electrophoresis.

Western-blot analysis

Cells were seeded at 0.5×10^6 cells in 6-cm dishes. OHT was added at least 4 h later for 16 h. After treatment, the cells were washed twice with ice-cold PBS and scraped off culture dishes in lysis buffer. The cell extracts were boiled 5 min, separated by SDS/PAGE and blotted on to a nitrocellulose membrane. The membranes were blocked with 5 % (w/v) non-fat dried milk powder in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and incubated with the appropriate antibody. The anti-(phospho-p38), anti-(phospho-c-jun) and anti-(phospho-MKK3/6) antibodies (all at a 1:1000 dilution) were diluted in 5 % (w/v) BSA in TBS-T and incubated overnight at 4 °C. The anti-Bcl-2 (1:100), anti-Bax (1:1000), anti-HA (1:3000) and anti-p38 (1:1000) antibodies were diluted in 5 % (w/v) non-fat dried-milk powder in TBS-T and incubated for 1 h at room temperature. The horseradish peroxidase-conjugated anti-rabbit (1:20 000) and anti-mouse (1:10 000) antibodies were incubated for 1 h at room temperature in 5 % (w/v) milk powder in TBS-T. Peroxidase activity was visualized with the Supersignal Chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

Transient transfection

For transfection, the cells were seeded at a density of 2.5×10^4 cells/3.5 cm-diameter dish and exposed 24 h later to a mixture of 0.5 μ g of the plasmid and 2.5 μ l of the GenePorterII lipofection reagent (Gene Therapy Systems, San Diego, CA, U.S.A.). In co-transfection studies, the cells were seeded at 6×10^4 cells/6 cm-diameter dish and transfected with a total of 1 μ g DNA mixed with 5 μ l of the GenePorterII lipofection reagent or by the calcium-phosphate method as described previously [31]. The OHT was added 24 h after transfection and the cells were used another 16 h later.

Kinase assay

After stimulation, the cells were scraped off the dishes in lysis buffer [20 mM Tris/HCl (pH 7.5), 12 mM β -glycerophosphate, 150 mM NaCl, 5 mM EGTA, 1 mM Na₃VO₄, 10 mM NaF, 1 % (v/v) Triton X-100, 3 mM DTT, 0.5 % deoxycholate sodium salt and 1 mM PMSF]. After vortexing, the extracts were centrifuged at 17 000 *g* for 10 min at 4 °C. The supernatants were incubated on ice with 5 μ l of anti-Ask1 antibody. After 1 h, Protein A-Sepharose [50 % (v/v) in lysis buffer; Amersham Biosciences]

was added for another 30 min. The samples were centrifuged for 15 s and washed twice with 500 μ l of wash buffer A [20 mM Tris/HCl (pH 7.5), 500 mM NaCl, 5 mM EGTA, 1 % Triton, 2 mM DTT and 1 mM PMSF] and twice with 500 μ l of wash buffer B [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 2 mM DTT and 1 mM PMSF]. The immunoprecipitates were used directly for kinase assays. Immunoprecipitated Ask1 was assayed using myelin basic protein (MBP) as substrate (5 μ g/sample). The assays were done in 20 μ l of kinase reaction buffer {20 mM Tris/HCl (pH 7.5), 30 mM MgCl₂, 150 μ M ATP and 5 μ Ci [γ -³²P]ATP}. The kinase activity was assayed for 12 min at 30 °C, stopped by boiling in SDS sample buffer and quantified by measuring the incorporation of radioactivity into the substrate by PhosphorImager analysis after electrophoresis.

Statistical analysis

Statistical analysis was performed on the means of the triplicates of at least three independent experiments using a two-tailed Student's *t* test.

RESULTS

MycER™ and Δ MycER™ cells express a fusion protein between c-Myc or Δ Myc (a transcriptionally inactive mutant of c-Myc) and a modified oestrogen receptor that responds to the synthetic ligand OHT. Addition of OHT to MycER™ cells, but not Δ MycER™ cells, generates physiological levels of deregulated c-Myc transcriptional activity [29]. MycER™ and Δ MycER™ cells were treated with OHT for 16 h in the presence of serum and then exposed to cisplatin. The functional activation of c-Myc by OHT rendered the cells extremely sensitive to the induction of apoptosis by cisplatin. In MycER™ cells, but not in Δ MycER™ cells, the number of cells showing nuclear fragmentation started to accumulate after approx. 4 h of treatment and peaked after 6 h (Figure 1A). Activation of p38 correlated perfectly with apoptosis and was also observed only in MycER™ cells, but not in Δ MycER™ cells (Figure 1B). Consistent with a causal role of p38 in the activation of the apoptotic process downstream of c-Myc, inhibition of p38 activity by treating the cells with the p38 α and p38 β inhibitor SB203580 prior to and during cisplatin exposure decreased the number of cells with a fragmented nucleus by approx. 60 % (Figure 1A). Similar results were obtained with other anticancer drugs, such as doxorubicin or daunorubicin, suggesting that this effect is not restricted to cisplatin (Figures 1B and 1C). At the concentration of SB203580 used in the present study, the *in vitro* phosphorylation of heat-shock protein 27 ('Hsp27'), a downstream target of p38 activity, was totally inhibited [21]. Higher concentrations of SB203580 were no more efficient in blocking apoptosis (results not shown).

