Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27

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SUMMARY

We have studied the contribution of the individual kinases of the MAP (mitogen-activated protein) kinase family, including ERK (extracellular-signal regulated kinase), JNK/SAPK (c-JUN NH2-terminal kinase/stress-activated protein kinase) and p38, to activation of the HSP27 (heat shock protein 27) kinase MAPKAP kinase-2/3 and to HSP27 phosphorylation in Chinese hamster CCL39 cells stimulated by either growth factors, cytokines or stressing agents. In vitro assays using fractionated cell extracts or immunoprecipitates indicated that only fractions containing ERK or p38, and not those containing JNK/SAPK, had the capacity to activate MAPKAP kinase-2/3. In vivo, however, it appeared that only p38 is an upstream activator of HSP27 phosphorylation after both stress or growth factor stimulation: expression of an interfering mutant of ras, which blocked the activation of ERK by both types of inducers, had no effect on HSP27 phosphorylation and p38 activation; and the cell-permeant specific inhibitor of p38, SB203580, blocked MAPKAP-kinase2/3 activation and HSP27 phosphorylation. HSP27 has been suggested to have a phosphorylation-activated homeostatic function at the actin cytoskeleton level. This raises the possibility that p38 might be directly involved in mediating actin responses to external stimuli. Accordingly, we observed that a prior activation of p38 increased the stability of the actin microfilaments in cells exposed to cytochalasin D. The effect was dependent on the expression of HSP27 and was totally annihilated by blocking p38 activity with SB203580. The results provide strong support to the idea that activation of p38 during adverse environmental conditions serves a homeostatic function aimed at regulating actin dynamics that would otherwise be destabilized during stress. Its activation during normal agonist stimulation may constitute an additional actin signaling pathway, the importance of which depends on the level of expression of HSP27.

Key words: Actin, p38 MAP kinase, Heat shock protein 27 (HSP27)

INTRODUCTION

HSP27 (heat shock protein 27) is a highly conserved oligomeric protein related to the α-crystallin proteins and a member of the heat shock protein family. Heat shock and other stress-inducing agents induce a rapid phosphorylation of HSP27, the transcriptional activation of the HSP27 gene and the accumulation of the protein (see Arrigo and Landry, 1994, for review). The physiological significance of the accumulation of HSP27 after stress has been demonstrated in gene transfection studies, which showed that overexpression of HSP27 confers resistance against heat shock and oxidative stress (Landry et al., 1989; Huot et al., 1991; Lavoie et al., 1993a; Mehlen et al., 1995; Huot et al., 1996). Overexpression of a non-phosphorylatable form of HSP27 was much less effective in mediating protection, suggesting that the function of HSP27 might be activated by phosphorylation (Lavoie et al., 1995; Huot et al., 1996). HSP27 behaves in vitro as a phosphorylation-regulated F-actin capping protein capable of inhibiting actin polymerization (Miron et al., 1991; Benndorf et al., 1994). This activity appears to be involved in the protective function of HSP27 in vivo, since overexpression of wild-type HSP27, but not a non-phosphorylatable HSP27, increases the stability of the actin microfilaments during heat shock or treatments with H2O2, menadione or cytochalasin D, and contributes to a faster recovery of the disrupted microfilaments after treatments (Lavoie et al., 1993a; Huot et al., 1995, 1996). Phosphorylation of HSP27 is also induced by growth/differentiation factors and occurs on the same serine residues that are phosphorylated after stress (Arrigo and Landry, 1994; Landry et al., 1992; Gaestel et al., 1991), suggesting that the same function of HSP27 is activated in both situations. Overexpression of wild-type HSP27 in serum-starved growth-arrested cells causes an enhanced F-actin accumulation in response to growth factors; overexpression of a non-phosphorylatable mutant partially blocks this response (Lavoie et al., 1993b). HSP27 phosphorylation thus appears to be involved in the regulation of microfilament dynamics, modifying the actin response to physiological agonists and stabilizing the actin cytoskeleton under stress conditions.

