Regulation by p38 Mitogen-activated Protein Kinase of Adenylate- and Uridylate-Rich Element-mediated Urokinase-Type Plasminogen Activator (uPA) Messenger RNA Stability and uPA-dependent in Vitro Cell Invasion

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ABSTRACT

MDA-MB-231 cells are highly metastatic breast tumor cells. Their high invasiveness is thought to be due to constitutively high levels of urokinase-type plasminogen activator (uPA) and its receptor. Previously (R. Nanbu et al., C. Eur. J. Biochem., 247: 160–174, 1997), we showed that uPA mRNA in these cells is stable and that mRNA degradation mediated by an AU-rich element (ARE) is impaired. Here we report that treatment of MDA-MB-231 cells with SB203580, an inhibitor of the stress-activated p38 mitogen-activated protein (MAP) kinase, strongly destabilized uPA mRNA in an ARE-dependent manner. In contrast, in LLC-PK1 and HeLa cells, uPA mRNA is unstable, and an ARE present in the 3′ untranslated region plays a role in its degradation. Enhanced ARE-mediated mRNA destabilization induced by SB203580 was also observed in both LLC-PK1 and HeLa cells with a globin chimeric mRNA harboring two copies of the ARE (globin-2ARE) from uPA mRNA. Overexpression of constitutively active MKK6, a p38 upstream activator kinase, increased the stability of the globin-2ARE message in LLC-PK1 cells, confirming the participation of p38 in the regulation of ARE-mediated mRNA decay. Interestingly, the half-life of the uPA mRNA in the three cell lines studied correlated with the basal levels of active p38. SB203580 treatment of MDA-MB-231 cells decreased cell-associated uPA activity and dramatically reduced in vitro cell invasiveness. These results suggest the participation of p38 in the control of invasiveness through regulation of the stability of uPA and uPA receptor mRNA, which is also destabilized by p38.

INTRODUCTION

The uPA is a secreted serine protease and, when bound to its cell-surface receptor, catalyzes the conversion of the ubiquitous humoralzymogen plasminogen into the active serine protease plasmin (Ref. 1 and reviewed in Ref. 2). Plasmin has a wide substrate specificity, including various extracellular matrix proteins and other protease precursors. Thus, its accumulation in the vicinity of the cell surface confers on the cell the ability to reorganize itself in the constraints of cell-cell and cell-matrix interactions (see review in Ref. 2). The uPA/uPAR system has been shown to play a key role in tumor invasion and dissemination in various malignancies, including breast, colorectum, and ovary cancers (3–6). A role for the fibrinolytic system in tumor malignancy has also been shown in uPA knockout mice (7). Although various conditions in which uPA and uPAR genes can be up-regulated have been studied and elucidated (see reviews in Refs. 8, 9), the mechanism resulting in their high expression in malignant tumor cells is not understood.

uPA mRNA is 2.4 kb long with 900 bp of 3′ UTR, conserved in several mammalian species, suggesting an essential function for this region (see review in Ref. 10). In the LLC-PK1 pig epithelial cell line, uPA mRNA has a half-life of about 1 h (11). Most of the information determining its instability resides in the 3′UTR with at least two regulatory regions acting independently of each other, one of which has an ARE (12). The ARE contains the minimum consensus element UUAUUUAUU (13) or UUAUUU(A/U)(A/U) (14). This element is usually present as multiple repeats in the 3′UTR of many mRNAs with short half-lives (reviewed in Refs. 15, 16). Whereas ARE-containing mRNAs exhibit basal instability, several recent reports showed that their half-lives can be modulated by external stimuli through mechanisms involving ARE sequences (17–22). We previously reported that, in LLC-PK1 cells, uPA mRNA stability increases after the down-regulation of protein kinase C by long treatment with TPA. This effect is mediated by impairment of an ARE-mediated mRNA degradation mechanism (12). In MDA-MB-231 cells, a human metastatic breast cancer cell line that constitutively expresses high levels of uPA and uPAR, uPA mRNA is extremely stable with a half-life of 17 h, and ARE-mediated mRNA degradation is impaired (23). These results raise the possibility that uPA mRNA stability is regulated through a particular signal transduction pathway, which may be differentially modulated in tumor cells.

