Cytoskeleton Reorganization Induces the Urokinase-type Plasminogen Activator Gene via the Ras/Extracellular Signal-regulated Kinase (ERK) Signaling Pathway*

José Pedro Irigoyen, Daniel Besser, and Yoshikuni Nagamine‡
From the Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

Urokinase-type plasminogen activator (uPA) expression is induced upon cytoskeletal reorganization (CSR) by a mechanism independent of protein kinase C and cAMP protein kinase in nontransformed renal epithelial (LLC-PK₁) cells. This CSR-dependent uPA gene activation is mediated by an AP-1-recognizing element located 2 kilobases upstream of the transcription initiation site. The phosphorylation of c-Jun, a component of AP-1, is induced by CSR, which seems to increase both the activity and stability of c-Jun (Lee, J. S., von der Ahe, D., Kiefer, B., and Nagamine, Y. (1993) Nucleic Acids Res. 21, 3365–3372). It has been shown that c-Jun is phosphorylated by members of the mitogen-activated protein kinase family, i.e. ERKs and JNKs. ERKs are activated through a growth factor-coupled Ras/Raf-dependent signaling pathway, while JNKs are activated through a stress-induced signaling pathway. Although CSR induces both ERK-2 and JNK activity, JNK does not seem to be involved in the uPA gene induction because UV irradiation, which activates JNK as efficiently as CSR, does not activate the uPA promoter. Further analysis showed the involvement of SOS, Ras, and Raf-1 in the pathway induced by CSR. Our results suggest that cells sense changes in cell morphology using the cytoskeleton as a sensor and respond by activating the ERK-involving signaling pathway from within the cell.

Extensive studies by numerous groups in the last decade have clarified many aspects of gene regulation induced by extracellular stimuli such as growth factors, peptide and steroid hormones, and cytokines (reviewed in Refs. 1–5). As gene expression occurs within layers of highly organized structures, it is reasonable to assume that it is also influenced by changes in various biological structures, such as chromatin, cytoskeleton, and cell shape.

Various experimental systems have demonstrated the impact of cell shape on gene expression. Cells plated on different substrata acquire different cell morphologies and exhibit different patterns of gene expression (reviewed in Ref. 6). Fluid shear stress in endothelial cells produces morphological changes and the induction of several genes (reviewed in Ref. 7). Disruption of the tight cell-cell interaction of epithelial cells using a specific antibody against E-cadherin induces cell disassociation accompanied by changes in cell morphology and expression of urokinase-type plasminogen activator (uPA) (8). However, despite the numerous observations that suggest a close link between cell morphology and gene expression, our understanding of their causal relationship at the molecular level is still limited.

A particular cell morphology is necessarily governed by a specific alignment of the cytoskeleton (reviewed in Refs. 9 and 10). The morphological change of a cell must therefore be accompanied or caused by cytoskeletal reorganization (CSR). There are at least three different types of cytoskeleton in the cell: microfilaments, microtubules, and intermediate filaments (10). Each of these can be reorganized independently by pharmacological agents: microfilaments are disrupted by cytochalasin, microtubules by colchicine or vinblastine, and intermediate filaments by acrylamide. Dynamic changes in cell shape can be obtained using these reagents, providing a means to study the coupling of cell morphology and gene expression.

uPA is an extracellular serine protease which converts plasminogen, a ubiquitous extracellular proenzyme, to plasmin, a serine protease with a wide spectrum of substrates (11). It is highly expressed in many transformed cells and cell lines (12–15), and the transformed phenotype is associated with changes in the cytoskeleton (16). uPA is also expressed in cells that exhibit dynamic changes in morphology such as those involved in embryogenesis (17) and wound healing (18). The increased production of uPA by these cells suggests that uPA expression is preceded and modulated by changes in the cytoskeleton.

We have previously shown that CSR brought about by colchicine or cytochalasin induces the uPA gene through a pathway distinct from those involving protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) in LLC-PK₁ pig kidney epithelial cells (19). We found that the cis-acting element responsible for CSR-dependent uPA gene activation is very closely related to an AP-1-binding consensus sequence located 2 kb upstream from the transcription initiation site, and that at least c-Jun phosphorylation is involved in the activation (20). However, it remained to be seen how CSR and AP-1 activation are causally linked at the molecular level.

