Role of LFB3 in Cell-specific cAMP Induction of the Urokinase-type Plasminogen Activator Gene*

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René Marksitzer, Aribert Stief, Pierre-Alain Menoud, and Yoshikuni Nagamine‡

From the Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

In previous work we suggested that a kidney-specific transcription factor LFB3 cooperates with cAMP-response element (CRE)-binding proteins within a cAMP regulatory unit comprised of three protein-binding domains and located 3.4 kilobase pairs upstream of the urokinase-type plasminogen activator (uPA) gene in LLC-PK₁ cells (Menoud, P.-A., Matthies, R., Hofsteenge, J., and Nagamine, Y. (1993) Nucleic Acids Res. 21, 1845–1852). The two domains contain a CRE-like sequence, and the third domain is recognized by LFB3. The absolute requirement of LFB3 as well as the cooperation among the three domains for cAMP regulation were confirmed by transient transfection assays in F9 teratocarcinoma cells, in which the level of LFB3 was negligible. Suspecting a possible feedback regulation of LFB3 mRNA expression during cAMP-dependent uPA gene induction in LLC-PK₁ cells, we measured LFB3 mRNA levels after cAMP treatment and found a strong reduction. This reduction was not due to a change in template activity of the LFB3 gene because run-on transcription showed significant changes in LFB3 gene transcription. RNA synthesis inhibitor-chase experiments indicated that the down-regulation was post-transcriptional. Interestingly, when the inhibitor was added at the same time as cAMP, the cAMP-induced decrease in LFB3 mRNA levels was abrogated, suggesting that ongoing RNA synthesis is required for the decrease. Similar effects on LFB3 mRNA metabolism were observed with all agents that induce uPA mRNA in LLC-PK₁ cells, including 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, colchicine, and cytochalasin. We discuss the significance of this regulation in uPA gene expression.

Signal transduction, a process of successive activation of various molecules, is subject to various levels of regulation. In many cases, for the sake of homeostasis, activated molecules are sequestered from the pathway by desensitization of membrane-bound receptors (1, 2), degradation of activated molecules (3, 4), inactivation of activated molecules by phosphatases (5, 6), or by a feedback mechanism (7). Cross-talk between different signaling pathways is also an important mechanism for bestowing flexible and versatile regulation on a given pathway. This can be either positive or negative and occurs at various steps in the pathway in a cell-specific manner (for reviews, see Refs. 8–10). Therefore, in addition to the identification of successively activated components of a signaling pathway and the elucidation of the mechanism of activation of each component, it is also very important to know how the activity of each component is modulated by molecules not immediately upstream in the pathway. In this way, the nature of a signaling pathway may be understood in a more physiologically relevant context.

We have been studying urokinase-type plasminogen activator (uPA) gene regulation in LLC-PK₁, a cell line derived from pig kidney epithelia (11). In these cells, the uPA gene is induced through independent signaling pathways by various signals such as cAMP (12), 12-O-tetradecanoylphorbol-13-acetate (TPA) (13), the protein phosphatase 1/2A inhibitor okadaic acid (14, 15), and cytoskeletal reorganization (13, 16). The pig uPA gene has a cAMP-inducible enhancer located 3.4 kb upstream of the transcription start site (12). This enhancer is comprised of three protein-binding domains, A, B, and C. Domains A and B contain a core sequence of the cAMP response element (CRE) and require the adjoining C domain to confer full cAMP responsiveness on a heterologous promoter (12, 17). The C domain has no CRE and cannot mediate cAMP responsiveness when used in isolation. We have purified the protein binding to the C domain (17) and found it to be the pig equivalent of mouse LFB3 (18). It is also known as HNF1b (19) or vHNF1 (20). LFB3 is a tissue-specific transcription factor highly expressed in kidney cells (18) with a structure closely related to the liver-specific transcription factor HNF1α. Both HNF1α and LFB3 recognize the same DNA sequence, at least in vitro (17, 21), although the domain C sequence is quite different from the consensus HNF1α recognition sequence. It is still not known which genes besides the uPA gene are the targets of LFB3 in kidney cells, or how the expression of LFB3 is regulated. As LFB3 is apparently involved in cAMP-dependent uPA gene regulation in LLC-PK₁ cells, we were interested to know whether cAMP-evoked signaling affected the expression of LFB3 in these cells. Indeed, we have shown that cAMP treatment strongly reduces LFB3 mRNA levels, suggesting a feedback mechanism via LFB3 in cAMP-dependent uPA gene regulation in LLC-PK₁ cells (17). In the present study, we verify the involvement of LFB3 in cAMP-induction of the uPA gene and show that not only cAMP but also other agents that induce uPA gene expression strongly reduce the amount of LFB3 mRNA. These agents are 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, colchicine, and cytochalasin B. Our results suggest the involvement of LFB3 in uPA gene regulation by cAMP at different levels.