Nuclear fragmentation induced by cisplatin in MycER™ cells was accompanied by caspase activation and inhibited by a pan-caspase inhibitor (results not shown). Staining with an antibody against the activated form of caspase 3 showed that inhibition of p38 with SB203580 blocked the activation of caspase 3 in 60 % of the cells, in the same cells in which nuclear fragmentation was also blocked (Figures 2A and 2C). The results indicated that p38 acts upstream of caspase activation in the apoptotic pathway potentiated by c-Myc. To determine whether apoptosis induced by cisplatin involved the mitochondrial pathway, we analysed the cells for the release of cytochrome *c* from the mitochondria by immunofluorescence microscopy. In control cells, cytochrome *c* staining showed a threadlike pattern, consistent with a mitochondrial staining. Cisplatin induced the

Figure 1 Inhibition of p38 activity antagonizes cisplatin-induced nuclear fragmentation

(A) OHT-treated MycERTM cells (squares) and ΔMycERTM cells (circles) were exposed to cisplatin (50 μM) for different time periods in the absence (closed symbol) or presence (open symbol) of SB203580 (5 μM). The percentage number of cells showing nuclear fragmentation was determined microscopically after DAPI staining. Data are means ± S.D. of four independent experiments. (B) OHT-treated MycERTM (myc) and ΔMycERTM (Δmyc) cells were treated for up to 6 h with cisplatin (50 μM; left panel) or for 3 h with solvent (CTL), etoposide (10 μg/ml; VP-16), doxorubicin (1 μM; Doxo) or daunorubicin (1 μM; Dauno). Total cellular extracts (20 μg) were analysed by Western blot for the expression of the phosphorylated form of p38 by using a phospho-specific antibody. A representative experiment of at least two is shown. (C) OHT-treated MycERTM cells were exposed to doxorubicin (1 μM; Doxo) or daunorubicin (1 μM; Dauno) for 6 h in the absence (open bars) or presence (closed bars) of SB203580 (5 μM). The percentage number of cells showing nuclear fragmentation was determined microscopically after DAPI staining. Data are means ± S.D. of three independent experiments. **P* < 0.05, ****P* < 0.001 for cells treated in the absence compared with cells treated in the presence of SB203580.

release of cytochrome *c* from the mitochondria into the cytoplasm only in MycERTM cells and with kinetics that parallel the appearance of fragmented nuclei (Figure 2B). A slight delay in nuclear fragmentation was apparent at 4 h, consistent with the fact that nuclear fragmentation occurs subsequent to cytochrome *c* release. At 6 h, the activation of caspase 3, the release of cytochrome *c* and the fragmentation of the nucleus occurred coincidentally in the same cells in more than 98% of the cases (Figure 2C). In the presence of SB203580, the number of cells with cytochrome *c* release was decreased to the same level as found for caspase 3 activity and nuclear fragmentation. Furthermore, inhibition of the three end-points occurred in the same cells. Hence, activation of p38 in response to cisplatin played a role upstream of cytochrome *c* release and caspase 3 activation.

We next determined the possibility that p38 might affect the function of Bax or Bcl-2. Since a modulation of the expression

Figure 2 Inhibition of p38 activity antagonizes cisplatin-induced caspase-3 activation and cytochrome *c* release

