Late Activation of Stress Kinases (SAPK/JNK) by Genotoxins Requires the DNA Repair Proteins DNA-PKcs and CSB

Gerhard Fritz and Bernd Kaina

Department of Toxicology, University of Mainz, D-55131 Mainz, Germany

Submitted July 7, 2005; Revised October 31, 2005; Accepted November 17, 2005
Monitoring Editor: J. Silvio Gutkind

Although genotoxic agents are powerful inducers of stress kinases (SAPK/JNK), the contribution of DNA damage itself to this response is unknown. Therefore, SAPK/JNK activation of cells harboring specific defects in DNA damage-recognition mechanisms was studied. Dual phosphorylation of SAPK/JNK by the genotoxin methyl methanesulfonate (MMS) occurred in two waves. The early response (≤2 h after exposure) was similar in cells knockout for ATM, PARP, p53, and CSB or defective in DNA-PKcs compared with wild-type cells. The late response however (≥4 h), was drastically reduced in DNA-PKcs- and Cockayne’s syndrome B (CSB)-deficient cells. Similar results were obtained with human cells lacking DNA-PKcs and CSB. Activation of SAPK/JNK by MMS was not affected upon inhibition of base excision repair (BER), indicating base damage itself does not signal to SAPK/JNK. Because SAPK/JNK activation was attenuated in nongrowing cells, DNA replication-dependent processing of lesions, involving DNA-PKcs and CSB, appears to be required. DNA-PKcs coprecipitates with SEK1/MKK4 and SAPK/JNK, supporting a role of DNA-PKcs in SAPK/JNK activation. In this process, Rho GTPases are involved since inhibition of Rho impairs MMS-induced signaling to SAPK/JNK. The data show that sensing of DNA damage by DNA-PKcs and CSB causes a delayed SEK1/MKK4-mediated dual phosphorylation of SAPK/JNK.

INTRODUCTION

Exposure of mammalian cells to genotoxic agents, including chemical genotoxins (e.g., alkylating environmental carcinogens and many anticancer drugs) as well as radiation (UV light, X-rays), induces complex cellular responses that affect cell cycle progression and cell survival (Canman and Kastan, 1996; Wang et al., 1996; Li and Karin, 1998; Zhou and Elledge, 2000; Arlt et al., 2001; Cortez et al., 2001; Durocher and Jackson, 2001). Stimulation of gene expression, that is central in this stress-induced program, can occur within minutes and lasts up to several hours after exposure. It is mediated by phosphorylation-induced activation of pre-existing transcription factors such as c-Jun, c-Fos, ATF2, SRF-TCP, CREB, and NF-kB. The response appears to be biologically highly relevant, because the lack of either one of these transcription factors dramatically impairs cell survival and genomic stability upon genotoxic stress. Thus, cells that are compromised in AP-1-mediated gene expression because of a lack of c-Fos are hypersensitive to a wide spectrum of genotoxic agents (Haas and Kaina, 1995; Schreiber et al., 1995; Wang et al., 1996; Kaina et al., 1997). Hypersensitivity to UV light was also reported for c-Jun knockout cells (Shaulian et al., 2000; Shaulian and Karin, 2002). Central players in the regulation of the activity of AP-1-like transcription factors (i.e., Jun/Fos and Jun/ATF heterodimers) are protein kinases belonging to the MAP kinase family, i.e., stress activated protein kinases/c-Jun-N-terminal kinases (SAPK/JNK), p38 kinase, and ERKs (Ichijo, 1999). Elucidating the regulation of cellular responses to genotoxic stress, a lot of attention has been paid to SAPK/JNK and p38 kinases. The majority of available data indicate that SAPK/JNK and p38 kinase triggered signaling stimulates apoptosis (Xia et al., 1995; Verheij et al., 1996; Sanchez Perez et al., 1998), although opposing reports also exist (Gjerset et al., 1999; Hayakawa et al., 2003). One reason for the proapoptotic function ascribed to SAPK/JNK lies in the expression of FasL, which is regulated by Ap-1 (Herr et al., 1997; Kolbus et al., 2000). Also, the activity of Bax/Bcl proteins is modulated by SAPK/JNK (Maundrell et al., 1997; Deng et al., 2001; Putcha et al., 2003), having additional impact on genotoxin-induced apoptosis. Protective effects reported for SAPK/JNK are thought to be due to the promotion of DNA repair mechanisms (Hayakawa et al., 2003).

