Differential Regulation of *fos* and *jun* Gene Expression and AP-1 cis-
Element Activity by Endothelin Isopeptides

POSSIBLE IMPLICATIONS FOR MITOGENIC SIGNALING BY ENDOTHELIN*

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Endothelins (ET) are potent vasoconstrictor peptides that also function as mitogens for numerous cell types. Although regulation of second messenger pathways by ET peptides has been extensively investigated, little is known about the pathways of nuclear signaling by which ET controls gene expression. The present experiments investigated whether *fos* and *jun* contribute to nuclear signaling and gene regulation by ET isopeptides. ET isopeptides induced a subset of *fos* and *jun* mRNAs in mesangial cells, including c-*fos*, fra-1, c-*jun*, and JunB. *fos* and *jun* mRNAs were induced as members of the immediate-early gene response. Activation of the high affinity ET receptor moderately increased c-*fos* and fra-1 mRNA, whereas activation of the low affinity receptor markedly induced both *fos* and *jun* mRNAs. Thus, different ET receptor subtypes evoke distinct patterns of *fos* and *jun* induction. Prominent isopeptide- and cell-specific differences in the magnitude and kinetics of *fos* and *jun* expression were observed. Most striking was the marked elevation of c-*fos* steady-state mRNA and protein by ET-1, as compared with ET-3. In addition, ET-1, but not ET-3, increased transcriptional activity conferred by an AP-
1 cis-element and directed collagenase gene expression. These results suggest that differential regulation of *fos* and *jun* expression and of AP-1 cis-element activity by ET isopeptides contributes to regulation of gene expression by ET. Furthermore, a role for AP-1 in mitogenic signaling by ET is suggested by the close correlation between AP-1 cis-element activity and cell growth.

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§ The abbreviations used are: ET, endothelin; AP-1, activator protein-1; BSA, bovine serum albumin; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase.

Endothelins (ET) are a family of acidic, 21-amino acid peptide hormones found in at least three distinct isoforms (1). Each isoform arises from an isopeptide-specific prepro-ET species and is encoded by separate genes. Both in structure and function, ET is closely related to sarafotoxin peptides isolated from venom of *Atractaspis engaddensis*, and it seems likely that the two gene families share a common evolutionary origin (2). Although ET was first thought to be synthesized exclusively by the vascular endothelium, it is now clear that numerous vascular and nonvascular cells secrete ET. ET secretion has been demonstrated in the central nervous system, lung, kidney, gut, pituitary, and hypothalamus, eye, and amnion (see Refs. 3 and 4 for review). Thus, it is not surprising that ET peptides regulate a wide range of biological actions, including contraction of vascular and nonvascular smooth muscle, neuromodulation and neurotransmission, secretion of biologically active compounds, such as prostaglandins and steroids, and control of cell growth. The pathways of transmembrane signaling that mediate short term actions of ET (i.e. contraction and secretion) have been extensively investigated (3). In contrast, the pathways of nuclear signaling that mediate long term actions of ET, such as mitogenesis, remain unclear.