(A) OHT-treated MycERTM cells were exposed to cisplatin (50 μM) for 6 h in the absence (Cis) or presence (Cis+SB) of SB203580 (5 μM). The percentage number of cells with activated caspase 3 was determined by immunofluorescence using an antibody directed against the activated form of caspase 3. Data are means ± S.D. of three independent experiments. (B) OHT-treated MycERTM cells (squares) and ΔMycERTM cells (circles) were exposed to cisplatin (50 μM) for different time periods in the absence (closed symbol) or presence (open symbol) of SB203580 (5 μM). The percentage number of cells with cytochrome *c* released into the cytoplasm was determined by immunofluorescence after staining with an anti-cytochrome *c* antibody. Data represent means ± S.D. of four independent experiments. (C) OHT-treated MycERTM cells were exposed to cisplatin (50 μM) for 6 h, fixed and double stained with anti-(activated caspase 3) and -cytochrome *c* antibodies, and the nuclei were stained with DAPI. Arrowheads indicate cells with activated caspase 3, cytoplasmic cytochrome *c* and nuclear fragmentation. ***P* < 0.01 for cisplatin compared with cisplatin + SB203580 treatment.

of Bax by c-Myc has been reported previously [32], we first analysed the level of expression of Bax by Western blotting. No change in Bax protein levels were detected as a function of the c-Myc status of the cells (MycERTM cells compared with ΔMycERTM cells) or between control and cisplatin-treated cells (Figure 3A). To determine whether Bax was involved in cisplatin-induced Myc-dependent apoptosis, we analysed its cellular localization and activity by immunofluorescence microscopy using a conformation-sensitive antibody as a function of c-Myc expression and cisplatin treatment. Immunofluorescence microscopy revealed a very light staining of Bax in the cytoplasm of sham-treated MycERTM cells and of both sham- and cisplatin-treated ΔMycERTM cells (Figure 3C, and results not shown). As expected for a treatment that induces a change in the

Figure 3 c-Myc- and p38-dependent activation of Bax and release of cytochrome *c*

(A) OHT-treated MycERTM cells (myc) or ΔMycERTM cells (Δmyc) were exposed for up to 6 h as indicated to cisplatin (50 μM). Total cellular extracts were prepared, separated by electrophoresis (30 μg/lane) and analysed by Western blot to determine Bax protein expression. (B) OHT-treated MycERTM cells (squares) and ΔMycERTM cells (circles) were exposed to cisplatin (50 μM) for different time periods in the absence (closed symbol) or presence (open symbol) of SB203580 (5 μM). After fixing and immunostaining for Bax, the percentage number of cells showing translocation of Bax from the cytoplasm to the mitochondria was determined microscopically as described in the Materials and methods section. Data are means ± S.D. of four independent experiments. (C) MycERTM (myc) or ΔMycERTM cells (Δmyc) were left untreated (CTL) or exposed to cisplatin (Cis) as in (B), fixed after 6 h and then processed for immunofluorescence staining of Bax and cytochrome *c* and nuclear staining with DAPI. Pictures on one line are of the same field. Arrowheads indicate cells with activated Bax, cytoplasmic cytochrome *c* and nuclear fragmentation. (D) MycERTM cells were treated with cisplatin (50 μM) for 6 h and stained with MitoTracker and an anti-Bax antibody. Pictures were taken by confocal imaging. The shared pixels were obtained by logical AND arithmetic analysis and indicate coincidence of the pixels turned on in both the MitoTracker and the Bax images (indicated by the arrowhead). (E, F) OHT-treated MycERTM cells (myc) or ΔMycERTM cells (Δmyc) were exposed to cisplatin (50 μM) for 0, 4 or 6 h as indicated. Cell lysates were centrifuged to obtain cytosolic (cyto) and particulate (pellet) fractions as described in the Materials and methods section. The cytosolic fraction (30 μg) and the pellet fraction (20 μg) were analysed by immunoblotting for Bax (E) and cytochrome *c* (F). A representative experiment of at least two is shown (A, C–F). ***P* < 0.01 for treatment with cisplatin compared with cisplatin + SB203580.

conformation/activity of Bax, a very strong signal was detected in approx. 30% of the MycERTM cells after 6 h of exposure to cisplatin, which was co-localized with the mitochondria, as revealed by double staining with the Bax antibody and the mitochondrion-selective stain MitoTracker (Figure 3D). Virtually all cells in which Bax was activated also showed cytochrome *c* release and nuclear fragmentation (Figure 3C).

The translocation of Bax to the mitochondria was confirmed by analysing the presence of Bax in cytosolic (soluble) and membrane (pellet) fractions prepared after treatment with cisplatin. In the pellet fraction containing the mitochondria, we found a clear increase in Bax protein content after cisplatin treatment in MycERTM cells, whereas we did not detect any increase of Bax in the pellet of ΔMycERTM cells (Figure 3E).