In mouse and Chinese hamster cells, HSP27 kinase activity purifies as two closely related polypeptide species of some 45
and 54 kDa, and was initially identified as the serine/threonine protein kinase MAPKAP kinase-2 (Stokoe et al., 1992a,b; Huot et al., 1995). It is not totally clear whether the p45-54 HSP27 kinases are two isoforms coded for by the same gene or are the products of two distinct genes. At least in human, a second HSP27 kinase, functionally similar and 70% identical at the amino acid level to MAPKAP kinase-2, has been cloned and termed MAPKAP kinase-3 (McLaughlin et al., 1996). MAPKAP kinase-2 and -3 are themselves activated by phosphorylation of serine residues by members of the mitogen-activated protein (MAP) kinase family of proteins (Stokoe et al., 1992a,b; Huot et al., 1995; Zu et al., 1995; Rouse et al., 1994; McLaughlin et al., 1996; Ben-Levy et al., 1995), implying that MAP kinases might be mediating, via HSP27, some of the responses of the actin cytoskeleton to external stimuli.

In mammalian cells, three groups of MAP kinases involved in three distinct signaling pathways have been identified (reviewed by Davis, 1994; Marshall, 1995; Waskiewicz and Cooper, 1995; Cobb and Goldsmith, 1995). The ERK1-2 (extracellular-signal-regulated kinase 1-2) or p44-42 MAP kinase signaling pathway is a critical regulatory element of cell growth and differentiation (Mansour et al., 1994; Cowley et al., 1994; Pages et al., 1993; Brunet et al., 1994). ERK1-2 activation has been characterized mostly in response to agonists of tyrosine kinase receptors, but it also occurs upon exposures to stressing agents such as heat shock, hyperosmolarity, oxidants, X-rays and UV light (Chung et al., 1992; Dubois and Bensaude, 1993; Fialkow et al., 1994; Terada et al., 1994; Matsuda et al., 1995; Guyton et al., 1996; Stevenson et al., 1994; Sachsenmaier et al., 1994; Radler-Pohl et al., 1993; Devary et al., 1992), where it may be an important surviving factor (Guyton et al., 1996). Stresses, however, more strongly induce the MAP kinases JNK/SAPK (c-JUN NH2-terminal kinase/stress-activated protein kinase) and p38, which were both implicated in the induction of apoptosis triggered by growth factor removal (Xia et al., 1995). JNK/SAPK was first described as a UV-light activated c-Jun kinase and suggested to trigger a protective response through the activation of genes coding for protective proteins (Devary et al., 1992; Kyriakis et al., 1994; Drijard et al., 1994). p38 is a mammalian homolog of the S. cerevisiae HOG1 kinase, an essential component of the yeast osmosensing signal transduction pathway (Han et al., 1994; Brewster et al., 1993; Rouse et al., 1994; Freshney et al., 1994). In mammalian cells, p38 appears to be involved in the production of cytokines by stimulated monocytes and to mediate the aggregation of platelets in response to collagen (Lee et al., 1994; Saktatvala et al., 1996).

The present study was aimed at determining the in vivo contribution of the three individual MAP kinases to activation of MAPKAP kinase-2/3, phosphorylation of HSP27, and HSP27-dependent regulation of microfilament dynamics. It has been suggested that p38 is the major physiological activator of MAPKAP kinase 2/3 during stress (Rouse et al., 1994; Freshney et al., 1994; Cuenda et al., 1995). However, ERK can also activate MAPKAP kinase-2/3 in vitro (Stokoe et al., 1992a,b; Huot et al., 1995; Zu et al., 1995; Rouse et al., 1994; McLaughlin et al., 1996; Ben-Levy et al., 1995) and thus might contribute in vivo to HSP27 phosphorylation, particularly when it is strongly activated by growth/differentiation agents. Our results, however, indicate that p38 can also be activated by non-stressing agonists and that only p38, not ERK or JNK/SAPK, contributes to HSP27 phosphorylation in all conditions tested. Moreover, using the piritidinyl imidazole SB203580 to specifically inhibit p38 activity in vivo, we further show that the activation of p38 participates, via HSP27 phosphorylation, in the regulation of actin microfilament dynamics.

**MATERIALS AND METHODS**

**Plasmids**

The plasmid pSVT7 was prepared using the vector pSVT7 and hamster HSP27 cDNA (Lavoie et al., 1990; Bird et al., 1987). The LT epitope (TPPPEPET) (MacArthur and Walter, 1984) was added at the C-terminal sequence of HSP27 by an insertional overlapping polymerase chain reaction; in this reaction, the HSP27 stop codon was replaced by the PstI restriction enzyme recognition sequence, adding two amino acids (Leu and Gln) between HSP27 and the LT epitope. A similar polymerase chain reaction procedure was used to prepare a pSVT7 expression vector containing the MAPKAP kinase-2 cDNA (Zu et al., 1994). The vectors pcDNA3-HA-p38 (Moscat et al., unpublished), HAP-MAP P44 (Meloche et al., 1992) and pEX-ERK2-TAG (Howe et al., 1992) were used for the expression of the N-terminal HA-tagged p38, HA-tagged ERK-1 and Myc-tagged ERK-2, respectively. The dominant negative form of the ras protein (N17-ras) was expressed using the vector pZIPrasAsn17 (Cai et al., 1990). The active form of the ras protein (HA-ras) was expressed using the vector pH06F1 (Shih and Weinberg, 1982).