MATERIALS AND METHODS

Reagents—TPA, colchicine, and cytochalasin B were obtained from Sigma; 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) from Fluka; 8-bromo-cAMP (Br-cAMP) from Boehringer Mannheim; and okadaic acid from Anawa. [α-32P]dATP (3000 Ci/mmol) was obtained from

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† To whom correspondence should be addressed. Tel.: 061-697-6669 or 061-697-4499; Fax: 061-697-3976.

‡ The abbreviations used are: uPA, urokinase-type plasminogen activator; TPA, 12-O-tetradecanoylphorbol-13-acetate; CRE, cAMP response element; DRB, 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole; Br-cAMP, 8-bromo-cAMP; UTR, untranslated region; kb, kilobase pair(s).
Amer sham Corp. The oligonucleotides used for electromobility shift assays were (only upper strands given): domain A, 5'-AATTCTGCTG-CTGACTCCACAC-3'; domain B, 5'-AATTCGCTCATTGGACTACCTGG-3'; domain C, 5'-GTGAAATGAATAAGGTAAGAATTATTC-GTTACA-3'; mPEA3/AP1, 5'-GATCGCCGCAAGATCATTGAGG-CATCCTG-3'; and SP1, 5'-GATCCACGGCTCGGCCCGTACGCTG-3'. The peptide P-2A3 is derived from the E3AP3' domain, the catalytic subunit of cAMP-dependent protein kinase. As shown in Fig. 1, expression vector of the catalytic subunit of cAMP-dependent protein kinase by transient transfection assays (Fig. 1A), the TATA box by transient coexpression assays in F9 cells, which may have a negligible level of endogenous LFB3. We used only the catalytic subunit to activate the signaling because endogenous cAMP-dependent protein kinase is not responsive to cAMP in F9 cells by an unknown mechanism (29). Fig. 1C shows that in F9 cells pABC-TATA was strongly induced by the catalytic subunit only when LFB3 was coexpressed. The control pTATA was not affected. These results unambiguously indicate the cooperation among three protein-binding domains and the involvement of LFB3 in cAMP regulation through the ABC site.

Effect of cAMP and Other uPA Inducers on LFB3 mRNA Levels—To confirm the previous observation that cAMP treatment reduces LFB3 mRNA in LLC-PK1 cells, we compared Br-cAMP to other agents shown to induce uPA mRNA in the same cells, TPA, colchicine, cytochalasin B, and okadaic acid. The cells were incubated for the time optimal for uPA mRNA induction, i.e., 2 h for Br-cAMP and TPA and 4 h for the rest. As shown in Fig. 2, Br-cAMP as well as other agents strongly reduced LFB3 mRNA levels; all of them induced uPA mRNA. The greatest reduction in LFB3 mRNA levels was obtained with TPA and okadaic acid (85% by 2 and 4 h) and the least with cytochalasin B (60% by 4 h).

With the exception of Br-cAMP, all the other agents induce uPA gene via the activation of AP1, acting on the PEA3/AP1 site located 2 kb upstream of the transcription initiation site (13, 15). Therefore, in the following experiments we compared in particular Br-cAMP and TPA.

Effect of Br-cAMP and TPA on Domain C Binding Activity—We tested whether the reduction of LFB3 mRNA levels after treatment with uPA inducers was reflected at the protein level. As specific antibodies against pig LFB3 were not available, we measured domain C binding activity in nuclear extracts. We performed electrophoretic mobility shift assays using crude nuclear extracts prepared from LLC-PK1 cells pretreated for 7 h with Br-cAMP or TPA. Using a 32P-labeled domain C oligonucleotide as a probe, we observed a single distinct band (Fig. 3), which could be competed by excess of the identical unlabeled oligonucleotide but not by an oligonucleotide carrying the same mutations as shown in Fig. 1A (data not shown). After treatment of the cells with TPA, the binding activity was reduced to about 50% with Br-cAMP to 40% and with TPA and Br-cAMP added together to about 30%. These data indicate that the LFB3 binding activity is reduced by Br-cAMP or TPA treatment, suggesting that the reduced mRNA level affects the protein level. To see whether the observed reduction is specific for domain C binding, we tested other oligonucleotides recognized by different transcription factors using the same nuclear extracts. Although domains A and B were required for cAMP induction, proteins binding to these sites were not affected by treatment of the cells with Br-cAMP or TPA. With the Sp1 oligonucleotide, two major specific bands were detected, but they did not change in intensity after this treatment. In contrast, with the mPEA3/AP1 oligonucleotide, which binds transcription factor AP1 (13), the intensity of the shifted band markedly increased on treatment with TPA and even more with Br-cAMP.

