SRC1, initially identified as a nuclear receptor coactivator, was found to interact with a member of the transcriptional enhancer factor (TEF) family of transcription factors, TEF-4. The interaction, which occurs in both intact cells and in a cell-free system, is mediated by the highly conserved basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) domain present in the N-terminal region of SRC1. Moreover, all three members of the p160 family of nuclear receptor coactivators, SRC1, TIF2, and RAC3, are able to potentiate transcription from a TEF response element in transient transfection experiments, and this activation requires the presence of the bHLH-PAS domain. These results suggest that the p160 proteins could be bona fide coactivators of the TEF family of transcription factors.

Transcriptional coactivators, recruited by sequence-specific transcription factors, enhance transcriptional activation of target genes via interactions with chromatin remodeling complexes and components of the basal transcriptional apparatus (1, 2). Three related 160-kDa proteins, SRC1, TIF2, and RAC3, encoded by separate genes, form the steroid receptor coactivator (SRC) or p160 family of coactivators (for a review, see Refs. 3 and 4). These proteins are highly homologous and were initially identified as factors that interacted with nuclear receptors (NRs) in the presence of ligand and were able to enhance receptor-dependent transcriptional activation (5–7). The p160 proteins have been reported to potentiate the activity not only of NRs but also a number of other transcription factors (8–14), although the mechanisms by which the p160s enhance the activity of other signaling pathways are less well characterized.

The p160 proteins contain conserved domains responsible for the interaction with NRs (15, 16), and protein interaction domains responsible for the recruitment of downstream effectors, such as histone acetyltransferases like CBP/p300 (17, 18) and p300/CBP-associated factor (PCAF) (19). In addition, the p160 coactivators have a highly conserved N-terminal basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) domain. The bHLH domain is a DNA binding and protein dimerization motif shared by many transcription factors (20), and in the bHLH-PAS subfamily an additional dimerization motif, called PAS domain, extends from the C-terminal end of the HLH domain (21). The bHLH-PAS domain present in the p160 proteins has a striking homology with those from the bHLH-PAS family of transcription factors, and it is also the most conserved region between the three members of the family. Nevertheless, its function remains unclear and it seems to be dispensable for the enhancing of the NR transcriptional activity in cotransfection studies (5). Therefore, the role of this putative protein dimerization motif in the stabilization of competent coactivator complexes, mediating accessory protein-protein interactions and/or the recruitment of p160 coactivators by other transcription factor remains to be established.

To understand the molecular mechanisms of SRC1 functions and identify its associated proteins, we performed a yeast two-hybrid screen using the bHLH-PAS domain of SRC1 as bait. In this report we present evidence supporting a role for the p160 proteins as coactivators for the transcriptional enhancer factor (TEF) family of transcription factors (22), which are implicated in the regulation of many developmental processes, such as the control of cardiac and skeletal muscle-specific gene expression (23–26), early gene expression in mouse development (27), and human chorionic somatomammotropin (hCS) gene expression in the placenta (28, 29).

**EXPERIMENTAL PROCEDURES**

Two-hybrid Screening—A region of SRC1 encoding the bHLH-PAS domain (amino acids 1–361) was cloned in frame 3′ of the DNA binding domain of LexA in pBETM116 to generate a bait fusion protein. A mouse embryo (9.5–12.5 dpc) cDNA library in the pASV3 vector (30) was used for screening according to the modified protocols described by Hollenberg et al. (31). The bait and the library were sequentially transformed into Saccharomyces cerevisiae strain L40a using the lithium acetate method. Polypeptides interacting with SRC1 bHLH-PAS domain were detected by the ability to activate transcription of HIS3 and lacZ reporter genes. Colonies able to grow on HIS-deficient medium containing 40 μM 3-amino-1,2,4-triazole were selected and tested for β-galactosidase expression. pASV3 plasmids from His+ lacZ− colonies were isolated, and cDNA inserts were determined by automated sequencing.