The finding that ET-1 induces expression of c-*fos* mRNA levels in glomerular mesangial cells suggests that AP-1 transcription factors might contribute to nuclear signaling by ET peptides (5). AP-1 complexes are prototypes for transcription factors that couple receptor-generated second messengers to long term responses requiring differential regulation of gene expression (6-8). AP-1 consists of a mixture of homo- and heterodimers that bind to cis-acting elements (5'-TGAC/ GTCA-3') in promoter and enhancer regions of numerous cellular and viral genes (9, 10). The AP-1 proteins are encoded by two gene families: (i) *fos* family genes including c-*fos*, fosB, ΔfosB, fra-1, and fra-2; and (ii) the *jun* family including c-*jun*, junB, and junD. Dimerization of Fos and Jun proteins occurs through a leucine zipper structural motif that involves parallel association of α-helical domains in a structure similar to a coiled-coil (11, 12). Dimerization is essential to align the basic DNA-binding regions and facilitate binding of AP-1 to its cognate cis-element. However, dimerization is tightly regulated and has important consequences for AP-1 activity. For example, Jun:Fos heterodimers are more stable than Jun:Jun dimers, and Fos proteins are unable to form stable heterodimers (13-15). Unlike the DNA-binding domains, which are highly conserved, the transactivator domains of AP-1 proteins are divergent. Thus, the dimer composition of AP-1 has been postulated to control transcriptional activity by increasing the combinatorial possibilities for protein:protein interactions with the RNA polymerase II preinitiation complex and with adjacent bound proteins on promoters and enhancers (7, 16). In at least one case (junB versus c-*jun*) AP-1 proteins have been reported to have different transcriptional properties (17, 18). Expression of c-*fos* and c-*jun* mRNA is one of the earliest genomic responses to cell stimulation by phorbol esters, growth factors, and serum (19-21). Moreover, the distinct pattern of *fos* and *jun* expression has been proposed to account in part for the specific phenotypic response to...
distinct stimuli. In PC12 pheochromocytoma cells, for example, mitogens, such as epidermal growth factor and nerve growth factor stimulate c-fos, c-jun, and junB expression, whereas only c-fos and junB are induced by membrane depolarization (22). Evidence from various hematopoietic cells also demonstrates agonist-specific patterns of fos and jun induction (23), but the biological consequences of agonist-specific patterns of fos and jun induction are not clear.

We designed a series of experiments to directly test whether fos, jun, and AP-1 cis-elements contribute to nuclear signaling by ET. In the studies reported here, we demonstrate that ET-1 and ET-3 differentially regulate the expression of fos and jun genes. Only ET-1, however, causes a significant increase in transcriptional activity conferred by an AP-1 cis-element. Moreover, the ability of ET isopeptides to increase AP-1 activity correlates closely with their ability to stimulate cell growth. These data are consistent with a role for AP-1 factors in nuclear signaling and transcriptional regulation by ET peptides.

EXPERIMENTAL PROCEDURES AND RESULTS

Evidence for fos/jun and AP-1 cis-Element Activity in Nuclear Signaling by ET Peptides—Although the ability of ET peptides to control short term actions by generating second messenger cascades has been extensively investigated (see Ref. 3 for review), virtually nothing is known about the pathways of nuclear signaling by which ET regulates gene expression. Recent evidence indicates that ET-1 increases expression of genes for several trans-acting factors, including c-fos and c-myc (3). Differential regulation of gene expression by ET-1 is thought to contribute to the long term biological actions of ET, such as mitogenesis and vascular remodeling in disease (3, 4, 44).

In the present study, we have examined the role of fos and jun expression and AP-1 cis-element activity in nuclear signaling by ET. Several lines of evidence suggest that AP-1 contributes to regulation of gene expression by ET peptides. First, ET isopeptides and serum induced expression of a subset of fos and jun mRNAs. c-fos, fra-1, c-jun, and junB were inducible in mesangial cells, whereas fosB and junD were never induced. Expression of fos and jun family genes regulates AP-1 activity by controlling the nuclear concentration of AP-1 protein and by directing the subunit composition of AP-1 dimers, which in turn determines in part the affinity for AP-1 cis-elements (6, 8). For example, induction of the c-fos gene by ET-1 would promote formation of stable c-Fos:Jun heterodimers with higher affinity for the AP-1 site than Jun:Jun homodimers (13-15). In addition, because AP-1 proteins contain unique trans-activating domains, the subunit composition of AP-1 dimers determines the combinatorial possibilities for gene regulation by protein:protein interactions with members of the RNA polymerase II preinitiation complex or with adjacent bound trans-activating factors (8, 16). Thus, differential regulation of fos and jun expression is a potential mechanism for regulation of AP-1 activity by ET.