Figure 4 Bcl-2 blocks cisplatin-induced c-Myc-dependent apoptosis, but not p38-activation

(A) OHT-treated MycERTM cells (myc) and Δ MycERTM cells (Δ myc) were exposed to cisplatin (50 μ M) for up to 6 h as indicated. Total cellular extracts (30 μ g/lane) were analysed by Western blot to determine Bcl-2 protein expression. (B) MycERTM cells were transfected with pEGFP-C3-hBcl2 (Bcl2-GFP) or with pEGFP (GFP). After incubation for 24 h, the cells were exposed for 16 h to OHT, followed by treatment in the absence (open bars) or presence of cisplatin for 6 h (50 μ M; closed bars). Cells were fixed and stained with DAPI. Nuclear fragmentation was analysed microscopically only in the GFP-positive cells as described in Materials and methods section. Data are means $\pm$ S.D. of four independent experiments. (C) MycERTM cells were transfected and treated as in (B), and immunostained for Bax and stained with DAPI. Arrowheads indicate two Bcl-2-GFP-positive cells. (D) MycERTM cells were transfected with pEBG-p38 and pcDNA3-hBcl2-Ha to express p38-GST and HA-tagged Bcl-2, with pEBG-p38 and an empty vector to express p38-GST alone, or with the empty vector only. After treatment with OHT for 16 h, the cells were treated in the absence (–) or presence (+) of cisplatin for 3 h (50 μ M), cellular extracts were prepared and analysed by Western blot with an antibody against phosphorylated p38 to detect phosphorylated p38-GST, an anti-p38 α antibody to determine the level of total p38-GST, or an anti-Ha antibody to detect Ha-tagged Bcl-2 (Ha). A representative experiment of at least two is shown (A, C, D). *** P < 0.001 for GFP compared with Bcl-2-GFP after cisplatin treatment.

This suggested that cisplatin-induced Bax translocation to the mitochondria was also strictly c-Myc-dependent. As a control, the same cellular extracts were analysed for the presence of cytochrome *c*. Cytochrome *c* appeared in the cytosolic fraction starting at 4 h after cisplatin treatment in MycERTM cells, which corresponded to a depletion of cytochrome *c* in the pellet. No detectable translocation of cytochrome *c* was induced in Δ MycERTM cells (Figure 3F). Therefore the results suggested that cisplatin-induced conformational change in Bax and its translocation to the mitochondria were c-Myc-dependent. Co-

staining of the cells for Bax, cytochrome *c* and with DAPI revealed a perfect correlation in 98% of the cells between the presence of activated Bax, cytoplasmic cytochrome *c* and nuclear fragmentation (Figure 3C). Accordingly, the kinetics for the increase in the number of cells with activated Bax was nearly identical with that measured for the other end-points of apoptosis (Figure 3B).

To test whether p38 activity was involved in Bax activation, cells were pre-exposed to 5 μ M SB203580 for 1 h prior to and during cisplatin treatment. SB203580 treatment caused a 60% decrease in the number of cells with activated Bax (Figure 3B). In these cells, cytochrome *c* translocation and nuclear fragmentation were also inhibited (results not shown). Thus p38 activation, which is sensitized by c-Myc transformation, induces a signal at the level or upstream of Bax, which leads to Bax activation and cytochrome *c* release. Consistent with a role of p38 upstream of Bax, we found that activation of Bax by overexpression induced apoptosis independently of p38 activity (results not shown).

As already shown for Bax, Western-blot analyses revealed no effect on Bcl-2 expression of either cisplatin treatment or c-Myc expression (Figure 4A). Nevertheless, Bcl-2 could, as expected, block apoptosis. We transfected cells with Bcl-2-GFP and examined the induction of apoptosis by cisplatin in these cells. Immunofluorescence microscopy revealed that nearly all cells expressing Bcl-2 were protected from cisplatin-induced nuclear fragmentation (Figures 4B and 4C), cytochrome *c* release (results not shown) and Bax activation (Figure 4C), whereas no protection was observed in cells transfected with GFP only. Therefore both Bcl-2 and the inhibitor of p38 blocked apoptosis at the level of, or upstream of, Bax. To determine whether Bcl-2 acted upstream of p38, Bcl-2 was co-transfected with p38-GST in MycERTM cells and the level of activation/phosphorylation of p38-GST was measured after treatment with cisplatin. Activation of p38-GST was not affected by Bcl-2 expression (Figure 4D), confirming that p38 was activated upstream of the Bcl-2 family members.

