Calmodulin (CaM) is the principal Ca\(^{2+}\) receptor protein inside the cell. When activated by Ca\(^{2+}\), CaM binds and activates target proteins, thus altering the metabolism and physiology of the cell. Under basal conditions, calcium-free CaM binds to other proteins termed CaM-binding proteins. Recently, we described endothelial differentiation-related factor (EDF)-1 as a protein involved in the repression of endothelial cell differentiation (Dragoni, I., Mariotti, M., Consalez, G. G., Soria, M., and Maier, J. A. M. (1998) J. Biol. Chem. 273, 31119–31124). Here we report that (i) EDF-1 binds CaM \textit{in vitro} and \textit{in vivo}; (ii) EDF-1 is phosphorylated \textit{in vitro} and \textit{in vivo} by protein kinase C; and (iii) EDF-1-CaM interaction is modulated by the concentrations of Ca\(^{2+}\) and by the phosphorylation of EDF-1 by protein kinase C both \textit{in vitro} and \textit{in vivo}. In addition, 12-O-tetradecanoylphorbol-13-acetate treatment of human umbilical vein endothelial cell stimulates the nuclear translocation of EDF-1. On the basis of the high homology of EDF-1 with multiprotein bridging factor-1, a transcriptional coactivator that binds TATA-binding protein (TBP), we also demonstrate that EDF-1 interacts with TBP \textit{in vitro} and \textit{in vivo} and in human endothelial cells. We hypothesize that EDF-1 serves two main functions in endothelial cells as follows: (i) to bind CaM in the cytosol at physiologic concentrations of Ca\(^{2+}\) and (ii) to act in the nucleus as a transcriptional coactivator through its binding to TBP.

Angiogenesis, the branching and sprouting of capillaries from pre-existing blood vessels, is a tightly controlled event crucial in development, reproduction, and wound healing (1). The consequences of abnormal angiogenesis are either excessive or insufficient blood vessel growth. Ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for wound healing, whereas up-regulated angiogenesis favors tumor growth and spreading, rheumatoid arthritis, and diabetic retinopathy (2). Angiogenesis is initiated by vascular endothelial cells and involves their orderly migration, proliferation, and morphogenesis into a new capillary network (3). Although considerable attention has been given to the mechanisms involved in the regulation of endothelial cell growth, the molecular events associated with the non-proliferative aspects of angiogenesis, i.e. the organization/differentiation of endothelial cells into capillaries, are poorly understood (4).

Recently, by RNA fingerprinting we isolated EDF-1,\(^1\) a novel gene product down-regulated in endothelial differentiation (5). EDF-1, which encodes a 16-kDa polypeptide, is the human homologue of the silkworm multiprotein bridging factor (MBF)-1, a transcriptional mediator that mediates transactivation by stabilizing the protein-DNA interaction (6). Interestingly, a neuronal calmodulin-binding peptide, termed CAP 19, was used to recover a full-length cDNA clone from rat brain (7). This cDNA is the rat homologue of EDF-1. By sequence comparison, we found that the human and rat proteins possess a putative IQ domain that could mediate their binding to CaM. Indeed, calmodulin-binding proteins share a conserved region of about 20 amino acids, designated the IQ motif, that contains a CaM-binding domain and a protein kinase C (PKC) phosphorylation site (8). The IQ motif was originally identified in neuromodulin that concentrates CaM at specific sites in neurons (9). Since then, several other proteins have been shown to possess this motif as follows: the GTPase-activating protein IQ-GAP (10), p68RNA helicase (11), neurogranin (12), the pro- oncogene EWS (13), among others. Calmodulin, which is the classical Ca\(^{2+}\) receptor protein inside cells (14), mediates calcium regulation of a number of enzymes such as adenyl cyclases, kinases, and phosphatases that are important components of signal transduction systems implicated in cell cycle progression and cytoskeletal rearrangement (15).

In this paper, we report \textit{in vitro} and \textit{in vivo} evidence that EDF-1 binds calmodulin in the absence of calcium. We also demonstrate that EDF-1-CaM interaction is tightly regulated by the levels of Ca\(^{2+}\) and the activation of PKC both \textit{in vitro} and \textit{in vivo}. To our knowledge, this is the first report about a protein that binds CaM and regulates its availability in endothelial cells. Interestingly, upon exposure to TPA, we can observe a dramatic increase in nuclear associated EDF-1. Moreover, native and phosphorylated EDF-1 interact with the TATA-binding protein (TBP), and EDF-1 and TBP communoprecipitate in endothelial cells.

MATERIALS AND METHODS

Cell Culture—HUVEC-C were from the ATCC and were cultured in HF12 containing 10% fetal calf serum, ECGF (150 μg/ml), and heparin (5 units/ml) on gelatin-coated dishes (5).