Full-length cDNA of Mouse TEF-4—The first 528 nucleotides of the partial TEF-4 clone 1.6 were amplified by PCR and 32P-labeled using the Multiprime DNA Labeling System (Amersham Pharmacia Biotech). This probe was used to screen a high density DNA Filter containing a mouse embryo (embryonic day 12.5) cDNA library using the protocols provided by the manufacturer (Resource Center/Primary Data Base, Max Planck Institut for Molecular Genetics, Heubnerweg 6, Berlin, Germany). The clone MPMGp1539M1368Q2 encodes the full-length cDNA of mouse TEF-4, identical to the TEF-4 in the data bases (GenBankTM accession number D50683).
A portion of the yeast two-hybrid interaction between TEF-4 and SRC1 bHLH-PAS domain. A, schematic representation of SRC1e, the LexA chimera used as bait in the two-hybrid screening and the VP16-tagged clone 1.6. Numbers refer to amino acids in the full-length proteins. Indicated are the bHLH and the PAS homology region (containing two imperfect repetitions named PAS A and PAS B regions), the nuclear receptor interacting domain (RID), and the activation domains 1 and 2 (AD1 and AD2, respectively) in SRC1e. B, the L40a yeast strain expressing either LexA-DBD or LexA-DBD fused to the SRC1 bHLH-PAS domain (PAS) was transformed with either the empty pASV3 plasmid or pASV3 expressing the clone 1.6 fused to the VP16 activation domain. β-Galactosidase activity in each yeast extract was measured in duplicate. Data represent the mean ± S.D. of two independent transfectants.
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Fig. 3. SRC1e activity on TEF response elements. A, SRC1e requires the bHLH-PAS domain to enhance the transcriptional activity from TEF response elements. HeLa cells were transiently transfected with the pGL3-MCAT/SV40 luciferase reporter, 30 ng of wild type or the N-terminal deletion mutant comprising the amino acids 381–1399 (ΔPAS-SRC1e) of SRC1e, and an internal control vector (pRL-EF1a, providing constitutive expression of Renilla luciferase). After transfection, cells were washed and incubated for 24 h. Subsequently, cells were assayed with a dual luciferase reporter system. Normalized values are expressed relative to the activity of ER in the presence of 10⁻¹² M E2. The experiment was repeated several times, and results of a representative example, assayed in duplicate, are shown as mean ± S.D. B, the deletion of the bHLH-PAS domain in SRC1e does not decrease protein expression. COS-1 cells were transiently transfected with expression vectors for wild type or ΔPAS-SRC1e. After 24 h of incubation the cells were harvested, and Western blot analysis was performed with an anti-SRC1 antibody. C, the bHLH-PAS domain is dispensable for the coactivation of estrogen receptor (ER) activity in transient transfections. HeLa cells were transfected with an expression vector for the ER, the luciferase reporter plasmid pGL3–2XERE-PS2, wild type SRC1e or ΔPAS-SRC1e, and the internal control pRL-EF1a. After transfection, cells were washed and incubated with vehicle (gray bars) or E2 (10⁻¹² M; black bars) for 24 h. Subsequently, cells were assayed with a dual luciferase reporter system. Normalized values are expressed relative to the activity of ER in the presence of 10⁻¹² M E2. The experiment was repeated several times, and results of a representative example, assayed in duplicate, are shown as mean ± S.D. D, the CBP-interacting domain of SRC1e is not required for the enhancing of TEF response elements. HeLa cells were transiently transfected with either the empty pGL3 Basic luciferase vector or the pGL3-MCAT/SV40 luciferase reporter and 30 ng of wild type p160s or deletion mutants as indicated. Data are presented as in A.

Note: The figure shows graphs A, B, C, and D, which illustrate the activity of SRC1e on TEF response elements. Graph A demonstrates the activity of SRC1e in comparison to wild type and deletion mutants. Graph B shows the effect of the deletion in the bHLH-PAS domain on protein expression. Graph C indicates the dispensability of the bHLH-PAS domain for coactivation with estrogen receptor. Graph D highlights the role of the CBP-interacting domain of SRC1e.

