Abstract—Osteopontin (OPN), an RGD-containing extracellular matrix protein, is associated with arterial smooth muscle cell (SMC) activation in vitro and in vivo. Many cytokines and growth factors involved in vessel wall remodeling induce OPN overexpression. Moreover, we recently demonstrated that the extracellular nucleotide UTP also induces OPN expression and that OPN is essential for UTP-mediated SMC migration. Thus, we set out to investigate the mechanisms of OPN expression. The aim of this study was to identify transcription factors involved in the regulation of OPN expression in SMCs. First, we explored the contribution of mRNA stabilization and transcription in the increase of UTP-induced OPN mRNA levels. We show that UTP induced OPN mRNA increases via both OPN mRNA stabilization and OPN promoter activation. Then, to identify transcription factors involved in UTP-induced OPN transcription, we located a promoter element activated by UTP within the rat OPN promoter using a gene reporter assay strategy. The −96 to +1 region mediated UTP-induced OPN overexpression (+276±60%). Sequence analysis of this region revealed a potential site for AP-1 located at −76. When this AP-1 site was deleted, UTP-induced activation of the −96 to +1 region was totally inhibited. Thus, this AP-1 (−76) site is involved in UTP-induced OPN transcription. A supershift assay revealed that both c-Fos and c-Jun bind to this AP-1 site. Finally, we demonstrate that angiotensin II and platelet-derived growth factor, two main factors involved in vessel wall pathology, also modulated OPN expression via AP-1 activation. (Circ Res. 2003;93:674-681.)

Key Words: osteopontin • smooth muscle cells • extracellular nucleotides • AP-1

Several studies suggest that migration and proliferation of arterial smooth muscle cells (SMCs) play a prominent role in vascular pathologies such as atherosclerosis, restenosis, and hypertension.1 SMC migration and proliferation can be induced by many factors, including growth factors and cytokines.2-4 We have previously shown that extracellular nucleotides are also able to induce cell-cycle progression of SMCs5,6 and their migration.7 Thus, we were interested in understanding the mechanisms by which UTP induces SMC migration. Nucleotide-induced SMC activation is mediated via G protein–coupled P2Y receptors. Their activation leads to phospholipase C activation and consequently to [Ca2+]i increase and protein kinase C activation. Our previous work demonstrated that P2Y2, P2Y4, and P2Y6 receptors are expressed in cultured SMCs8 and that P2Y2 is overexpressed in balloon-injured rat carotids.9 Moreover, we have shown that these receptors are involved in UTP-induced migration.8 We also demonstrated that UTP induces the expression of the extracellular matrix protein osteopontin (OPN)6 and that UTP-induced migration is dependent on OPN expression and binding to αvβ3 integrin.7

OPN is an RGD-containing extracellular matrix (ECM) phosphoprotein involved in cell attachment10 and migration11,12 and prevention of apoptosis.13 OPN expression is induced by many growth factors, hormones, and cytokines involved in vascular remodeling, such as angiotensin II (Ang II), basic fibroblast growth factor,14 platelet-derived growth factor (PDGF),15 and interleukin-1.1 Moreover, in vitro studies have shown that OPN is associated with proliferation16 and migration7 of rat cultured SMCs. OPN is detected in SMCs and macrophages of atherosclerotic plaques, and its expression is upregulated in neointimal thickenings of rat vessels induced by balloon angioplasty.17,18 Moreover, UTP-induced OPN expression and SMC migration are mediated by mitogen-activated protein kinase extracellular signal–regulated kinase 1/2 activation.7 Therefore, the regulation of OPN expression seems to play a key role in SMC migration. The aim of this study was to identify transcription factors and promoter elements involved in UTP-induced OPN expression. Previous studies have shown that OPN expression is regulated by various inducers at the transcriptional level (USF-1 is associated with differentiation19 and AP-1 is involved in glucose signaling20). Various transcription factors have been shown to induce OPN expression in osteoclasts, including vitamin D receptor,21 estrogen receptor α,22 Smad,23 Cbfa,24 Ets-1,25 and Tcf-1 in mammary cells.26 In contrast, transcription factors involved in growth factor–induced OPN expression in SMCs have not been identified. However, several transcription factors such
as NFAT, AP-1, CREB, SRF, STAT, and nuclear factor-kB, are activated by UTP in SMCs.27

In the present study, we first demonstrate that the increase in OPN mRNA levels observed in SMCs after UTP stimulation is mediated both by mRNA stabilization and transcription. Then, by a systematic analysis of OPN promoter-luciferase constructs, we identify the AP-1 factor as a regulator of UTP-mediated OPN transcription in cultured SMCs.

