Role of Accessory Factors and Steroid Receptor Coactivator 1 in the Regulation of Phosphoenolpyruvate Carboxykinase Gene Transcription by Glucocorticoids*

John M. Stafford, Mary Waltner-Law, and Daryl K. Granner‡

From the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine and the Nashville Veterans Administration Hospital, Nashville, Tennessee 37232

In the liver, glucocorticoids induce a 10–15-fold increase in the rate of transcription of the phosphoenolpyruvate carboxykinase (PEPCK) gene, which encodes a key gluconeogenic enzyme. This induction requires a multicomponent glucocorticoid response unit (GRU) comprised of four glucocorticoid accessory factor (AF) elements and two glucocorticoid receptor binding sites. We show that the AFs that bind the gAF1, gAF2, and gAF3 elements (hepatocyte nuclear factor [HNF]/chicken ovalbumin upstream promoter transcription factor 1 and HNF3β) all interact with steroid receptor coactivator 1 (SRC1). This suggests that the AFs function in part by recruiting coactivators to the GRU. The binding of a GAL4-SRC1 chimeric protein completely restores the glucocorticoid induction that is lost when any one of these elements is replaced with a GAL4 binding site. Thus, when SRC1 is recruited directly to gAF1, gAF2, or gAF3, the requirement for the corresponding AF is bypassed. Surprisingly, glucocorticoid receptor is still required when SRC1 is recruited directly to the GAL4 site, suggesting a role for the receptor in activating SRC1 in the context of the GRU. Structural variants of GAL4-SRC1 were used to identify requirements for the basic-helix-loop-helix and histone acetyltransferase domains of SRC1, and these are specific to the region of the promoter to which the coactivator is recruited.

Mammals must have a readily adaptable mechanism for maintaining glucose homeostasis in the face of fluctuations in nutrient supply. Hormones such as glucocorticoids control transcription of genes that encode enzymes that help provide the adaptive response to these environmental changes (3–5). These hormone responses were originally thought to be mediated through a direct interaction of the hormone receptor and a simple DNA element, the hormone response element (HRE)1 (6–8). In recent years it has become apparent that more complex structures are involved. Hormone response units, comprised of HREs and accessory factor (AF) elements have been found in many genes involved in metabolic regulation (9–14). AFs are DNA binding transcription factors that act in concert with the hormone receptor to provide the flexible regulation of gene transcription required for metabolic homeostasis (5, 6, 15, 16).

In addition to their function in hormone-mediated gene transcription, AFs also play a central role in determining how metabolites regulate transcription. For instance, glucose-stimulated transcription of the L-PK gene requires a glucose response element that binds transcription factors of the upstream stimulating factor family. This glucose response, however, is regulated positively or negatively by the binding of HNF4 or COUP-TF, respectively, at an adjacent accessory element (17–20). In addition, Osborne and co-workers show that, in the absence of cholesterol, SREBP activates transcription of the low density lipoprotein receptor and hydroxymethylglutaryl-CoA reductase gene promoters (21–23). An SREBP response element (SRE) alone, however, is not sufficient for activation of either gene. The activation of the low density lipoprotein receptor gene promoter by SREBP requires not only a sterol response element but also an SP1 binding element (21, 24). SP1 binding to this promoter correlates with transcription of the gene in the absence of cholesterol (9). Similarly, the activation of hydroxymethylglutaryl-CoA reductase gene expression by SREBP requires both a sterol response element and an element that binds nuclear factor Y (9, 22, 23).

The role of AFs is well characterized in the regulation of PEPCK gene transcription by glucocorticoids. In adipose tissue, where PEPCK is involved in glycerogenesis, glucocorticoids repress basal and cAMP-induced PEPCK gene transcription (25, 26). By contrast, the PEPCK gene is stimulated by glucocorticoids in the liver, where PEPCK is an important control point for gluconeogenesis (16, 27). In the liver, glucocorticoids induce an ∼10-fold increase in the rate of transcription of the PEPCK gene through a multicomponent glucocorticoid response unit (GRU) located in the promoter region (28). The GRU consists of a tandem array of four AF elements (gAF1–3 and the cAMP response element) that bind HNF4/COPD-TF, HNF3β, COPD-TF, and C/EBPβ, respectively, and two nonconsensus glucocorticoid receptor binding sites (GR1, GR2) (13, 28–32). Interestingly, neither HNF3 nor HNF4 is expressed in adipose tissue, whereas both are expressed at high levels in the liver (33–35). Thus, the accessory factors HNF3 and HNF4...
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| Oligonucleotides used; sense strand 5’ to 3’ |
|--------------------------------------------|
| GR2 block                                  |
| GR1 block*                                |
| ΔHLH                                       |
| ΔHAT                                       |
| ΔNRIV                                      |
| ACGAGGCCAGCCGCGCTATGAAATACTAAGGCGGCTCCGGCC |
| GACAGCCCCACGCGCAGCAATAATTGAACGGGCGGCTATG   |
| GACAGCTTGTACGTGAGCGGTTACGCTTCG            |
| CTGCCAAATCTTGCTAGGTGTGAACGCAG            |
| CCCAGCCCCAGCCAGAAATACCACTTTTGAAG         |

* Used with GR2 block construct as a template.