Site-directed Mutagenesis—Oligonucleotides were designed to mutate EDF-1 by polymerase chain reaction and to clone the construct in the KpnI/BamHI sites of pQE30. To generate the single mutant Thr-91 → Asp, we used the following primers: 5′-AAG GGG CTT GAC CAG AAC GAC CTG -3′ and 5′-CAG GTC CTT CTG GTC AAG CCC CTT -3′. To mutate Thr-40 → Asp, we used the following primers: 5′-GAT GGT GAG GAT TCC AAG AAA-3′ and 5′-TTT CGA TTT GCA ATC CAC

\(^1\) The abbreviations used are: EDF, endothelial differentiation-related factor; CaM, calmodulin; MBF, multiprotein bridging factor; PKC, protein kinase C; TBP, TATA-binding protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; HUVEC, human umbilical vein endothelial cell; PS, 1,4-phosphatidyl-1-serine.
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ATC-3' To mutate Thr-58 → Asp, we used the following primers: 5'-ACC AAG AAC GAT GCC AAG CTC-3' and 5'-CAG CTT GGC ATC GTT CTG-3'. To mutate Ser-111 → Asp, we used the following primers: 5'-GAT TAT GAG GAA CGG GCC-3' and 5'-GGC CGG TCC ATC CTC ATA GTC-3'. The external primers used to generate restriction sites are 5'-CTA GGT ACC AAA GAA CGG CCGG AAC-3' and 5'-CTA GGA TCC GCC ATG GCC AGG GAC-3'.

Phosphorylation of EDF-1 by PKC—Since EDF-1 contains conserved PKC phosphorylation sites, one of which is within the IQ domain, we examined whether the phosphorylation of EDF-1 by PKC affects its binding to CaM. EDF-1 was phosphorylated in vitro by PKC in the presence of Ca2+ and phospholipids (Fig. 3A). It is interesting to note that, once phosphorylated, EDF-1 did not interact with calmodulin-agarose, and the 32P-labeled protein was all recovered in the flow-through fraction of the column (Fig. 3B).

We also determined whether Thr-91, which is the PKC phosphorylation site within the IQ motif, plays a role in modulating EDF-1 interaction with CaM.

To this purpose, because the introduction of a negative charge mimics a phosphorylated residue, the Thr-91 → Asp mutation was introduced. Although the substitution of Thr-91 to CaM-agarose column, and we eluted with buffers containing increasing concentrations of Ca2+. Fig. 1A shows that EDF-1 interacted with CaM and eluted with 1 and 2 mM Ca2+.

No interaction was instead observed between EDF-1 and agarose (Fig. 1A). We then evaluated whether EDF-1 and CaM interact also in vivo. To this purpose, HUVEC lysates were immunoprecipitated with an antibody against CaM, and Western blot was performed utilizing an immunopurified antibody against EDF-1 (Fig. 1B). Similar results were obtained when lysates were immunoprecipitated for EDF-1, and Western blot was performed using anti-CaM antibodies (data not shown).

No bands were detectable after immunoprecipitation with nonimmune IgGs (Fig. 1B).

EDF-1 interacts with CaM, and this binding is regulated by the levels of Ca2+.

**RESULTS**

Interaction between EDF-1 and CaM—To determine whether EDF-1 interacts with CaM, we loaded recombinant EDF-1 on a calmodulin-agarose column, and we eluted with buffers containing increasing concentrations of Ca2+. Fig. 1A shows that EDF-1 interacted with CaM and eluted with 1 and 2 mM Ca2+. No interaction was instead observed between EDF-1 and agarose (Fig. 1A). We then evaluated whether EDF-1 and CaM interact also in vivo. To this purpose, HUVEC lysates were immunoprecipitated with an antibody against CaM, and Western blot was performed utilizing an immunopurified antibody against EDF-1. In HUVEC, EDF-1 and CaM coimmunoprecipitated (Fig. 1B). Similar results were obtained when lysates were immunoprecipitated for EDF-1, and Western blot was performed using anti-CaM antibodies (data not shown).

No bands were detectable after immunoprecipitation with nonimmune IgGs (Fig. 1B).

Fig. 2 shows that EDF-1 and CaM also colocalize both in the nucleus and in the cytosol. Interestingly, upon exposure to TPA (100 nM), EDF-1 was mainly localized in the nucleus (see below), whereas the subcellular localization of CaM was not significantly altered (Fig. 2).

To evaluate whether different levels of intracellular Ca2+ affected the interaction between CaM and EDF-1 in HUVEC, we exposed HUVEC for 20 min to the calcium ionophore A23187 (1 μM) to increase intracellular Ca2+ (21). We therefore coimmunoprecipitated EDF-1 and CaM. Fig. 1C shows that in the presence of A23187 the interaction between EDF-1 and CaM was reduced.