A central question that still needs to be answered is whether stress kinases are mainly stimulated by receptor activation or DNA damage-related mechanisms. It has been suggested that rapid activation of signaling pathways related to MAP kinase and NF-kB by genotoxins such as UV irradiation and alkylating agents is independent of DNA damage, because 1) their activation was observed in both intact and denucleated cells (Devaray et al., 1993; Wilhelm et al., 1997), 2) genotoxic agents are able to induce the phosphorylation of growth factor receptors similar to physiological ligands (Coffer et al., 1995; Huang et al., 1996b; Knebel et al., 1996; Gross et al., 1999; Kitagawa et al., 2002), and 3) functional inactivation of growth factor receptors impacts on signaling to MAP kinases (Rosette and Karin, 1996). Based on this, it is believed that various cellular receptors for growth factors and cytokines act as cellular sensors for genotoxins, provoking the rapid activation of MAP kinases and...
NF-κB in that it can trigger reprogramming of gene expression (Canman and Kastan, 1996; Rosette and Karin, 1996). Supporting evidence for the involvement of DNA damage is the finding that the activation of MAP kinases (Nehme et al., 1997, 1999) and NF-κB (Bender et al., 1998) differs between wild-type cells and cell lines containing reduced capacity for the repair of cisplatin and UV-C-induced DNA lesions. It should also be noted that EGF receptor-deficient mutants were similarly effective as wild-type cells in the UV-C-induced activation of AP-1 (Huang et al., 1996a). Thus, collectively, the contribution of DNA damage-dependent and -independent mechanisms as to the activation of MAP kinase-related signaling pathways is still enigmatic.

In the present study we addressed the question of whether cellular functions related to DNA damage processing and repair may contribute to the activation of SAPK/JNK. Kidani et al., 2000). We show that MMS provokes a long-lasting dual phosphorylation of SAPK/JNK at Thr183/Tyr185, with an early and a late component. We also show that the late SAPK/JNK phosphorylation requires DNA-PKcs and Cockayne syndrome B (CSB) protein and that it is dependent on Rho GTPases. The essential requirement of the DNA repair proteins DNA-PKcs and CSB for genotoxic-induced SAPK/JNK phosphorylation indicates that DNA damage-related processes are able to act as a trigger for signaling involving SAPK/JNK.

MATERIALS AND METHODS

Materials

MMS, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), lovastatin, and repair inhibitors (i.e., prunasin, 3-aminoazobenzamide, 3-methoxyamine) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Rho-inactivating toxin B from Clostridium difficile was generously provided by I. Just (Hannover, Germany). PARP-/-/- mouse cells originated from G. de Murcia (Ilkirch Cedex, France), ATM-/-/- mouse cells were from P. Leder (Boston, MA), and CSB-/-/- mouse cells originate from G. T. van der Horst (Rotterdam, The Netherlands). MPG-/-/- and corresponding wild-type MEFs were generously provided by R. Elder (Manchester, United Kingdom). Human DNA-PKcs-proficient and -deficient glialoma cells (MO59K and MO59J) were kindly provided by C. Dib et al. (Hannover, Germany). The DNA-PKcs protein was purchased from Promega (Mannheim, Germany). PARP substrate for DNA-PKcs, immunoprecipitated DNA-PKcs or 0.5 g of recombinant HA-JNK2 protein as a substrate. To examine whether JNK is a substrate for DNA-PKcs, immunoprecipitated DNA-PKcs or 0.5 μg of purified DNA-PKcs protein was incubated with 1 μg of recombinant HA-JNK2 protein (Kinase Detect ApS) for 20 min at 30°C in 40 μl of DNA-PKcs reaction buffer (50 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol [DTT], 50 μM ATP, and 1 μCi γ-32P-ATP) containing either GST-Jun (1 μg) or DNA-PKcs (0.5 μg) as a substrate. To examine whether JNK is a substrate for DNA-PKcs, immunoprecipitated DNA-PKcs or 0.5 μg of purified DNA-PKcs protein was incubated with 1 μg of recombinant HA-JNK2 protein (Kinase Detect ApS) for 20 min at 30°C in 40 μl of DNA-PKcs reaction buffer (50 mM HEPES, 100 mM KC1, 10 mM MgCl2, 0.2 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 10 μg/ml sonicated calf thymus DNA, 50 μM ATP, and 1 μCi γ-32P-ATP). Products of kinase reactions were separated by SDS-PAGE and visualized by autoradiography of the dried gel.

RESULTS

MMS-induced DNA Damage Quantitatively Correlates with Dual Phosphorylation of SAPK/JNK

The potent DNA alkylator MMS induces SEK1/MKK4 triggered phosphorylation of SAPK/JNK at positions Thr183/Tyr185 in a dose- and time-dependent manner (Figure 1, A and B). SAPK/JNK phosphorylation was paralleled by an increase in DNA strand break formation (Figure 1A). Furthermore, phosphorylation of SAPK/JNK correlates inversely with the level of MMS-induced blockade of DNA replication as observed in a time- and dose-response analysis (Figure 1, B and C). Because DNA strand breaks and replication inhibition are caused by MMS-induced DNA alkylation, the findings indicate that DNA damage-related mechanisms are involved in MMS-triggered signaling to SAPK/JNK.

DNA Replication

To assay the blockade of DNA replication, incorporation of BrdU was quantified by ELISA as described previously (von Bardeleben et al., 2002). Exponentially growing cells were treated with MMS for 1 h, carefully rinsed, postincubated at 37°C for the times indicated, and harvested for analysis. Fifty cells were analyzed per measurement as to their olive tail moment (OTM) for calculation of the mean value.