Materials and Methods

Cell Culture
Rat aortic SMCs were prepared from thoracic aortas of Wistar rats and cultured as previously described.28 SMCs from passages 10 to 20 were used. Quiescent SMCs were obtained by a 72-hour incubation in serum-free DMEM.

Plasmid Constructs
The rat OPN promoter cloned in the pGL2 basic vector was kindly provided by Dr. A. Ridall29 (University of Texas Houston–Health Science Center, Houston, Tex). The luciferase-reporter plasmids containing different lengths of the 5′ region of the rat OPN promoter (−1599 luc, −1240 luc, −1051 luc, −551 luc, −294 luc, and −96 luc) were constructed by a serial deletion strategy using restriction enzyme digestion. The plasmid containing the AP-1 potential site was deleted, as follows: −96ΔAP-1 luc (PCR amplification of the Eagl (−96)–XhoI (−66) fragment using mutated primer).

<table>
<thead>
<tr>
<th>Name of the Construct</th>
<th>Rat OPN Promoter Fragment</th>
</tr>
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<tbody>
<tr>
<td>−1599 luc</td>
<td>NsiI (−1599)–XhoI (+66) fragment</td>
</tr>
<tr>
<td>−1240 luc</td>
<td>AflII (−1240)–XhoI (+66) fragment</td>
</tr>
<tr>
<td>−1014 luc</td>
<td>PmlI (−1014)–XhoI (+66) fragment</td>
</tr>
<tr>
<td>−551 luc</td>
<td>SnaBI (−551)–XhoI (+66) fragment</td>
</tr>
<tr>
<td>−294 luc</td>
<td>Construct provided by A.L. Ridall</td>
</tr>
<tr>
<td>−96 luc</td>
<td>Eagl (−96)–XhoI (+66) fragment</td>
</tr>
<tr>
<td>−96ΔAP-1 luc</td>
<td>PCR amplification of the Eagl (−96)–XhoI (+66) fragment using mutated primer</td>
</tr>
</tbody>
</table>

Rat OPN fragments of different sizes were obtained by enzymatic digestion of the OPN promoter sequence (AF017274 S78412).28 These fragments were cloned into the pGL2 basic vector. Sites for restriction enzymes were located relative to the transcription initiation site.

Transient Transfection of SMCs and Reporter Assays
Rat SMCs were transfected in 380-mm² wells using SuperFect according to the protocol proposed by the provider (Qiagen). Cells were cotransfected by pGL2 basic vectors (Promega) containing rat OPN promoter fragments (0.4 μg) and by pHook-LacZ (Invitrogen) (0.4 μg). For experiments using the plasmids encoding the dominant negative to Fox or Jun, A-Fos, or A-Jun,29 cells were cotransfected by 0.27 μg −96 luc construct, 0.27 μg A-Fos, A-Jun or pcDNA3 (Invitrogen), and 0.27 μg pHook-LacZ. After a 24-hour incubation in serum-free DMEM, cells were stimulated by 100 μmol/L UTP, 10 μmol/L Ang II, or 50 ng/mL PDGF BB for 6 hours.

Luciferase activities were measured using the Luciferase assay system (Promega). Luciferase activity was normalized for transfection efficiency by β-galactosidase activities determined by a spectrophotometric method using ONPG as substrate.

Because stimulation by UTP induced an increase in basal luciferase activity in SMCs transfected with the pGL2 vector, luciferase activities of the OPN promoter constructs were normalized to pGL2 basic luciferase activity stimulated or not by UTP.

RNA Extraction, Northern Blot, mRNA Stability Studies
Total RNAs were isolated from SMCs by the guanidinium isothiocyanate/phenol/chloroform extraction method.28 RNAs (20 μg) were submitted to a 1% agarose gel electrophoresis and blotted onto a nylon membrane (Immobilon-Ny+, Millipore). Blots were first hybridized with an OPN-specific cDNA probe as previously described31 and then stripped and hybridized again with an 18S ribosomal RNA oligonucleotide probe.

Electrophoretic Mobility Shift Assays
Nuclear extracts from 20 10⁶ quiescent SMCs were prepared as previously described,32 missing the end dialysis. For binding assays, probe 1 containing the AP-1 potential site was 5′-CTAGAACCCTCATGACATCACTCC-3′ and probe 2 containing CCAAT box, SP-1, Ets-1, and c-myb potential sites was 5′-CTAGACTCCGGCCTCCTGGTTGG-3′.