These results indicate that EDF-1 interacts with CaM, and this binding is regulated by the levels of Ca2+.

**FIG. 1.** EDF-1 is a CaM-binding protein. A, EDF-1 was applied to a CaM-agarose or an agarose alone column, washed, and eluted in the indicated buffers. B, HUVEC lysates were immunoprecipitated with a monoclonal antibody anti-CaM (lane 1) or with nonimmune IgGs (lane 2). Western blot was performed using anti-EDF-1 IgGs (upper panel) or an anti-CaM polyclonal antibody (lower panel). C, HUVEC were exposed to A23187 (1 μM) for 20 min and immunoprecipitated with a monoclonal antibody anti-CaM or with nonimmune IgGs. Western blot was performed using anti-EDF-1 IgGs (upper panel) or an anti-CaM polyclonal antibody (lower panel). FT, flow-through fraction.
IgG. Merge of the anti-EDF-1 and anti-CaM staining is shown in the isothiocyanate-labeled swine anti-goat IgG and rhodamine anti-rabbit IgG for 1 h. Cells were then fixed and stained with either rabbit anti-EDF-1 to CaM-agarose.

A, in vitro phosphorylated EDF-1 was incubated with CaM-agarose, washed, and centrifuged, and the resin was eluted in sample buffer. The eluates were analyzed by Western blot. Lanes 1–3, recombinant EDF-1, EDF-1T91D, and EDF-1D4, respectively; lanes 4–6, recombinant EDF-1, EDF-1T91D, and EDF-1D4, respectively, eluted from CaM-agarose.

Fig. 5. TPA stimulates the phosphorylation of EDF-1 in HUVEC. A, HUVEC were metabolically labeled with 50 μCi/ml carrier-free 32P. Then TPA (100 nM) or A23187 (1 μM) alone or in combinations was added for 20 min. The cells were lysed and immunoprecipitated with anti-EDF-1 or nonimmune (last lane) antibodies as described above. Immunoprecipitates were divided in two aliquots. One aliquot was utilized for autoradiography (upper panel), and the remaining aliquot was used in Western analysis with anti-EDF-1 antibodies (lower panel). B, HUVEC were treated with TPA for 1 h. Cells were lysed and immunoprecipitated with a monoclonal antibody anti-CaM, and Western blot was performed using anti-EDF-1 IgGs (upper panel) or an anti-CaM polyclonal antibody (lower panel).

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EDF-1, which is unphosphorylated in untreated HUVEC, was readily phosphorylated upon exposure to TPA. The degree of phosphorylation was increased when HUVEC were cotreated with TPA and A23187, probably due to the fact that when intracellular Ca²⁺ concentration increases, more EDF-1 is released from CaM and available to be phosphorylated. To evaluate whether in vivo phosphorylated EDF-1 bound or not to CaM, we communoprecipitated EDF-1 and CaM in HUVEC exposed to TPA. As shown in Fig. 5B, a lower amount of EDF-1 communoprecipitated with CaM in TPA-treated cells when compared with controls. These results show that EDF-1 is a substrate of PKC, and PKC phosphorylation prevents its binding to CaM.

It is noteworthy that, although EDF-1 was localized both in the nucleus and in the cytosol in control cells, EDF-1 was mainly associated with the nucleus of phorbol TPA-treated cells (Fig. 2). No relocalization of CaM was observed in TPA-treated cells.

EDF-1 Interacts with TBP—Since EDF-1 is highly homologous to MBF-1 which binds TBP, we investigated whether EDF-1 interacted with TBP. As shown in Fig. 6A, EDF-1 and its mutants bound TBP to a similar extent in in vitro protein-protein interactions assays. This interaction was detected by immunoprecipitation with anti-EDF-1 antibodies followed by Western blot utilizing an anti-TBP monoclonal antibody. Analogously, we found that in vitro phosphorylated EDF-1 interacted with TBP (data not shown). We also determined whether this interaction occurred in vivo in HUVEC treated or not with...
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DISCUSSION

CaM is the principal Ca\(^{2+}\) receptor protein inside cells (15). The calcium-rich form of CaM activates a large array of enzymes that are part of intracellular signaling cascades controlling cellular homeostasis (16). Because of the central role of CaM in signal transduction, there is interest in modulatory proteins that alter the way in which CaM senses calcium levels. CaM-binding proteins share a loosely defined IQ motif that directs binding to CaM in its calcium-free form (8). In some cases, this binding is regulated by the PKC pathway (22). For instance, neuromodulin and neurogranin binding to CaM is reversed when they are phosphorylated by PKC (22).