Our data also demonstrate significant cell- and ligand-specific differences in fos and jun induction. Although serum induces fosB mRNA in 3T3 fibroblasts (32), the present experiments show that fosB was not induced by serum, ET-1, or ET-3 in mesangial cells. Previous experiments demonstrate that fosB is not expressed in stimulated hepatoma cells despite the marked induction of c-fos and c-jun (45). Collectively, these data suggest that cell-specific differences in fosB expression exist. Similarly, fra-1 is differentially induced by serum in PC12 and 3T3 cells (30). Ligand-specific differences exist in both the dynamics and pattern of fos and jun induction by ET-1 and ET-3. The enhanced ability of ET-1 to increase both c-fos steady-state mRNA and protein was the most prominent isopeptide-specific difference. Collectively, these data are consistent with the hypothesis that AP-1 complexes consist of a continuously changing population of homo- and heterodimers (6, 16) and that stimulation by ET peptides can shift the dynamic equilibrium of AP-1 formation and perhaps affect the transcriptional activity of AP-1. Our present data imply that in quiescent mesangial cells, AP-1 consists mostly of homo- and heterodimers without c-fos. After activation by ET-1, AP-1 complexes probably consist mostly of c-Fos:Jun heterodimers, and the proportion of c-Fos:Jun dimers declines until 4 h when c-Fos protein is no longer present. However, further experiments are necessary to characterize the population of AP-1 dimers following activation of ET receptors.

The dose-response relationship of fos and jun mRNA induction suggests that activation of distinct ET receptor subtypes gives rise to different patterns of AP-1 gene induction. We note that activation of the high affinity receptor stimulated an increase in only c-fos and fra-1 steady-state mRNA. Activation of this receptor in mesangial cells stimulates a ligand-operated Ca²⁺ channel and does not activate phospholipase C (5, 36). Thus, stimulation of c-fos mRNA is likely to occur via the CRE/CaRE element (CAMP and Ca²⁺ response element) in the c-fos promoter, which is sensitive to increments in cytosolic free [Ca²⁺] (46). Consistent with these results, the c-jun promoter is not known to have a Ca²⁺-sensitive element. On the other hand, activation of the low affinity ET receptor stimulates even greater increases in c-fos and fra-1 while also inducing c-jun and junB. The low affinity ET receptor in mesangial cells activates numerous effectors in mesangial cells, including phospholipase C, protein kinase C, and phospholipase A₂ (see Ref. 3). We surmise, therefore, that activation of different signaling cascades by ET receptor subtypes leads to unique patterns of fos and jun induction. As discussed below, these differences might be reflected in the ability of the low affinity, but not the high affinity receptor, to stimulate mitogenesis in mesangial cells.

One of the unexpected results of our experiments is that ET-3 fails to increase transcriptional activity conferred by an AP-1 cis-element, despite increasing mRNA levels for fra-1, c-jun, and junB. A significant increase in CAT expression from the −73/+63 Coll-CAT constructs was observed only with ET-1. Significant induction of the native collagenase gene was also observed only with ET-1. These results not only confirm the regulatory data observed with the −73/+63 Coll-CAT constructs but also demonstrate that ET-1 directs expression of the collagenase gene. Expression of the collagenase gene by ET-1 in mesangial cells might have important implications for the role of ET-1 as a proinflammatory mediator (4, 44). Several hypotheses might explain why ET-1, but not ET-3, increases AP-1 activity. First, AP-1 activity in vivo appears to be regulated by poorly characterized accessory proteins (47, 48) that might be subject to positive regulation by ET-1 but not ET-3. Post-translational modification by reversible phosphorylation/dephosphorylation is also known to be an important mechanism regulating AP-1 activity (49, 50). Perhaps ET-1 stimulates post-translational modification of AP-1 proteins at a greater extent than ET-3. Consistent...
with this hypothesis, ET-1 is a much stronger stimulus for phospholipase C activation and Ca\(^{2+}\) signaling than ET-3 (3, 36). The importance of ligand-specific post-translational regulation of AP-1 is supported by recent studies in U937 monoblastic leukemia cells, where single doses of diacylglycerol, as opposed to phorbol ester, induced c-fos and c-jun mRNA but failed to elevate AP-1 cis-element activity (51). Thus, similar to our results in mesangial cells with ET isopeptides, simple induction of fos and jun mRNA does not always increase AP-1 activity and suggests that ligand-dependent post-transcriptional mechanisms might play an important role. Finally, part of the explanation for isopeptide-specific regulation might lie in the potency of ET-1 to increase c-fos expression. Induction of c-fos gives rise to AP-1 dimers with enhanced stability, moreover, the transactivation domain of c-fos could contribute to unique protein:protein interactions that somehow enhance the initiation rate of RNA polymerase II. If this interpretation is correct, it points to regulation of c-fos expression as a critical step in nuclear signaling by ET-1. Further experiments are necessary to distinguish between these possible explanations.

