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The Level of Intracellular Glutathione Is a Key Regulator for the Induction of Stress-Activated Signal Transduction Pathways Including Jun N-Terminal Protein Kinases and p38 Kinase by Alkylating Agents

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Monofunctional alkylating agents like methyl methanesulfonate (MMS) and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) are potent inducers of cellular stress leading to chromosomal aberrations, point mutations, and cell killing. We show that these agents induce a specific cellular stress response program which includes the activation of Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), p38 mitogen-activated protein kinase, and the upstream kinase SEK1/MKK4 and which depends on the reaction mechanism of the alkylating agent in question. Similar to another inducer of cellular stress, UV irradiation, damage of nuclear DNA by alkylation is not involved in the MMS-induced response. However, in contrast to UV and other inducers of the JNK/SAPKs and p38 pathways, activation of growth factor and G-protein-coupled receptors does not play a role in the MMS response. We identified the intracellular glutathione (GSH) level as critical for JNK/SAPK activation by MMS: enhancing the GSH level by pretreatment of the cells with GSH or *N*-acetylcysteine inhibits, whereas depletion of the cellular GSH pool causes hyperinduction of JNK/SAPK activity by MMS. In light of the JNK/SAPK-dependent induction of c-*jun* and c-*fos* transcription, and the Jun/Fos-induced transcription of xenobiotic-metabolizing enzymes, these data provide a potential critical role of JNK/SAPK and p38 in the induction of a cellular defense program against cytotoxic xenobiotics such as MMS.

Monofunctional alkylating agents are versatile environmental mutagens and carcinogens (61). Once formed or activated, they induce diverse aspects of severe cellular stress, including chromosomal aberrations, sister chromatid exchanges, point mutations, and cell killing. The agents react with a variety of nucleophilic sites in DNA and protein, generating various types of adducts (70). The relative amount of alkyl adducts varies with the type of agent; it is largely determined by its electrophilicity (71).

DNA-damaging agents as well as other agents causing cellular stress, such as UV irradiation, heat shock, or protein synthesis inhibitors, cause a specific cellular stress response. This program includes the transcriptional induction of the proto-oncogene c-*jun*. c-Jun is a member of a multiprotein family that has been implicated in a number of signal transduction pathways associated with cellular growth, differentiation, neuronal excitation, and cellular stress (2, 3, 24, 25, 34, 37, 56, 75) and is thought to be required for cellular defense against toxicity (68). Induction of c-*jun* in response to genotoxic agents and cellular stress is mediated by two AP-1-like sites in its promoter that are recognized by either c-Jun homodimers (4, 20), ATF-2 homodimers, or, preferentially, c-Jun–ATF-2 heterodimers (77, 78). The activity of these complexes is stimulated by hyperphosphorylation of the transactivation domain of promoter-associated c-Jun and ATF-2 (31, 46, 62, 73, 74, 78). Both proteins are efficiently phosphorylated by a single type of protein kinase termed JNK (**J**un **N**-terminal protein **k**inase), also known as SAPK (**s**tress-**a**ctivated **p**rotein **k**inase), which exists in several isoforms (22, 32, 33, 42) that are members of the mitogen-activated protein kinase (MAPK) group of protein kinases (19). Similar to activation of the MAPKs extracellular signal-regulated kinases 1 and 2 (ERK-1 and ERK-2), which are activated predominantly by growth factors and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, JNK activation requires phosphorylation at two residues, Thr-183 and Tyr-185, by JNK kinase, also termed SEK1/MAPK kinase 4 (MKK4) (23, 44, 67), a dual-specificity kinase which is structurally related to MAPK/ERK kinases (MEKs). MKK4 is activated by phosphorylation mediated by one of several upstream protein kinases, for example, MEK kinase 1 (MEKK1) (51, 82). Additional upstream kinases involved in this protein kinase cascade are PAK protein kinases, which are activated by the small GTP binding proteins Rac1 and Cdc42 (6, 16, 17, 52, 59) and possibly Pyk2 (76).

While these kinase cascades induced by tumor necrosis factor alpha (TNF-α) or UV have been analyzed extensively, neither the upstream kinases induced by alkylating agents leading to the activation of JNK/SAPKs nor the primary sensors of the cell that trigger the stress response have been identified. Here we show that monofunctional alkylating agents, such as methyl methanesulfonate (MMS) and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG), whose biological activities are characterized by efficient induction of chromosomal aberrations, sister chromatid exchanges, and cell killing, are very potent inducers of two subclasses of MAPKs, p46/54 JNK/SAPKs and p38, but, in contrast to UV, affect neither ERK-1 nor ERK-2. Interestingly, ethylnitrosourea (ENU), the most potent alkylating agent to induce mutagenesis and carcinogenesis, failed to induce either one of these kinases. Efficient induction of JNK/SAPKs was also observed in enucleated cells,

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demonstrating that damage of genomic DNA is not required to trigger the MMS response. MMS-induced signalling includes SEK1/MKK4 and possibly MEKK1. Despite the fact that MMS and UV share the ability to induce p38- and JNK/SAPK-dependent signalling pathways regulating expression of c-Jun–ATF-2-dependent target genes, such as c-*jun*, activation of growth factor receptors required for the UV response is not involved in the initiation of MMS-dependent signal transduction pathways. We identified the redox state of the cell as critical for JNK/SAPK and p38 activation by MMS: while induction of JNK/SAPKs and p38 was almost completely blocked by pretreatment of the cells with reducing agents, such as glutathione (GSH) and *N*-acetylcysteine (NAC), depletion of the intracellular pool of GSH resulted in superinduction of JNK/SAPK activity by MMS. In light of the AP-1-dependent induction of GSH synthesis in response to alkylating agents, these data suggest a potential critical role of JNK/SAPKs and p38 activation in the regulation of the cellular defense program against the harmful consequences of MMS-induced cellular toxicity.