No Change in Transcription Rate of the LFB3 Gene—The
Cooperative role of domain C with neighboring domains A and B in uPA gene induction by cAMP signaling.

a, luciferase gene constructs containing different parts of a cAMP-inducible enhancer of the uPA gene which is composed of domains A, B, and C. The positions of apparent protein contacts as determined by methylation interference experiments are indicated by stars. Mutated domains and sequences are indicated by lowercase letters.

b, Transient transfection assays in LLC-PK1 cells. Luciferase constructs (1 μg) were induced either by 1 mM 8-Br-cAMP or by transfecting together with 0.5 μg of pCEV (CEV), a vector expressing a catalytic subunit of the cAMP-dependent protein kinase.

c, the role of LFB3 was tested by transient cotransfection assays in F9 cells using luciferase constructs (pTATA or pABC-TATA; 1 μg) and LFB3 expression vector (1 μg) with or without pCEV (CEV) (0.5 μg). Assays were done in duplicate and mean values are shown with error bars.
mechanism leading to the reduction of LFB3 mRNA by uPA inducers may involve either transcriptional or post-transcriptional regulation of the LFB3 gene. To distinguish between these possibilities, we first performed nuclear run-on transcription to assess changes in the LFB3 gene transcription rate. The results shown in Fig. 4 indicate that the transcription rate of the LFB3 gene did not change when cells were treated with TPA, Br-cAMP, colchicine, or cytochalasin B. As expected, these agents significantly enhanced the uPA gene transcription rate. Thus, the decrease in LFB3 mRNA levels seems not to be due to decreased de novo synthesis of LFB3 mRNA, suggesting that the reduction of LFB3 mRNA is a post-transcriptional event.

Induced Instability of LFB3 mRNA—If a post-transcriptional step is responsible for the induced decrease in LFB3 mRNA level, the most obvious mechanism could be an effect on mRNA stability. The stability of LFB3 mRNA was assessed by DRB-chase experiments. Since DRB specifically inhibits the synthesis of eukaryotic heterogeneous nuclear RNA and mRNA (30), chase of mRNA levels after its addition allows estimation of the decay rate of the mRNA. Because inhibition of mRNA synthesis may have some indirect influence on mRNA stability (31), we did chase experiments using two different schemes: in one experiment DRB was added at the same time as Br-cAMP or TPA, and in the other DRB was added 1 h after Br-cAMP or TPA treatment. The effect on LFB3 mRNA was independent of the presence of Br-cAMP when DRB was added at the beginning of the experiment (Fig. 5a). However, LFB3 mRNA decayed faster in the presence of Br-cAMP when DRB was added 1 h after Br-cAMP (Fig. 5b). Similar results were obtained using TPA (Fig. 5, c and d). These results indicate that the stability of LFB3 mRNA is reduced by uPA inducers, and that this requires on-going RNA synthesis for at least 1 h at the beginning of the treatment.

Effect of the Decrease in DNA Binding Activity of LFB3 on cAMP Induction—TPA and cAMP treatment reduced the DNA binding activity of LFB3. To test the biological relevance of this decrease in cAMP induction we asked whether TPA pretreatment could affect the cAMP-induction of pABC-TATA. As shown in Fig. 6, TPA pretreatment by itself had little effect on
basal expression but significantly reduced cAMP induction of the luciferase gene driven by ABC sites.

**DISCUSSION**

LFB3 is an enhancer-binding protein augmenting basal expression of a gene that contains its cognate cis-element. We found in the induction of the uPA gene by cAMP in LLC-PK₁ cells that LFB3 is a positive regulator cooperating with CRE-binding proteins within a composite cAMP-responsive enhancer (17) (this work). Our results also suggest that LFB3 is involved in a down-regulating phase of cAMP-induced uPA gene expression. We have previously shown that uPA gene induction by cAMP is transient; the rate of uPA gene transcription reaches optimal after 2-4 h of cAMP treatment but declines thereafter (32). It may be that in uPA gene regulation LFB3 acts as a negative feedback regulator by decreasing its own concentration in response to cAMP. This throws new light on LFB3, which has been implicated as a factor coupling hormonal regulation and tissue-specific regulation of uPA gene expression in kidney epithelial cells (17).

The decrease in domain C binding activity seems to be due to a decrease in LFB3 protein levels. The decrease was also observed with TPA, and it may also be the case for colchicine, cytochalasin B, and okadaic acid, which all decreased LFB3 mRNA levels (see below). These agents induce uPA gene expression in LLC-PK₁ cells via activation of the transcription factor AP1, although the mechanism of AP1 activation by each agent is different (13-15). Thus, in addition to the features mentioned above, LFB3 may mediate negative cross-talk between cAMP-dependent signaling and AP1-activating signaling pathways in uPA gene regulation. Indeed, pretreatment with TPA significantly reduced cAMP induction of the luciferase gene driven by an enhancer consisting of domains A, B, and C. The decrease in DNA binding by LFB3 in the cells seems to be due to the reduction in the protein levels. We cannot formally exclude the possibility that the decrease is due to a post-translational modification of the protein; however, this is in any case not the main cause because we also detected a strong reduction in LFB3 mRNA levels. The possible role of LFB3 in cAMP-dependent uPA gene regulation through the ABC site revealed by this work is summarized in Fig. 7.