DNA Strand Break Analysis

To quantify the level of MMS-induced DNA strand breaks, the alkaline comet assay was used as previously described (von Bardeleben et al., 2002). Exponentially growing cells were treated with MMS for 1 h, carefully rinsed, postincubated at 37°C for the times indicated, and harvested for analysis. Fifty cells were analyzed per measurement as to their olive tail moment (OTM) for calculation of the mean value.

Immunoprecipitation

Cells were harvested in SDS sample buffer. After SDS gel electrophoresis, communoprecipitated proteins were identified by Western blot analysis (see above).

In Vitro Kinase Assays

The phosphorylation of DNA-PKcs and SAPK/JNK was analyzed in vitro. Immunoprecipitation of JNK1 from MMS-treated and nontreated cells, phosphorylation of GST-Jun 1/166 (as a control) and purified DNA-PKcs (Promega) was analyzed by immune complex kinase assay as described previously (Grad et al., 2000). Briefly, phosphorylation was performed for 20 min at 30°C in 40 μl of JNK reaction buffer (25 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol [DTT], 50 μM ATP, and 1 μCi γ-32P-ATP) containing either GST-Jun (1 μg) or DNA-PKcs (0.5 μg) as a substrate. To examine whether JNK is a substrate for DNA-PKcs, immunoprecipitated DNA-PKcs or 0.5 μg of purified DNA-PKcs protein was incubated with 1 μg of recombinant HA-JNK2 protein (Kinase Detect ApS) for 20 min at 30°C in 40 μl of DNA-PKcs reaction buffer (50 mM HEPES, 100 mM KC1, 10 mM MgCl2, 0.2 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 10 μg/ml sonicated calf thymus DNA, 50 μM ATP, and 1 μCi γ-32P-ATP). Products of kinase reactions were separated by SDS-PAGE and visualized by autoradiography of the dried gel.

Cell Culture Conditions

Primary human fibroblasts were grown in F10/Dulbecco (+10% fetal calf serum). All other cell lines used in this study were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

MAP Kinase Activation

Activation of MAP kinases by phosphorylation was measured by Western blot analysis using phospho-specific antibodies that detect the dual phosphorylated (i.e., activated) form of SAPK/JNK (Thr183/Tyr185) and p38 kinase (Thr180/Tyr182). Phospho-specific antibodies were also used for measuring activation of SEK1/MKK4 (Thr261) and Chk1 (Ser345). Cell extracts were prepared by lysing an identical number of cells in boiling SDS sample buffer as recommended by the manufacturer (New England Biolabs GmbH). Proteins were separated by SDS gel electrophoresis (10% gels) and transferred onto nitrocellulose membrane. After blocking of the membrane (5% dry milk in Tris-buffered saline [TBS]/0.1% Tween, 2 h at RT) phosphorylation status of kinases was analyzed using the corresponding antibody (1:1000, 5% BSA in TBS/0.1% Tween, overnight incubation at 4°C). After washing, blots were incubated with secondary horseradish peroxidase-coupled anti-rabbit-anti-body (1:5000, 2 h, RT). Subsequently, phosphorylated kinases were visualized by chemiluminescence using ECL detection reagent (Amersham, Freiburg, Germany).

Molecular Biology of the Cell852

G. Fritz and B. Kaina

852
Figure 1. The level of MMS-induced dual phosphorylation of SAPK/JNK quantitatively correlates with DNA strand break formation and DNA replication blockade. (A) Six hours after exposure of mouse fibroblasts (BK4) to different concentrations of MMS, cells were harvested, and the level of phosphorylated SAPK/JNK was analyzed by Western blot analysis (autoradiography inserted). In parallel, DNA strand break formation was assayed using the comet assay described in Materials and Methods. Shown is the result of one representative experiment. After densitometrical analysis of the autoradiogram, relative p-JNK phosphorylation obtained with 1 mM of MMS was set to 1.0. (B) After MMS exposure (1 mM), cells were postincubated for different periods of time before SAPK/JNK phosphorylation was analyzed (autoradiography inserted). Determination of DNA replication was performed as described below. Replication data shown are the mean ± SD from one representative experiment performed in quadruplicate. Relative p-JNK phosphorylation obtained 6 h after MMS exposure was set to 1.0. (C) Mouse fibroblast cells (BK4) were exposed to different concentrations of MMS. After incubation period of 6 h, cells were harvested for analysis of SAPK/JNK phosphorylation (autoradiography inserted) or were pulse-labeled for 30 min with BrdU for determination of DNA replication. Relative replication in nonexposed cells was set to 100%. Replication data shown are the mean ± SD from one representative experiment performed in quadruplicate.