MATERIALS AND METHODS

Chemicals. Stock solutions of MMS and MNNG (obtained from Serva, Heidelberg, Germany) were prepared as described by Kaina et al. (38). L-Buthionine-*S*,*R*-sulfoximine (BSO), human epidermal growth factor (EGF), NAC, and TNF-α were purchased from Sigma Chemical Co.; GSH was purchased from Fluka.

Stock solutions of suramin (Germanin; Bayer Leverkusen) and ENU (3× recrystallized; kindly provided by Manfred Rajewsky) were freshly prepared in phosphate-buffered saline and diluted into the cell culture to final concentrations of 0.3 mM and 1 mM, respectively.

Cell culture and treatment of cells. HeLa, Jurkat, 293, NIH 3T3, and mouse L cells were grown at 37°C and 6% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfection of cells was performed as described previously (78).

For UV irradiation of cells, the culture medium was removed and UVC irradiated (40 J/m^2) where indicated, and the original culture medium was added back to the cells. Chemicals were added in the culture medium from concentrated stock solutions.

Enucleation of cells was performed as described previously (11). Briefly, 10^6 cells were seeded into a 25-cm^2 tissue culture flask 24 h prior to enucleation. For enucleation, the culture flask was filled with prewarmed medium (37°C) and cytochalasin B was added to a final concentration of 10 μg/ml. Flasks were immediately placed into the prewarmed (37°C) bottle cavities (GSA rotor of a Sorvall R2C centrifuge), and cells were spun down at 8,500 rpm for 30 min. After centrifugation, the karyoplast pellet was removed, and the cytoplasts were washed once with culture medium and then placed in regular culture medium to allow recovery for 14 to 16 h prior to induction. To test the efficiency of enucleation, the cells and cytoplasts were stained with the fluorescent DNA dye Hoechst 33528.

Protein kinase assays. In-gel kinase assay was performed as described previously (78). The activation of MAPKs (ERK-1 and -2) was determined either by Western blot analysis as described by Radler-Pohl et al. (64) or by an immune complex kinase assay using myelin basic protein as a substrate. Phospho-p38 was detected by Western blot analysis using a phospho-p38-specific polyclonal antibody (New England Biolabs) according to the manufacturer's instructions. For the immune complex kinase assay, cells were left untreated or stimulated with various agents. Subsequently, cells were washed twice with ice-cold phosphate-buffered saline and lysed at 0°C in lysis buffer (20 mM Tris [pH 7.5], 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 10 μg of pepstatin per ml). The lysate was centrifuged (13,000 rpm, 0°C for 20 min), and 1.5 μl of polyclonal antibodies raised against JNK1 (C-17; Santa Cruz Biotechnology) and JNK2 (FL; Santa Cruz), 2 μl of polyclonal antibody raised against ERK-1 (K-23; Santa Cruz), 1.5 μl polyclonal antiserum raised against the Flag epitope (D-8; Santa Cruz), or 1 μg monoclonal antibody against the hemagglutinin (HA) epitope (Boehringer Mannheim) was added to the supernatant, together with 50 μl of a 50% suspension of protein A-Sepharose beads. After 2 h of incubation at 4°C, the beads were washed twice with lysis buffer and once with kinase buffer. The immune complex kinase assays were performed in 25 μl of kinase buffer, consisting of 25 mM HEPES (pH 7.4), 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 5 μCi of [γ-^{32}P]ATP containing 2 μg of either glutathione *S*-transferase (GST)–Jun 1/166, GST-SAPK, or myelin basic protein. After 20 min at 30°C, sample buffer was added, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and phosphorylated substrates were visualized by autoradiography.

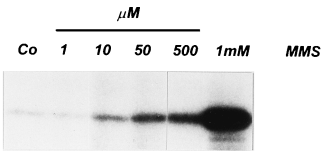

FIG. 1. Dose-dependent activation of JNK/SAPKs. Human 293 cells were left untreated (Co) or treated with the indicated concentrations of MMS. The cells were harvested 2 h after stimulation, and whole-cell extracts were prepared. Activation of JNK/SAPKs was determined by an immune complex kinase assay using GST-Jun 1/166 as the substrate.

RESULTS

Induction of JNK/SAPKs by specific classes of alkylating agents. A number of transcription factors such as c-Jun, c-Fos, and p62 TCF/Elk-1 have been shown to be phosphorylated in vitro and in vivo by distinct members of the MAPK family (21, 22, 28–30, 42, 62, 63, 81, 84) triggered by a large variety of extracellular conditions, in particular by agents that induce cellular stress. We have shown previously that one group of the MAPK family, the JNK/SAPKs, are activated in response to the alkylating agent MMS in many cell lines, e.g., HeLa cells, Jurkat cells, immortalized mouse fibroblasts, and 293 cells (reference 78, Fig. 1, and data not shown), in a dose-dependent manner (Fig. 1). Alkylating agents react with a variety of nucleophilic sites in DNA and protein, leading to different types of adducts. MMS and MNNG are known to react almost exclusively with the ring nitrogens of the purine bases, particularly N-7 of guanine (7), which correlates with the induction of cell killing, chromosomal aberrations, and sister chromatid exchanges (58). Only about 0.3% of the total alkyl adducts in DNA are O^6-methylguanine, which is the lesion critical in mutagenesis and malignant transformation. In contrast, ENU, which produces about 9% O^6-ethylguanine, is the most potent alkylating agent with respect to mutagenicity and carcinogenicity. The relative frequency of the different types of adducts depends in particular on the electrophilicity and therefore on the chemical reaction pattern of the alkylating agent in question.

To determine whether the activation of JNK/SAPKs is a general response to alkylating agents, both MNNG and ENU, representing either one of the two classes of monofunctional alkylating agents, were tested by immune complex kinase assays. MMS induced a transient activation of JNK/SAPK activity peaking 2 h after stimulation (Fig. 2A). Similarly, MNNG was a very potent inducer of JNK/SAPK activity (Fig. 2B). In-gel kinase assays confirmed that both p54 and p46 JNK/SAPK isoforms are activated upon MMS or MNNG treatment (data not shown).

