Accessory Factors Facilitate the Binding of Glucocorticoid Receptor to the Phosphoenolpyruvate Carboxykinase Gene Promoter*

John M. Stafford‡, John C. Wilkinson§, Joseph M. Beechem‡¶ and Daryl K. Granner**

From the ‡Department of Molecular Physiology and Biophysics, §Department of Biological Sciences, Vanderbilt University School of Medicine and ¶Department of Veterans Affairs Medical Center, Nashville, Tennessee 37232

Glucocorticoid induction of the phosphoenolpyruvate carboxykinase (PEPCK) gene requires a glucocorticoid response unit (GRU) comprised of two non-consensus glucocorticoid receptor (GR) binding sites, GR1 and GR2, and at least three accessory factor elements (gAF1–3). DNA-binding accessory proteins are commonly required for the regulation of genes whose products play an important role in metabolism, development, and a variety of defense responses, but little is known about why they are necessary. Quantitative, real time homogenous assays of cooperative protein-DNA interactions in complex media (e.g. nuclear extracts) have not previously been reported. Here we perform quantitative, real time equilibrium and stopped-flow fluorescence anisotropy measurements of protein-DNA interactions in nuclear extracts to demonstrate that GR binds to the GR1-GR2 elements poorly as compared with a palindromic or consensus glucocorticoid response element (GRE). Inclusion of either the gAF1 or gAF2 element with GR1-GR2, however, creates a high affinity binding environment for GR. GR can undergo multiple rounds of binding and dissociation to the palindromic GRE in less than 100 ms at nanomolar concentrations. The dissociation rate of GR is differentially slowed by the gAF1 or gAF2 elements that bind two functionally distinct accessory factors, COUP-TF/HNF4 and HNF3, respectively.

The balance between glucose production and disposal must be tightly regulated to ensure glucose homeostasis. Many genes, whose products are control points in metabolism, are regulated through multicomponent hormone response units in which DNA-binding accessory proteins modulate hormone receptor function (1–3). Similar arrangements are found in genes whose products play an important role in development and in a variety of host defense responses (1–7). Little is known, however, about how accessory factors function in these hormone response units.

Glucocorticoid induction of the PEPCK† gene, which encodes a key enzyme involved in gluconeogenesis, is accomplished through a glucocorticoid response unit (GRU) comprised of two non-consensus glucocorticoid receptor binding sites (GR1, GR2) and at least three accessory factor elements (gAF1–3) that bind HNF4/COUP-TF, HNF3β, and COUP-TF, respectively (Fig. 1a) (1, 8–10). HNF3β and HNF4 are expressed in a tissue-specific manner that correlates with the ability of glucocorticoids to induce this gene (11, 12). The GR1 and GR2 elements correspond to the consensus GR binding element at only 7 of 12 and 6 of 12 nucleotides, respectively, and bind GR with very low affinity relative to the consensus glucocorticoid response element (GRE) (13). The GR1 and GR2 elements, alone or in combination, are not able to confer glucocorticoid responsiveness to a heterologous promoter-reporter construct (13). A mutation of any one of the accessory elements results in a 50–60% reduction of glucocorticoid-stimulated PEPCK gene transcription in H4IIE rat hepatoma cells (1, 13, 14). Any combination of two mutations of gAF1/gAF3 or gAF2 abolishes the response (1, 14). This series of observations suggests that the accessory factors required for the glucocorticoid response may facilitate GR binding to the GR1 and GR2 elements.

Here we perform quantitative, real time equilibrium and stopped-flow fluorescence anisotropy measurements of protein-DNA interactions in nuclear extracts to demonstrate that the multicomponent complexes that regulate PEPCK gene transcription form and dissociate with kinetics appropriate for the rapid changes in the transcription of this gene mediated by hormones (15). Further, two functionally distinct accessory factor elements, which bind HNF3β and HNF4, differentially promote the stability of GR in the GRU complex. These results suggest that the magnitude of gene regulation in response to glucocorticoids may be achieved by modulation of GR binding by accessory factors.

**EXPERIMENTAL PROCEDURES

Synthesis, Labeling, and Annealing of Fluorescently Labeled Oligonucleotides—Oligonucleotides 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. A 5′-amino group on a 6-carbon atom linker arm (Glen Research Catalog No. 1916-02) was added to the oligonucleotides labeled with a fluorescent dye, Rhodamine X (Molecular Probes Catalog No. X-491) and 5- and 6-carboxyfluorescein (Molecular Probes Catalog No. C-1311) were attached to the linker, purified, and annealed as previously described (16). This method ensures that only complete oligonucleotides are labeled. No single-stranded fluorescently labeled oligonucleotides were detected by non-denaturing gel electrophoresis after annealing. Double-stranded oligonucleotides used for competition have the same sequence and were prepared in the same way as the corresponding fluorescently labeled oligonucleotides, but the fluorescent dye was not included in the reaction.