SB203580 inhibits two isoforms of p38, namely p38 α and p38 β . To better support the essential role of p38 in drug-induced apoptosis and to determine which isoform of p38 was implicated, MycERTM cells were transfected with plasmids expressing dominant-negative mutants of either p38 α (p38 α -AGF) or p38 β (p38 β -AGF) and then exposed to cisplatin or etoposide. Immunofluorescence microscopy revealed that nearly all cells that were positive for p38 α -AGF were refractory to cisplatin- and etoposide-induced Bax activation (Figure 5B, and results not shown) and nuclear fragmentation (Figures 5A and 5B). In contrast, expression of the dominant-negative mutant of p38 β led by itself to a significant increase in the number of apoptotic cells and, in two out of three experiments, to a slight sensitization towards apoptosis, which, however, was not significant overall. The possibility that the two isoforms of p38 might have opposite effects on apoptosis was evaluated further by investigating the effect of SB203580 on the response of p38 α -AGF-transfected cells to cisplatin. As expected for an opposing action of the two isoforms, adding SB203580 to block p38 β in the p38 α -AGF-expressing cells produced a sensitization, rather than a protection, to cisplatin treatment (Figure 5C). To evaluate the capacity of p38 α -AGF to block apoptosis in different cell lines and species, we compared the response of HeLa cells with HeLa/p38 α (AGF) cells, a clonal derivative that stably expresses the interfering mutant of p38 α [30]. At the dose of cisplatin used, nuclear fragmentation was observed in approx. 30% of control HeLa cells (Figure 5D), of which more than 95% also showed cytochrome *c* release and Bax activation (results not shown). In these cells, all three parameters were inhibited by SB203580 by approx. 30%. As in Rat1 cells, p38 α -AGF was a much more

Figure 5 Inhibition of p38 α , but not p38 β , blocks cisplatin-induced nuclear fragmentation in Rat1 and in HeLa cells

(A) MycERTM cells were transfected with pCMV-HA-p38 α AGF (p38 α AGF) or pcDNA3-p38 β -AGF (p38 β AGF). After 24 h, the cells were exposed for 16 h to OHT followed by a 6 h treatment with cisplatin (50 μ M; black bars), etoposide (10 μ g/ml; grey bars) or left untreated (white bars). Cells were fixed and processed to detect the HA epitope or human p38 β , and were stained with DAPI. The percentage number of cells with a fragmented nucleus was determined by immunofluorescence microscopy in the HA-, p38 β -positive or non-transfected cells. Data are means $\pm$ S.D. of three independent experiments. (B) MycERTM cells were transfected with pCMV-HA-p38 α AGF (Ha-p38 α AGF) and exposed for 16 h to OHT followed by a 6 h treatment with cisplatin (50 μ M). The cells were immunostained for Bax and the HA epitope and the nuclei were stained with DAPI. Pictures were taken of the same field. Arrowheads indicate two Ha-p38 α AGF-positive cells. (C) MycERTM cells were transfected with pCMV-HA-p38 α AGF as in (A) and then exposed to cisplatin (50 μ M) for 6 h in the absence (Cis) or presence (Cis+SB) of SB203580 (5 μ M), or left untreated (CTL). Nuclear fragmentation was evaluated microscopically after DAPI staining. Data are means $\pm$ S.D. of three independent experiments. (D) Control HeLa/HIVcat (HIVCAT) and HeLa/p38 α AGF (p38 α AGF) cells were treated with cisplatin (50 μ M) for 16 h in the absence (white bars) or presence (black bars) of SB203580 (5 μ M). Nuclear fragmentation was evaluated microscopically after DAPI staining. Nuclear fragmentation in untreated cells was less than 3% in both cell lines. Data are means $\pm$ S.D. of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 comparing similarly treated transfected cells with non-transfected cells (A) or as indicated (C, D).

Figure 6 c-Myc-dependent induction of p38, MKK3/6 and Ask1 by cisplatin exposure

OHT-activated MycERTM (myc) and Δ MycERTM (Δ myc) cells were left untreated (CTL) or exposed to 50 μ M cisplatin (Cis) for 3 h, 43.5 $^{\circ}$ C heat shock (HS) for 12 (B) or 20 (A) min (HS) or 0.5 mM H₂O₂ for 1 h. Total cellular extracts (20 μ g) were analysed by Western blot for the expression of the phosphorylated forms of p38 and MKK3/6, using phospho-specific antibodies against p38 and MKK3/6 (A), or processed to immunoprecipitate Ask1 and determine its kinase activity using MBP as substrate (B). Ask1 activity (incorporation of ³²P into MBP) was visualized after electrophoresis by PhosphorImager analysis. A representative experiment of three is shown (A, B).