In this report, we show that EDF-1, recently described as a factor involved in the repression of human endothelial cell differentiation (5), contains an IQ motif, interacts with CaM, and is phosphorylated by PKC. Phosphorylation of EDF-1 by PKC prevents the protein from binding CaM, thus suggesting that PKC phosphorylation and CaM binding are mutually exclusive. In addition, upon PKC activation in HUVEC, EDF-1 is translocated to the nucleus where it may exert its function as a transcriptional activator. Indeed, we demonstrate by in vitro protein-protein interaction that EDF-1 binds TBP. Moreover, TBP and EDF-1 coimmunoprecipitate in HUVEC, and this interaction is not affected in HUVEC exposed to TPA. We therefore suggest that TPA potentiates the accumulation of EDF-1 in the nucleus where a large amount of EDF-1 would be available to interact with TBP, thus regulating transcription. We therefore hypothesize that EDF-1 serves two principal roles. In the cytosol EDF-1 interacts with CaM in the presence of low Ca\(^{2+}\), and in the nucleus it interacts with TBP and modulates transcription. Accordingly, during the course of this work, Kabe et al. (23) showed that the human homologue of MEF-1, which is identical to EDF-1, interacts with TBP and Ad4BP/SF1, a mammalian counterpart of FTZ-F1, by gel shift.

The nuclear staining of EDF-1 is intriguing, since EDF-1 does not possess a nuclear localization sequence (24). Indeed, EDF-1 is detected both in the cytosol and in the nucleus of control cells. Upon PKC activation, nuclear translocation of EDF-1 is enhanced. It is conceivable to think that EDF-1 is driven to the nucleus by a shuttling protein (25) and that PKC potentiates this shuttling mechanism. In partial disagreement with our results, Kabe et al. (23) show that, when ectopically expressed in COS-1, EDF-1 was mainly localized in the cytoplasm, and coexpression with Ad4BP/SF1 induced nuclear localization of EDF-1. It should be recalled that we localize endogenously synthesized EDF-1 in human endothelial cells, whereas Kabe et al. (23) perform their experiments in a totally different system, i.e., transiently transfected COS-1. However, we agree on the fact that the fate of EDF-1 is to be translocated to the nucleus where it may connect the DNA-binding domain of transcription factors and TBP.

To our knowledge, this is the first demonstration about the expression of a CaM-binding protein that may modulate CaM activation in endothelial cells. When activated CaM influences various metabolic pathways, among which is the synthesis of nitric oxide (NO) through endothelial NO synthase (26). NO not only is a potent vasodilator, thus regulating arterial pressure (27), but it is also involved in the nonproliferative events of angiogenesis (28). It is tempting to speculate about a relation between EDF-1 and NO levels and its implication in vascular disease and in angiogenesis.

We show that in vitro EDF-1 has a rather high affinity for CaM-agarose and elutes with 1 mM Ca\(^{2+}\). It is unlikely that 1 mM levels of free Ca\(^{2+}\) may be reached within the cell; however, it is conceivable that free Ca\(^{2+}\) accumulates in highly specialized areas. It is reported that the Ca\(^{2+}\) ionophore A23187 transiently increases Ca\(^{2+}\) by 4–5-fold in endothelial cells (21). We show that this increase of intracellular Ca\(^{2+}\) levels is sufficient to release, at least in part, EDF-1 from CaM. Under these experimental conditions, we hypothesize that calcium-CaM would activate a large array of enzymes, whereas EDF-1 would be available for phosphorylation and/or bind a shuttle protein to be transported to the nucleus where it would mediate transactivation by stabilizing the protein-DNA interaction. Interestingly, a range of Ca\(^{2+}\) concentrations between 2 and 5 mM is required to release two prototypic neural-specific CaM-binding proteins, neuromodulin and neurogranin, from CaM-agarose (29). Recently, it has been hypothesized that neurogranin and neuromodulin may function to concentrate CaM at specific sites in neurons and release free CaM in response to increased Ca\(^{2+}\) and PKC activation (22).

It is tempting to speculate about the significance of EDF-1-CaM interaction. More experiments are required to address this issue.

In the case of an endothelial cell exposed to angiogenic factors, we hypothesize the following scenario. After binding to their cognate receptors, angiogenic factors lead to the release of Ca\(^{2+}\) from internal stores and diacylglycerols, which stimulate PKC (30–31). These two collaborate to sustain high levels of calcium-CaM and calcium/CaM-dependent enzyme activity. Moreover, Ca\(^{2+}\) reduces the affinity of EDF-1 for CaM, and PKC blocks the re-binding of CaM by phosphorylating EDF-1. This leads to an increase in free EDF-1, part of which is phosphorylated by PKC and promptly translocated to the nucleus where EDF-1 would modulate gene expression.

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