†The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; GRU, glucocorticoid response unit; GR, glucocorticoid receptor; GRE, glucocorticoid response element; COUP-TF, chicken ovalbumin upstream promoter transcription factor; HNF, hepatocyte nuclear factor.
Nuclear Extract Preparation—H4IIIE hepatoma cells were cultured as previously described (17). Cells were treated with 500 nM dexamethasone for 45 min and collected, and nuclear extracts were prepared as described previously (8, 18), aliquoted, and stored at −70 °C. Aliquots were only used one time to avoid the effects of freeze-thawing on binding. These nuclear extracts have abundant amounts of GR and the necessary accessory factors.

Steady State Anisotropy Measurements—A SPEX (Edison, NJ) 1681 fluorolog spectrophotometer was used to make fluorescence anisotropy measurements of the GRE-containing oligonucleotides in the presence or absence of GR from the nuclear extracts (for more background on fluorescence anisotropy see Ref. 19). Anisotropy was calculated as previously described (20). The assay is homogenous, without distinct aqueous and solid-support phases, as in filter binding and electrophoretic mobility shift assays. No separation of free and complex-bound DNA is required; thus the equilibrium conditions necessary to study cooperative binding are easily maintained (21, 22). A 10 nM concentration of rhodamine-labeled oligonucleotide provides a strong fluorescent signal. Samples were excited with vertically polarized light (Oriel dichroic sheet polarizers) with a wavelength of 585 nm. Emission at 620 nm was detected through two photo detectors, assembled in T format, which discriminate axial and planar excitation (rhodamine X and fluorescein, respectively). Using this technique, 95% confidence intervals of individual measurements are typically less than 0.0001 anisotropy unit; thus even small changes in anisotropy can be measured.

The binding buffer used contained 20 mM Tris, pH 7.6, 100 mM NaCl, 250 μM ZnSO4, 6% glycerol, 6.25 mM EDTA, 10 mg/ml bovine serum albumin, and 500 nM dexamethasone, and was prepared fresh for each experiment. The concentrations of zinc and dexamethasone used provide for optimal binding of GR (24). There is no difference in the binding capacity of GR if the extract is preincubated at 25 °C for 30 min or kept on ice for the duration of the experiment, presumably because of the presence of dexamethasone in both cell culture and binding buffer (25). Nonspecific binding was determined by two complementary methods; first, by competition with unlabeled GRE and secondly by binding of nuclear extract to a labeled oligonucleotide with point mutations in the GRE and without necessary flanking sequences for GR binding (26, 27).

Nonspecific binding was determined by subtracting nonspecific binding from total binding. Only specific binding is shown in the remaining steady-state experiments. 16 kinetic measurements, or shots, were only used one time to avoid the effects of freeze-thawing on binding. These nuclear extracts have abundant amounts of GR and the necessary accessory factors.

**RESULTS AND DISCUSSION**

**High Affinity Binding of GR from Nuclear Extracts as Measured by Changes in Fluorescence Anisotropy**—The dynamics of assembly of the GRU were studied using fluorescence anisotropy...
 Compete for this binding as well as the palindromic GRE (data not shown); thus GR from the nuclear extract closely recapitulates the activity of the purified protein. The observed binding of GR from the nuclear extract is not restricted to the palindromic GRE. A consensus GRE with flanking sequences that maximize affinity for GR (26, 32) binds GR with a $K_d$ of 5 nM. Binding of purified GR to this palindromic GRE has a $K_d$ of 7.5 nM (data not shown); thus GR from the nuclear extract closely recapitulates the activity of the purified protein. The observed binding of GR from the nuclear extract is not restricted to the palindromic GRE. A consensus GRE with flanking sequences that maximize affinity for GR (26, 32) binds GR with a $K_d$ of 1.6 nM GR (Fig. 1c, bars 5 and 6, and Fig. 2a, filled circles). Preincubation of the nuclear extract with an antibody against the DNA binding domain of GR significantly reduces the ability of GR to associate with the consensus GRE, whereas an antibody raised against the mineralocorticoid receptor does not inhibit binding (Fig. 1c, bars 8 and 9). The observed binding of GR to the palindromic and consensus GRE elements correlates with the measured affinity of GR for these sites (32, 33).