For competition, Ets-1 consensus probe was 5′-CTCAGTTAAGCAGAGGATGACTAAC-3′. GC-rich probe was 5′-CTAGTTGTTGTGCCCCCTCCGTCG-3′.

Binding probes were labeled by the Klenow fill-in method with α-³²P dCTP (NEN). For DNA binding reaction, 10 μg of nuclear proteins was incubated with 80 000 cpm of radiolabeled oligonucleotides in binding buffer (glycercolly 10%, HEPES 20 mmol/L, pH 7.9, NaCl 75 mmol/L, MgCl2, 2 mmol/L, poly dI.dC 1 μg, BSA 1 μg) for 60 minutes at room temperature before loading onto nondenaturating 5% polyacrylamide gel (200 V for 1.5 hours). Competitive probes or antibodies against c-Fos or c-Jun (Santa-Cruz Biotechnology) were added to the nuclear proteins 15 minutes before the addition of labeled probes.

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation (ChIP) assay was performed from quiescent SMCs treated by 100 μmol/L UTP for 2 hours, essentially according to the protocol described at www.upstate.com/misc/protocols.asp?prot=chips using anti–c-Fos or anti–c-Jun antibodies. Solution used was Sds lysis buffer (1% SDS, 10 mmol/L EDTA, 50 μmol/L Tris HCL, pH 8, 1 mmol/L PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl, pH 8, 167 mmol/L NaCl, containing the protease inhibitor cocktail). Other solutions are described in Current Protocols in Molecular Biology.33

The OPN promoter fragment containing the AP-1 potential site was PCR amplified using 5′-AAAAACCCAGAGTGGCGAGT-3′ and 5′-GAATGCTTGCAGGGAGAC-3′ primers. The Sp-1 gene fragment control was amplified using 5′-AGAAGCGCACA-GTCCTGTT-3′ and 5′-GGGACAGCTTGCGTGG-3′ primers.

Results

UTP-Induced OPN mRNA Increase Is Mediated Both by Stabilization of mRNA and Transcription
Previously, we showed that UTP increased OPN mRNA levels in a dose-dependent manner from concentrations as low as 10⁻⁸ mol/L.7 Figure 1 shows that the OPN mRNA steady-state level reached its maximum between hours 4 and 8 after UTP stimulation. To determine whether OPN mRNA was regulated at the transcriptional level, postranscriptional level, or both, we first evaluated the contribution of UTP-induced mRNA stabilization using actinomycin D to block transcriptional activity.
When quiescent SMCs were incubated in serum-free medium in the presence of actinomycin D 20 μg/mL, the mRNA OPN half-life was 15 hours (Figure 2B). When SMCs were incubated in the presence of actinomycin D and 100 μmol/L UTP, the OPN mRNA level remained quite stable for at least 26 hours (Figure 2A). Consequently, UTP induced OPN mRNA stabilization in quiescent SMCs. It is noteworthy that autoradiography was performed for 1 week to detect the basal OPN mRNA level. Nevertheless, at the 6th hour after UTP stimulation, when the OPN mRNA level reached its maximum (Figure 1), OPN mRNA stabilization represented no more than 30% of the OPN mRNA rate increase when the OPN mRNA level without UTP stimulation was taken as reference (Figure 2B). Therefore, at that time, OPN mRNA stabilization played a minor role in the UTP-induced increase in OPN mRNA, because the OPN mRNA level was increased at least 7-fold after UTP stimulation (Figure 1).

The effect of UTP on the transcriptional activity of the OPN gene was studied by a reporter gene strategy. Rat SMCs were transiently transfected with a vector containing the luciferase reporter gene downstream of the rat OPN promoter. After a 24-hour incubation in serum-free medium, SMCs were stimulated for 6 hours by UTP at concentrations from 10^-9 to 10^-3 mol/L. Then luciferase activity was measured in cell extracts. The data demonstrated that UTP induced a dose-dependent activation of the rat OPN promoter (Figure 2C). At 100 μmol/L, UTP induced a maximal effect with a 7-fold activation of OPN transcription. This concentration was then used for the following experiments.