Inhibition of Repair of MMS-induced DNA Alkylation Lesions Does Not Affect SAPK/JNK Phosphorylation

It was tempting to speculate that DNA alkylation lesions act as a direct trigger of SAPK/JNK phosphorylation. Therefore, we studied the response under conditions of inhibition of specific repair functions involved in removal of alkylation lesions. Inhibition of base excision repair (BER) by genetic knockout of methylpurine-DNA-glycosylase (MPG; Figure 2A) or by pharmacological inhibitors such as the PARP inhibitor 3-aminobenzamide (Figure 2B), the DNA polymerase β inhibitor prunasin (Figure 2B), and the BER incision inhibitor 3-methoxyamine (Figure 2C) did not influence MMS-stimulated phosphorylation of SAPK/JNK. Obviously, neither the DNA lesions themselves nor the process of BER impacts on MMS-induced signaling to SAPK/JNK. Therefore, we addressed secondary DNA lesions induced by MMS as inducer. Critical secondary lesions might be DNA double-strand breaks (DSBs) formed in response to MMS. To assess whether DSBs on their own are sufficient for stimulating dual phosphorylation of SAPK/JNK, we used ionizing radiation (IR) as an inducer. IR did not cause dual phosphorylation of SAPK/JNK, which is in line with other report (Lee et al., 1998), whereas UV light provoked a dramatic response (Figure 2D). Obviously, the presence of DSBs is not sufficient in triggering Thr183/Tyr185 phosphorylation of SAPK/JNK.

MMS-induced DNA Replication Blockade Contributes to SAPK/JNK Phosphorylation

MMS-induced genotoxicity requires DNA replication. Therefore, we checked whether DNA replication is required for MMS-provoked signaling to SAPK/JNK. To this end, we compared MMS-stimulated dual phosphorylation in growing versus nongrowing cells. As shown in Figure 3A, dual phosphorylation of SAPK/JNK but not of p38 kinase, was reduced by ~50% in nonreplicating cells. This indicates that replication mediated processes participate in signaling to SAPK/JNK upon MMS treatment. DNA replication inhibition itself might be considered as a trigger. Indeed, inhibition of DNA replication by aphidicolin (Figure 3B) and

Figure 2. Dual phosphorylation of SAPK/JNK is independent of processing of DNA alkylation damage and formation of DSBs. (A) MPG–/– and corresponding wild-type MEFs (MPG+/+) were exposed to MMS (1 mM). Two and 4 h after exposure, cells were harvested for analysis of SAPK/JNK phosphorylation as described in Materials and Methods. (B) Mouse wild-type cells that had been pretreated or not with the repair inhibitors 3-aminobenzamide (3-AB; 2 mM) or prunasin (1 mM) were exposed to MMS (1 mM). Six (for 3-AB) and 3 h (for Prunasin) later, cells were harvested for determination of SAPK/JNK phosphorylation. (C) Wild-type mouse fibroblasts were pretreated or not with BER repair inhibitor 3-methoxyamine (5 mM) before MMS (1 mM) exposure. Up to 6 h after treatment SAPK/JNK phosphorylation was assayed. (D) Dual phosphorylation of SAPK/JNK was analyzed up to 6 h after induction of DSBs by radiation treatment (20 Gy) of wild-type mouse fibroblasts (right part of the figure). Wild-type mouse fibroblast (WT) and DNA-PKcs–/– defective cells (Scid) were irradiated with 20 Gy or, as a positive control, with UV-C light (40 J/m²). Up to 15 min after exposure dual phosphorylation of SAPK/JNK was analyzed (left part of the figure).
DNA-PKcs Is Essential for Late MMS-triggered Phosphorylation of SAPK/JNK

An attractive hypothesis would be that blockage of DNA replication together with MMS-induced secondary lesions, notably DNA strand breaks, provide the ultimate signal for triggering dual phosphorylation of SAPK/JNK. This hypothesis can be studied best with DNA repair-defective mutants. Therefore, we examined cell lines that are defective in DNA repair processes upon MMS exposure might generate an additional signal to SAPK/JNK. To this end we studied CSB Is Essential for Late Phosphorylation of SAPK/JNK in ATM−/−, PARP−/−, and DNA-PKcs-deficient Scid cells was similar to that of wild-type BK4 cells (Figure 4A; and several other wild-type mouse fibroblast cell lines; unpublished data), indicating that early processing of DNA strand breaks is not involved in signaling to SAPK/JNK. However, 6 h after MMS exposure (designated as “late response”), Scid cells showed a clearly reduced phosphorylation of SAPK/JNK compared with ATM−/−, PARP−/−, and wild-type fibroblasts (Figure 4B). This was supported by a more detailed kinetic analysis (Figure 5A), revealing that MMS-induced phosphorylation of SAPK/JNK occurring within 1–2 h after exposure was similar in normal fibroblasts (BK4) and Scid cells, whereas at later times the phosphorylation level of SAPK/JNK further increased in wild-type cells but not in Scid (Figure 5A). A similar response was observed for the highly potent alkylating mutagen MNNG (Figure 5B). Compared with SAPK/JNK, the phosphorylation of p38 kinase upon MMS and MNNG treatment was only slightly affected in Scid cells (Figure 5, A and B). It should be noted that wild-type and Scid fibroblast responded similarly to SAPK/JNK phosphorylation upon UV-C treatment (Figure 5A), which points to the specificity of the observed stress response elicited by alkylating genotoxins. It is important to note that attenuation of late phosphorylation of SAPK/JNK in Scid cells was also detectable at the level of SEK1/MKK4 (Figure 5C). Yet, the MMS-induced increase in phosphorylation of checkpoint kinase 1 (Chk1) was even prolonged in Scid compared with wild-type cells (Figure 5C), showing that Scid cells are not generally impaired in DNA damage-triggered stress response. Overall, the data indicate that DNA-PKcs, which becomes activated by DNA strand breaks, is specifically required for alkylation-induced dual phosphorylation of SAPK/JNK by SEK1/MKK4.