In contrast, ENU at a concentration (1 mM) which is mutagenic in cultured mammalian cells (for a review, see reference 69), did not affect JNK/SAPK activity as measured by immune complex kinase assay (data not shown). Even a 10-fold higher concentration of ENU (10 mM), which led to efficient cell killing within 3 h, did not induce, and even slightly reduced, JNK/SAPK activity (Fig. 2C). In line with the lack of JNK/SAPK activation, we did not observe transcriptional activation of the c-*jun* gene upon ENU treatment (data not shown).

These results demonstrate that the activation of the stress response in the cells depends on the type of alkylating agent. Only substances like MMS and MNNG, which react preferentially with receptor atoms with high nucleophilic strength, are

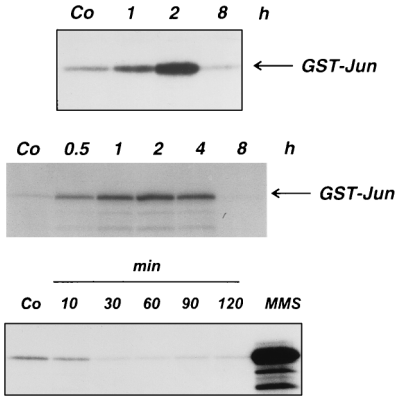

FIG. 2. Activation of JNK/SAPK by different types of alkylating agents. (A) Human 293 cells were left untreated (Co) or treated with MMS (1 mM). The cells were harvested at the indicated time points after stimulation, and whole-cell extracts were prepared. Activation of JNK/SAPKs was determined by an immune complex kinase assay using GST-Jun 1/166 as the substrate (B). Whole-cell extracts from untreated 293 cells (Co) or cells treated with MNNG (70 μM) for the indicated times were analyzed by an immune complex kinase assay as described for panel A (C). Human 293 cells were left untreated or treated with ENU (10 mM) for the indicated times, or MMS (1 mM) for 2 h, and whole-cell extracts were prepared. Activation of JNK/SAPKs was determined by an immune complex kinase assay with GST-Jun 1/166 as the substrate. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

potent activators of the JNK/SAPK signal transduction pathway. In contrast, ENU, which reacts with low-nucleophilic-strength sites, failed to do so.

Protein kinase cascades induced by MMS. To determine whether the activation of MAPKs by MMS is restricted to the JNK/SAPK subfamily, we measured the activities of the two other members of the MAPK family, ERKs (p44 ERK-1 and p42 ERK-2), and p38, in response to MMS treatment. JNK/SAPKs, ERKs, and p38 represent the three subfamilies, which are characterized by distinct dual phosphorylation motifs (23, 57) and which differ in their substrate specificities. ERKs are activated predominantly by growth factors or phorbol esters (reviewed in reference 48); SAPKs (JNKs) and p38 kinase are both activated by inflammatory cytokines and cellular stress, such as heat and osmotic shock, or UV irradiation (27, 42, 65).

To determine the activation of ERK-1 and ERK-2, we performed Western blot analysis using antibodies that recognize both ERK-1 and -2, exploiting the mobility shift of these kinases, which is indicative of their phosphorylation and activation status (43). Both ERK-1 and ERK-2 were activated within 15 min after EGF treatment, whereas no mobility shift was observed after MMS treatment up to 4 h (Fig. 3A). Induction of ERK-1 and ERK-2 activity by EGF (2.9-fold), but not MMS, was confirmed by an immune complex kinase assay using myelin basic protein as a substrate (Fig. 3B).

Similar to ERK-1 and ERK-2, p38 MAPK is activated by phosphorylation of Thr and Tyr residues, which can be monitored by Western blot analysis using an antiserum specific for phospho-p38. As shown in Fig. 3C, MMS treatment leads to enhanced phosphorylation of p38, reaching maximal levels 2 h poststimulation. To measure directly p38 MAPK activity, an expression vector encoding an epitope-tagged p38 protein was transiently transfected into 293 cells, and cells were treated with MMS or UV (40 J/m^2). Exogenously expressed p38 was purified by immunoprecipitation, and p38 activity was determined by an immune complex kinase assay using an antibody against the Flag epitope and bacterially expressed GST–ATF-2 as the substrate. Similar to the activation by UV irradiation, treatment of the cells with MMS led to the activation of Flag-p38. The kinetics of induction of p38 and JNK/SAPKs were very similar, reaching maximal levels 2 h poststimulation (Fig. 3D and data not shown). These data demonstrate that both JNK/SAPKs and p38 MAPK, but not ERK-1 and -2, are components of signal transduction pathways initiated by the alkylating agent MMS.

In general, signal transduction pathways represent cascades of protein kinases which are subsequently activated after the initiating primary signal. For example, JNK/SAPKs can be activated by phosphorylation at two residues, Thr-183 and Tyr-185, by MKK4, also termed SEK1 or JNKK. MKK4/SEK1 is able to activate both JNK/SAPKs and p38 MAPK (23). It is closely related to MKK3, a specific activator of p38 MAPK. MKK3 activity is enhanced by UV irradiation, inflammatory cytokines, and osmotic shock (23). MKK4/SEK1 itself is phosphorylated and activated by upstream kinases, such as MEKK1. The mechanism of MEKK1 activation is still unclear.