**Cell culture and transfection**

Hu27-6#6, Hu27-#V and neo-3# cells are clonal cell lines derived from the Chinese hamster CCL39 cell line. All three cell lines express the neo gene and a normal level of endogenous HSP27 (1.5 ng of HSP27 per μg of total protein). In addition, Hu27-6#6 cells express 3.4 ng of wild-type human HSP27 per μg of total proteins and Hu27-#V cells express 3.7 ng/μg of total proteins of a mutant human HSP27 in which all three phosphorylation sites were replaced by glycine residues (Lavoie et al., 1993b; Lavoie et al., 1995). The clonal cell line CHSP27D does not express a detectable amount of either Chinese hamster or human HSP27. It is also a derivative of the CCL39 cell line and was obtained serendipitously while screening HSP27 transfected cell clones. CCL39 and their derivatives were maintained at 37°C in a 5% CO2 humidified atmosphere, in Dulbecco’s modified Eagle’s medium containing 2.2 g/l NaHCO3, 4.5 g/l glucose, supplemented with 5% fetal calf serum. Clonal cell lines were used at a passage number lower than 15. 3T3-NIH and CCL39 cells were transfected using 5 μg/106 cells of the desired plasmid and 35 μg/106 cells of carrier DNA. The cells were replated 24 hours later and used at 44 hours (exponentially growing cells) or 68 hours (quiescent cells) after transfection.

**Immunoprecipitation**

MAPKAP kinase-2/3 and ERK2 were immunoprecipitated using the rabbit anti-GST-MAPKAP kinase-2 and anti-C-terminal ERK2 peptide antibodies, respectively (Huot et al., 1995). The immunoprecipitating antibodies against p38 (#473 and #474) and JNK/SAPK were raised in rabbits against synthetic peptides corresponding to the C-terminal sequence PPLQEEMES of murine p38 (Han et al., 1994) and ASTGPLEGCR of rat JNK2/SAPKα (Kyriakis et al., 1994), respectively. Myc-, HA- and LT-tagged proteins were immunoprecipitated with the monoclonal antibodies 9E10, 12CA5 and KT3, respectively. After stimulation, exponentially growing cells or serum-starved cells, deprived for 24 hours (for mitogen stimulation), were scraped and extracted in lysis buffer (20 mM 3-(N-morpholino) propanesulfonic acid, pH 7, 10% glycerol, 80 mM β-glycerophosphate, 5 mM EGTA, 0.5 mM EDTA, 1 mM sodium vanadate, 5 mM sodium
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RESULTS

MAPKAP kinase-2/3 reactivating activity is associated exclusively with Mono-Q fractions containing ERK1-2 and p38 MAP kinase activities

Extracts of thrombin- or H2O2-treated CCL39 fibroblasts were fractionated by Mono Q anion exchange chromatography and the various fractions were tested for their capacity to reactivate the HSP27 kinase activity of inactive purified MAPKAP kinase-2/3. Three peaks of MAPKAP kinase-2/3 reactivating activities were detected, whether the cells were stimulated by thrombin (Fig. 1B) or by H2O2 (Fig. 1C). The first two peaks at 220 and 270 mM NaCl also had MBP kinase activities (arrow in Fig. 1B) and co-eluted with the fractions containing p44 ERK1 and p42 ERK2 (Fig. 1A). The third peak at 350 mM NaCl co-fractionated with the MAP kinase p38 as detected with the anti-p38 antibody (Fig. 1A). Similar results were obtained with extracts from CCL39 cells exposed to heat shock, serum or FGF (fibroblast growth factor), or with extracts from NIH 3T3 cells or HeLa cells stimulated with H2O2 or heat shock (data not shown). In every case, all MAPKAP kinase-2/3 reactivating activities co-fractionated with ERK1-2 and p38.