It appears to play an additional important role in facilitating tissue-specific induction of PEPCK gene transcription. A mutation of any one of the accessory elements results in a 50–60% reduction of glucocorticoid-stimulated PEPCK gene transcription in H4IIE hepatoma cells (28, 32, 36, 37). Any combination of two mutations of gAF1/gAF3 or gAF2 nearly abolishes the response (28, 37). The physiologic importance of these gAFs is evident in their role in glucose production. Overexpression of an HNF3 variant that reduces the response of the PEPCK gene to glucocorticoids by ~50% nearly abolishes glucocorticoid-induced glucose production in hepatoma cells (14).

These gAF elements are not all functionally equivalent in the context of the PEPCK gene promoter (28, 31, 38). gAF1 and gAF3 can be exchanged, a finding consistent with the observation that COUP-TF binds to both elements. However, gAF1/gAF3 cannot be replaced with gAF2 and vice versa (37). Furthermore, the spacing between gAF2 and GR1 must be maintained, whereas this is not as important for gAF1 and gAF3 (37). HNF3, the AF that binds gAF2, interacts with GR in vitro (37). Thus, direct contact between HNF3 and GR may be important for stabilizing GR binding to the GR1 and GR2 elements of the PEPCK gene promoter, which bind GR with low affinity relative to a consensus GRE (36). In fact, these nonconsensus elements (GR1 and GR2), either alone or in combination, cannot by themselves mediate a glucocorticoid response when placed in the context of a heterologous promoter (36). Thus, the glucocorticoid response of the PEPCK gene is completely dependent on the gAF elements and the factors that bind to these sites. However, the mechanism by which AFs control the direction and magnitude of PEPCK gene transcription in response to glucocorticoids remains largely unknown.

The recruitment of coactivator molecules provides a crucial step in the integration of the functions of steroid hormone receptors and AFs with the basal transcription machinery (2, 39, 40). Coactivators generally do not bind DNA, but instead associate with DNA-bound activator proteins. Some coactivators such as CREB (cAMP-response element-binding protein)-binding protein (CBP) mediate the functions of a diverse group of transcription factors. Others, such as steroid receptor coactivator 1 (SRC1) and glucocorticoid receptor interacting protein (GRIP1), are functionally more specific in that they interact with activator proteins involved in steroid hormone-mediated gene transcription (41, 42).

In this study, we show that the AFs bound to the gAF1 and gAF2 elements of the PEPCK gene promoter recruit the coactivator molecule SRC1. HNF4 and COUP-TF interact with SRC1 through the domains required for their AF activity (1, 2). We show here that HNF3 also interacts with SRC1 through the same domain required for its AF activity in the PEPCK gene promoter. Mutations of either gAF1 or gAF2 that prevent HNF4/COUP-TF or HNF3 from binding, including replacement of the gAF element with a GAL4 binding site (gAF→GAL4), result in a 50–60% reduction of the response of the PEPCK gene to glucocorticoids (43). The binding of a GAL4-SRC1 chimeric protein to a gAF1→GAL4 or a gAF2→GAL4 element in a PEPCK luciferase reporter gene construct completely restores the glucocorticoid induction. Thus, when SRC1 is recruited directly to gAF1 or gAF2, the requirement for the corresponding AF is functionally bypassed. Surprisingly, even when SRC1 is recruited to the PEPCK gene promoter in this manner, active involvement of ligand-bound GR is still required, suggesting a role of the receptor in activating SRC1 in the context of this enhanceosome. Finally, we show that the bHLH and HAT domains of SRC1 function differently in the context of the PEPCk gene GRU to which the coactivator is recruited.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The reporter construct PEPCK-LUC was made by excising the PEPCK gene promoter (from -467 to +65) from a pGL2-B Luciferase Reporter Vector (Promega) and inserting it into the multiple cloning sites of pGL3 basic (Promega). The same procedure was used to generate the gAF1→GAL4 PEPCK-LUC, gAF2→GAL4 PEPCK-LUC, and gAF3→GAL4 PEPCK-LUC reporter constructs from their respective chloramphenicol acetyltransferase reporters, as described previously (43). The gAF1→GAL4 PEPCK-LUC constructs with block mutations in the GR1 and GR2 elements were constructed using the Quick Change site-directed mutagenesis kit (Stratagene). The GAL4-SRC1 deletion constructs were also made by the Quick Change site-directed mutagenesis kit using a GAL4-SRC1 construct as the template. Oligonucleotides used to generate these constructs were made by an Expedite 8909 oligonucleotide synthesizer (Perspective Biosystems, Framingham, MA). The sequences of all oligonucleotides used in this study are shown in Table I. All constructs were verified by sequencing. The reporter plasmid (GAL4)-E1B-LUC has been described previously (1, 2). The GAL4-HNF3β constructs were also described previously and are expressed at approximately equal levels in transient transfection experiments (43).