Several kinases have been reported to phosphorylate and activate the c-Jun protein, including ERK (21–24) and JNK (25). It has also been suggested that activation of MAP kinase is linked to cell shape change (26), and, in quiescent fibroblasts, CSR induced by colchicine or vinblastine activates MAP kinase (27). Shear stress forces on endothelial and other cells induce

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‡To whom correspondence should be addressed: Friedrich Miescher Institute, P.O. Box 2543, CH 4002 Basel, Switzerland. Tel.: 41-61-697-6669; Fax: 41-61-697-3976; E-mail: nagamine@fmi.ch.

1 The abbreviations used are: uPA, urokinase-type plasminogen activator; CSR, cytoskeletal reorganization; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; TPA, 12-O-tetradecanoylphorbol 13-acetate; MAP, mitogen-activated protein; MKP-1, MAP kinase phosphatase 1; kb, kilobase(s); DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum.
the reorganization of actin stress fibers (28, 29) and remodeling of the microtubule system (30, 31) resulting in activation of MAP kinase in a Ca\(^{2+}\)- and PKC-independent manner (32).

Here we present evidence that CSR induces the uPA gene by activating the Ras-MAP kinase signaling pathway, suggesting an interesting relationship between cell shape modulation, the cytoskeleton, the development of metastatic capacity, and expression of the uPA gene.

EXPERIMENTAL PROCEDURES

**Materials**—Luciferin was obtained from Chemie Brunschwig AG and bovine serum albumin (fraction V) from Boehringer Mannheim. The protein assay system was from Bio-Rad, skim milk powder from Fluka, and 12-O-tetradecanoylphorbol 13-acetate (TPA) from Pharmacon Bio-tech Inc., 8-Br-cAMP, colchicine, cytochalasin D, and myelin basic protein were purchased from Sigma. \([\alpha^-32P]dATP\) (500 mCi/mmol), \([\gamma^-32P]ATP\) (800 mCi/mmol), and the enhanced chemiluminescence immunodetection system were from Amersham.

**Cell Culture**—LLC-PK1 cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS; AMIMED), 0.2 mg/ml streptomycin, and 50 units/ml penicillin in a humidified atmosphere at 37°C with 5% CO\(_2\).

**Probes and Plasmids**—The uPA probe for Northern blot hybridization was prepared by the random oligonucleotide-primed reaction (33) using a cDNA insert from the porcine uPA cDNA plasmid pYN15 (34). The plasmid pGL2-puPA-4.6 was constructed by inserting an 4.6 kb fragment from the porcine uPA gene promoter containing 4.6 kb of the 5’-flanking region plus part of the first exon upstream of the luciferase-coding region of the promoterless plasmid vector pGL2-basic (Promega) at the NheI site. The control plasmid, pGL2-control (Promega), contains the SV40 enhancer and promoter.

**Western Analysis**—Cells (1.75 × 10\(^6\) well) were plated in 35-mm dishes with 2 ml of DMEM containing 10% FCS. After 14 h, cells were treated as indicated, total RNA was isolated as described (42), and 5 µg was subjected to Northern blot analysis (43). Levels of specific mRNA were measured by scanning filters in a PhosphorImager system (Molecular Dynamics).

**Transfection and Analysis of Gene Expression**—LLC-PK, cells (0.5 × 10\(^6\)) were plated in 6-well plates (35-mm) tissue culture plates with 2 ml of DMEM containing 10% FCS and transfected 20 h later by the calcium phosphate precipitation method (Pharmacia) with 1.0 µg of reporter gene and varying amounts of coexpressed plasmids (if indicated).

**Activity Assays for ERK**—Cells (1.75 × 10\(^6\)) were plated in 60-mm dishes with 5 ml of DMEM containing 10% FCS and treated the next day as indicated. Cell extracts were prepared and analyzed by Western blotting as described (35, 44).

**Activity Assays for JNK**—Cells (1.75 × 10\(^6\)) were plated in 60-mm dishes with 5 ml of DMEM containing 10% FCS and treated the next day as indicated. The preparation of whole-cell extracts and determination of ERK activity were performed as described by Graus-Porta et al. (45).

**RESULTS**

**CSR Induces Modifications of c-Jun, JunD, and ERK-2**—We have previously shown that an AP1-like site 2.0 kb upstream of the transcription initiation site is responsible for CSR-dependent uPA gene induction, and that CSR induces a mobility shift of c-Jun consequent to its hyperphosphorylation, which can be reversed by protein phosphatase 2A (20). The same AP1-like site mediates the induction by TPA, but unlike induction by CSR, full induction by TPA requires the adjacent PEA3 site (21) and JunD (25). Phosphorylation of JunD by MAP kinase has not been studied in detail, but JunD contains two serine residues, Ser\(^{66}\) and Ser\(^{70}\), corresponding to Ser\(^{63}\) and Ser\(^{67}\) of c-Jun, which are targets of MAP kinases.