To examine whether MMS-induced signalling also includes these known members of the JNK/SAPK kinase cascade, we

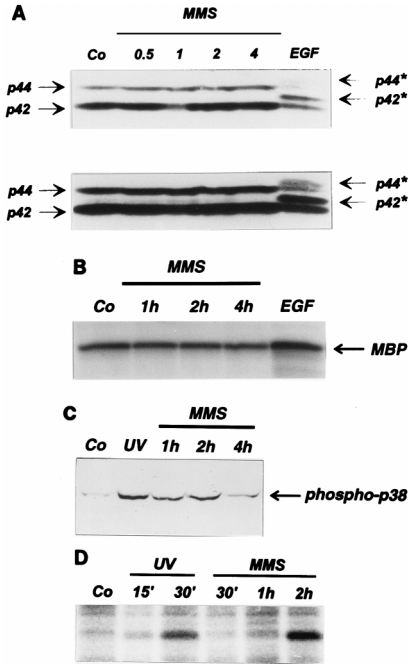

FIG. 3. Activation of p38 MAPK, but not ERK-1 and -2, by MMS. (A) Cell lysates were prepared from 293 cells at the indicated time points after MMS (1 mM) or EGF (10 ng/ml) treatment. Co, extract from untreated cells. Thirty micrograms of total cell lysate was resolved by SDS-PAGE (10% gel), and the ratio of activated and nonactivated forms of p42 and p44 MAPKs was measured by Western blot analysis using p42/44MAPK-specific polyclonal rabbit antibodies (66). Activation of the kinases is measured by the appearance of the slower-migrating forms of p42 and p44 MAPKs. The lower panel is a five-times-longer exposure of the upper panel to visualize activation of p44 MAPK. (B) Activation of ERK-1 and ERK-2 was determined by an immune complex kinase assay using myelin basic protein (MBP) as the substrate. (C) Thirty micrograms of total cell lysate was resolved by SDS-PAGE (10% gel), and the amount of phospho-p38 was detected by Western blot analysis using phospho-p38-specific polyclonal rabbit antibodies. Cells were left untreated (Co), irradiated with UV (40 J/m^2) and harvested after 30 min, or treated with MMS (1 mM) for the indicated times. (D) Human 293 cells transiently transfected with an epitope-tagged p38 MAPK expression vector were left untreated (Co), irradiated with UV (40 J/m^2), or treated with MMS (1 mM) for the indicated times. The p38 MAPK was immunoprecipitated with Flag-probe rabbit polyclonal antibody D-8 (Santa Cruz). Immune complex kinase assays were performed with GST–ATF-2 as the substrate. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

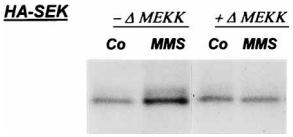

FIG. 4. MEKK-dependent activation of SEK by MMS. Human 293 cells were cotransfected with an expression vector encoding HA-tagged SEK together with an empty expression vector (−) or a vector encoding transdominant-negative MEKK (+ ΔMEKK). The cells were not treated (Co) or treated with MMS (1 mM), and whole-cell extracts were prepared 2 h poststimulation. Exogenously expressed SEK was immunoprecipitated with the HA-specific monoclonal antibody, and the kinase activity was measured by immune complex assay using [γ-^{32}P]ATP and GST-SAPK as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

first studied the effect of MMS on the activation status of SEK1. An expression vector encoding an HA-tagged SEK1 protein was transfected into 293 cells. Extracts from untreated and MMS-treated cells were prepared, and expression of equal amounts of HA-SEK in untreated and MMS-treated cells was confirmed by Western blot analysis using an HA-specific monoclonal antibody (data not shown). Immunoprecipitated HA-SEK immobilized on protein A-Sepharose was assayed for its ability to phosphorylate bacterially expressed GST-SAPK in vitro. As shown in Fig. 4, HA-SEK was activated by MMS within 2 h. To investigate the involvement of MEKK1 in the MMS response, transfection experiments were performed in the presence or absence of an expression vector encoding a transdominant-negative mutant of MEKK1 (51). The activation of SEK1 by MMS could be blocked by overexpression of the transdominant-negative mutant, suggesting that MEKK1, or a closely related kinase, is also part of the signal transduction pathway induced by MMS.

The MMS response is independent of a nuclear signal. Having identified p38, JNK/SAPKs, SEK1, and MEKK1 (or a closely related protein kinase) as being activated by MMS, we wanted to determine the primary cellular targets.

The experiments described above showed that the specific initiation of the stress-activated signal transduction pathway leading to activation of JNK/SAPK and p38 MAPK clearly depends on the electrophilic reactivity of the alkylating agents and, thereby, the affected alkylation products, either nucleic acids, lipids, or proteins. Most recently, DNA damage has been suggested to be the initiating signal in response to *cis*-platinum and mitomycin in activating signal transduction pathways that include activation of c-Abl and JNK/SAPKs (39). However, in agreement with recent findings (45), mitomycin activated c-Abl but not JNK/SAPKs, whereas MMS was a very poor inducer of c-Abl but efficiently activated JNK/SAPK activity (data not shown). On the other hand, induction of JNK/SAPKs by UV irradiation has been found to be independent of DNA damage (26). To determine whether modification of DNA and/or other cellular components are essential intermediates of the cellular response against alkylating agents, such as MMS and MNNG, we first investigated the requirement of nuclear signals generated by alkylation of genomic DNA in activation of the JNK/SAPK pathway.

To directly analyze the possible role of alkylation of the nuclear DNA in the activation of the JNK/SAPK cascade, we measured JNK/SAPK activity in cells lacking nuclei (cytoplasts). Mouse L cells were enucleated by centrifugation in the presence of cytochalasin B, and the efficiency of enucleation was estimated by staining intact cells and cytoplasts with a fluorescent dye for nuclear DNA. As shown in Fig. 5A, almost 100% of the cells were enucleated. The JNK/SAPK activity in intact cells and cytoplasts, which were left untreated or treated with MMS, was determined by an immune complex kinase assay. As described previously (26), we usually observed an increase in basal JNK/SAPK activity in cytoplasts, compared to intact cells, possibly due to the cytochalasin B treatment. Nevertheless, in three independent experiments treatment with MMS always induced JNK/SAPK activation in cells lacking their nuclei with an activation factor (4.3-fold) similar to that in intact cells (4.8-fold) (Fig. 5B). In agreement with previous findings (26), under these conditions we also observed JNK/SAPK activation by UV irradiation in cytoplasts (data not shown).