**Transient Transfection**—The maintenance and transfection of H4IE cells and the measurements of luciferase activity were performed as described previously (44, 45), except that H4IE cells were maintained in alpha-modified Eagle's medium 10% fetal bovine serum growth medium during incubation with calcium phosphate-precipitated DNA for 3 h before the Me2SO shock. Subsequently, the cells were washed and incubated overnight in serum-free medium with or without 500 nM dexamethasone, as described in the figure legends. In experiments performed with H4IE cells, the glucocorticoid response varied from 7- to 12-fold (without dexamethasone). The variation is passage- and batch-dependent. For this reason, we expressed the data in each experiment as a percentage of the wild-type glucocorticoid response, which allowed for comparison of experiments done in cells grown at different times. For experiments that involved mapping the domains of GAL4-SRC1 required for accessory factor activity, 5 μg of the reporter construct (gAF1→GAL4 PEPCK-LUC or gAF2→GAL4 PEPCK-LUC) were cotransfected with 2.5 μg of GR expression vector and 0.25, 0.5, or 1.0 μg of expression plasmid encoding the various GAL4-SRC1 structural variants. The total amount of DNA transfected in each experiment was kept constant by using varying amounts of the empty vector. These experiments were repeated 3–5 times, and the optimal hormonal responses from each titration experiment were pooled for the results shown in Figs. 5a and 6. The optimal concentration of GAL4-SRC1 was consistently 0.25 μg, but the optimal concentration of the structural variants varied from 0.25 to 1.0 μg. Luciferase activity was determined using the Promega luciferase assay system and normalized to the protein concentration of whole cell extracts. Transient transfections of HeLa and COS cells were performed as described previously (2, 40). The expression vector for GAL4-SRC1 was provided by Dr. Peter Kushner, University of California, San Francisco. Statistical analysis was performed using Student's t test, assuming two-tailed variance.

**Gel Mobility Shift Assay**—The gel mobility shift assays were performed as described previously, except that 7.5 μg of nuclear extracts were used for incubations rather than whole-cell extracts (43). Nuclear extracts were prepared as described (30, 46). Briefly, extracts were
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incubated in the presence or absence of GALA-DNA binding domain (GALA-DBD) antibody or Lex A antibody as a control (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at room temperature in a 10-μl reaction and then mixed with 10 μl of a reaction buffer containing 30,000 cpm of a 32P-labeled DNA probe (5'-CACACGGAGGACTGTC-CTCCGACCA-3'), 20 mM HEPES, pH 7.6, 100 mM NaCl, 5 mM MgCl2, 10 μM ZnSO4, 6% glycerol, 0.6 μg poly(dI-dC) (Amersham Pharmacia Biotech), and 2 μg of salmon sperm DNA for another 15 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel in 0.5× Tris-buffered EDTA and electrophoresed at 20 mA for 150 min at room temperature. The gels were dried and exposed to x-ray film (Eastman Kodak Co.).

RESULTS

Direct Recruitment of a Coactivator, GALA-SRC1, Can Replace the AF Function of HNF4 or COUP-TF at gAF1—Previous studies of the PEPCK gene GRU show that each gAF element is specifically required and that a precise organization of these elements in the GRU is necessary for a full glucocorticoid response (28, 37). Glucocorticoids cause a 15-fold induction of reporter enzyme activity when a PEPCK gene promoter is fused to the gAF1 element (Fig. 1 and Ref. 43). By contrast, the binding of chimeric proteins consisting of the GALA-DBD and either HNF4 or COUP-TF restores the glucocorticoid response (1, 43). It is possible that HNF4 and COUP-TF recruit coactivator molecules to form a glucocorticoid-specific enhanceosome, whereas the GALA-DBD does not. These observations suggest that even when SRC1 is constitutively recruited by a GALA binding element, active involvement of ligand-bound GR is still required, suggesting a role of the receptor in activating SRC1 in the context of the enhanceosome. We and others also show that the coactivator cAMP-response element-binding protein (CREB)-binding protein (CBP) interacts with HNF4 (2, 48). A fusion construct expressing a GALA-CBP chimeric protein also restores the lost glucocorticoid response through a gAF1→GALA PEPCK-LUC construct (Fig. 1). Thus, the requirement for the AF is bypassed when SRC1 is recruited directly to gAF1. Surprisingly, there is little increase in basal promoter-reporter activity with GALA-SRC1 expression in the absence of glucocorticoids (Fig. 1). This implies that, even when SRC1 is constitutively recruited by a GALA binding element, active involvement of ligand-bound GR is still required, suggesting a role of the receptor in activating SRC1 in the context of the enhanceosome. We and others also show that the coactivator cAMP-response element-binding protein (CREB)-binding protein (CBP) interacts with HNF4 (2, 48). A fusion construct expressing a GALA-CBP chimeric protein also restores the lost glucocorticoid response through a gAF1→GALA PEPCK-LUC construct (data not shown). Thus, one role of the AF bound at the gAF1 element appears to be recruitment of its cognate coactivators.