CSB Is Essential for Late Phosphorylation of SAPK/JNK by MMS

Genotoxins also impair transcription. Therefore, we addressed the question of whether impaired transcription related repair processes upon MMS exposure might generate an additional signal to SAPK/JNK. To this end we studied cells that lack Cockayne syndrome group B protein (CSB−/− cells). The CSB protein is known to be a central player in the regulation of transcription-coupled repair of oxidative and UV-induced DNA damage (van der Horst et al., 1997; van Gool et al., 1997; Le Page et al., 2000). As analyzed 4 h after exposure to different doses of MMS, SAPK/JNK phosphorylation was largely reduced in CSB−/− cells compared with normal mouse fibroblasts (BK4; Figure 6A) and CSB+/- MEFs (Figure 6B, right...
Phosphorylation of SEK1/MKK4 and p38 kinase was also reduced in CSB-deficient cells (Figure 6A). At early times (≤2 h) after MMS exposure, phosphorylation of SAPK/JNK and p38 kinase was not impaired in CSB-deficient cells (Figure 6B). Moreover, activation of SAPK/JNK and p38 kinase was also impaired in CSB-defective human cells compared with normal primary human fibroblasts, which were included as a control (Figure 6C). Thus, both mouse and human cells impaired in either DNA-PKcs or CSB display a defect in the late MMS-triggered phosphorylation of SAPK/JNK. Notably, the effect of DNA-PKcs and CSB on MMS-induced p38 kinase activation is different between the human and the mouse system. p53 is not involved in late signaling to SAPK/JNK upon MMS exposure because early and late activation of SAPK/JNK was very similar in p53 wild-type and p53 knock-out cells (unpublished data).

DNA-PKcs Interacts with SAPK/JNK

Having shown that DNA-PKcs and CSB are essential players in MMS-induced signaling from damaged DNA downstream to SAPK/JNK, the question arose as to the mechanism involved. To examine whether a direct interaction exists between the DNA repair proteins involved and SAPK/JNK, immunoprecipitation experiments were performed.

Figure 6. Cockayne’s syndrome B protein (CSB) is required for late MMS-stimulated phosphorylation of SAPK/JNK. (A) Wild-type cells (BK4) and CSB-deficient cells (Csb−/−) were exposed to different concentrations of MMS. After an incubation period of 4 h, cells were harvested for determination of phosphorylation status of SAPK/JNK, SEK1, and p38 kinase. Rehybridization with ERK2-specific antibody was used as a loading control. (B) Up to 6 h after MMS exposure (1 mM), cells were harvested for determination of phosphorylation of SAPK/JNK and p38 kinase. Data obtained using the wild-type littermates (Csb+/+) of the knockout MEFs (Csb−/−) are shown on the right part of the figure. (C) Mouse fibroblast cells (BK4) and CSB-deficient cells (Csb−/−) were irradiated with different doses of UV-C light. After an incubation period of 30 min, cells were harvested for determination of SAPK/JNK phosphorylation. On the right part of the figure, time response analysis using wild-type littermates (Csb+/+) of the knockout MEFs (Csb−/−) is shown.
formed. They revealed that DNA-PKcs coprecipitates with both nonphosphorylated SAPK/JNK (JNK) and phosphorylated SAPK/JNK (p-JNK) as well as with p-SEK (Figure 8, A and B) and the mitogen-activated kinase phosphatase (MKP-1) (Figure 8B), which was described to act on stress kinases (Wu and Bennett, 2005). No coprecipitation was observed if heat inactivated JNK antibody or antibody directed against p38 kinase was used (Figure 8A). MMS treatment did not increase the level of coprecipitated DNA-PKcs (Figure 8A). It is well established that DNA-PKcs interacts with Ku proteins (Ku70 and Ku86). Therefore, to corroborate the results of the immunoprecipitation experiments, we additionally checked for coprecipitation of Ku86 with SAPK/JNK. As shown in Figure 8, A and B, immunoprecipitation of JNK, p-JNK, p-SEK1, and MKP-1 also caused coprecipitation of Ku86 protein. Yet, coprecipitation was not observed for the nuclear mismatch repair protein MSH2 (Figure 8B), demonstrating the specificity of the observed interaction of DNA-PKcs and Ku-86. In contrast to BK4 cells, which harbor wild-type DNA-PKcs, Scid cells are mutated in their DNA-PKcs gene, therefore showing only very low residual amount of DNA-PKcs protein expression and activity (Blunt et al., 1996; Beamish et al., 2000). Correspondingly, the level of DNA-PKcs that coprecipitated with JNK from Scid cells was very low compared with wild-type BK4 cells (Figure 8C). SAPK/JNK not only binds to but also phosphorylates DNA-PKcs (Figure 8D). Most importantly, MMS treatment stimulates phosphorylation of DNA-PKcs by SAPK/JNK to a similar extent as that observed for c-Jun, which is the classical substrate for SAPK/JNK (Figure 8D). On the other hand, recombinant SAPK/JNK was neither substrate for phosphorylation by immunoprecipitated DNA-PKcs nor by commercially available purified DNA-PKcs protein (unpublished data).