Accessory Factor Elements Restore Affinity of GR for Non-consensus PEPCGR GR1 and GR2 Elements—GR binds with very low affinity to the PEPCGR GR1 and GR2 elements (13). To test whether the accessory factors enhance binding of GR for the low affinity GR1 and GR2 elements, we constructed a double-stranded rhodamine X-labeled oligonucleotide that spans the gAF2, GR1, and GR2 elements from the PEPCGR gene promoter (Fig. 1a). The presence of the gAF2 element restores the affinity of the GR-accessory factor complex binding to this promoter segment to a level comparable with that exhibited by GR for the consensus GRE (compare the filled circles in Fig. 2, a and b). This binding is completely dependent on association of the requirements for GR binding to a simple response element in vitro have been well characterized (26, 31). We used this knowledge to demonstrate that GR in a nuclear extract can specifically bind to a palindromic GRE contained within a double-stranded oligonucleotide 29 base pairs in length (29-mer) labeled at the 5′ end with the fluorescent dye rhodamine X (filled circles, Fig. 1b). Addition of GR-containing nuclear extract shows the expected saturation (open triangles, Fig. 1b), and nonspecific binding increases linearly (dashed line, Fig. 1b).

Competition with an unlabeled palindromic GRE eliminates nearly all specific binding (Fig. 1, b and c, bar 3). Addition of a nonspecific oligonucleotide that does not bind GR has little effect (Fig. 1c, bar 4). A competitor containing a consensus GRE competes for this binding as well as the palindromic GRE (data not shown); thus competition is dependent on affinity of the competitor for GR and is specific. The binding seen in Fig. 1b can be recapitulated using partially purified GR (data not shown). We quantified the GR content in the nuclear extract preparations using this partially purified GR as a standard. There are $\sim0.16$ pmol of GR/µg of nuclear extract, providing an approximate $K_d$ of binding to the palindromic GRE of 5 nM. Binding of purified GR to this palindromic GRE has a $K_d$ of 7.5 nM (data not shown); thus GR from the nuclear extract closely recapitulates the activity of the purified protein. The observed binding of GR from the nuclear extract is not restricted to the palindromic GRE. A consensus GRE with flanking sequences that maximize affinity for GR (26, 32) binds GR with a $K_d$ of 1.6 nM GR (Fig. 1c, bars 5 and 6, and Fig. 2a, filled circles). Preincubation of the nuclear extract with an antibody against the DNA binding domain of GR significantly reduces the ability of GR to associate with the consensus GRE, whereas an antibody raised against the mineralocorticoid receptor does not inhibit binding (Fig. 1c, bars 8 and 9). The observed binding of GR to the palindromic and consensus GRE elements correlates with the measured affinity of GR for these sites (32, 33).

Accessory Factor Elements Restore Affinity of GR for Non-consensus PEPCGR GR1 and GR2 Elements—GR binds with very low affinity to the PEPCGR GR1 and GR2 elements (13). To test whether the accessory factors enhance binding of GR for the low affinity GR1 and GR2 elements, we constructed a double-stranded rhodamine X-labeled oligonucleotide that spans the gAF2, GR1, and GR2 elements from the PEPCGR gene promoter (Fig. 1a). The presence of the gAF2 element restores the affinity of the GR-accessory factor complex binding to this promoter segment to a level comparable with that exhibited by GR for the consensus GRE (compare the filled circles in Fig. 2, a and b). This binding is completely dependent on association of
the accessory factor with the gAF2 element, as a block mutation in gAF2, which prevents accessory factor binding and reduces the glucocorticoid effect (10), leaves only GR1 level affinity (compare open triangles in Fig. 2, a and b).

The gAF2-GR1-GR2 construct (a 76-mer) has a higher free anisotropy value than the palindromic GRE (a 29-mer); thus we predict that binding of a large protein complex would be needed to produce the observed anisotropy changes seen in Fig. 2b (19). If the association of accessory factors with the gAF2 element does indeed restore affinity of GR for this complex, the GR should represent most of the bound mass on the gAF2-GR1-GR2 promoter segment. This prediction is based on the presumption that the complex is comprised of HNF3 (40 kDa) and four monomers of GR (each 90 kDa), as shown schematically in Fig. 1a. Preincubation of the nuclear extract with the anti-GR antibody prevents ~70% of the mass assembled to this complex from forming, based on the anisotropy changes seen (Fig. 2d, bar 3). Thus, the complex formed in vitro recapitulates the complex that is required for functional induction of PEPCK gene transcription. The anti-GR antibody reduces the effective concentration of GR able to participate in this complex. The remaining mass on this promoter segment may indicate that this antibody is not 100% effective but likely represents the remaining mass of the gAF2-bound accessory factor, which binds to its response element with high affinity in the absence of GR (data not shown).