In a set of experiments similar to those shown in Fig. 1, we found that GALA-SRC1 can also replace COUP-TF at gAF3 in a construct where the gAF3 element was changed to a GALA binding element, gAF3→GALA PEPCK-LUC (data not shown). This result is not surprising since the gAF1 and gAF3 elements are interchangeable, and both bind COUP-TF. The amino acid region from 408–423 of COUP-TFI is necessary for this protein to function as an AF in the PEPCK gene GRU (1). This same region of COUP-TFI is also essential for the interaction with SRC1 (1). These observations suggest that GALA-SRC1 replaces some essential function of the AFs that bind to these elements in the native promoter.

Fig. 1. GALA-SRC1 mediates accessory factor activity through the gAF1 element. H4IIE cells were transfected with 5 μg of the PEPCK-LUC reporter construct (wild-type PEPCK gene promoter, from position −467 to +65 relative to the transcription start site, fused to LUC) and 2.5 μg of an expression plasmid encoding either the GALA-DBD or a construct where the gAF3 element was changed to a GALA DNA binding element (gAF1→GALA4). These cells were cotransfected with 0.5 μg of an expression plasmid encoding either the GALA-DBD or GALA-SRC1. Transfected cells were incubated in serum-free medium with or without hormone overnight. Cells were harvested, and luciferase assays were performed as described under “Experimental Procedures.” These results represent the mean ± S.E. of at least three experiments. Dex, dexamethasone.

provide for this response. The presence of large amounts of these wild-type proteins, however, makes it difficult to analyze the effects of their transiently expressed variants on PEPCK gene transcription. Mutations of the gAF1 element that prevent HNF4/COUP-TF binding, including replacement of gAF1 with a GALA binding site (gAF1→GALA4), result in a 50–60% reduction of the response of the PEPCK gene to glucocorticoids, as noted previously (Fig. 1 and Refs. 28 and 43). The GALA binding site can bind chimeric proteins that contain domains of interest and, thus, can serve as a surrogate gAF element. This is useful because the endogenous PEPCK AFs cannot function through this surrogate gAF element; thus, they are effectively removed from the analytical system.

The reduction of the glucocorticoid response caused by replacement of the gAF1 element with a GALA element cannot be restored by expression of the GALA-DBD, indicating that the GALA-DBD cannot act as an AF through this surrogate gAF1 element (Fig. 1 and Ref. 43). By contrast, the binding of chimeric proteins consisting of the GALA-DBD and either HNF4 or COUP-TF restores the glucocorticoid response (1, 43). It is possible that HNF4 and COUP-TF recruit coactivator molecules to form a glucocorticoid-specific enhanceosome, whereas the GALA-DBD does not. These observations suggest that even when SRC1 is constitutively recruited by a GALA binding element, active involvement of ligand-bound GR is still required, suggesting a role of the receptor in activating SRC1 in the context of the enhanceosome. We and others also show that the coactivator cAMP-response element-binding protein (CREB)-binding protein (CBP) interacts with HNF4 (2, 48). A fusion construct expressing a GALA-CBP chimeric protein also restores the lost glucocorticoid response through a gAF1→GALA PEPCK-LUC construct (data not shown). Thus, one role of the AF bound at the gAF1 element appears to be recruitment of its cognate coactivators.

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2 J. M. Stafford, M. Waltner-Law, and D. K. Granner, unpublished observation.
Coactivator Recruitment Does Not Relieve the Requirement for Active Involvement of Ligand-bound GR in the Glucocorticoid Response—The ligand-bound GR could serve primarily as a signal transducer for recruitment of coactivators and assembly of a glucocorticoid-specific enhanceosome. If so, ligand-bound GR would no longer be required after this enhanceosome is assembled. The low basal reporter gene activity in cells expressing GAL4-SRC1 was surprising, as the coactivator is constitutively bound to the promoter, yet the reporter gene is inactive in the absence of glucocorticoid treatment (Fig. 1). We therefore reasoned that there must be active involvement of ligand-bound GR for GAL4-SRC1 to function as a surrogate AF. In a set of experiments performed concomitantly with those described in Fig. 1, PEPCK promoter reporter gene constructs were made in which block mutations were created in either GR1 or GR2 elements (Fig. 2, inset). Since GR1 is more important than GR2 in the glucocorticoid response (36), we were able to create a graded system in which only 40% (GR2 mutated) or all of the function of GR is eliminated (GR1 and GR2 mutated), respectively. Mutation of GR2 or both GREs severely blunts or eliminates the glucocorticoid response, respectively, even when GAL4-SRC1 is tethered to the promoter through the surrogate gAF1 element (Fig. 2).