Taken together, the data discussed above demonstrate a role for AP-1 in nuclear signaling by ET peptides and support the hypothesis that isopeptide-specific activation via AP-1 cis-elements can differentially regulate transcriptional responses.

Possible Role for AP-1 in Mitogenic Signaling by ET-1—AP-1 transcription factors are thought to play an important role in mitogenic signaling. First, v-fos and v-jun are potent transforming oncogenes (42, 52), which suggests that the cellular homologues of these proteins play a fundamental role in normal growth control. Moreover, transient transfection with a variety of oncogenes (i.e. src and H-ras) increases AP-1 transcriptional activity (43, 50). AP-1 genes are induced by a wide variety of growth factors and mitogenic stimuli (6, 8). Rapidly cycling cells express higher levels of c-jun mRNA than serum-starved cells (53). In addition, blockade of c-fos expression by antisense c-fos RNA (54, 55) or microinjection of anti-fos antibodies (56) attenuates the mitogenic response to ET. However, the exact outcome of the phenotypic response to ET isoproteins most likely depends on other concurrent events that have yet to be identified.

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Endothelin and AP-1 cis-Elements
Supplemental Material To Differential Regulation of the Ras and JAK-STAT Pathways by AP-1 cis-Elements by Michael E. Dustin

EXPERIMENTAL PROCEDURES

Materials: ET-1 and ET-3 were obtained from Peptide Institute (Tokyo, Japan), methyl-3H-thymidine (2 Ci/mM), L-[35S]methionine (5 Ci/mM), C-3H-thymidine (2 Ci/mM), [3H]thymidine (2 Ci/mM) and other (3H)-labeled nucleotides (5 Ci/mM) were from New England Nuclear (Boston, MA). (gallium31)methionine (2.2Tc/mM) was from ICN Biomedicals (Costa Mesa, CA). Rabbit polyclonal antibody raised against rat c-fos (67 C) was provided by Oncogene Science (Manhasset, NY). Except where indicated, all oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer in the Molecular Biology Core Facility at Case Western Reserve University.

Mammalian Cell Culture and Measurement of Cell Growth: Mammalian cell lines were propagated in medium (Earle's MEM + 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml of insulin, 5% CO2, and 5% CO2, all obtained from GIBCO, Grand Island, NY). Mammalian cells at appropriate cell concentrations were seeded next to observe changes in cell morphology and were used for metabolism studies in serum-free RPMI supplemented with 10% dialysed fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml of insulin, and 5% CO2. Mammalian cells at approx. 5 x 10^3 cells/cm² were infected with virus for 4 h and then washed four times with serum-free RPMI supplemented with 0.1% BSA. As previously reported (30), serum-free treatment did not compromise mammalian cell viability. For measurements of cell growth, growth studies were performed with cell plates coated with 1 mg/ml of bovine serum albumin (BSA) containing type 1 (1 mg/ml of BSA) or type 3 (1 mg/ml of BSA) with ET-1, ET-3, or ET-3. After a 24 h preincubation period, the culture medium was replaced with serum-free RPMI supplemented with 0.1% BSA and the cell growth was measured by the MTT assay. A cell growth assay was performed exactly as described (30).