These experiments rule out the absolute requirement of a nuclear signal generated by alkylation of genomic DNA for induction of JNK/SAPK activity by MMS. Although we have not eliminated the possibility that mitochondria are involved in JNK/SAPK activation, as suggested for heat shock-dependent JNK/SAPK activation (1), we favor the concept that, as for UV (1), the primary signal induced by MMS that initiates the JNK/SAPK signal transduction pathway is established in the cytoplasm or cell membrane.

The MMS response does not involve cell surface receptors. Having excluded genomic DNA as a prerequisite for the MMS response, we wanted to know if the primary signal is initiated at the cell membrane and whether it involves cell surface receptors. Candidate receptors which are possibly affected by MMS are those for interleukin-1, TNF-α, EGF, and G-protein-coupled receptors, which have been found to be involved in common routes of signal transduction pathways leading to JNK/SAPK activation (42, 51, 65, 72, 80).

Examples of G-protein-coupled receptors which are primary targets for initiation of JNK/SAPK-specific pathways are the m1 and m2 muscarinic receptors (16, 17). Activation of this pathway involves $\beta\gamma$ subunits of heterotrimeric G proteins. This activation can be nearly abolished by expression of β-adrenergic receptor kinase (βARK), which sequesters free $\beta\gamma$ complexes when dissociated from Gα subunits upon receptor stimulation, thus blocking $\beta\gamma$-dependent pathways (18).

To analyze whether MMS induces the cellular response by mimicking activation of G-protein-coupled receptors, we determined the involvement of $\beta\gamma$ subunits of heterotrimeric G proteins in the MMS response in mouse L cells which were stably expressing the βARK. For activation of a G-protein-coupled receptor, we used lysophosphatidic acid (LPA), an albumin-bound phospholipid mitogen that is released from activated platelets (for reviews, see references 36, 54, and 55). Treatment of the parental cell line with LPA activated JNK/SAPKs within 15 min, and this activation was nearly abolished in the stably transfected clones expressing the transdominant negatively acting βARK protein. In contrast, JNK/SAPK activity induced by MMS, UV, and TNF-α was even slightly enhanced in the stably transformed cells compared to their wild-type counterparts (Fig. 6). These experiments rule out that MMS activates JNK/SAPKs via $\beta\gamma$ subunits of heterotrimeric G proteins, at least in L cells.

It has been shown for UV irradiation that among the primary targets are growth factor receptors, such as the EGF receptor (66). The direct involvement of growth factor receptors in UV-induced signalling was demonstrated by exploiting the effects of suramin, which is a general inhibitor of the function of membrane-positioned receptors (9, 35, 83). To address the question of whether MMS and UV irradiation have the same primary target, activation of growth factor receptors, we determined activation of JNK/SAPKs by UV irradiation and MMS treatment in the presence or absence of suramin. Cells were pretreated with suramin prior to induction with MMS or UV irradiation, and JNK/SAPK activity was

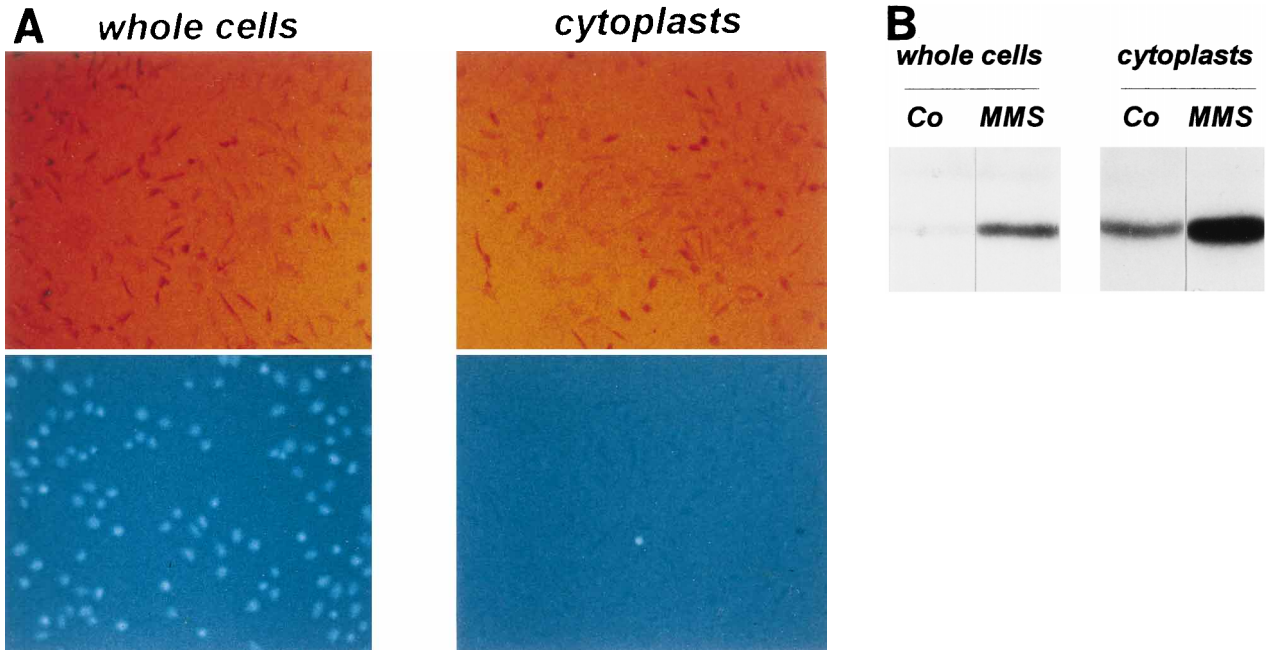

FIG. 5. Activation of JNK/SAPKs in cytoplasts. Mouse L cells were left untreated or treated with cytochalasin B (10 μg/ml) and left intact or enucleated by centrifugation. (A) Cytoplasts and intact cells were stained with the fluorescent DNA dye Hoechst 33528 (lower panel). (B) Adherent cytoplasts and intact cells were either left untreated (Co) or exposed to MMS (1 mM). After 2 h, cells and cytoplasts were harvested, and total cell extracts were prepared. JNK/SAPKs were immunoprecipitated with rabbit polyclonal antibodies, and the protein kinase activity was measured in the immune complex with GST-Jun and [γ-^{32}P]ATP as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

determined by an in-gel kinase assay. The data are clear-cut: activation of JNK/SAPKs by UV irradiation was markedly reduced by pretreatment of cells with suramin, whereas the induction by MMS was not affected. Suramin alone had no effect on JNK/SAPK activity (Fig. 7). This result shows that although MMS and UV irradiation activate common routes of signal transduction pathways, they differ in their primary targets.