In this work, we present data indicating that the inhibition of p38 MAPK reduced the stability of uPA mRNA through its ARE in LLC-PK1 and MDA-MB-231 cells. We further analyzed p38 MAPK-dependent regulation of invasion in vitro and found that the inhibition of p38 also suppressed the invasiveness of MDA-MB-231 cells without affecting their proliferation.

MATERIALS AND METHODS

Materials. Actinomycin D was purchased from Sigma; high-molecular-weight human uPA (human urine; HMW) from Alexis Corporation; plasminogen from Boehringer Mannheim; SB203580 from Novartis AG; phospho-p38 MAPK (Thr180/Tyr182) and phospho-JNK (Thr183/Tyr185) antibodies from New England Biolabs; and p38 MAPK antibodies from Santa Cruz Biotechnology.

Cells. LLC-PK1 (24), HeLa, and MDA-MB-231 (25) cells were maintained in DMEM supplemented with 10% FCS at 37°C in a humidified CO2 incubator.

Expression Vectors for Chimeric Globin mRNAs. The expression vectors for chimeric globin mRNAs have been described (12). The parent vector, pCMVβgalXho, expresses rabbit intron-1-less globin mRNA containing a unique XhoI site immediately upstream of the poly(A) addition signal under the control of the cytomegalovirus promoter. globin-2ARE and globin-1ARE were derived by inserting either synthetic oligonucleotides or a PCR fragment corresponding to the 3′UTR of uPA mRNA without the ARE into the XhoI site.

RNA Isolation and Northern Blot Analysis. Total RNA preparation and Northern blotting were performed as described previously (12). Equal RNA loading and transfer to nylon membranes were confirmed by staining with RNA on the membranes with methylene blue (26). Hybridization was performed with the QuickHyb hybridization solution (Stratagene) and 32P-labeled cDNAs as probes. Levels of specific RNA were measured in a Molecular Dynamics PhosphorImager.

Nuclear Transcription. The isolation of nuclei, nuclear run-on transcription, and quantitation of specific transcripts by hybridization were performed as described previously (27), using 2 μg of empty vector (pBluescript), 32P-labeled cDNA probes.
pBluescript-uPA, and EF1α CDNA denatured and slot-blotted onto nitrocellulose (Schleicher & Schuell).

**Reporter Gene Expression.** The c-fos gene expression was measured in transient transfection assays with pfos-luc containing a human c-fos promoter (−711/+45) linked to the firefly luciferase gene (28). Expression vectors for wild-type MKK7 (29) and constitutively active MKK6 (30) have been described previously. The transient transfection assays were done using FuGENE (Boehringer Mannheim). Stable transfection of MKK6 or pcDNA3-HA vector in LLC-PK1 cells was carried out with the calcium phosphate precipitation method (Pharmacia Biotech).

**Cytoplasmic Lysates and Western Blot Analysis.** Cells cultured on a plastic dish (2 × 10⁶ cells) were washed with PBS and lysed by adding 100 μl of buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% v/v SDS, 10% glycerol, and 50 mM DTT. Samples (80 μg of protein) were subjected to SDS-PAGE and Western blotting (as described by New England Biolabs). For uPAR detection, 2 × 10⁶ cells were washed with PBS and lysed in 200 μl of 0.1 M Tris-HCl (pH 8.1), 1% Triton X-114, 10 mM EDTA, 10 mM p-amino, and 1 mM phenylmethylsulfonyl fluoride. The samples were warmed at 32°C, and phases were separated by centrifugation in a microfuge for 20 s at 10,000 rpm. 3-(3-Cholamidopropyl)dimethylammonio)-1-propane-sulfonate was added to the antigen phase at a final concentration of 0.25% (w/v). Aliquots (11 μl) of the detergent-phase extract were subjected to SDS-PAGE and Western blot analysis using a rabbit antihuman uPAR IgG (399R; American Diagnostica Inc).