potent inhibitor of apoptosis than SB203580, apoptosis being inhibited by 75% in the HeLa/p38 α (AGF) cells. Also, as in Rat1 cells, treatment of HeLa/p38 α (AGF) cells with SB203580 prior to and during cisplatin treatment resulted in an augmentation of the apoptotic features, reversing back to the level observed in control cells in the presence of SB203580. The results suggested that p38 β plays an anti-apoptotic role, whereas p38 α is pro-apoptotic.

p38 is activated in a cascade of phosphorylation reactions involving the MAP kinase kinase MKK3/6 and a MAP kinase kinase which, in the case of cisplatin, has been suggested to be Ask1 [33]. Further analyses of the upstream activator of p38 showed that, like p38, MKK3/6 was activated strongly by cisplatin in MycERTM cells, whereas little induction was seen in Δ MycERTM cells (Figure 6A). Well-known activators of p38, such as heat shock and H₂O₂, however, were able to activate MKK3/6 independently of c-Myc expression, suggesting that the capacity of control cells to activate these signalling pathways was not generally disrupted. Analysis of Ask1 activity following cisplatin exposure also revealed a strong activation in MycERTM cells, whereas Δ MycERTM cells did not show any activation after cisplatin treatment. Again, Ask1 was activated in Δ MycERTM cells by heat shock, indicating that Ask1 was activatable in these cells and functional (Figure 6B). To determine whether Ask1 was required for the activation of p38, cells were transfected with p38-GST together with a dominant-negative mutant of Ask1 (Ask-KM). Ask1 has been shown recently [24] to mediate heat-shock-induced activation of p38 and, as a control, we confirmed that expression of Ask-KM inhibited the activation of p38-GST after heat shock. Similarly, Ask-KM blocked the activation of p38-GST by cisplatin to nearly control levels, whereas the phosphorylation of endogenous p38 was normal (Figure 7A). Endogenous p38 was measured as an internal control for the activation of p38 in the cells that did not express any trans-genes. Transfection of MycERTM cells with Ask-KM and subsequent

Figure 7 Activation of p38 via Ask1, but not JNK, is responsible for nuclear fragmentation

(A) MycERTM cells were transfected with pEBG-p38 alone (p38-GST) alone or together with pcDNA3-Ha-ASK-KM (p38-GST+Ask-KM). After 24 h, the cells were exposed for 16 h to OHT and then left untreated (CTL), or treated with 50 μ M cisplatin (Cis) for 3 h or with 43.5 °C heat shock (HS) for 20 min. Cellular extracts were analysed by Western blot using a phospho-specific anti-p38 antibody to detect phosphorylated p38 and p38-GST (left panel). Right panel, to control for protein expression, the same samples from untreated cells (CTL) were analysed for expression of transfected pcDNA3-Ha-ASK-KM (Ask-KM) using the anti-Ha antibody, and transfected p38-GST and endogenous p38 using the anti-p38 antibody. A representative experiment of three is shown. (B) MycERTM cells were transfected with pEGFP (GFP), pcDNA3-Ha-Ask1 (Ask-WT) or pcDNA3-Ha-ASK-KM (Ask-KM). After 24 h, the cells were exposed to OHT for 16 h and treated (black bars) or not (white bars) with 50 μ M cisplatin for 6 h. After fixation and staining, the percent number of GFP- or HA-positive cells showing a fragmented nucleus (DAPI) was determined microscopically. Data are means $\pm$ S.D. of three independent experiments. (C) OHT-treated MycERTM cells were left untreated (CTL) or treated (Cis) with 50 μ M cisplatin for 3 (inset) or 6 h in the absence or presence of 2 μ M D-JNKI1 added 2 h before the beginning of the cisplatin treatment. The cells were either fixed and stained with DAPI to determine the number of cells showing nuclear fragmentation or processed to determine the level of c-jun phosphorylation by Western-blot analysis using an antibody directed against the phosphorylated form of c-jun (inset). Data are means $\pm$ S.D. of three independent experiments. * P < 0.05, Cis+D-JNKI1 compared with Cis; ** P < 0.01, Ask-KM compared with GFP treated with cisplatin.

analysis of nuclear fragmentation after cisplatin treatment showed that inhibition of Ask1 activation completely blocked apoptosis in the transfected cells (Figure 7B), suggesting that the action of c-Myc is at the level or upstream of Ask1.