Inhibition of the MMS response by GSH and NAC. Although the primary target of MMS is still unknown, modulation of poly(ADP-ribose) polymerase may be important for MMS-induced signalling. Activation of this enzyme, which is a component of the cellular DNA repair machinery, has metabolic consequences, particularly the consumption of NAD resulting in a depletion of ATP. An inhibitor of poly(ADP-ribose) polymerase, 3-aminobenzamide (ABA), prevented the depletion of NAD and ATP and the subsequent cell killing by MMS (53). On the other hand, there is evidence that the intracellular level of GSH, regulating the redox state of the cell (for a review, see reference 49), may be an important sensor for the initiation of the cellular response to alkylating agents. GSH and thiols play a crucial role in the detoxification of oxidative stress by carcinogenic xenobiotics (15), and an elevated level of GSH has been associated with acquired alkylating resistance (10).

To assess the role of oxidative stress and poly(ADP-ribosyl)ation in the activation of JNK/SAPKs and p38 by MMS, we studied the effects of pretreatments of cells with GSH, NAC, and ABA prior to MMS induction in mouse fibroblasts, in Jurkat cells, and in 293 cells. In all three cell lines, activation of JNK/SAPKs, measured by an immune complex kinase assay, was found to be inhibited by both GSH and NAC pretreatment, whereas the poly(ADP-ribose) polymerase inhibitor ABA had no effect (Fig. 8 and data not shown). Similarly, activation of p38 by MMS was blocked in the presence of GSH (data not shown). GSH and NAC alone did not affect the activation status of JNK/SAPKs or p38 in a time period of up to 4 h (data not shown).

To obtain additional support for a critical role of GSH in the JNK/SAPK activation by MMS, the cellular GSH pool was depleted by treatment of the cells with BSO. BSO is a specific inhibitor of γ-glutamylcysteine synthetase, the enzyme responsible for the first and rate-limiting step of GSH synthesis (50). Immune complex kinase assays revealed that treatment of the cells with 500 μM BSO alone did not induce JNK/SAPK activity (data not shown). Induction of JNK/SAPKs with suboptimal doses of MMS (500 μM [Fig. 9]; see also Fig. 1) was dramatically enhanced by pretreatment with BSO.

These results demonstrate that the cellular GSH content is a critical parameter for the stress signaling cascade induced by MMS. To test if the inhibition of JNK/SAPK activity by GSH and NAC is specific for induction by alkylating agents, we investigated the effect of pretreatment of 293 cells with GSH and NAC on the JNK/SAPK activation by UV irradiation or treatment with TNF-α. In agreement with previous findings (1,

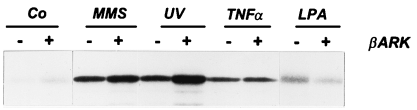

FIG. 6. The MMS response does not include activation of G-protein-coupled receptors. Wild-type mouse L cells (−) and stably transformed derivatives expressing βARK (+) were left untreated (Co), treated with MMS (1 mM, 2 h), TNF-α (5 ng/ml, 15 min), or LPA (10 μg/ml, 15 min), or irradiated with UV (40 J/m^2, 30 min), and whole-cell extracts were prepared. JNK/SAPKs were immunoprecipitated with rabbit polyclonal antibodies, and the protein kinase activity was measured in the immune complex with GST-Jun and [γ-^{32}P]ATP as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

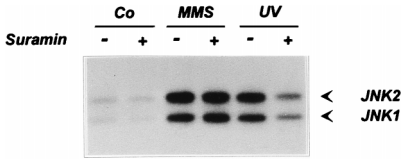

FIG. 7. The MMS response does not involve growth factor receptors. Cell extracts from untreated human 293 cells (Co) or from cells treated with MMS (1 mM, 2 h) or irradiated with UV (40 J/m^2, 30 min), either without (−) or with (+) pretreatment with suramin (0.3 mM, 40 min), were subjected to an in-gel kinase assay using GST-Jun as the substrate.

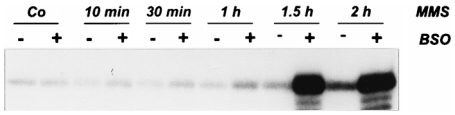

FIG. 9. Depletion of endogenous GSH leads to superinduction of JNK/SAPKs by MMS. Human 293 cells were left untreated (−) or treated with 1 mM BSO for 24 h, followed by treatment with MMS (500 μM). Whole-cell extracts were prepared at the indicated times after addition of MMS, and JNK/SAPKs were immunoprecipitated. The protein kinase activity was measured in the immune complex, using GST-Jun and [γ-^{32}P]ATP as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

25), both GSH or NAC are potent inhibitors of UV-mediated JNK/SAPK activation (Fig. 10 and data not shown), whereas activation by TNF-α is not impaired (Fig. 10), suggesting the existence of specific cellular components involved in the JNK/SAPK activation in response to different forms of cellular stress.

DISCUSSION

Monofunctional alkylating agents like MMS and MNNG induce a specific program of gene expression, known as the cellular stress response, which is commonly activated by a variety of environmental cues, such as chemical carcinogens and UV irradiation. This program includes the activation of JNK/SAPK activity required for transcriptional activation of c-*fos* and c-*jun*, whose gene products have been proposed to be required for the cellular defense against genotoxic agents such as UV (68).