**uPA Detection.** MDA-MB-231 cells were first acid-washed to remove the endogenously bound uPA as described previously (31), trypsinized, and plated in the presence or absence of SB203580 on 35-mm dishes. For the ELISA for uPA, 1 × 10⁶ cells were plated, and the medium was collected after 24 h. The uPA protein levels were determined using TintElize uPA (Biopool). For the uPA zymography, 1.2 × 10⁶ cells were plated. After 72 h, cells were lysed in 500 μl lysis buffer and lysates (10 μl each) were analyzed as described previously (32). For cell-associated uPA activity, 500 cells were plated. After 24 h, the cells were processed for cell overlay assays as described previously (33). Depending on the treatment, 1 mM amiloride (Sigma) or SB203580 was added to the overlayingagarose gel containing 2% skim milk and 20 μg/ml plasminogen. After incubation for several hours, the overlays were stained with 0.5% amidoblack in 50% methanol plus 10% acetic acid and destained with 45% methanol plus 10% acetic acid.

**Motility and Invasion.** These assays were carried out as described previously (34) with slight modifications. Before trypsinization, MDA-MD-231 cells were acid-washed as described above. For the motility assay, 1.25 × 10⁶ MDA-MB-231 cells in 200 μl DMEM plus 10% FCS ± 10 or 20 μM SB203580 were plated on 8.0-M polycarbonate filters in 24-well plates. For the invasion assay, the transwell filters were coated with 100 μl of Matrigel (Collaborative Research). MDA-MB-231 cells (2 × 10⁶) in 200 μl DMEM plus 10% FCS were plated on the upper chamber.

**Cell Proliferation.** Cells (4.4 × 10⁴) were plated on 24-well plates, and 24 h later, cells were treated with or without 10 or 20 μM SB203580. At different times, cells were rinsed in PBS, fixed with 1% glutaraldehyde in PBS, and stained with 0.1% crystal violet in water. After staining in water, cells were solubilized in 300 μl of 0.2% Triton X-100, and the absorbance was measured at 590 nm.

**RESULTS**

**Destabilization of uPA mRNA by SB203580 Depends on the ARE.** We examined the effect on uPA mRNA stability in MDA-MB-231 cells of several reagents known to influence protein phosphorylation directly or indirectly and to interfere with certain signal transduction pathways: A3, H7, okadaic acid, wortmannin, and SB203580. Among these, SB203580, an inhibitor of p38 MAPK (35), induced significant destabilization of uPA mRNA.

To determine mRNA half-life, a chasing approach using the RNA method (Pharmacia Biotech).

We previously showed that the uPA mRNA in LLC-PK1, and HeLa cells has a short half-life and that insertion of either the ARE or another region upstream of the ARE renders globin mRNA less stable (12; and data not shown). To determine which region mediates the effect of SB203580, we expressed chimeric globin mRNAs containing either two copies of the ARE (2ARE) of uPA mRNA or the 3’UTR of uPA mRNA without the ARE (ΔARE) in the 3’UTR of otherwise stable globin mRNA (Fig. 1C; 12) and examined the effect of the inhibitor on the decay of these chimeric mRNAs. Each expression vector was stably transfected into MDA-MB-231 cells and more than 100 transformants were pooled and characterized. The chimeric globin-2ARE mRNA was stable but its stability dropped to a half-life of 3.2 h in the presence of SB203580 (Fig. 1D). In contrast, globin-ΔARE was unstable, with a half-life of 4.2 h, but its stability was not influenced by the presence of the inhibitor (Fig. 1E). These results suggest that the effect on uPA mRNA stability was mediated by the ARE.

The slowing in degradation of uPA mRNA after 2 h in the presence of actinomycin D and SB203580 (Fig. 1A) may reflect different uPA mRNA subgroups with distinct sensitivities to SB203580 or a time-dependent indirect effect of the inhibitor through changing cellular metabolism. There have been several reports of a requirement for ongoing RNA synthesis in the decay of some mRNAs (18, 36, 37). Hence, we compared the influence of SB203580 on uPA mRNA levels in the presence and absence of actinomycin D. Destabilization of uPA mRNA by the inhibitor was stronger in the absence of actinomycin D, which suggested the involvement of a labile protein or RNA in this induced uPA mRNA decay (Fig. 2A).