**Figure 8.** DNA-PKcs interacts with stress kinases and is subject to phosphorylation by SAPK/JNK. (A) SAPK/JNK was immunoprecipitated (IP) from untreated (Con) or MMS-treated (MMS, 1 mM, 6 h) DNA-PKcs-proficient (WT) cells. Coprecipitation of DNA-PKcs and Ku-86 was analyzed by Western blot analysis. As controls, immunoprecipitation was performed with heat-inactivated (95°C, 5 min) JNK antibody and antibody directed against p38 kinase. (B) p-JNK, p-SEK, and MKP-1 protein was immunoprecipitated from untreated (Con) and MMS-treated (1 mM, 6 h) DNA-PKcs-proficient (WT) cells by use of the corresponding antibody. Coprecipitation of DNA-PKcs and Ku-86, and the mismatch repair protein MSH2 was analyzed by Western blot analysis. (C) SAPK/JNK was immunoprecipitated from untreated (Con) DNA-PKcs-proficient (BK4) and -deficient (SCID) cells. As a control, immunoprecipitation was performed using heat-inactivated JNK antibody as described in A. Coprecipitation of DNA-PKcs and Ku-86 was analyzed by Western blot analysis. (D) SAPK/JNK was immunoprecipitated from untreated (Con) or MMS (1 mM, 4 h) treated mouse fibroblasts. Afterward, purified DNA-PKcs protein or recombinant GST-Jun protein was added, and SAPK/JNK-mediated 32P-phosphorylation of DNA-PKcs and GST-Jun was assayed in vitro as described in Materials and Methods. Shown is the autoradiography of a representative experiment.

**Ras-homologous GTPases Are Essential for Early and Late Activation of SAPK/JNK by MMS**

Early SEK1/MKK4-regulated dual phosphorylation of SAPK/JNK can result from activation of growth factor and cytokine receptors (Coffer et al., 1995; Cannman and Kastan, 1996) and requires Ras-homologous (= Rho) GTPases (Coso et al., 1995; Minden et al., 1995). Therefore we were interested in elucidating whether Rho GTPases are also essential for MMS-induced late signaling to SAPK/JNK that originates from damaged DNA. Inhibition of Rho-dependent signaling by lovastatin, which blocks Rho by attenuating its C-terminal isoprenylation (Laufs and Liao, 1998; Gnad et al., 2000) largely abrogated both the early and the late MMS-stimulated phosphorylation of SAPK/JNK as well as SEK1/MKK4 and, to a lesser extent, also p38 kinase (Figure 9A). Similar results were obtained using Toxin B (ToxB) from *C. difficile* (Figure 9A, right panel), which specifically inactivates Rho GTPases by glycosylation (Just et al., 1995; Aktories et al., 2000). Importantly, even if cells were treated with ToxB 1 h after MMS exposure, a strong attenuation of the late phosphorylation of SAPK/JNK was detected (Figure 9B). The same holds true for phosphory-
SAPK/JNK in DNA-PKcs- and CSB-defective cells is due to reduced late phosphorylation of SAPK/JNK. It might be argued that reduced late phosphorylation of SAPK/JNK is due to protein kinase phosphatases such as MKP-1 (Wu and Bennett, 2005). As shown in Figure 10A, MKP-1 protein expression increased in wild-type, Scid and Csb−/− cells upon MMS treatment, with wild-type cells showing the strongest response. MMS-induced expression of MKP-1 was completely abrogated in all cell lines by Ro-318220 (10 μM). Afterward, cells were exposed to MMS (1 mM). After 2–8 h of postincubation, cells were harvested for determination of MKP-1 expression. Pretreatment with Ro-318220 (10 μM) was performed for 30 min. As a loading control, filter was reprobed with ERK2-specific antibody. Shown is the autoradiography. (B) Effect of Ro-318220 pretreatment (10 μM) on SAPK/JNK phosphorylation of wild-type (WT), Scid, and Csb−/− cells as measured 6 h after MMS exposure.