According to their ability to grow in a medium lacking histidine. The positive transformants were identified and tested for β-galactosidase activity. Sequence analysis revealed that one clone (1.6) encoded a truncated TEF-4 protein, with the N-terminal region (amino acids 37–176) fused to the C-terminal region (amino acids 401–445), when compared with the published sequence (40) (Fig. 1A). To test the specificity of the interaction, the plasmid expressing the truncated TEF-4 fused to the VP16 AD, or the isolated VP16 AD, were re-transformed into yeast expressing the LexA-DBD fused to the bHLH-PAS domain or the isolated LexA-DBD. The interactions were studied by determining the levels of lacZ reporter expression in yeast extracts using β-galactosidase assays (Fig. 1B). We found a background activity indicating a weak interaction between the bHLH-PAS domain and the VP16 AD, but the presence of the TEF-4 polypeptide increased this activity 8-fold, indicating that the N-terminal region of SRC-1 and the TEF-4 clone 1.6 are able to interact in vivo.

The polypeptide encoded by the clone 1.6 lacks the N-terminal part of TEF-4 (amino acids 1–36) and also has an internal deletion of the region 177–401. This internal deletion raises the possibility that the TEF-4 pre-mRNA might undergo alternative splicing. To investigate this possibility we screened a mouse embryo cDNA library with the partial TEF-4 clone as a probe and found 18 clones encoding partial or full-length TEF-4. However, in all cases, the cDNA sequences were identical to that described previously, suggesting that alternatively spliced variants rarely if ever occur.

We next investigated whether the in vivo interaction between SRC1 and TEF-4 that we observed in yeast was also detectable in vitro, using GST pull-down assays. The truncated TEF-4 encoded by the clone 1.6 bound to the N-terminal region of SRC1 (1–807) fused to GST (Fig. 2A), in agreement with the in vivo interaction between SRC1 and the clone 1.6. This in vivo interaction was characterized in more detail using the bHLH-PAS domain alone (region 1–450), and the full-length TEF-4 protein, and we found that they were able to interact (Fig. 2B). Another member of the TEF family of transcription factors, TEF1-A (34), also was able to bind to SRC1 in the same assay (Fig. 2B), indicating that the interaction is not restricted to a single member of the TEF family.

SRC1, TIF2, and RAC3 are well established coactivators that are recruited to the ligand activated NRs, enhancing their transcriptional activity (3, 4). Having demonstrated a physical interaction between TEF transcription factors and SRC1 both in vivo and in vitro, we investigated whether SRC1 was able to potentiate transcriptional activation from a TEF response element in transiently transfected cells. Using the luciferase reporter plasmid pGL3-MCAT/SV40 (37) we found that the expression of full-length SRC1e consistently showed 2–3-fold induction of the MCAT/SV40 luciferase reporter (Fig. 3A). When we cotransfected a truncated SRC1e deletion mutant, lacking the bHLH-PAS domain, the ability of SRC1e to activate the transcription from the TEF response element was completely abolished (Fig. 3A). This lack of activation cannot be explained by a lower expression of the SRC1 deletion mutant, as immunoblot analysis of transfected cell extracts using an anti-SRC1 antibody showed that the SRC1 mutant is efficiently expressed (Fig. 3B). Moreover, this SRC1 deletion mutant is able to potentiate the transcriptional activity mediated by the
estrogen receptor α in a similar transient transfection experiment using the 2XERE-PS2 luciferase reporter (Fig. 3C), showing that the mutant is able to go to the nucleus and interact with its downstream effectors. When the empty pGL3-basic, lacking the TEF binding elements, was used in the transient transfection assay, SRC1 showed no activation of the luciferase reporter gene (Fig. 3D), indicating that the effect was specific of the sequences in the TEF artificial promoter. Overexpression of either full-length TEF-4 or TEF-1A repressed the activity of the TEF response element as has been reported previously in a variety of cell lines (34, 41–43), and this repression was not relieved by the cotransfection of SRC1 (data not shown).