In nuclear run-on assays, SB203580 pretreatment did not affect uPA gene transcription (Fig. 2B). As we had shown previously in NIH3T3 and LLC-PK1 cells, the uPA gene is activated by various extracellular signals through the Ras/ERK signaling pathway (38–42), we also examined as a control the effect of an inhibitor (PD98059) of MEK1, which is the upstream kinase of ERK. As expected, there was a strong decrease in uPA gene transcription by PD98059 treatment (Fig. 2B).

As in MDA-MB-231 cells, the presence of SB203580 rendered globin-2ARE mRNA more unstable in both LLC-PK1 (Fig. 3A) and HeLa (data not shown) but had no effect on globin-ΔARE mRNA (Fig. 3B). Thus, the specific effect of SB203580 on ARE-mediated uPA mRNA degradation is not confined to MDA-MB-231 cells.

**Destabilizing Effect of SB203580 in LLC-PK1 and MDA-MB-231 Cells is by Inhibition of p38 MAPK.** SB203580 was developed as a specific inhibitor of p38 MAPK (35). Recently it was shown to inhibit JNK MAPK at 10 μM or higher in vitro, but it had no effect on JNK MAPK at 10 μM in vivo in CHO cells (43). To determine whether ARE-mediated mRNA destabilization induced by SB203580 is due to the inhibition of p38, we examined in stably cotransfected cells the effect of the ectopic expression of a constitutively active form of MKK6(Glu), an upstream kinase of p38 (30, 44, 45), on the stability of globin-2ARE and globin-ΔARE chimeric mRNAs. As shown in Fig. 3, C and D, the stability of globin-2ARE mRNA was increased by MKK6 coexpression in LLC-PK1 cells, whereas that of globin-ΔARE was not affected. This suggests that p38 activation protects mRNA from ARE-mediated degradation. As a complementary experiment, we tried to overexpress in MDA-MB-231 cells...
dominant negative forms of p38 (46), MKK6 or MKK3 (47), the latter two being upstream kinases of p38 (30, 45). However, cells stably expressing these proteins were not recovered.

To determine the specificity of SB203580 in MDA-MB-231 cells in vivo, we examined its effect on c-fos reporter gene expression in cells cotransfected with an expression vector for constitutively active MKK6 or wild-type MKK7 (Fig. 4). Overexpression of MKK7 has been shown to activate JNK (29, 48). The c-fos promoter was activated by MKK6 or MKK7 coexpression. MKK6-induced activation was dose-dependently inhibited by SB203580 with saturation at 10 μM, whereas MKK7-induced activation was less inhibited even at 20 μM (Fig. 4, A and B). Thus, 20 μM SB203580 significantly blocks the p38 MAPK but only slightly affects the JNK MAPK in MDA-MB-231 cells in vivo.

uPA mRNA was destabilized at 3 μM and globin-2ARE mRNA as low as 1 μM SB203580 (Fig. 4B), whereas in the presence of actinomycin D the effect was seen at 6 μM (data not shown). Destabilization of uPA mRNA in the presence of low SB203580 concentrations confirms that p38 and not JNK is responsible for the higher levels of uPA mRNA in MDA-MB-231 cells.

The basal levels of total p38 are higher in MDA-MB-231 than in LLC-PK1 and HeLa cells (Fig. 4C). Using antibodies that recognize the phosphorylated (i.e., activated) forms of p38 and JNK, we observed that the active form of p38 is more abundant in MDA-MB-231 than in the other two cell lines studied. Interestingly, the basal levels of total and active p38 MAPK, but not JNK, were positively correlated with the stability of uPA and globin-2ARE mRNAs in these cells.
cells, supporting the idea that p38 MAPK plays a role in the stabilization of uPA mRNA.

Inhibition of p38 Suppresses Cell-associated uPA Activity. Because SB203580 destabilized uPA mRNA, we asked whether the uPA activity in living cells was concomitantly decreased and whether, as a consequence, the invasiveness of MDA-MB-231 cells was also affected. The activity of cell-associated uPA was assessed by casein overlay assays (33) after treating cells with low concentrations of SB203580. The results in Fig. 5A show that caseinolytic activity was solely dependent on uPA, inasmuch as it was completely suppressed by the specific uPA inhibitor amiloride (49), and 3 μM SB203580 strongly reduced this uPA activity. These results were confirmed by uPA zymography of cell lysates (Fig. 5B). The levels of uPA protein in the culture medium also showed a significant reduction after SB203580 treatment (Table 1).