RNA Extraction and Northern Blot Analysis: As previously reported (31), total cellular RNA was isolated by the acid guanidinium thiocyanate procedure with minor modifications. For Northern analysis RNA was denatured and fractionated by electrophoresis through a 1.2% agarose gel (0.7% agarose) and transferred to a nitrocellulose membrane

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Endothelin and AP-1 cis-Elements

**RESULTS**

ET-1 and ET-3 differentially regulate fos and jun mRNA expression. To begin identifying the mechanisms whereby ET peptide regulates AP-1, we investigated whether ET-1 and ET-3 differentially regulate fos and jun expression. ET-1 and ET-3 (100 nM) had the same low-affinity (ETAR) receptor in neonatal cells and activated phospholipase C and other effects (2,28). In neonatal cells and in neonatal mRNA levels were differentially regulated by serum and both ET isoforms (Fig. 1 A and Table 1). Only a subset of fos and jun genes were inducible in neonatal cells: c-fos, f-jun, and jun B were induced whereas Jun D and fos B were not induced. Although serum induces fos mRNA in NIH 3T3 fibroblasts (29), we were unable to document increased fos mRNA by serum in neonatal cells (Fig. 1). Consistent with these data, previous experiments have also shown that jun D mRNA is not induced by serum or TPA in quiescent 3T3 cells (29). In neonatal cells we consistently observed two c-jun transcripts (2.8 and 3.1 kb) in response to both ET isoforms, which are similar in size to the two transcripts observed in NIH 3T3 cells (2.7 and 3.1 kb) (29). Hybridization with the 3′-1 kilobase-end cDNA probe revealed two transcripts induced with similar kinetics: an abundant transcript at 1.8 kb that corresponds to c-jun (1.8) and a less abundant band at approximately 0.9 kb (Fig. 1). This less abundant transcript is not fos 2.2 in the oligonucleotide probe corresponds to a unique sequence in the fos mRNA not conserved in fos 2.2 (30,31). Moreover, the 2.6-kb mRNA transcript is 6.8 kb as compared to the 0.9-kb transcript observed in our studies (Fig. 1).

Fos and jun gene expression in neonatal cells were induced with different kinetics and magnitude in response to both serum and ET isoforms. As expected, c-fos and c-jun were transiently induced whereas the induction of fos 1 and jun B was sustained (Fig. 1 and Table 1). Similar steady-state levels of c-fos and c-jun mRNA were induced to a greater degree than fos 1 and jun B. These data also reveal ligand-specific patterns of AP-1 induction. In general, serum and ET-1 induced a greater fold increase in fos and jun mRNA that did ET-3. The increase in jun B mRNA was also sustained longer by ET-1 than ET-3. Most strikingly, however, is the finding that ET-1, but not ET-3, induces c-fos mRNA. Taken together, these data demonstrate that fos and jun mRNA levels are differentially regulated by serum and by ET isoforms. Moreover, there are common inositol- and cell-specific differences in the patterns of fos and jun gene expression.

**Activation of high- and low-affinity ET receptors by ET-3 differentially induce fos and jun mRNA expression.** In neonatal cells, ET-1 had a high-affinity (ETAR) receptors and low-affinity (ETBR) receptors that can be differentially induced by equilibrium binding studies (32,33). The high-affinity receptor couples to a ligand-operated Ca²⁺ channel whereas the low-affinity receptor couples to numerous effectors including phospholipase C, protein kinase C, and phosphatase A (32,33). We therefore tested the possibility that activation of different ET receptor subtypes leads to differential expression of fos and jun mRNA. At low doses of ET-3 (0.1 nM), which activate the high-affinity receptor, c-fos and jun B mRNA were moderately induced whereas jun D and fos B mRNA levels were not elevated (Fig. 3). By contrast, at concentrations of ET-1 (1.0 nM through 10 nM) that activate the low-affinity receptor, c-fos and Jun B were markedly induced as were jun D mRNA and jun B. This, these dose-response curves for c-fos and jun B induction were topographically. These data imply that fos and jun gene expression are differentially regulated by different ET receptor subtypes.