GAL4-SRC1 Can Also Replace the AF Function of the gAF3-bound Factor—The gAF1/3 and gAF2 elements are not interchangeable in the PEPCK gene GRU (36, 37). As expected, about 60% of the glucocorticoid response is lost when gAF2 is changed to a GAL4 binding element (gAF2→GAL4 PEPCK-LUC) (Fig. 3 and Ref. 43). This AF activity cannot be restored by coexpression of the GAL4-DBD but is completely restored by GAL4-HNF3b (43). Despite the functional distinction between gAF1/3 and gAF2, GAL4-SRC1 recruitment to gAF2→GAL4 PEPCK-LUC completely restores the glucocorticoid response lost by mutation of this element (Fig. 3). Thus, GAL4-SRC1 can act as a surrogate AF through gAF2 as well, and in so doing, it provides a functional bypass of the requirement for HNF3b.

HNF3b Interacts with SRC1 in Vivo—As mentioned previously, HNF4 and COUP-TF, the AFs that bind gAF1 and gAF3, both interact with SRC1. We proposed that GAL4-SRC1 could serve as a surrogate AF through gAF1 and gAF3 when these elements are changed to a GAL4 binding element, respectively, because SRC1 is recruited to the promoter at an appropriate position (this paper and Refs. 1 and 2). If SRC1 replaces a distinct function of HNF3b in the GRU, it is possible that HNF3b also interacts with SRC1. To test this, an expression vector for SRC1 was cotransfected into HeLa cells with GAL4-HNF3b or variants thereof and a luciferase reporter plasmid that contained a minimal E1b promoter positioned downstream of five GAL4 binding elements [(GAL4)5-E1b-LUC]. All GAL4 constructs were expressed at low levels (0.5 μg of vector) to allow for maximal coactivation by SRC1. Cotransfection of SRC1 potentiates transactivation mediated by GAL4-HNF3b an additional 7-fold relative to expression of an empty control vector (Fig. 4). Coexpression of SRC1 does not coactivate through the GAL4-DBD, as seen in previous work (Fig. 4 and Refs. 1 and 2).

The region of HNF3b encompassing amino acids 361–458 is required for the AF activity of this protein (43). The interaction of SRC1 with GAL4-HNF3b361–458 results in a 12-fold coactivation, whereas GAL4-HNF3b361–442, which cannot act as an AF (43), is ineffective (Fig. 4). Thus, the region of HNF3b required for AF activity in the GRU may act by recruiting SRC1, a finding consonant with the ability of GAL4-SRC1 to replace the function of HNF3 on the gAF2→GAL4 PEPCK-LUC reporter construct.

Promoter- and Element-specific Requirements for the bHLH/PAS and HAT Domains of GAL4-SRC1—It is difficult to analyze the effect of transiently expressed SRC1 variants in the presence of significant amounts of the endogenous, wild-type protein. However, the system described in the previous figures,
in which GAL4-SRC1 is recruited to the GAL4 element of the gAF3 GAL4 PEPCK and (GAL4)5-E1b promoters, respectively, is useful because variants of GAL4-SRC1 can be specifically targeted to these promoters, effectively removing endogenous SRC1 from the analytical system. This system can be used to identify the minimal functional domains of SRC1 required for the PEPCK glucocorticoid response.

SRC1 was originally identified from a cDNA that lacked the region encoding the 41-most N-terminal amino acids (40). The protein encoded by this cDNA does not function as a coactivator for HNF4, although it still presumably binds HNF4 through the nuclear receptor binding motifs, NRBI-IV, which are present in the truncated protein. By contrast, full-length SRC1 does serve as a coactivator for HNF4, and as a GAL4-chimeric protein, it can replace HNF4 as an AF on a gAF13GAL4 PEPCK-LUC construct (Fig. 1). Expression of a variant of GAL4-SRC1 that lacks the N-terminal 45 amino acids (GAL4-SRC1-DbHLH) does not replace HNF4 as an AF (Fig. 5a). This GAL4-SRC1-DbHLH protein provides no more AF activity than does the GAL4-DBD alone or a deletion of the gAF1 element (Fig. 5a and Ref. 28). Thus, the ability of GAL4-SRC1 to serve as a surrogate AF through the gAF1 element correlates with its ability to coactivate HNF4, the AF that binds this element in the wild-type promoter (30, 43).