Ask1 is also an upstream regulator in the JNK signalling pathway and, as expected, JNK was activated in a c-Myc-dependent manner by cisplatin (results not shown). To determine whether JNK contributed to cisplatin-induced apoptosis, we treated the cells with an inhibitor of JNK signalling, D-JNKI1, 2 h prior and during cisplatin treatment. D-JNKI1 is a cell-permeant peptide that is thought to compete with physiological substrates for binding to JNK [34]. As expected, Western-blot analyses with an antibody directed against the phosphorylated form of c-jun revealed lack of phosphorylation of c-jun in cells treated in the presence of D-JNKI1. However, in the same experiment, immunofluorescence analysis revealed that nuclear fragmentation was not inhibited by inhibiting JNK and might even have been slightly increased (Figure 7C), thus suggesting that JNK is not implicated in apoptosis induced by cisplatin in transformed cells, and supporting the important role of the Ask1/p38 axis.

DISCUSSION

Recent reports have shown that c-Myc transformation promotes apoptosis by amplifying or relaying the signal from death receptors or toxic stress to the mitochondria. This action appears to be mediated by either the activation or the change in expression of Bcl-2 protein members and results in the facilitated release of cytochrome *c* from the mitochondria [17,18,32,35,36]. In the present study, we now report that p38, and more precisely p38 α , is an essential mediator of cisplatin-induced activation of Bax at the mitochondria in c-Myc-transformed cells. We have shown that c-Myc-deregulation-dependent apoptosis induced by cisplatin also involves the classical mitochondrial pathway, which includes the activation of Bax at the mitochondria, the release of cytochrome *c*, the activation of caspases and the fragmentation of the nuclei, and that blocking p38 α activity inhibits Bax activation. Furthermore, we have shown that the step sensitive to c-Myc-deregulation is located upstream of Ask1 in the signalling kinase cascade leading to p38 activation. Results obtained with other DNA-damaging agents suggested that this mechanism may apply to other apoptogenic stressing agents.

Activation of Bax is regulated positively by Bcl-2 homology (BH) 3-only proteins that are produced/activated in specific manners by distinct apoptotic stimuli, and are negatively regulated by anti-apoptotic members of this family such as Bcl-2 or Bcl-X_L. Some BH3-only proteins appear to directly bind Bax to induce conformational changes. Other BH3-only proteins rather interact with Bcl-2/Bcl-X_L proteins, blocking their inhibitory action on Bax. [37,38]. In both cases, Bcl-2/Bcl-X_L and Bax (and Bak) are presumably competing for BH3-only proteins therefore making the ratio between the pro- and anti-apoptotic members of the Bcl-2 family an important index of the susceptibility to apoptosis [37–39]. It is believed that the binding of BH3-only proteins to Bax causes a conformational change that leads to the exposure of the N-terminus and would then be followed by the translocation of Bax to the mitochondria. However, activation and translocation may not occur following the same strict temporal sequence in different cell lines or experimental conditions. In some cell systems, Bax is located to the mitochondria in the absence of activation, a process which, by itself, does not lead to the exit of cytochrome *c* and toxicity. In Rat1 cells subjected to etoposide or taxol treatments, Bax translocation to the mitochondria was reported to be induced in a c-Myc-independent manner, whereas Bax activation and apoptosis occurred in a c-Myc-dependent manner [18]. In the case of cisplatin, our data showed that both the translocation and activation processes required a c-Myc-dependent event mediated through the activation of p38, suggesting that the two events are coupled. A p38-dependent translocation of Bax to the mitochondria has also been demonstrated in neurons exposed to nitric oxide [40].

There are a number of possible mechanisms by which Bax activation can be regulated downstream of p38. p38 can regulate the activity of various transcriptional factors and could in theory mediate some of the reported effects of c-Myc on the expression of Bax, Bcl-X_L or Bcl-2 [19,32,35,41]. However, our Western-blot analyses revealed no change in the expression of either Bax or Bcl-2 induced by c-Myc expression or cisplatin treatments. A more likely possibility is that one of the Bcl-2 protein family members, including Bax itself, or a protein that regulates their activity, is a direct or indirect target of p38-mediated phosphorylation. Although there is no evidence that Bax can be phosphorylated, a number of proteins of the Bcl-2 family can be regulated either positively or negatively by phosphorylation during apoptosis. For example, the BH3-only protein Bad can be phosphorylated at multiple sites by several distinct kinases [38], including mitogen- and stress-activated kinase 1 ('MSK1'), a kinase activated downstream of p38 [42]. This phosphorylation, however, mediates a survival, rather than pro-apoptotic, mechanism. Interestingly, phosphorylation of Bcl-2 can mediate either pro- or anti-apoptotic effects and can be regulated by a few kinases, including JNK and p38. JNK can phosphorylate Bcl-2 on Ser⁷⁰, a residue whose phosphorylation appears essential for the protective activity of Bcl-2 [43]. However, Bcl-2 phosphorylation on Ser⁷⁰ by JNK has also been reported to promote apoptosis after treatment with paclitaxel or Fas activation [44,45]. The discrepancy could be partly explained by the capacity of JNK to phosphorylate Bcl-2 also at Ser⁸⁷ under these conditions [45]. On the other hand, p38 can phosphorylate Bcl-2 on Ser⁸⁷ and Thr⁵⁶ during growth factor deprivation. p38-mediated phosphorylation of Bcl-2 caused mitochondrial leakage of cytochrome *c* and was therefore pro-apoptotic [46]. Bcl-2 is thus a good candidate for mediating the effect of p38 at the mitochondria.