**Fin and jun mRNA levels are induced by ET-1 over members of the immediatene-cisisent gene network.** Because some genes encoding AP-1 proteins are induced as members of the immediate-early gene response (34), we addressed this possibility by using superinduction of fos and jun mRNA by protein synthesis inhibition. Proteolysis with cycloheximide (CHX) led to block of the increase in c-fos, f-jun, and jun B mRNA by ET-1 (Fig. 3). For example, in the presence of CHX, a 6.5-h incubation with ET-1 induced c-fos and jun B mRNA (5, 9.3 ± 6.4-fold, respectively) compared to 9.2 and 5.4-fold in the absence of CHX, i.e., Table 1 (35). In contrast, the induction of the c-jun by ET-1 was somewhat attenuated in the cells treated with CHX. These data indicate that induction of fos and jun mRNA by ET-1 does not require new protein synthesis. Similar to other immediate-early genes, induction of protein synthesis solely induced expression of c-fos, c-jun, and jun B mRNA without having little or no effect on c-jun expression (Fig. 2). The data with the fos 1 are consistent with a previously published report demonstrating variability of the induction and superinduction of fos 1 mRNA in PC12 cells and fibroblasts (36). The present study suggests that c-fos, f-jun, and jun B are induced by ET-1 as part of the immediate-early gene response. Moreover, the finding of fos, jun, and fos mRNA in PC12 cells and fibroblasts is characteristic of immediate-early gene expression with the same kinetics (37) further suggests that AP-1 genes are induced by ET-1 in the immediate-early response. However, the finding that fos 1 was not induced by CHX alone suggests that fos mRNA cells distinct mechanisms might exist for regulating the steady-state mRNA levels of different fos genes.
Endothelin and AP-1 cis-Elements

ET-1 increases immunoreactive c-fos protein. At saturating concentrations of ET-1 and ET-3 (100 nM), both peptides occupy the low-affinity (ET-1) receptor in murine cells. We were surprised, therefore, to find that ET-1, but not ET-3, increased steady-state c-fos mRNA (Fig. 1, and Table I). To determine whether the inability of ET-3 to increase c-fos mRNA was reflected at the level of protein expression, we assayed c-fos protein levels in murine cells following activation by ET-1 and ET-3. Immunoreactive c-fos protein was undetectable in quiescent murine cells. Following activation by ET-1, c-fos protein was markedly elevated with a nuclear and perinuclear localization (Fig. 4A, single arrow). c-Fos protein was present at 1 h and declined rapidly thereafter, consistent with the short half-life for c-fos mRNA and protein (42). In contrast, c-fos protein was only minimally elevated following treatment with ET-3 (Fig. 4B). Specificity of the immunostaining was documented by comparison with an excess of the peptide immunogen and by omission of the primary antibodies (Fig. 4B). Higher magnification photomicrographs at 1 h following treatment with ET-1 and ET-3 confirm that ET-1 markedly elevates c-fos protein with a nuclear and perinuclear localization. We note that the alignment of cytoplasmic immunostaining into discrete "raps" (arrowheads) towards the nucleus (Fig. 4C, double arrows) suggests the nuclear import of c-fos is tightly regulated. These findings further demonstrate that in contrast to ET-3, ET-1 markedly increases c-fos expression. These findings also lend further support to the concept of isoform-specific differences in expression of proteins comprising the AP-1 complex.

Figure 4. Assessment and localization of immunoreactive c-fos protein expression following activation by ET-1 and ET-3. (A) Quiescent murine cells were incubated with ET-1 and ET-3 to RPMI 1640 medium with 10% FBS at 37°C. At the times indicated the monolayers were fixed and c-fos protein was localized by immunostaining using an antiperoxidase antisera as described in Experimental Procedures. Exposure was identical to facilitate quantitative comparisons of staining intensity. Specific properties of staining patterns, identified by arrows, are described in the text. Magnification X 1010. Identical results were obtained at two independent experiments. (B) To control for specificity, the primary antibodies were preincubated for 1 h at 37°C with the peptide antigen used to generate the antibody before addition to fixed and permeabilized monolayers. In several experiments the first antibody was also omitted. (C) Higher magnification (X 1020) of the perinuclear localization of c-fos protein and the formation of "raps" suggesting regulated nuclear import of c-fos protein.
Endothelin and AP-1 cis-Elements
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