No MAPKAP kinase-2/3 reactivating activity was ever associated with the fractions (70 mM NaCl) which contained the c-jun kinase activity (arrowhead in Fig. 1B) and which reacted with the anti-JNK/SAPK antibody (Fig. 1A). The inability of JNK/SAPK to activate MAPKAP kinase-2/3 was confirmed by comparing the activity of immunoprecipitated JNK/SAPK from control or H2O2-treated CCL39 cells to that of immunoprecipitated p38. H2O2 stimulated strongly the MAPKAP kinase-2/3 reactivating activity associated with p38 and the c-jun kinase activity of JNK/SAPK. However, activated p38 had no c-jun kinase activity and JNK/SAPK had no MAPKAP kinase-2/3 reactivating activity (data not shown).

The induction of MAPKAP kinase-2/3 activity by mitogens or stressing agents correlates in vivo with the activation of p38

Activation of MAPKAP kinase-2/3 by both ERK1-2 and p38 may explain how a large number of agents activate this kinase
in vivo. We thus measured, in the same experiment, the activation of ERK1-2, p38 and MAPKAP kinase-2/3 following exposure of CCL39 cells, either serum-starved or exponentially growing, to a number of mitogens or stressing agents known to induce HSP27 phosphorylation or ERK activation to varying degrees. With each agent, time and dose were chosen to yield approximately maximal induction of MAPKAP kinase-2/3. p38 was, in general, more sensitive to stressing agents than ERK (Fig. 2A and C) whereas ERK was more responsive to growth factors than p38. Heat shock, H$_2$O$_2$, the oxyradical-generating cytokine IL-1β, HOS (hyperosmotic shock) and ansomycin, weakly activated ERK but were the best activators of p38. In contrast, FGF strongly activated ERK and weakly p38. Thrombin, serum and arsenite, however, induced both ERK and p38. The activation of MAPKAP kinase-2/3 correlated with the activation of p38 ($p>0.8$), whereas it did not show any correlation with the activation of ERK ($p>0.002$) (Fig. 2D and E).

In Fig. 2, the activity of MAPKAP kinase-2/3 was measured after immunoprecipitating cell extracts with an anti-GST-MAPKAP kinase-2 antibody (Huot et al., 1995). Typically this antibody immunoprecipitated some 90% of all HSP27 kinase activity present in the extract. Nonetheless, to ensure that the activity induced by the various agonists and stressing agents corresponded to the same protein kinase, cell extracts were fractionated by Fast Flow-S chromatography, and the fractions containing HSP27 kinase activity were pooled and processed to identify polypeptides with HSP27 kinase activity by in-gel
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As shown in Fig. 3A, HSP27 kinase activity was always associated with two bands of 45 and 54 kDa, corresponding to MAPKAP kinase-2/3, whether mitogenic or stressing agents were used. In the same experiment, we also measured ERK activation in three different ways. We observed the same lack of correlation between ERK and HSP27 kinase activation, regardless of whether ERK activity was estimated by assaying its MBP kinase activity, by evaluating its degree of phosphorylation by western blotting (reduced $M_r$ in SDS-PAGE) or by measuring its MAPKAP kinase-2/3 reactivating activity (Fig. 3B,C,D). There was always a good relationship between the MBP kinase activity, the levels of phosphorylated

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**Fig. 2.** Correlation between activation of p38, MAPKAP kinase-2/3 and ERK–2 by mitogens, cytokines and stressing agents. Exponentially growing (open bars and symbols) or quiescent (close bars and symbols) CCL39 cells were left untreated (CON) or stimulated with fibroblast growth factor (FGF, 10 ng/ml for 5 minutes), fetal calf serum (FCS, 2% for 10 minutes), thrombin (THR, 1 U/ml for 5 minutes), interleukin-1β (IL1, 20 ng/ml for 10 minutes), anisomycin (ANIS, 10 μg/ml for 30 minutes), arsenite (ARS, 200 μM for 30 minutes), heat shock (HS, 44°C for 15 minutes), sorbitol (HOS, 300 mM for 15 minutes) and hydrogen peroxide (H$_2$O$_2$, 5 mM for 15 minutes). Cell extracts were prepared and kinase activities were tested in immunoprecipitates of p38 (A), MAPKAP kinase-2/3 (B) and ERK2 (C). The values from A, B and C were used to plot correlation curves between the induced activities of p38 (D) or ERK (E) and MAPKAP kinase-2/3.
ERK isoforms and the MAPKAP kinase-2/3 reactivating activity of ERK, but no strict correlation was found between these activities and the activity of MAPKAP kinase-2/3 in vivo.