The decrease in DNA-binding activity evoked by treatment with the uPA inducers in these cells was specific to the domain C binding protein, LFB3, and not a general effect, because DNA binding of the proteins recognizing domains A and B and of the ubiquitous transcription factor SP1 remained constant. Furthermore, the DNA-binding activity to the mutated PEA3/AP1-oligonucleotide, which contains an active AP1 site mediating the action of TPA, colchicine, cytochalasin and okadaic acid, was increased by Br-cAMP as well as by TPA. We have not elaborated the mechanism of the increase in PEA3/AP1-binding activity, i.e. whether it is transcriptional or post-transcriptional. It is worthwhile to mention that the peptide hormone calcitonin, which raises intracellular cAMP concentrations, strongly enhances de novo synthesis of c-Fos and c-jun (13), raising the interesting possibility of a cross-regulation of the TPA-dependent signaling pathway by the cAMP-dependent signaling pathway at the transcription step. The cAMP signal by itself does not utilize the PEA3/AP1 site to increase uPA gene expression (13). We do not know yet whether the enhancement of c-Fos together with c-jun un levels exerts positive effects on PEA3/AP1 site-mediated uPA gene expression, because the overexpression of c-Fos had no effect on uPA gene induction in NIH3T3 cells (33).

The decrease in LFB3 mRNA levels is mainly attributable to induced mRNA instability. We did not detect changes in the LFB3 gene transcription rate, but we did observe that LFB3 mRNA degradation increased in the presence of TPA or Br-cAMP. Interestingly, however, enhanced instability was observed only when DRB was added 1 h after TPA or Br-cAMP treatment, suggesting that some RNA transcripts or their translation products are involved in LFB3 mRNA metabolism. It may be that TPA or Br-cAMP induces a factor, RNA or protein essential for LFB3 mRNA degradation, or that an RNA or a protein of short half-life is involved in LFB3 mRNA degradation, at least at an early stage. A requirement for on-going RNA synthesis in mRNA degradation has been reported for several mRNAs, such as those for c-fos, (34), c-myc (35), collagenase (36), and the transferrin receptor (37). We have shown that an RNA instability-regulating site in the 3'-UTR of uPA mRNA requires on-going RNA synthesis for its activity (31) and that the importance of this site in overall uPA mRNA degradation may depend on cell type (38). In none of these cases is it known how on-going RNA synthesis contributes to mRNA degradation.

Several instability-determining sequences have been identified in many mRNAs. These include sequences located in the 3'-untranslated region, such as the iron-responsive element in the transferrin receptor mRNA (37, 39), sequences in the un-
stable yeast MFA2 mRNA (38) and AU-rich sequences in various oncogene and lymphokine mRNAs (40–44). But instabili-
determining elements have also been identified in coding
regions, e.g. c-myc (35) and c-fos (45) mRNAs. We tested the
3′-UTR and protein-coding regions of LFB3 mRNA in a system
developed for the study of uPA mRNA degradation by inserting
these sequences in an otherwise stable globin mRNA (31); however, the stability of recombinant globin mRNAs was not
affected by TPA or Br-cAMP. It may be that this regulatory
sequence resides in 5′-UTR or the 3′ extremity which we have not
tested or that the globin mRNA context interfered with TPA-
and Br-cAMP-induced mRNA degradation.

Whether the cAMP and TPA signals utilize the same mech-
anism to induce LFB3 mRNA destabilization is not yet estab-
lished, although it is plausible considering that induced LFB3
mRNA instability by either agent requires ongoing RNA syn-
thesis and that signal transductions induced by the two agents
are related. In the cell, CAMP and TPA activate distinct signal-
ning pathways but are otherwise quite related. Both agents
trigger signaling by activating serine/threonine kinases, and the transcription factors that are eventually activated by these
signals are also related; the cAMP and TPA signals activate
CREB/ATF and AP1, respectively, which are highly related
transcription factors containing basic/leucine zipper domains,
recognize highly similar sequences, and can cross-dimerize (for
reviews, see Refs. 8 and 46). A protein responsible for induced
LFB3 mRNA degradation could be phosphorylated and regu-
lated by cAMP-dependent protein kinase as well as by protein
kinase C. Alternatively, the two different but related transcrip-
tion factors may exert their effects at a post-transcriptional
step by interacting with the same RNA sequence or RNA-

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