Activation of ERK and JNK involves their phosphorylation on both threonine and tyrosine residues. Their possible involvement in the induction was investigated in the following experiments. Total protein extracts prepared from cells pretreated with the inducers for 20 and 60 min were analyzed by Western blotting using specific antibodies against ERK-1 and ERK-2. Fig. 1B shows that the mobility shift of ERK-2 was induced after 60 min with colchicine and after only 20 min with cytochalasin D and TPA, whereas no shift of ERK-1 was observed.

**CSR Activates Both ERK-2 and JNK**—To correlate the observed modification shifts with phosphorylation and subsequent activation of ERK-2 and to investigate whether JNK kinase is activated, in vitro kinase assays were performed. Cells treated as above were lysed, and equal amounts of total protein extracts were examined for specific ERK and JNK kinase activities, using as substrates myelin basic protein and c-Jun, respectively. Fig. 2 shows that ERK-2 activity was induced after 60 min with colchicine and after 20 min with cytochalasin D and TPA, whereas ERK-1 activity was only slightly induced by cytochalasin D and TPA and not by colchicine, showing good agreement with the mobility shift results.
As shown in Fig. 3, JNK activity was also enhanced after 60 min of treatment with colchicine, cytochalasin D, and less efficiently with TPA. As reported in other cells (25), UV treatment showed strong activation of JNK in LLC-PK1 cells (Fig. 3).

**MAP Kinase Involvement in uPA Gene Induction**—Involvement of a MAP kinase in uPA gene induction was further confirmed by coexpression of MAP kinase phosphatase 1 (MKP-1). MKP-1 is a dual-specificity protein phosphatase, highly specific for ERK (41) and JNK (46) which it inactivates. In transient transfection assays, expression of the luciferase gene linked to the uPA gene promoter was induced by treating cells with colchicine, cytochalasin D, TPA, and Br-cAMP. Induction of the uPA promoter by the first three treatments requires the AP1 site 2 kb upstream from the cap site, but that by Br-cAMP does not (20). MKP-1 coexpression almost completely suppressed the induction by colchicine, cytochalasin D, and TPA, but only partially suppressed the induction by Br-cAMP (Fig. 4), suggesting that the induction by the first three inducers requires a MAP kinase. Partial suppression of Br-cAMP induction may be due to interference of protein synthesis because the activity of initiation factor 4E is modulated by an inhibitor whose activity is modulated by ERK-dependent phosphorylation (47). JNK is supposed to phosphorylate and activate c-Jun (25). Although UV was a good activator of JNK in LLC-PK1 cells (Fig. 3), it had a smaller inductive effect on the uPA promoter in transient transfection assays (Fig. 5A) and Northern analysis (Fig. 5B) than colchicine and cytochalasin, suggesting that JNK is not the main MAP kinase that is responsible for uPA gene activation by colchicine and cytochalasin.

In the above we showed strong activation of ERK-2 but not ERK-1. Involvement of ERK-2 was corroborated by the reduction of colchicine and TPA induction by coexpression of a dominant negative mutant of ERK-2 (40) (Fig. 6). The dominant negative ERK-1 (39) showed no (colchicine) or a slightly negative (TPA) effect. Coexpression of dominant negative JNK-1 showed no effect on the induction by colchicine and cytochalasin (data not shown).

**Involved of SOS, Ras, and Raf Proteins in uPA Gene Induction by CSR**—How is ERK-2 activated by CSR? In the cell, ERKs are activated via a growth factor-induced signaling pathway comprising various signaling molecules (4, 48), and CSR might utilize this pathway. Another possibility, not mutually exclusive with the first possibility, is that CSR causes the release of sequestered ERK or modulators of ERK from the cytoskeleton, a model suggested by Ben-Ze’ev (6).

If a signaling pathway is involved in the CSR-induced activation of ERK, the induction of the uPA promoter would be suppressed in transient transfection assays by coexpression of dominant negative mutant forms of signaling molecules located upstream of ERK in the signaling pathway. First, we tested the...
effect of the dominant negative mutant of Raf-1, (38), and, as shown in Fig. 7A, it strongly suppressed the induction by colchicine, cytochalasin D, and TPA. Wild-type Raf-1 (37) did not interfere with the induction. Activated Raf-1 activates MEK, which in turn activates ERK but not JNK (49). These results further support the claim that the MAP kinase induced by CSR and responsible for uPA gene induction is ERK-2 but not JNK and also suggest that the activation of ERK-2 by CSR is via a signaling pathway and not by release of ERK-2 or its modulator from the cytoskeleton.