**Contribution of ERK and p38 to HSP27 phosphorylation**

The results presented above clearly established that phosphorylation, MBP kinase activity and MAPKAP kinase-2/3 reactivating activity associated with ERK, could all be strongly induced without generating much MAPKAP kinase-2/3 activity and HSP27 phosphorylation in vivo. Conversely, MAPKAP kinase-2/3 activity and HSP27 phosphorylation (data not shown) could be strongly induced in vivo in the absence of a strong activation of ERK. These results suggested that ERK1-2 proteins are not involved in signaling to HSP27. This conclusion was substantiated in a more direct manner by taking advantage of the known function of ras as an upstream modulator of ERK in vivo. We examined the effect of expressing an activated form of ras (HA-ras) or an interfering mutant of ras (N17-ras) on HSP27 phosphorylation induced by stress and mitogens. LT-tagged HSP27 was transfected alone or in combination with Ha-ras or N17-Ras in CCL39 (Fig. 4) or in NIH-3T3 cells (data not shown). The transfected cells were serum-starved, incubated in the presence of radioactive phosphate (32PO4) for 2 hours and then subjected to stress (heat shock or H2O2 treatment) or stimulated with the mitogen EGF (epidermal growth factor) or with serum. The radioactivity incorporated in equal amounts of immunoprecipitated LT-tagged HSP27 was determined after SDS-PAGE. Results showed that neither stress- nor mitogen-induced phosphorylation of HSP27 was significantly affected by either forms of ras. In contrast, as reported previously in several cell lines, Ha-ras and N17-ras, respectively, enhanced and blocked the induction of Myc-tagged ERK2 by serum, thrombin or EGF (data not shown), or by stress (see below).

We next investigated the effect of Ha-ras and N17-ras expression on the activation of ERK2 and p38 by stress (Fig. 5A,B). Transfected epitope-tagged ERK2 or p38 were immunoprecipitated and the activity of the kinases was determined using MBP or ATF2 as substrates, respectively. As in the case of mitogenic stimulation, stress-induced activation of ERK2 was strongly modulated by ras expression. Ha-ras potentiated induction of ERK2 by H2O2 or sorbitol and N17-ras blocked the induction. The same results were obtained with ERK1 (data not shown). In contrast to ERK1-2, but as found in the case of HSP27 phosphorylation, active and dominant negative forms of ras did not modify p38 induction by stress (Fig. 5) or mitogens (data not shown).

We next looked at the effect of SB203580, a specific inhibitor of p38, and SKF106978, a non-functional structural analog of SB203580 (Lee et al., 1994; Cuenda et al., 1995), on the induction of HSP27 phosphorylation in response to serum.

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**Fig. 3.** Activation of ERK and MAPKAP kinase-2/3 by mitogens and stressing agents. Quiescent CCL39 cells were left untreated (CON), heat shocked at 44°C for 15 minutes (HS) or stimulated for 5 minutes with 10% fetal calf serum (FCS), 1 U/ml thrombin (THR), 10 ng/ml fibroblast growth factor (FGF), 1 μM phorbol ester (PMA) or for 30 minutes with 1 mM H2O2. Samples of the cell extracts were processed to identify the in-gel HSP27 kinase activities (A). Other samples were fractionated by SDS-PAGE and blotted with anti-ERK1/2 antibodies (B) or immunoprecipitated with an anti-ERK2 antibody and tested for MBP kinase activity (C) or MAPKAP kinase-2/3 reactivating activities (expressed as percentage of maximum) (D). Asterisks indicate phosphorylated isoforms of ERK1-2.

**Fig. 4.** Phosphorylation of HSP27 in response to stress and mitogens is Ras-independent. CCL39 cells were transfected with the LT-tagged HSP27 vector alone (−) or with either the active (HA-ras) or dominant negative (N17-ras) ras expression vectors (+). Serum-starved cells were incubated in phosphate-free medium containing H1[32P]O4 (50 μCi/ml) for 2 hours and then stimulated with H2O2 (5 mM for 15 minutes), heat shock (HS, 44°C for 15 minutes), epidermal growth factor (EGF, 2.5 ng/ml for 10 minutes) or serum (FCS, 10% for 10 minutes). Equal amounts of LT-tagged HSP27 were immunoprecipitated from cell extracts and the radioactivity incorporated into LT-tagged HSP27 was quantified after SDS-PAGE. Results are presented as the -fold increase in incorporation of [32P] into HSP27 of stimulated cells as compared to control unstimulated cells.
thrombin, heat shock and sorbitol. In the presence of SB203580 at a concentration of 10 μM (Fig. 6C,D) or 25 μM (Fig. 6A,B) the activities of MAPKAP kinase-2/3 and the phosphorylation of HSP27 remained at basal uninduced levels for all four inducers. SKF106978 had no effect either on MAPKAP kinase-2/3 activity or on HSP27 phosphorylation.