Inhibition of p38 MAPK Suppresses in Vitro Invasiveness of MDA-MB-231 Cells. MDA-MB-231 cells express high levels of uPA and uPAR and are very invasive in vitro (50) and in vivo (51). The addition of a monoclonal antibody that interferes with the uPA:uPA-receptor interaction has been shown to diminish migration of the cells through Matrigel (50). We examined the effect of SB203580 on cell invasiveness. As shown in Fig. 6A, the invasiveness was reduced by 26% at 6 μM SB203580 and almost completely at 20 μM. The inhibition of invasiveness was not due to cell toxicity, because cell proliferation was barely affected by SB203580 over a period of 3 days (Fig. 6C). Cell motility was also inhibited by the chemical, although to a much lesser extent than invasion (30% at 20 μM). It has been reported that uPA plays a role in endothelial and myoblast cell motility on vitronectin matrix without involving its proteolytic activity (52, 53), and this may well explain the inhibitory effect of the chemical on cell motility that we observed. Nevertheless, combined reduction in cell motility and proliferation cannot account for the strong reduction in invasiveness observed, which requires high uPA activity for the degradation of the Matrigel. Differences in the inhibitory effects of the chemical on cell motility and cell invasiveness may reflect the K_m value in each process for cell-associated uPA, the latter process requiring higher concentrations of uPA. To test whether the inhibition of cell invasiveness by SB203580 is due to reduction of uPA levels, we added 6.4 IU/ml uPA to inhibitor-treated cultures, which is 10 times higher than that expressed by MDA-MB-231 cells (50). The exogenous uPA did not restore invasiveness (Fig. 6B). Because cell-association of uPA is mediated by a specific uPA receptor (uPAR; reviewed in Ref. 2), we then examined whether uPAR mRNA was also affected by the p38 inhibitor. As shown in Fig. 7, A and B, uPAR mRNA was constitutively expressed at high levels and was destabilized by treatment with 6 μM SB203580, which led to a decrease in uPAR protein levels. These results argue that p38 MAPK is also involved in uPAR mRNA stabilization in these cells. As with uPA mRNA, the destabilizing effect was more pronounced in the absence of actinomycin D (data not shown).

DISCUSSION

In the present study, using SB203580, we provide evidence that p38 MAPK stabilizes uPA mRNA via the ARE in the 3’UTR of the mRNA. The regulation of uPA mRNA by p38 MAPK through an ARE may be general because a destabilizing effect of SB203580 on globin-2ARE was also observed in HeLa and LLC-PK1 cells. p38 is a member of the MAPK family and has been implicated in environmental stress response and inflammation induced by cytokines in a variety of cell types (Refs. 46, 54, 55 and reviewed in Ref. 56), through a signaling pathway in which MKK3 (57) and MKK6 (30, 45)
are the immediate upstream kinases of p38, p38α and its isoforms p38β and p38β2 are inhibited by SB203580 at low micromolar concentrations without affecting the activity of the other MAPK family members, p38γ, p38δ, ERKs, and JNKs (35, 43, 58, 59). In this context, it is interesting to note that uPA is highly expressed during inflammation (60, 61). This enhanced uPA expression may also involve p38-mediated uPA mRNA stabilization.

We previously showed (23) that uPA mRNA is constitutively expressed in MDA-MB-231 cells at high levels and is more stable than that in LLC-PK1 and HeLa cells. In accordance with this, p38 MAPK activity is higher in MDA-MB-231 cells than in the other cell lines, and overexpression of MKK6 in LLC-PK1 cells increased the stability of the globin-2ARE transcript. At the moment, we do not know which protein regulates uPA mRNA stability via the ARE as the substrate of p38 MAPK. We previously reported a cytoplasmic protein of Mr 40,000 (p40) that bound specifically to the ARE and whose binding activity was higher in MDA-MB-231 cells or PKC-down-regulated LLC-PK1 cells than in control LLC-PK1 cells (23). This suggested a role for p40 in the protection of mRNA from ARE-mediated degradation. Interestingly, treatment of cytoplasmic extracts with alkaline phosphatase strongly reduced p40-binding to the ARE. We observed that ARE-binding by p40 in the cytoplasm, as detected by UV cross-linking, was transiently increased by 50% on treatment with SB203580 and was never lower than the control (data not shown). Therefore, if p40 is involved in the regulation of uPA mRNA degradation, it is likely that its function is differently regulated by p38 MAPK and PKC down-regulation.