HNF3 interacts with GR in vitro (14). Furthermore, the helical spacing between the gAF2 element and the GR1 element is critical for the glucocorticoid response (14), suggesting that a physical interaction between HNF3 and GR may be responsible for the facilitated binding seen here. In support of this hypothesis, we show that although GR represents the majority of the protein mass assembled to this complex (Fig. 2d, bar 3), there is significantly less competitor-induced dissociation of GR from this complex than from the palindromic GRE (compare Fig. 2d, bars 2 and 4, with Fig. 1c, bars 2 and 3), indicating that this complex is quite stable with regard to GR binding. Whereas the gAF2 element can bind both HNF3 and C/EBP in vitro (10), the facilitated binding of GR seen here requires HNF3, as the TTR HNF3 element can substitute for gAF2, whereas a gAF2 element with point mutations that only permit C/EBP binding, cannot (data not shown). This correlates precisely with the ability of these proteins to function as accessory factors in vivo (10).

**Fig. 2.** Accessory factor elements restore affinity of GR for non-consensus PEPCK GR1 and GR2 elements as determined by steady-state anisotropy. a, specific binding to a consensus GRE (filled circles) is compared with the PEPCK GR1 element (open triangles). Kd is expressed in units of μg of nuclear extract. This corresponds to an approximate Kd of 1.6 nM GR for the consensus GRE and 48 nM GR for the PEPCK GR1 element or a 30× affinity difference. b, specific binding to a 76-base pair, rhodamine-labeled segment of the PEPCK gene GRU containing the gAF2 accessory factor element and the GR1 and GR2 GR-binding elements (filled circles). A corresponding segment with a block mutation that prevents accessory factor binding to gAF2 does not promote complex formation (open triangles). c, a 76-base pair segment of the GRU spanning GR1-GR2 and gAF1 also forms a complex with high affinity (filled circles). A block mutation in gAF1 that prevents accessory factor binding markedly reduces complex formation (open triangles). d, the gAF2-GR1-GR2 complex is more stable with regard to GR binding than GR1-GR2-gAF1. Most of the mass associated with the gAF2-GR1-GR2 fragment can be prevented by preincubating the nuclear extracts with an anti-GR antibody (bar 3); however, competition with a 50× excess consensus GRE can eliminate only approximately one-third of specific binding (bar 4). By contrast, competition of binding of GR from GR1-GR2-gAF1 is efficient (bar 8), indicating that these complexes have a different capacity to retain bound GR. Preincubation of nuclear extract with an anti-GR antibody prevents most of this complex from forming (bar 7).
A complete set of accessory factors is required for induction of PEPCK gene transcription by glucocorticoids (1, 13, 14). By contrast, when the GR1 element is replaced with a palindromic GRE, the gAF2 element is still required for the glucocorticoid response, but the gAF1 and gAF3 elements are not (13). This observation suggests that two functionally distinct classes of accessory factors are required for the PEPCK gene glucocorticoid response. The gAF1 element binds both HNF4 and COUP-TF, whereas the gAF3 element binds only COUP-TF (Fig. 1a) (8, 9). There is no functional consequence of replacing gAF3 with gAF1 and vice versa (14). We took advantage of this observation to create a GRU segment that contains the GR1 and GR2 elements but where the associated accessory factor element is gAF1 (Fig. 1a). This allowed us to generate an oligonucleotide of the same length and free anisotropy value of gAF2-GR1-GR2. As with the gAF2 element, the association of gAF1 also restores affinity of the GR-accessory factor complex to the PEPCK gene promoter to a level comparable with the binding of GR to the consensus GRE (compare filled circles in Fig. 2, c and a). Again, GR contributes the majority of the mass in this protein-DNA complex (Fig. 2d, bar 7). Whereas GR binding to the GRU is facilitated by either class of accessory factor element, the GR1-GR2-gAF1 complex is less stable with regard to GR binding than is gAF2-GR1-GR2, as competition with an unlabeled consensus GRE oligonucleotide effectively eliminates GR binding from the former (compare Fig. 2d, bars...
4 and 8). A block mutation that prevents HNF4 and COUP-TF binding is much less effective in recruiting GR to GR1-GR2 (open triangles, Fig. 2c, and Ref. 8). Although it is not known if HNF4 or COUP-TF can interact with GR, the set of experiments shown in Fig. 2 suggests that the factors bound to the gAF1 element facilitate binding of GR by a mechanism different from that employed by HNF3 at gAF2.