The −96 to +66 Promoter Region Mediates UTP-Induced Transcription

Next, our aim was to identify the cis-regulating elements of the OPN promoter that regulated OPN gene transcription induced by UTP in rat aortic SMCs. Therefore, we con-
constructed a set of pGL2 plasmids carrying 5′ deletions of the rat OPN promoter cloned by Ridall (Table and Figure 3A). Luciferase reporter activity was determined in SMCs transiently transfected with these deleted constructs and then stimulated or not by 100 μmol/L UTP (Figure 3B). It is noteworthy that unstimulated SMC luciferase activity of the shortest construct (−96 luc) was 10 times that of the pGL2 basic in the same conditions, so the first 96 bp of the OPN promoter was sufficient to give a promoter activity. UTP-stimulated SMCs had 2- to 3-fold higher luciferase reporter activity than unstimulated SMCs for all constructs tested containing −1599 to −96 bp of OPN promoter (Figure 3C), whereas deletion of the −96 to +66 fragment led to the loss of UTP stimulation. Thus, it could be hypothesized that a cis-regulating element responding to UTP was located in the −96 to +66 region of the OPN promoter.

### EMSA Analysis of the −96 to +66 Region of the Rat OPN Promoter

Sequence analysis of the −96 to +66 region revealed several potential cis-regulated sequences, including an AP-1 site located at −76 (this site had already been identified on the mouse OPN promoter34), a GC-rich sequence located at −65 (described on the human OPN promoter35), an Ets-1 site located at −59, a CCAAT box located at −57 (identified on the mouse OPN promoter36), and a c-myb site located at −55. To check whether some transcription factor bound to these sites in UTP activated cells and could thus be involved in OPN promoter regulation, EMSA analyses were performed using two 25-bp probes. Probe 1 (−83 to −66) contained the AP-1 potential site, and probe 2 (−70 to −50) contained the GC-rich (for Sp-1), Ets-1, CCAAT box, and c-myb potential sites.

When nuclear extracts were incubated in the presence of 32P-labeled probe 1, two complexes were formed. These complexes disappeared when an excess of cold probe 1 (but not a nonspecific probe) was added with the labeled probe 1 (Figure 4A), thus demonstrating that the AP-1 potential site on the rat OPN promoter was recognized by specific tran-
Supershift assays using anti-c-Fos and anti-c-Jun antibodies were performed to identify transcription factors constituting the AP-1 complex. When c-Fos or c-Jun antibody was added to the nuclear extract, a supershift band was observed. In contrast, no supershift was observed when an anti–Tcf-1-antibody was used (Figure 6A). This experiment suggested that the transcription factors c-Fos and c-Jun are both parts of the UTP-activated complex. ChIP assay was performed to verify that c-Fos and c-Jun effectively bind the AP-1 site in the entire cell. PCR analysis demonstrated that OPN promoter DNA was communoprecipitated together with c-Fos and c-Jun (Figure 6B). Altogether, these results demonstrate that c-Fos and c-Jun bind the AP-1 site not only in nuclear extract but also in the cell.

The role of c-Fos and c-Jun on the AP-1 site–dependent transcription was evaluated using a dominant-negative strategy to inhibit Fos and Jun proteins. SMCs were cotransfected with both the −96 luc construct deleted or not for the AP-1 site and the pRc/CMV vector expressing either the A-Fos or the A-Jun dominant negative. In this way, it appeared that in the presence of A-Fos or A-Jun, the UTP-induced luciferase activity of the −96 luc construct was reduced by 27±16% or 28±11%, respectively (Figure 6C). Thus, these results confirmed definitively that c-Fos and c-Jun are involved in UTP-induced OPN expression.

The AP-1 Site Is Also Involved in Ang II and PDGF-Induced OPN Expression

Then we investigated whether Ang II and PDGF, two main growth factors involved in vascular remodeling, also regulated OPN expression via the stimulation of the AP-1 site (−76). To this end, SMCs were transiently transfected with the −96 luc or the −96 AP-1 luc constructs. After 24 hours in serum-free medium, the transfected cells were stimulated by Ang II 10 μmol/L or PDGF 50 ng/mL for 6 hours. Both Ang II and PDGF induced luciferase activity in the cells transfected with the −96 luc construct, and their activity depends on the AP-1 site (Figure 7).

Discussion

In a previous study, we demonstrated that nucleotides induce an increase in OPN mRNA steady-state levels in SMCs cultured in the presence or absence of serum.6 The present work shows that the UTP-induced OPN mRNA increase in rat SMCs results from both OPN mRNA stabilization and OPN gene transcription.