SRC1, with its resident acetyltransferase activity and protein-protein interaction surfaces, could serve both as a structural adapter molecule in the GRU enhanceosome and also to promote transcription by the acetylation of chromatin compo-
nents and transcription factors (49–51). A deletion construct of GAL4-SRC1, in which the acetyltransferase domain was deleted (GAL4-SRC1ΔHAT), does not restore the glucocorticoid responsiveness of the reporter gene (Fig. 5a). A chimeric construct in which the five C-terminal amino acids encompassing NRBI-IV were deleted (GAL4-SRC1ΔIV) provides a glucocorticoid response comparable with that of GAL4-SRC1 (Fig. 5a). This region is important for the interaction of SRC1 with GR, although NRBI-III also contributes to this physical interaction (52).

The expression of the GAL4-SRC1 variants was confirmed in a gel mobility shift experiment in which nuclear extracts prepared from GAL4-SRC1 fusion protein-expressing cells were tested. The empty vector was used as a control to ensure that no endogenous mammalian proteins bind to the GAL4 binding element. COS cells were used for this experiment because of the very low transfection efficiency of H4IIE hepatoma cells. All of the structural variants of GAL4-SRC1 are expressed efficiently and at approximately equal levels (Fig. 5b).

The identity of these protein-DNA complexes as the GAL4-SRC1 variants was determined by preincubation of the nuclear extract with a polyclonal antibody raised against the GAL4-DBD. This preincubation with the anti-GAL4-DBD antibody prevents each complex from forming, but an unrelated antibody (anti-Lex A) is without effect (Fig. 5b). No protein-DNA complexes are seen with nuclear extracts prepared from COS cells transfected with pRC-RSV, the vector control for GAL4-SRC1 (Fig. 5b).

The gAF1 and gAF2 elements are functionally distinct in the PEPCK gene GRU (36, 37); thus, we speculated that there might be different requirements for SRC1 when it is recruited...
through these respective sites. As seen at gAF1, the acetyltransferase activity of SRC1 is also required at gAF2, since GAL4-SRC1 \( \Delta HAT \) does not mediate AF activity (Fig. 6). As at the gAF1 element, the NRBIV region of GAL4-SRC1 is also dispensable at gAF2 (Fig. 6). However, by contrast to the functional requirements of GAL4-SRC1 at gAF1, where the bHLH/PAS domain is critical to the surrogate-AF activity provided by this protein, the bHLH/PAS region is completely dispensable at gAF2 (Fig. 6). The distinct requirements of various regions of GAL4-SRC1 at gAF1, as compared with gAF2, supports the contention that the gAF1 and gAF2 elements have distinct represent the mean ± S.E. of at least three experiments. The asterisks represent statistically significant differences (\( p < 0.05 \)) from GAL4-SRC1. Dex, dexamethasone. Panel B, the expression and DNA binding of various GAL4-SRC1 fusion proteins was determined in COS cells transfected with 20 μg of expression plasmids that encode various GAL4-SRC1 fusion constructs. Nuclear extracts were prepared, and the gel mobility shift assay was used to analyze expression levels (as described under "Experimental Procedures"). The arrows indicate the various GAL4-SRC1 fusion proteins. Each protein complex was eliminated by precipitation of the extracts with a polyclonal antibody directed against the GAL4-DBD but not an unrelated antibody raised against Lex A.

**Fig. 5.** The bHLH and HAT domains of SRC1 are required at gAF1 for the glucocorticoid response. Panel A, experiments were performed as described in Fig. 1, except that equal amounts of GAL4-DBD, GAL4-SRC1, or the structural variants indicated were used. The results are represented relative to the fold induction of wild-type PEPCK-LUC by glucocorticoids, which is taken as 100%. These results represent the mean ± S.E. of at least three experiments. The asterisks represent statistically significant differences (\( p < 0.05 \)) from GAL4-SRC1.

**Fig. 6.** The HAT domain of SRC1 is required for the glucocorticoid response at gAF2, but the bHLH domain is not. Experiments were performed as described in Fig. 3, except that equal amounts of GAL4-DBD (+G4-DBD), GAL4-SRC1 (+G4-SRC1), or the structural variants indicated were used. This result demonstrates element-specific requirements of SRC1 in the PEPCK gene GRU, and it further confirms that GAL4-SRC1–bHLH is not transcriptionally inactive. Results are represented relative to the fold induction of wild-type PEPCK-LUC by glucocorticoids, which is taken as 100%. These results represent the mean ± S.E. of at least three experiments. The asterisk represents a statistically significant difference (\( p < 0.05 \)) from GAL4-SRC1.
The precise role of ligand-bound GR in mediating this response is less clear. A previous in vivo footprint analysis showed that all of the gAF elements in the GRU are occupied in vivo (25, 26). Upon exposure to the ligand, the GR may help promote the formation of a functional enhanceosome by recruiting coregulatory molecules rather than ubiquitously expressed factors. We envision that, together, the AFs provide a surface for recruitment of a coactivator complex that includes SRC1, CBP, and probably other proteins. The PEPCK gene GRU shares DNA elements with the hormone response units that provide a response to retinoic acid, cAMP, and insulin (38), but different transcription factors are recruited to the AF elements in response to these hormones (37, 54, 55). It is probable that the constellation of coregulators recruited by each of these effectors is also different, yet somehow this array of hormone response units provides a coordinated response to the metabolic challenges imposed by hyperglycemia, when the PEPCK gene is activated, and hyperglycemia, when the gene is repressed.