**DISCUSSION**

The cellular response to genotoxic stress can be classified into a response that originates from damaged DNA (Durocher and Jackson, 2001) and a response that originates from other sources such as receptors for cellular growth factors and cytokines (Canman and Kastan, 1996). Both responses are mechanistically poorly understood. This is especially true for the DNA damage response. For the immediate-early response involving rapid activation of stress-activated protein kinases and subsequent activation of AP-1-like transcription factors, DNA damage-independent mechanisms appear to be of utmost importance (Sachsenmaier et al., 1994; Knebel et al., 1996; Rosette and Karin, 1996; Gross et al., 1999). DNA damage-related activation of c-Jun (Nehme et al., 1994; Rosette and Karin, 1996; Gross et al., 1999), indicating that MMS- but not UV-C-induced damage, as measured by DNA strand break formation and blockage of DNA replication, was quantitatively related to Thr183/Tyr185 phosphorylation of SAPK/JNK. A similar correlation was not observed for UV-C irradiation (unpublished data), indicating that MMS- but not UV-C-induced DNA lesions trigger phosphorylation of SAPK/JNK.

Because the main cellular target of MMS is the DNA, specific nonrepaired DNA methylation lesions or repair intermediates were initially considered as candidates responsible for the initiation of the signal cascade resulting in SAPK/JNK phosphorylation. Because most of the methylation lesions are repaired by BER (Christmann et al., 2003), we examined whether modulation of this repair pathway influences activation of SAPK/JNK by MMS. N-methylpurines are removed by MPG. Yet, MPG knockout MEFs did...
not differ from the corresponding wild-type cells as to MMS-induced phosphorylation of SAPK/JNK. Furthermore, chemical inhibitors of BER were also ineffective in changing the response. Obviously, DNA methylation lesions themselves, even if they remain unrepaird, do not trigger signaling to SAPK/JNK. Also DNA DSBs, which can be induced by MMS (presumably due to labilization of phosphate backbone by methyl phosphotriesters), are very likely not responsible for triggering Thr183/Tyr185 phosphorylation of SAPK/JNK because IR, a well-known inducer of DSB, is a rather poor activator of dual phosphorylation of SAPK/JNK (shown here and by others; Lee et al., 1998). Nevertheless, IR triggers a JNK-mediated phosphorylation of c-Jun as measured by the complex kinase assay method (Lee et al., 1998). Apparently stimulation of SAPK/JNK activity by IR is independent of Thr183/Tyr185 phosphorylation of the kinase. Alkylating agents need DNA replication in order to provoke genotoxic effects (Kaina, 2003). Therefore, we hypothesized some other DNA damage-related lesions formed during DNA replication to be involved. Inhibition of DNA replication itself or inhibition of transcription should also be taken into consideration. Comparing MMS-induced SAPK/JNK phosphorylation in growing versus nongrowing cells, we found phosphorylation to be reduced by ~50% in nonreplicating cells. This indicates that replication-mediated lesions, or the block of DNA replication itself, is involved in signaling SAPK/JNK activation. This is in line with the time kinetic and dose-response analyses that showed a correlation between both end points. Inhibition of DNA replication by the DNA polymerase inhibitors aphidicolin and hydroxyurea (unpublished data) also caused SAPK/JNK phosphorylation, supporting the view that replication blockage is critical in signaling to SAPK/JNK. MMS largely potentiated aphidicolin-induced signaling to SAPK/JNK, whereas p38 kinase phosphorylation was only slightly promoted by combination treatment. Apparently, MMS-induced signal mechanisms eventually resulting in activation of SAPK/JNK and p38 kinase are different. Based on the data, it is pertinent to conclude that, in addition to replication-related processes, alkylation specific mechanisms such as collapse of replication forks and the generation of secondary lesions, presumably transient DNA breaks of specific structure, contribute to the signaling to SAPK/JNK.

Replication-dependent formed DNA lesions may be recognized by sensor molecules. A key player might be proteins that recognize and bind to DNA breaks that appear as repair and replication intermediates. Therefore, we investigated whether lack of sensors of DNA strand breaks such as ATM and DNA-PKcs which become activated by DSBs and are the most important players in DNA damage-triggered signaling, checkpoint control, and repair (Liu et al., 2000; Zhou and Elledge, 2000; Cortez et al., 2001; Durocher and Jackson, 2001), or PARP have impact on SAPK/JNK phosphorylation by MMS. We found that DNA-PKcs-mutated cells (Scid), but not ATM−/− and PARP−/− fibroblasts, exhibited significantly reduced SAPK/JNK phosphorylation, which was observed ≥4 h after MMS exposure. At early times (<2 h) after MMS treatment Scid cells did not differ from the wild type. Because the time course of UV-C-stimulated SAPK/JNK phosphorylation was similar in wild-type and Scid cells, we conclude that DNA-PKcs is specifically required for the late MMS response giving rise to SAPK/JNK phosphorylation. Abrogation of the late SAPK/JNK phosphorylation in DNA-PKcs-deficient cells was also observed when another alkylating agent, the potent mutagen MNNG, was used for treatment. The effect is not restricted to MEFs. Comparing DNA-PKcs-proficient and -deficient human glioblastoma cells, essentially identical results were obtained. Overall, our findings indicate that alkylating genotoxins provoke late SEK1/MKK4-dependent signaling to SAPK/JNK, which requires DNA-PKcs. Because MMS-induced signaling to Chk1 was not attenuated (it was rather enhanced) in DNA-PKcs-deficient cells, DNA-PKcs appears to be required for signaling to SAPK/JNK but not to checkpoint kinases. Bearing in mind that genotoxin-induced activation of SAPK/JNK has been shown to increase the repair of DNA adducts (Potapova et al., 1997; Hayakawa et al., 2003, 2004), it is tempting to speculate that DNA-PKcs-defective cells, which lack a putatively protective activation of SAPK/JNK, show a MMS-hypersensitive phenotype. Initial experiments indicate that this is indeed the case (unpublished data). Therefore, we suggest that late DNA-PKcs-dependent activation of SAPK/JNK contributes to a MMS-resistant phenotype.