The MAPK family includes the p38, JNK, and ERK MAPKs, and the engagement of these kinases in transcriptional regulation has been well documented (reviewed in Ref. 62). Work by our group and others has shown that all three of the MAPK family members also play a role in the regulation of mRNA stability. In the present report, we show the involvement of p38 MAPK in ARE-mediated uPA mRNA metabo-
It was recently reported that IL-6 mRNA is destabilized by p38 inhibition, although the RNA sequence mediating this effect has not been characterized (63). Chen et al. (64) and Ming et al. (65) reported that bicyclic imidazole SB202190, which presents the same pattern of protein kinase inhibition as SB203580, destabilized IL-2 mRNA in Jurkat cells and IL-3 mRNA in PB-3c mast cells. In these cases, destabilization was through JNK inhibition. Because high concentrations of SB203580 also slightly inhibit JNK in MDA-MB-231 cells, we cannot completely exclude the involvement of JNK in the regulation of uPA mRNA stability. At the same time, the possibility of p38 MAPK- and ARE-independent mechanisms by which the stability of uPA mRNAs is controlled need to be kept in mind because globin-DAU mRNA is unstable, and its stability is not affected by SB203580. Recently, we found that the inhibition of ERK MAPK destabilized uPA mRNA in several metastatic melanoma cell lines. So far, no proteins have been identified as substrates of these kinases that influence mRNA stability. Although this is the first report of the involvement of p38 in the regulation of ARE-mediated mRNA degradation, this kinase has already been shown to participate in other aspects of mRNA metabolism, e.g., it plays an essential role in the initiation of the translation of the tumor necrosis factor α mRNA in lipopolysacharide-treated human monocytic cells (66).

Recently, Simon et al. (67) showed that, in a head and neck cancer cell line in which p38 is constitutively activated, SB203580 had an inhibitory effect on in vitro invasion and on TPA-induced production of type IV collagenase matrix metalloprotease-9. It is not known whether this effect on matrix metalloprotease-9 mRNA was through transcriptional or posttranscriptional regulation or both.

Holst-Hansen et al. (50) showed that MDA-MB-231 cells express high levels of uPA and uPAR and that blocking the interaction between uPA and uPAR by an α-uPAR monoclonal antibody causes dose-dependent inhibition of cell migration through the basement membrane. This suggested a role for the uPA/uPAR system in invasiveness. The in vitro invasiveness of three cancer cell lines (MCF-7, MDA-MB-435, and MDA-MB-231) correlated well with levels of uPA expression (50). We showed here that p38 inhibition leads to the enhanced decay of uPA mRNA in MDA-MB-231 cells and concomitantly diminished uPA protein levels, cell-associated uPA casein-
lytic activity, and cell invasiveness without affecting cell proliferation. The suppressed invasiveness, however, was not restored by the exogenous addition of uPA. Because uPA is recruited on the cell surface by way of its specific receptor, we asked whether uPAR expression was also affected and found that the inhibitor also strongly reduced the stability of uPAR mRNA and consequently uPAR protein levels. The AU-rich region in the 3’UTR of uPAR mRNA is highly conserved in humans, cattle, rats, and mice (68), which suggests that ARE-mediated degradation is a conserved mechanism for uPAR mRNA in many species. In Jurkat cells, uPAR mRNA has a short half-life and is stabilized on treatment with an antibody that recognizes the adhesion receptor LFA-1; the basal instability as well as the induced stabilization is mediated by the ARE (68). Taking this into account, the destabilization of uPAR mRNA by SB203580, which we observed in MDA-MB-231 cells, may also be mediated by the ARE.

Our data suggest that p38 MAPK is a potential target for inhibiting uPA/uPAR-dependent cell invasiveness of metastatic cells.

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p38 MAPK-REGULATED uPA mRNA STABILITY AND CELL INVASION


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