The ability of GAL4-SRC1 to replace the function of the AFs bound to gAF1, gAF2, or gAF3 in the GRU shows that the precise factor that binds these elements is less critical than is the ability of these proteins to assemble a functional enhanceosome. There is, however, specificity associated with these AFs in the GRU. The observation that the PEPCK gene GRU uses liver-enriched factors such as HNF4 and HNF3β to recruit coregulatory molecules rather than ubiquitously expressed factors provides insight into how tissue specificity of gene transcription in response to glucocorticoids may arise. In this regard, it is noteworthy that in adipose tissue, which does not express GR, glucocorticoids may medi-ate this response.

By contrast to the PEPCK gene GRU, where GAL4-SRC1ΔHAT does not act as an AF at either gAF1 or gAF2, this protein increases expression through the simple promoter about 100× more effectively than does the DBD (Fig. 7). The heightened activation produced by deletion of the HAT domain may reflect different protein targets of this enzyme activity in the two promoters. Deletion of NRBI1 has no effect on basal transcription mediated by GAL4-SRC1 (Fig. 7).

DISCUSSION

The accessory factors HNF4, COUP-TF, and HNF3β are required for maximal induction of PEPCK gene transcription by glucocorticoids (13, 28–32), and each interacts with the coactivator SRC1 through exactly the same regions required for their AF function (this paper and Refs. 1 and 2). This series of observations strongly suggests that recruitment of SRC1 is important for the glucocorticoid response. Recruitment of SRC1, however, does not exclude recruitment of other coactivators such as CBP to, or by, the same AFs. We envision that, together, the AFs provide a surface for recruitment of a coactivator complex that includes SRC1, CBP, and probably other proteins. The PEPCK gene GRU shares DNA elements with the hormone response units that provide a response to retinoic acid, cAMP, and insulin (38), but different transcription factors are recruited to the AF elements in response to these hormones (37, 54, 55). It is probable that the constellation of coregulators recruited by each of these effectors is also different, yet somehow this array of hormone response units provides a coordinated response to the metabolic challenges imposed by hyperglycemia, when the PEPCK gene is activated, and hyperglycemia, when the gene is repressed.

The precise role of ligand-bound GR in mediating this response is less clear. A previous in vivo footprint analysis showed that all of the gAF elements in the GRU are occupied in the absence of glucocorticoids (56). Here we show that GAL4-SRC1 is only able to function as a surrogate AF when GR is present (Figs. 1–3). Upon exposure to the ligand, the GR may help promote the formation of a functional AF by interacting with SRC1 to recruit components of the basal transcription machinery. GR may also activate SRC1 once the latter has been recruited to the GRU by AFs such as HNF4, COUP-TF, and HNF3β. The activation of coactivators by nuclear receptors is not unprecedented. The function of the peroxisome proliferator-activated receptor-γ coactivator, PGCl, is stimulated by its association with peroxisome proliferator-activated receptor-γ (57). This protein-protein interaction pro-
motes a conformational change in PGC1 that permits recruitment of additional coactivators SRC1 and CBP/p300 (57). We propose that the binding of GR to the PEPCK gene GRU promotes activation of SRC1 and assembly of a functional enhansome in a similar fashion. The interaction between GR and SRC1 appears to be facilitated by localization of GR to its DNA binding elements. When both the GR1 and GR2 elements are intact, GAL4-SRC1 is able to serve as a surrogate AF at gAF1. As the ability of GR to be recruited to the promoter is reduced or lost by introducing mutations in the GR2 or GR1 and GR2 elements, respectively, the ability of GAL4-SRC1 to serve as a surrogate AF is reduced or lost in a coordinate manner (Fig. 2).