As MMS may also impair transcription and transcription-related processes such as transcription-coupled repair (TCR), we investigated whether absence of CSB, which is known to be a central player in TCR (van Gool et al., 1997), impacts on the phosphorylation level of SAPK/JNK. Similar to the findings with DNA-PKcs-defective cells, lack of CSB dramatically impaired late phosphorylation of SAPK/JNK. The early phosphorylation step remained unaffected. Again, similar to Scid cells, the effect was alkylation specific. It was not observed for UV-C that triggered activation of SAPK/JNK in wild-type and CSB−/− fibroblasts to a similar extent. Identical results were obtained with human CSB-deficient fibroblasts. The data show that CSB is required for MMS-induced late signaling giving rise to SAPK/JNK phosphorylation. Because CSB-defective cells reveal a MMS-hypersensitive phenotype (unpublished data), we assume that late SAPK/JNK activation upon MMS exposure has a protective function. It is worthwhile to note that p53−/− cells do not differ from p53 wild-type cells as to the phosphorylation of SAPK/JNK after MMS treatment, showing that this DNA damage response is independent of p53 (unpublished data).

The kinase function of DNA-PKcs is known to be activated upon binding of the protein to DNA double-strand breaks. Therefore, we asked whether activated DNA-PKcs directly phosphorolyses SAPK/JNK. For direct phosphorylation a physical contact would be postulated. To substantiate a physical interaction of DNA-PKcs with stress kinase, immunoprecipitation experiments and in vitro kinase assays were performed. The immunoprecipitation experiments showed a specific coprecipitation of DNA-PKcs and Ku-86 with SAPK/JNK, SEK1/MKK4, and MKP-1. Yet, DNA-PKcs was not found to phosphorylate recombiant SAPK/JNK in vitro. However, SAPK/JNK was able to phosphorylate DNA-PKcs in vitro. This reaction was stimulated by MMS to a similar extent as phosphorylation of c-Jun, which is the classical substrate of SAPK/JNK. Overall, the data show for the first time that there is a physical interaction between stress kinases (i.e., SEK1/MKK4 and SAPK/JNK) and DNA-PKcs, which supports the view that DNA-PKcs is a key player in DNA damage-induced cellular responses. DNA-PKcs and CSB may be considered as DNA damage sensors that deliver signaling downstream to AP-1-governed gene expression. The mechanism appears to be complex because signaling also requires Rho GTPases as an essential component for late phosphorylation of SEK1/MKK4 and SAPK/JNK. Whether there is a DNA damage-triggered de novo activation of individual Rho proteins or whether Rho GTPases have a permissive function for DNA damage-trig-
MMS-induced complex structural DNA distortions occurring during replication and transcription, finally mediating signaling to SAPK/JNK. Because DNA-PKcs was shown to be an in vitro substrate for SAPK/JNK, regulation of DNA-PKcs function by SAPK/JNK is feasible as well, indicating regulatory feedback mechanisms (see Figure 11). Members of the Rho family of small GTPases are also essential for MMS-induced DNA damage-dependent phosphorylation of SAPK/JNK by SEK1/MKK4, suggesting a cross-talk of Rho signaling with signaling triggered by DNA repair proteins.

ACKNOWLEDGMENTS

We thank G. T. van der Horst (Rotterdam, The Netherlands) for generously providing mouse CSB−/− MEFs, T. Stevnsner (Aarhus, Denmark) for human CSB cells, P. Leder (Boston, MA) for ATM−/− MEFs, G. de Murcia (Illkirch Cedex, France) for PARP−/− MEFs, and R. H. Elder (Manchester, United Kingdom) for MPG−/− MEFs. We also thank I. Just (Hannover, Germany) for providing ToxinB and B. Boldyreff (Odense, Denmark) for recombinant SAPK/JNK protein, T. Brachetti for technical assistance, and W. Roos for proofreading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (FR 1241/5-1).

REFERENCES


Huang, C., Ma, W., Bowden, G. T., and Dong, Z. (1996a). Ultraviolet B-induced activated protein-1 activation does not block epidermal growth factor receptor but is blocked by a dominant negative PKClambda/iota. J. Biol. Chem. 271, 31262–31268.