Dissociation of GR from the PEPPCK Gene GRU Is Modulated by Accessory Factors—The increases in anisotropy seen by binding of GR to the consensus GRE and PEPPCK gene GRU segments occur too rapidly to be measured by steady-state techniques (Figs. 1 and 2). Pre-steady-state stopped-flow analysis provides the additional advantages of rapid mixing and detection to these experiments, so that complex assembly and disassembly can be observed in real time. For the palindromic GRE, binding is effectively complete in less than 100 ms (Fig. 3a). A slower second phase of association is observed but is of undetermined significance (Fig. 3a). Competition with an unlabeled palindromic GRE gives a rapid decrease in anisotropy, again with most dissociation complete in less than 100 ms (Fig. 3b). The association-dissociation rates measured here represent multiple rounds of binding and dissociation of GR to the palindromic GRE before equilibrium is reached (34).

The experiments shown in Fig. 2 demonstrate that association of the PEPCCK accessory factor elements facilitates GR binding to the GRU. Using fluorescein-labeled oligonucleotides that span GR1–GR2, and including either gAF2 or gAF1 (Fig. 1a), we sought to determine the association rates of the protein-DNA complexes. The observed formation of an enhanceosome-like structure by either gAF2 or gAF1 occurs rapidly (Fig. 3, c and e). Both GRU segments form complexes with kinetics of binding similar to that observed when GR binds to the palindromic GRE (Fig. 3a).

Whereas all of the complexes formed equally rapidly within the detection limits of this system, dissociation of GR is dramatically slowed by the presence of the adjacent accessory factor elements. Indeed, there is substantially less dissociation of GR from the complex bound to gAF2-GR1-GR2 (Fig. 3d). These results are qualitatively similar to the steady-state composition of this complex, where there is very little GR dissociation (Fig. 2d, bar 4), despite GR contributing ~70% of the mass of this complex (Fig. 2d, bar 3). The physical association of GR with HNF3 may account for the stability of this complex (14). By contrast, GR readily dissociates from the GR1-GR2-gAF1 complex (Fig. 2d, bar 8), albeit about 10 times more slowly than dissociation of GR from the palindromic GRE (compare b and f in Fig. 3).

Binding to an oligonucleotide containing only the gAF2 element is also complete within 100 ms (data not shown); thus it may be that prior association of the accessory factors creates a high affinity binding environment to the GR1-GR2 elements that, as a complex, functions as well as the palindromic GRE. Indeed, in this study we show that high affinity binding of GR to the PEPPCK gene promoter is strictly dependent on associated accessory factor elements. It was not possible to test the individual roles of HNF3, HNF4, and COUP-TF as accessory factors in these biophysical experiments (as described for GR in Fig. 2) because antibodies that prevent DNA binding for these factors are not currently available. However, in support of this model, we have shown by the chromatin immunoprecipitation assay that the accessory factors described here are constitutively bound to the PEPPCK gene promoter in vivo, creating a high affinity binding environment for GR, which only binds to the promoter in the presence of glucocorticoids (35). Addition-ally, we show that GR binding to a GRE is intrinsically rapid but can be altered by association with accessory factors.

We speculate that even small changes in the dynamics of the structures involved in gene regulation could have a large impact on the physiological end points of the processes controlled by these gene products, such as glucose production. Simultaneous changes in the regulation of multiple genes whose products are involved in the same pathway have a large combined impact on glucose production (36). For instance, overexpression of an HNF3 variant that reduces the responses of the PEPCCK and glucose-6-phosphatase genes to glucocorticoids by ~50% nearly abolishes glucocorticoid-induced glucosuria in hepatoma cells. Although GR binding to a GRE is dynamic (Fig. 3 and Ref. 37), and such binding is stabilized by accessory factors, these complexes still form and dissociate within a few seconds (Fig. 3). We propose that dynamic structures such as the PEPCCK gene GRU provide for the adaptable regulation of metabolic genes that is seen in vivo.

The maintenance of glucose homeostasis in the face of the gradient of nutrient availability imposed by the extremes of the fasting and fed states is created by changes in the rates of glucose production and utilization. These adaptive responses are achieved by the products of a concert of genes that require accessory factors for hormonal regulation of their expression. Complex hormone response units, in which receptor binding and dissociation are modulated by accessory factors, provide variable rates of gene transcription and could be a mechanism by which a gradient of glucose production is generated. Gradi-

tents of gene products necessary for processes such as development, differentiation, and host defense responses may also be achieved by this mechanism.

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