Taken together, the results indicated that activation of ERK is neither required nor sufficient for induction of HSP27 phosphorylation in vivo, whereas activation of p38 is necessary.

HSP27-dependent stabilization of the actin microfilament is enhanced by and requires activation of p38

We previously reported that overexpression of wild type but not of a phosphorylation mutant of HSP27 partially stabilizes the actin microfilament during exposure to heat shock and oxidants (Lavoie et al., 1995; Huot et al., 1995, 1996). This suggested that p38, via HSP27 phosphorylation, may be involved in modulating the dynamics of the microfilaments.

To test this idea further, we sought to examine the consequence of stimulating cells with a strong activator of p38 and inducer of HSP27 phosphorylation on the stability of the actin microfilaments in cells exposed to cytochalasin D, a specific inhibitor of actin polymerization (Yahara et al., 1982; Sampath and Pollard, 1991; Kolega et al., 1991). We used sodium arsenite to activate p38, based on preliminary results showing that arsenite had little short-term toxic effect on actin; as shown in Fig. 7A, the proportion of cells retaining some stress fibers was not changed during the first 60 minutes of exposure to 200 μM arsenite. Less than 30% of control cells exposed to cytochalasin D for 30 minutes maintained some stress fibers (Fig. 7 and 8A). Pretreatment with arsenite induced a strong dose-dependent protection against cytochalasin D; 50% of the cells treated with cytochalasin D immediately after a 30 minute arsenite treatment maintained stress fibers. This proportion further increased to 70% when the cells were conditioned by a 60-minute arsenite treatment before exposure to cytochalasin D (Figs 7 and 8C). To establish that this protection was HSP27-dependent, the effect of arsenite pre-treatment was investigated in four different CCL39 cell derivatives expressing varying levels of HSP27: HU27-#6 and Hu27-#V cells express normal levels of endogenous Chinese hamster HSP27 (1-2 μg/mg total protein) plus about 4 μg/mg of wild-type or nonphosphorylatable human HSP27, respectively; neo-#3 is a control cell line which expresses the endogenous Chinese hamster HSP27 and the neo marker gene; CHSP27D cells express no significant amount of either Chinese hamster or human HSP27. In contrast to neo-#3 cells in which arsenite pre-treatments induced a level of protection similar to that found in CCL39 cells, the resistance of the actin fibers to cytochalasin-D was not affected by arsenite pretreatments in CHSP27D cells (Fig. 7B). Hu27-#V cells behaved...
Fibroblasts were treated with cytochalasin D for 30 minutes. After the treatment and fixation, the cells were stained with FITC-labeled phalloidin and examined under a fluorescence microscope to enhance the actin polymerization observed after cytochalasin D treatment and completely blocked actin-induced polymerization in both neo-3 cells (Fig. 7B) and HuC-D6 cells (Fig. 8E,F). The effect was most dramatic in HuC-D6 cells. After cytochalasin D treatment, these cells became totally resistant to a 30 minute cytochalasin D treatment. However, in the presence of SB203580, the protective effect of both the increased concentration and arsenite-induced phosphorylation of HSP27 was abolished. The intensity of the phalloidin staining was severely reduced in cells exposed to SB203580 plus cytochalasin D as compared to those incubated with cytochalasin-D alone. In those cells, F-actin remained present only in small punctate structures, mainly located at the ventral face of the cells and in the cortical area under the membrane and protrusions.