Our suggestion that p38 α , but not p38 β (also called p38 β 2 [27,28]), is essential for Bax activation is based mainly on the

finding that the interfering mutant of p38 α , p38 α AGF, could almost totally block all examined markers of apoptosis in both transformed Rat1 cells and HeLa cells, whereas p38 β AGF had no effect. Furthermore, SB203580, which blocks both p38 α and p38 β activities, had only a partial (50–60%) inhibitory effect on apoptosis (even at concentrations much higher than required to block *in vivo* phosphorylation of downstream targets of p38; results not shown) and, in fact, sensitized the cells in which p38 α activity was already inactivated with p38 α AGF. Although a more specific study is required to provide a definitive proof, the results are consistent with the interpretation that p38 α and p38 β have opposite effects on apoptosis. Numerous reports previously associated activation of p38 to either protection from or induction of apoptosis. It is not always possible in these studies to determine whether p38 α or p38 β was involved, inasmuch as the activities of interfering mutants were not directly compared with that of chemical inhibitors. Anyway, when determined, p38 α was generally associated, as in the present study, with pro-apoptotic functions, whereas p38 β seems instead to mediate protective pathways [47,48]. Our conclusion is also plausible considering that p38 α and p38 β appear to form distinct signalling complexes and to have distinct substrate specificities [27,28].

Our results confirmed the previously suggested role of Ask1 in cisplatin-induced apoptosis and are consistent with several reports proposing a major role of Ask1 in apoptosis induced by several agents [33,49,50]. We found that Ask1, like p38, was activated by cisplatin only in cells with deregulated expression of c-Myc, and that this activation was responsible for the subsequent activation of p38. Consequently, inhibition of Ask1 completely inhibited the conformational change of Bax, cytochrome *c* release and nuclear fragmentation. Sensitization of Ask1 activation by cell transformation can be a general mechanism of sensitization to apoptosis induced by a variety of agents or conditions. In addition to cisplatin and other DNA damaging agents, Ask1 can be activated by a number of agonists and toxic agents and may in fact constitute an integrator on which several signalling pathways converge to activate the stress-activated protein kinases JNK and p38 [51,52]. Several distinct regulators of Ask1 activity have been described, each mediating activation from distinct stimuli. The mechanism involved in the case of cisplatin is not known. The soluble tyrosine kinase c-abl is activated by cisplatin and is responsible in another cell system for the activation of p38 and JNK [53]. Since Ask1 can activate both kinases, it is possible that c-abl is the link upstream of p38 between DNA damage induced by cisplatin and a c-Myc-dependent process of Ask1 activation. This c-Myc-dependent pathway may be a general pathway used by DNA-damaging agents, since p38 is also activated in a c-Myc-dependent manner by several other chemotherapeutic agents that damage DNA, such as etoposide, doxorubicin or daunorubicin. One possible mechanism of sensitization of Ask1 activation by c-Myc transformation involves the production of reactive oxygen species. Ask1 is strongly activated by oxidative stress. The activation is mediated by the oxidation of thioredoxin, an endogenous inhibitor of Ask1 which, in its oxidized state, dissociates from Ask1 [54]. Interestingly, overexpression of c-Myc and other oncoproteins has recently been associated with an increased production of reactive oxygen species in response to apoptotic stimuli, which was responsible for activation of p38 and JNK [55,56].

Although less studied than JNK, there are now several reports linking p38 activity to apoptosis. In the light of the results in the present study, one may anticipate that the failure to activate p38 could be acquired during tumour development in order

to overcome c-Myc- and other oncogene-dependent apoptosis and, therefore, may represent a mechanism of drug resistance in many tumours. This is in line with the recent reports that overexpression of the p38 phosphatase Wip1/PPM1D facilitates cell transformation and that amplification of the gene coding for Wip1 occurs in human breast tumours [57,58].

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