To date, few studies have tried to establish the mechanisms of OPN mRNA increase. In particular, it has been shown that neither transforming growth factor-β, a growth factor activating serine/threonine kinase receptors, nor intracellular receptor agonists, such as glucocorticoids and retinoic acid,35,36 induce OPN mRNA stability.3 In this study, we demonstrate that OPN mRNA level is regulated by UTP via a mRNA stabilization mechanism. Ang II, another agonist of G-protein–coupled receptors, can also stabilize OPN mRNA but not PDGF (data not shown). The sequence analysis of OPN mRNA reveals one potential sequence involved in mRNA stability in the 3′ UTR, TAAAT (located at 1084 to 1088). However, this OPN mRNA stabilization did not lead to a major increase in OPN mRNA level over a short period (6 hours), probably because of the long half-life of OPN mRNA (~10 to 15 hours) (Figure 2B).
We also found that UTP increases OPN transcription. The exploration of the pathways regulating OPN transcription demonstrated that an AP-1 cis-regulating site located at −76 on the OPN promoter is involved in UTP-induced OPN transcription. Moreover, supershift and ChIP assays together with the use of a dominant-negative strategy to inhibit Fos and Jun demonstrated that both c-Fos and c-Jun transcription factors are responsible for AP-1 activation. Previously, we demonstrated that c-fos mRNA levels are increased by UTP.5 In this study, we show that the c-Fos transcription factor is necessary for OPN transcription. This AP-1 site (TGA-CACA) seems important because it is conserved in rat, mouse, and human. In mouse, it is involved in okadaic acid–induced OPN expression in osteoblasts39 and in glucose-induced OPN expression in SMC.20

By using EMSA analysis, we found that protein complexes bound to the AP-1 site, the GC-rich region, and the Ets-1 site located respectively at −76, −65, and −59, thus suggesting that these sites are potentially active on the rat OPN promoter. However, only the AP-1 binding complex is modulated by UTP, suggesting that the other sites are not involved in UTP-induced OPN transcription. The latter might be involved in basal transcription of OPN. Indeed, the GC-rich region binds the Sp1 factor, an ubiquitous transcription factor believed to be important for the basal transcription of most genes. Moreover, the GC-rich region of the human OPN promoter (located at −69) is involved in OPN transcription in human malignant astrocytoma cell lines.35 Although Ets-1 has been shown to be induced by Ang II and PDGF,40 it seems that in our conditions this factor is not involved in Ang II- and PDGF-induced OPN expression, because the −96 luc construct deleted from the AP-1 site was not activated by Ang II or PDGF (Figure 7). The AP-1 site is not involved in basal transcription of OPN, because when deleted, basal luciferase activity of the −96 luc construct was not modulated (Figure 5).

The prominent role of Ang II and PDGF in vascular remodeling has been widely underlined. It has been demonstrated that these factors are involved in SMC migration and proliferation.41 The present work demonstrates that the AP-1
site is also involved in Ang II- and PDGF-induced OPN expression, thus suggesting that AP-1 is a common pathway for exogenously induced OPN expression in SMCs and that it could mediate SMC migration and vascular remodeling.

Various studies have suggested that the transcription factor AP-1 plays a major role in proliferation and migration of SMCs in vitro. Indeed, an oligonucleotide AP-1 decoy inhibited SMC proliferation and migration induced by FCS and glucose. This hypothesis is confirmed in vivo, because AP-1 has been shown to be overexpressed in SMCs after vascular lesion and because neointimal formation after balloon injury of rat carotid artery was limited when cells were transfected by an oligonucleotide AP-1 decoy.

OPN has also been shown to be associated with SMC proliferation and migration. Moreover, OPN is overexpressed during neointimal formation in rat artery and in human atherosclerotic plaques. Therefore, AP-1 and OPN seem to be key factors involved in vascular remodeling, and the present study establishes a clear link between AP-1 and OPN expression. Our results strongly suggest that growth factors overexpressed during vascular injury and nucleotides released by perivascular nerves, activated platelets, endothelial cells under shear stress, activated or injured SMCs, and released by perivascular nerves, activated platelets, endothelial factors overexpressed during vascular injury and nucleotides promote vascular cell adhesion and inhibits SMC proliferation and migration induced by FCS and glucose.

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AP-1 Is Involved in UTP-Induced Osteopontin Expression in Arterial Smooth Muscle Cells


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