SRC1 contains at least two activation domains, AD1 and AD2 (6, 32) (Fig. 1A). AD1 has been demonstrated to recruit the general coactivator CBP/p300, and this domain is required for the transcriptional activation by the NRs (6, 32, 33). To test whether AD1 was also required for the potentiation of the TEF promoter, we cotransfected an expression vector encoding a SRC1 mutant with an internal deletion of that region (Δ900–950). Interestingly, that deletion did not affect SRC1 potentiation from TEF response element (Fig. 3D).

The other two members of the p160 family, TIF2 and RAC3, were also able to potentiate the pGL3-MCAT/SV40 reporter in the transient transfection assay, and this activation was even greater than that observed with SRC1 (Fig. 3D).

**DISCUSSION**

The TEF family of transcription factors is characterized by a conserved DNA binding domain, TEA/ATT (44), which recognizes several cis-regulatory motifs like Sp1, GT-IIC, and M-CAT (23). The mechanism of their action appears to be complex and likely to require interactions with specific coactivators. Several proteins have been shown to interact with TEF proteins and enhance their transcriptional activity, these include the bHLH protein Max (45), the Drosophila protein Vestigial (46), the human functional homologous TONDV (47), and the chromatin-modifying protein PARP (48).

In this report we demonstrate that a member of the p160 family of transcriptional coactivators, SRC1, is able to interact physically in vivo and in vitro with a TEF transcription factor, TEF-4, using the predicted bHLH-PAS protein dimerization motif present in its N-terminal end. Moreover, SRC1 is able to enhance the transcriptional activation from a TEF response element in transient transfection experiments, and this activation requires the presence of the bHLH-PAS domain. We speculate that this activation occurred through the interaction of SRC1 with endogenous TEF proteins present in the HeLa cells. Another TEF transcription factor tested, TEF-1A, was also able to bind directly to the SRC-1 bHLH-PAS domain, suggesting a general role of SRC1 as a coactivator for members of the TEF family. This possibility has already been suggested, based on the fact that SRC1 is able to enhance in transient transfection experiments the SV40 viral promoter, which contains multiple TEF binding sites (48). TIF2 and RAC3 were also able to potentiate the TEF response element, indicating that the highly conserved bHLH-PAS domain may play a similar role for all the p160 proteins in the recruitment of these coactivators to the TEF family of transcription factors.

The typical repression observed when exogenous TEF proteins were overexpressed was not relieved by cotransfected SRC1, suggesting that other factors than the p160 coactivators are also limiting for TEF transcriptional activity. Repression through direct interaction between TEF proteins and the TATA-binding protein could explain that result, as has been reported for several promoters (43).

The SRC1 bHLH-PAS domain is absolutely required for enhancing the TEF reporter; in contrast, it is dispensable for potentiation of transcription by NRs. In the case of the estrogen response element the deletion mutant ΔPAS-SRC1 was an even better coactivator than the full-length protein. This could be explained in terms of competition for a limiting pool of coactivators, shared between the NRs, which interact with the p160 proteins via leucine motifs (15, 16) and other transcription factors that would recruit the p160 coactivators via the bHLH-PAS domains. Recently it has been shown that GRIP-1, the murine homologous of TIF2, uses its bHLH-PAS domain to interact with another transcription factor involved in skeletal muscle differentiation, MEF-2C (14). Our results give additional evidence to the role of the bHLH-PAS domain as a protein-interacting motif used to recruit the p160 proteins to different transcription factors.

The potentiation of the NRs transcriptional activity by the p160 proteins requires the recruitment of CBP. For example, deletion of the CBP-interacting domain in SRC1e (residues 900–950) completely abolished its enhancement of the androgen receptor transcriptional activity (33), but the same mutant was still able to potentiate the TEF reporter, indicating that SRC1 must use alternative mechanisms to activate the TEF-dependent transcription. It is conceivable that the recruitment of protein methyltransferases via the C-terminal activation domain 2 (19), or its intrinsic histone acetyltransferase activity (50), are involved in this activation.

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