To understand the primary reactions of the cell in response to alkylating agents, the mechanisms responsible for the activation of stress-related protein kinases need to be identified. Here we show that MMS and MNNG, like other cellular stress-inducing agents, such as UV irradiation, heat shock, and protein synthesis inhibitors, activate both the JNK/SAPKs and another member of the MAPK family, the HOG1 homolog p38 MAPK. This activation was independent of the cytotoxic properties of MMS: even at a concentration of MMS (10 μM) that does not affect cell viability (40b), JNK/SAPK activity was significantly induced. Moreover, ABA, an inhibitor of poly(ADP-ribose) polymerase which prevents depletion of NAD and ATP and the subsequent killing by alkylating agents (53), had no effect on the activation of JNK/SAPK by MMS.

The activity of the third subgroup of MAP kinases, consisting of ERK-1 and ERK-2, is not affected by MMS. This specific activation of different subfamilies of MAPKs reveals the existence of parallel MAPK cascades that can be activated independently and simultaneously, representing a common mechanism of signal transduction that has been adapted to couple different stimuli to distinct physiological responses.

Despite the slower kinetics of induction of JNK/SAPKs and p38 by MMS and MNNG (compared to UV), reaching maximal levels between 1 and 2 h poststimulation, efficient induction of JNK/SAPK activity in enucleated cells (cytoplasts) strongly suggests a direct activation of protein kinase and/or inhibition of active phosphatase cascades which does not require MMS-induced transcriptional activation of cellular genes and enhanced de novo protein synthesis. Recently, members of protein kinase cascades induced by extracellular stimuli such as UV and TNF-α have been identified. By transient transfection assays, under conditions where UV-induced activation of SEK was not yet detectable, we observed enhanced kinase activity of an epitope-tagged SEK1, also known as MKK4 or JNKK, in response to MMS, which could be blocked by overexpression of a transdominant negative form of MEKK1. MEKK1 has been reported to function preferentially in the JNK/SAPK pathway, specifically controlling SEK1/MKK4 activity (51, 82), but not MKK3, the direct upstream activator of p38 MAPK (23). Since p38 MAPK is also activated by MMS, the inhibition by the transdominant negative MEKK1 protein may be explained by the existence of a closely related protein kinase which shares the binding sequence and phosphorylation motif for their upstream protein kinase. This MEKK1-related kinase may phosphorylate and thereby activate MKK3. The existence of MEKK1-related protein kinases critical for the MMS response is also supported by our findings that the transactivation of JNK/SAPKs by MEKK1 cannot be further enhanced by MMS treatment (data not shown). Interestingly, the activation of these pathways does not represent a general cellular response to different classes of alkylating agents. MMS and MNNG, which show similar reaction kinetics and similar affinities to nucleophilic sites, are very potent activators of JNK/SAPK activity. In contrast, ENU, which acts by a different reaction mechanism (S_N1 instead of S_N2), and thereby preferentially attacks other nucleophilic sites leading to different alkylation products, was unable to activate JNK/SAPK activity. The differences in the adducts are well defined with respect to the alkylation of DNA. Nevertheless, we provide evidence that

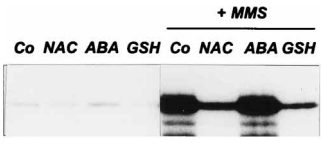

FIG. 8. Inhibition of the MMS response by GSH and NAC. Human 293 cells were left untreated (Co) or treated for 2 h with GSH (15 mM), ABA (10 mM), or NAC (15 mM) and cultured in the presence or absence of MMS (1 mM, 2 h). Whole-cell extracts were prepared, and JNK/SAPKs were immunoprecipitated. The protein kinase activity was measured in the immune complex, using GST-Jun and [γ-^{32}P]ATP as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

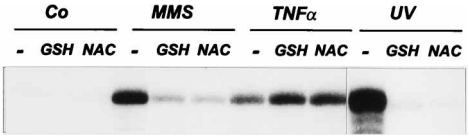

FIG. 10. GSH and NAC inhibit JNK/SAPK activation by MMS and UV but not by TNF-α. Human 293 cells were left untreated (Co) or treated with GSH (15 mM) or NAC (15 mM). Subsequently, cells were left untreated, treated with MMS (1 mM, 2 h) or TNF-α (5 ng/ml, 15 min), or irradiated with UV (40 J/m^2, 30 min). Whole-cell extracts were prepared at the indicated time points, and JNK/SAPKs were immunoprecipitated. The protein kinase activity was measured in the immune complex, using GST-Jun and [γ-^{32}P]ATP as substrates. The products of the phosphorylation reaction were resolved by SDS-PAGE and detected by autoradiography.

alkylation of genomic DNA is not critical for induction of the MMS response. Most importantly, by preparing cytoplasts, we found that induction of JNK/SAPK activity by MMS did not require alkylation of nuclear DNA. In addition, c-Abl, a nonreceptor tyrosine kinase which has been suggested to be activated selectively by DNA-damaging agents (ionizing radiation, mitomycin, and *cis*-platinum) and whose activation correlates with the activation of JNK/SAPKs (39), seems not to be involved in the MMS response (reference 45 and data not shown). Although we cannot completely rule out the involvement of alkylation of mitochondrial DNA, taken together, these data favor the concept that specific alkylation of components in the cytoplasm or cell membrane by MMS and MNNG, but not ENU, triggers the initiation of signal transduction pathways involving JNK/SAPKs and p38.