DISCUSSION

In many cell types, the phosphorylation of HSP27 is induced not only when quiescent cells are stimulated by mitogens or growth factors, but also when cells, either quiescent or in growth phase, are exposed to cytokines or various stressing agents (for a review, see Arrigo and Landry, 1994). HSP27 phosphorylation occurs as a result of the activation of MAPKAP kinase-2/3, a p45-54 HSP27 kinase which phosphorylates HSP27 on serine residues (Gaestel et al., 1991; Landry et al., 1992; Stokoe et al., 1992a; McLaughlin et al., 1996). In the present study, we have investigated the contribution of each member of the MAP kinase family to phosphorylation of HSP27 in a cell line in which both stress and mitogenic stimulation activate MAPKAP kinase-2/3. As shown before in stress-stimulated cells (Rouse et al., 1994), we found in both stress- or mitogen-stimulated CCL39 (Fig. 1), HeLa and NIH3T3 cells (data not shown), that all MAPKAP kinase-2/3 reactivating activity was associated with the Mono Q fractions containing ERK1, ERK2 and p38 MAP kinases. No activity was associated with fractions containing JNK/SAPK, and active JNK/SAPK could not activate MAPKAP kinase-2/3 in vitro. Moreover, blocking the ERK pathway with an interfering mutant of ras, or enhancing ERK activation with an active ras, had no effect on HSP27 phosphorylation strongly suggesting that ERK, even when strongly stimulated by growth factors, did not contribute to HSP27 phosphorylation in vivo. These results, together with the observation that SB203580, a specific inhibitor of p38, blocked the activation of MAPKAP kinase-2/3 and the phosphorylation of HSP27 during H2O2 or sorbitol treatment as well as during stimulation by serum or thrombin, strongly suggest that p38 is the major upstream activator of HSP27 phosphorylation, not only after stress but also after agonist stimulation. Thus, although the p38 pathway is mostly sensitive to stress and cytokines, it can also convey some of the signals generated by mitogens and agonists of growth and differentiation.

Several reasons can explain the failure of ERK to contribute to the phosphorylation of HSP27 in vivo. ERK and MAPKAP kinase-2/3 may exist in different cell compartments in vivo. Another possibility, suggested by recent results showing that MAPKAP kinase-2/3 can be immunoprecipitated with p38 (data not shown, and McLaughlin et al., 1996) and that MAPKAP kinase-3 interacts with p38 in the yeast two-hybrid system (McLaughlin et al., 1996), is that some MAPKAP kinase-2/3 is already associated with inactive p38, facilitating its activation by p38 upon cell stimulation and preventing activation by ERK. Such an association has also been described between ERK and Rsk (MAPKAP kinase-1) and between ERK and Elk-1 (Rao and Reddy, 1994; Hsiao et al., 1994), and may be a general mechanism to limit cross-talk between different signaling pathways. It has recently been suggested that the N-terminal SH3-binding proline-rich domain of MAPKAP kinase-2 may be important for suppressing the activation of MAPKAP kinase-2 by ERK (Ben-Levy et al., 1995).

Both in vitro and in vivo results suggest that phosphorylation, by modifying the suprastructure of HSP27 (Kato et al., 1994; Lavioe et al., 1995), modulates a function of the protein at the level of the actin cytoskeleton. In vitro, phosphorylation represses the ability of HSP27 to block actin polymerization (Benndorf et al., 1994). In vivo, phosphorylation of HSP27
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appears to be an important element in the modulation of F-actin dynamics following mitogenic stimulation and exposure to stresses (Lavoie et al., 1993b, 1995; Huot et al., 1996). Most of these conclusions were obtained from experiments based on the lack of function of phosphorylation-mutants of HSP27, and it could not be totally excluded that structural alterations, unrelated to phosphorylation, caused the loss of function. Here, our finding that blocking the p38 pathway with SB203580 totally eliminates the effect of HSP27 overexpression on actin, provides independent support to the proposal that the HSP27 function is activated by phosphorylation. Our results also imply that activation of p38, at least in cells that express high levels of HSP27, leads to major changes in the dynamics of actin filaments.

A link between p38 activation and actin dynamics may be of major importance during stress and also normal agonist stimulation. During stress an increased stability of the actin cytoskeleton mediated by activation of p38 may constitute an important arm of the adaptive cell response to external stress. We previously reported that expression of HSP27, but not phosphorylation-mutant HSP27, protects against cytochalasin D-induced growth inhibition and cell death (Lavoie et al., 1995). HSP27 expression also confers enhanced cell resistance to heat shock, H₂O₂ and various cytotoxic agents commonly used in the treatment of cancer (Landry et al., 1989; Huot et al., 1991, 1996; Mehlen et al., 1995). In normal physiology, activation of p38 may be biologically significant, particularly for cells such as those of the vascular system which are naturally exposed to high concentration of oxidants. In this regard, in human umbilical vein endothelial cells, a cell type which expresses high levels of HSP27 (5-7 μg of HSP27 per mg of total protein as compared to 1-2 μg in CCL39 cells), we recently observed that oxidative stress induced a major p38-mediated reorganization of the actin microfilaments (J. Huot et al., unpublished).