We then asked how far upstream in the Raf-1-linked signaling pathway is the site of input of the CSR-induced signal. Raf-1 is activated by Ras, which in turn is activated by the GTP-GDP exchange factor SOS (4, 48). Dominant negative mutants of Ras (36) (Ras17N, Fig. 7A) and of SOS (67) (ΔSOS, Fig. 7B) suppressed the CSR induction. Interestingly, TPA induction was partially suppressed by ΔSOS. Again, the wild-type molecules tended to show stimulatory effects.

DISCUSSION

There are three types of cytoskeleton: microtubules, which are polymers of tubulin molecules; microfilaments of actin; and intermediate filaments of various molecules. Their distribution and extent of polymerization are cell type-specific (9, 10). The cytoskeleton provides a framework for a specific cell shape, but its role is not limited to a static structural function. The non-homogeneous distribution of the cytoskeleton anchors mRNAs, proteins, and organelles to specific cell sites (50, 51). Through a dynamic balance of the polymerization and depolymerization of its components, regulated by many accessory proteins (52, 53), the cytoskeleton can engage in various cellular activities involving morphological changes, such as differentiation (54), scattering (55), chemotaxis (56), phagocytosis, and secretion (57). Cytosplasmic domains of growth factor receptors and integrins are linked indirectly to the terminal of microfilament bundles (58) and reviewed in Ref. 59), and cell motility induced by growth factors and cytokines is tightly coupled to CSR (55, 60), although the exact molecular mechanism of the reorganization remains to be elucidated.

We have shown that CSR is not only a consequence of the cellular response to extracellular signals, but can also be the trigger to generate signal transduction within the cell. To induce CSR, we used the pharmacologic agents colchicine and cytochalasin D, which induce the reorganization of microtubules and microfilaments, respectively. Both agents induced uPA gene expression by activating ERK-2, not as the result of the release of the kinase or a modulating factor from the cytoskeleton, but via activation of the signaling pathway at least as far upstream as SOS. It is well documented that this pathway is induced by growth factors (4, 48).

In NIH 3T3 cells, we have shown that FGF-2 induces the uPA gene through the same signaling pathway (35). SOS complexes with Grb2, and this complex is translocated to the membrane mediating the signal of a ligand binding to its receptor protein-tyrosine kinase and the subsequent activation of Ras. Grb2 can bind directly, or indirectly via SHC, to the autophosphorylated receptor (61, 62). We do not yet know where the CSR signal is integrated in this growth factor-induced signaling pathway: via SHC or a receptor, and if a receptor, which type. Another interesting possibility is that the Grb2-SOS complex is activated by CSR through activation of c-Src, because activated Src is closely associated with microfilaments via cortactin (63) and can induce the uPA gene through the SOS/Ras/ERK signaling pathway in NIH 3T3 cells (64). Activation of SOS and other steps upstream take place on the inner surface of the plasma membrane. The cytoplasmic domains of integrins and receptors for growth factors and cytokines are closely associated with the ends of microfilament bundle. Reorganization of microfilaments may influence the environment of these integrins and receptors, thereby facilitating the triggering of a signaling pathway.

From the pattern of inhibition of CSR induction of the uPA promoter obtained using various dominant negative mutants, it seems that the reorganization of microtubules and of microfilaments activates the same signaling pathway. It may not be the case, however, that the reorganization of microtubules...
leads to that of microfilaments or vice versa, because immuno-
cytological studies have shown that the effects of colchicine
and cytochalasin are restricted to the corresponding cytoske-
elons.2 The two cytoskeleton systems may colocalize at specific
places in the cell such as focal adhesion sites, where activation
of the ERK-linked signaling pathway might be initiated (65).
Further studies are necessary to elucidate whether the two
cytoskeleton components employ the same mechanism to trig-
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overgrowth of tumor cells will cause structural stress, then CSR,
of neighboring cells. This may contribute to the high uPA expres-
sion demonstrated in stroma cells surrounding tumor cells in
colon adenocarcinoma (69).

We also examined TPA because it uses the same AP1 site as
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they have different patterns of AP1 activation: TPA modules
JunD, while CSR modules both c-Jun and JunD (Fig. 1B).

JunK activation, because CSR activates JNK better than TPA

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