Although MMS and UV irradiation share common routes of signal transduction pathways, such as activation of JNK/SAPK and p38 activity, hyperphosphorylation of c-Jun and ATF-2, and transcriptional activation of c-*jun* and c-*fos* expression, the primary cellular targets of UV and MMS are clearly different. Among the targets of UV are cell surface receptors in the cell membrane, which are activated immediately after irradiation of the cells and can be blocked by pretreatment of the cells with the inhibitor suramin (66). In agreement with these findings, we observed an efficient reduction of JNK/SAPK activation in response to UV. In contrast, we did not find an effect of suramin on the activation of JNK/SAPK activity by MMS. In line with these findings, we did not obtain evidence for MMS-induced hyperphosphorylation of the EGF receptor (40a), which is a characteristic of UV-induced immediate-early events (66). The involvement of membrane-associated receptors in the UV response was also supported by the reduction of UV-induced activation of JNK/SAPKs in the presence of low concentrations of Triton X-100 (1). In contrast, Triton X-100 treatment did not affect MMS-induced JNK/SAPK activation (81a). In addition to the resistance of the MMS response to suramin, we were able to rule out the involvement of βγ subunits of heterotrimeric G proteins coupled to cell surface receptors in the MMS response, at least in L cells. Stimulation of such G-protein-coupled receptors, e.g., m1 and m2 muscarinic receptors, was found to activate JNK/SAPKs through the βγ subunit of heterotrimeric G proteins (16, 17). Here we show that LPA, recently emerging as an intercellular phospholipid messenger with a wide range of biological activities (for reviews, see references 54 and 55), can activate the JNK/SAPK pathway. This activation is mediated by the βγ subunit of the heterotrimeric G protein activated by its specific receptor. Accordingly, induction could be blocked by overexpression of a CD8-βARK chimeric protein which sequesters free βγ complexes when dissociated from Gα subunits (18). In contrast, activation of JNK/SAPK activity by MMS was unaffected by the CD8-βARK fusion protein.

Despite exclusion of many components of signal transduction pathways induced by other types of cellular stress-inducing agents, the primary target molecule(s) responsible for initiation of MMS-specific signal transduction remains to be identified. Possibly, MMS directly methylates one or multiple upstream components of signal transduction pathways, e.g., MEKK1, PAKs, Cdc42, Rho, and/or Rac1 (16, 17, 51, 52, 67, 79, 82), and thereby activates these proteins by changing their conformation. In fact, overexpression of a transdominant negative mutant of Rac1, but not Rho, reduced MMS-dependent induction of JNK/SAPK activity (45). Since ENU, in contrast to MMS and MNNG, does not activate the JNK/SAPK pathway, the specific modifications of these critical proteins created by MMS and MNNG and by ENU must be different. Although protein modifications induced by monofunctional alkylating agents have not been analyzed in great detail, qualitative and quantitative differences have been observed in vitro (61). It is also possible that MMS inhibits a specific phosphatase responsible for downregulation of one, or multiple, activated upstream protein kinases. Inhibition of a constitutive dual-specificity JNK phosphatase has been found to be responsible for JNK/SAPK and p38 activation by arsenite (14). Because MMS leads to induction of the JNK/SAPK kinase SEK1, which can be inhibited by a transdominant-negative mutant of MEKK, inactivation of JNK phosphatase cannot account for JNK/SAPK activation in response to MMS. A similar scenario of phosphatase-dependent activation of signal transduction pathways has been established recently for the activation of the EGF and platelet-derived growth factor receptors by UV (40). Inhibition of a protein tyrosine phosphatase by UV is due to direct modification of catalytically important SH groups of tyrosine phosphatases which can be blocked in the presence of reducing agents, such as NAC (40). This is in agreement with previous findings demonstrating that oxidative stress contributes in part to the transient JNK/SAPK activation and induction of c-*jun* transcription by UV (1, 14, 25). In contrast to UV, we did not obtain evidence for direct modification of SH groups of tyrosine phosphatases by MMS (40a). In fact, in vitro MMS preferentially modifies lysine and histidine residues, rather than cysteines (60). Nevertheless, we found the redox state of the cell very critical for induction of the MMS response. JNK/SAPK activation by MMS is inhibited by pretreatment of the cells with GSH or NAC, which acts by raising the intracellular concentrations of cysteine and hence of GSH (5). Vice versa, depletion of the intracellular pool of GSH resulted in superinduction of JNK/SAPK activity. GSH and other thiols play a crucial role in the detoxification of carcinogenic xenobiotics (15). Although GSH and NAC interfere with both UV- and MMS-induced signalling, the mechanisms of interference are different. While the direct modification of SH groups of growth factor receptor tyrosine phosphatases by UV leading to inactivation is blocked by NAC, the lack of EGF receptor phosphorylation by MMS and the resistance of MMS-induced JNK/SAPK activation against suramin and Triton X-100 strongly suggest that the targets of GSH and NAC interference in UV- and MMS-induced signalling are different. It has been shown that GSH can directly react with alkyl methanosulfonates, probably via GST-mediated catalysis (41). Accordingly, both MMS and MNNG cause a rapid decrease in cellular GSH (53). This manipulation of the cellular GSH content may be the trigger for induction of oxidative stress which causes, via a yet unknown sensor, the activation of JNK/SAPKs. However, neither depletion of GSH by treatment with BSO nor treatment of cells with GSH or NAC activated JNK/SAPKs. Thus, it is likely that GSH and NAC, by interacting directly or through enzyme-catalyzed reactions with electrophilic compounds, resemble a dominating competitive pathway reducing the number of reactions of alkylating agents with critical target molecules.

Regardless of the primary receptor for MMS and MNNG, these agents activate JNK/SAPK- and p38-specific signal transduction pathways, resulting in hyperphosphorylation of c-Jun, ATF-2, and TCF/Elk (12, 13, 65, 78, 81, 84). In turn, c-*jun* and c-*fos* transcription is induced, leading to newly synthesized c-Jun and c-Fos proteins, the major components of transcriptionally active AP-1. AP-1 has been found to bind DNA-regulatory elements in the promoters of several xenobiotic-metabolizing enzymes: GSTs, glucuronyltransferases, and NAD(P)H:quinone reductase (8). Transcriptional activation of these genes leads to an enhanced GSH level in the cells within 4 h

(47). This rise in GST content may allow inactivation of MMS and may therefore be responsible, at least in part, for down-regulation of JNK/SAPK activity 4 to 6 h poststimulation. Our data suggest that induction of JNK/SAPKs and p38 may be an important mechanism for controlling the genetic program which cells use to actively and immediately respond to environmental cues by induction of proteins that are ultimately responsible for detoxification of cytotoxic xenobiotics.

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