HSP27-dependent modulation of actin filament dynamics by p38 activation may also be biologically significant during normal agonist cell stimulation. Although more strongly induced by stress, p38 is also activated and HSP27 is phosphorylated upon cell stimulation with serum, growth factors and cytokines. Previous studies have shown that overexpression of HSP27 enhanced the accumulation of F-actin in response to serum, thrombin or PGE in Chinese hamster fibroblasts, whereas expression of a phosphorylation mutant of HSP27 blocked this early response of actin to growth factors (Lavoie et al., 1993b). The members of the Rho family of small

Fig. 8. SB203580 blocks HSP27 concentration- and phosphorylation-dependent stabilization of actin filaments in cells exposed to cytochalasin D. Control neo-#3 cells (A-D) and HU27-46 cells (E,F) were plated onto fibronectin-coated dishes and allowed to attach and enter exponential growth phase for 24 hours. SB203580 was then added at a final concentration of 0 (vehicle only, DMSO, A,C,E) or 10 μM (B,D,F) for a total of 2.5 hours. In C-F, arsenite (200 μM) was added for 1 hour, 1 hour after addition of SB203580. All groups were exposed to cytochalasin D (0.5 μM) during the last 30 minutes. After the treatments the cells were fixed, stained with FITC-labeled phalloidin and examined by confocal microscopy.
GTases Rac, RhoA and Cdc42, play major roles in regulating actin filament level and organization in response to external stimuli. In response to agonists, Rac, RhoA and Cdc42 mediate the formation of membrane ruffles and lamellipodia, stress fibers and filopodia, respectively (Ridley et al., 1992; Ridley and Hall, 1992; Nobes and Hall, 1995). The relationship between the Rho signaling pathway and the p38/HSP27 pathway in mediating the response of the microfilaments deserves further investigation. It is intriguing that, in Hela cells, expression of active forms of Rac and Cdc42 leads to activation of p38, whereas interfering mutants blocked the activation of p38 by IL-1 (Zhang et al., 1995). This would suggest that HSP27 is one effector of the action of these small GTases. In CCI39 cells, however, we were unable to activate p38 or to induce phosphorylation of HSP27 by expressing active Rac or Cdc42 under conditions in which they strongly activated JNK/SAPK (data not shown). Hence, at least in CCI39 cells, p38 activation appears to affect actin responses in a pathway parallel to the Rho GTase-activated pathway.

The mechanism of action of HSP27 at the level of actin remains unknown. In conditions where actin polymerization activity was highly reduced by cytochalasin D, we showed that the stability of actin microfilaments was enhanced in a HSP27 concentration- and phosphorylation-dependent manner. Actin polymerization/depolymerization is regulated by a number of mechanisms involving proteins that, for example, affect the concentration of free actin monomers, the number of nucleation sites, the GDP-GTP exchange on G-actin and the affinity of G-actin for barbed ends (Hall, 1994; Pantaloni and Carlier, 1993; Zigmond, 1996). Cytochalasin D interacts with both F-actin and G-actin and reduces polymerization activities, probably through a combination of factors including partial end capping, changes in the nucleotide composition of G-actin, and modification of the interactions of F- and G-actin with actin-binding proteins (Sampaio and Pollard, 1991). In vitro, HSP27 in its unphosphorylated state, but not when phosphorylated, behaves as an actin-capping protein and inhibits polymerization (Miron et al., 1991; Benndorf et al., 1994). Hence, in cells expressing high level of the protein, HSP27 is likely to contribute to the equilibrium between polymerization and depolymerization activities. A sudden phosphorylation of HSP27, via activation of p38, may markedly modify the equilibrium in favor of polymerized actin, thereby contributing to the maintenance of the microfilament network.

In conclusion, our results provide strong evidence to implicate HSP27 as an effector downstream of the p38 MAP kinase in a signal transduction pathway regulating actin filament dynamics. Considering the role played by the microfilaments in various aspects of cellular physiology such as cell-cell interaction, cell migration, proliferation and secretion, activation of p38 may thus exert pleiotropic effects on several cellular processes. It will be of interest to determine whether this actin-related function of p38 is linked to previously reported roles of p38 in cytokine synthesis and platelet aggregation (Lee et al., 1994; Saklatvala et al., 1996).

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