All of the p160 family of coactivators contain three central LXXLL motifs (NRB-III) that are important for interaction with nuclear receptors (58, 59). SRC1a, used in these experiments, has an additional C-terminal LXXLL motif (NRBIV) that exhibits strong in vitro binding to class I nuclear receptors such as estrogen receptor and GR (59, 52, 59, 60). One might imagine that the NRBIV domain could help stabilize GR binding to the low affinity GR1 and GR2 elements. If so, deletion of the NRBIV region of SRC1 (GAL4-SRC1ΔIV) should reduce the effectiveness of GAL4-SRC1 as a surrogate AF. Deletion of this region is without effect, however, as GAL4-SRC1ΔIV functions comparably with the full-length chimeric protein (Figs. 5 and 6). Kalkhoven et al. show that, although NRBIV is the nuclear receptor interaction motif that interacts most strongly with estrogen receptor in vitro, deletion of this region has little functional consequence in estrogen receptor activation, providing the NRBI-III region is intact (60). This suggests that multiple LXXLL motifs facilitate the in vitro effects of SRC1 on nuclear receptors. Rather than stabilizing GR binding, the SRC1-GR interaction may be more important for the activation of SRC1. By this model, AFs such as HNF4, COUP-TF1, and HNF3β would serve to recruit SRC1, which in turn is only activated by the additional presence of ligand bound GR.

The recruitment of GAL4-SRC1 or variants of this protein to promoter constructs with GAL4 binding elements (gAF→GAL4 PEPCK and (GAL4)5-E1b promoters, respectively) allowed us to look for promoter- and DNA element-specific requirements of SRC coactivation. The bHLH region of GAL4-SRC1 is required for AF activity at the gAF1 element (Fig. 5a). GAL4-SRC1-ΔbHLH also provides a low rate of basal transcriptional activation through the simple E1b promoter (Fig. 7). The bHLH/PAS domain of SRC1 is highly conserved among the p160 family of coactivators, and the PAS motif serves as a protein-protein interaction surface for the transcription factors, Per, ARNT, and SIM (61, 62). The importance of the bHLH/PAS domain of SRC1 in the context of the PEPCK gene GRU may indicate the requirement for interaction with an additional PAS-containing factor that is necessary for SRC1 function. Interestingly, this region is not required when GAL4-SRC1 is recruited to gAF2 (Fig. 6). This result supports the idea that these gAF elements and associated factors have distinct roles in the response of the PEPCK gene to glucocorticoids.

The acetyltransferase domain of GAL4-SRC1 is required at both gAF1 and gAF2 (Figs. 5a and 6); however, the target of this acetyltransferase activity in the PEPCK gene GRU is unknown. Acetylation of chromatin components and transcription factors correlates with activation of gene transcription (49, 50, 63–65). The chromatin structure of the PEPCK gene promoter is already free of nucleosomes before exposure to hormone in H4IIE cells (66); thus, it is unlikely that this is the target of the acetyltransferase activity required of SRC1. There are several other potential targets for the acetyltransferase activity of SRC1 in the GRU. HNF4 can be acetylated by CBP, a reaction that promotes DNA binding and nuclear retention of HNF4 (64). The androgen receptor can also be acetylated by CBP (67); thus, it is possible that other nuclear receptors, such as GR, may also be acetylated. Different targets of the acetyltransferase activity of SRC1 (chromatin components versus transcription factors) may also explain why GAL4-SRC1ΔHAT strongly activates a simple promoter but is not functional at any of the PEPCK gAF elements tested (Figs. 5a, 6 and 7).

The use of the GAL4-SRC1 chimeric protein to recruit this coactivator to specific DNA elements allowed us to demonstrate both promoter-specific requirements of SRC1 (between the simple and PEPCK gene promoters) and also element-specific requirements for domains of SRC1 at gAF1 and gAF2 in the PEPCK gene GRU. A summary of these differences is illustrated in Fig. 8. HNF4/COUP-TF and HNF3β mediate distinct functions as AFs, yet they are both capable of interacting with the same coactivator, SRC1. SRC1 may serve both as a scaffolding molecule and an activator through its acetyltransferase activity. Which of these functions is required seems to be determined by the local promoter environment to which SRC1 is recruited. Furthermore, the function of SRC1 as a coactivator is not simply determined by the site of its recruitment to the GRU but also depends upon activation by GR. This role of
recruitment and activation of coactivators may have implications for other genes that employ AFs to mediate responses to metabolic signals. Indeed, the AFs required for the glucocorticoid response of the PEPC gene are involved in regulating the hormone responses of many genes that encode enzymes required for metabolic homeostasis (10–14, 68, 69). Thus, the functional interactions between HNF3 and HNF4 with SRC1, as described in the PEPC gene GRU, may have a broad role in metabolic adaptation.

Acknowledgments—We thank Dr. Peter Kushner (University of California, San Francisco) for providing the expression vector for GAL4-SRC1, Dr. Jen-Chywan Wang for helpful advice, Cathy Caldwell for technical assistance, and Deborah Caplenor Brown for helping prepare this manuscript.

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John M. Stafford, Mary Waltner-Law and Daryl K. Granner

J. Biol. Chem. 2001, 276:3811-3819. doi: 10.1074/jbc.M009389200 originally published online November 7, 2000

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