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Sequence-specific DNA binding by glucocorticoid receptor "zinc finger peptides"

(zinc finger/glucocorticoid response element/estrogen response element)

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ABSTRACT **Steroid hormone receptors can activate or repress transcription from responsive loci by binding to DNA. We have examined the mechanism of DNA binding by individually synthesizing the putative "zinc finger peptides" from the rat glucocorticoid receptor. Atomic absorption studies show that the peptides will bind zinc on an equimolar basis, and circular dichroism experiments demonstrate a significant alteration in secondary structure in the presence of zinc. The results from a series of experiments establish that metal ion is required for binding to DNA and that the amino-terminal zinc finger shows a significantly greater affinity for glucocorticoid response element-containing DNA over control DNA. These observations indicate that a single synthetic "zinc finger peptide" is able to bind to DNA in a sequence-specific manner.**

The ability of steroids to regulate transcription from specific genes has been intensively studied (1–3). In the case of glucocorticoids, the hormone binds to a cytosolic non-DNA-binding form of its specific receptor, which then translocates into the nucleus. This "activated" receptor is now able to bind to specific sites on chromatin and either enhance or repress transcription (1). With the isolation and sequencing of cDNA clones for the various steroid receptors, it has become apparent that they form a superfamily of transcription factors (4–7). The receptors share a common organizational plan, with the most conserved region of the molecules being a cysteine-rich DNA binding domain (8–10), designated region C.

The experiments of several investigators have demonstrated that this conserved portion of the molecule is absolutely required for binding to DNA (11–13). Recent studies have demonstrated that this domain of the receptor, expressed in bacteria independent of flanking amino and carboxyl sequences, is sufficient to bind specifically to DNA (14). Analysis of the predicted protein sequence of region C revealed an intriguing homology with the DNA-binding transcription factor III A from *Xenopus laevis* (TFIIIA) (15). These findings led to the proposal that the receptor folds to form two subregions, CI and CII, that are analogous to the "zinc fingers" of TFIIIA and that interact with the DNA in a manner that is similar to the well-established binding of TFIIIA (16).

Cloning and sequencing of the human estrogen receptor gene revealed that the DNA binding domain is encoded by two exons, one for each finger, raising the possibility that the fingers could be functionally distinct (17). Support for this idea has come from experiments that demonstrated that the amino-terminal finger, CI, can be experimentally exchanged between the estrogen receptor and glucocorticoid receptor, with corresponding changes in the binding specificity of the chimeric receptors (18). More recent experiments have demonstrated that changing the specificity of a steroid receptor may be accomplished by more subtle alterations (19–21). The alteration of two or three amino acids in the CI finger of either a glucocorticoid receptor or an estrogen receptor is sufficient to change the DNA sequence recognized by the mutant receptor (19). An implication from these experiments is that the carboxyl terminal finger, CII, is not involved in determining the specificity of the receptor DNA interaction but is necessary for tight binding to the cognate site.

We have examined this question by chemically synthesizing as individual peptides the CI and CII "zinc fingers" from the glucocorticoid receptor. The peptides have been used in DNA binding studies with glucocorticoid response elements (GREs) and nonrelated response elements. The results of these experiments establish that individual finger peptides are capable of sequence-specific DNA binding in the presence of zinc. In addition we have synthesized a mutant CI peptide, CI_M, in which the conserved Cys-440 is mutated to an alanine. This peptide will chelate zinc but is unable to bind DNA, suggesting that the precise coordination of the metal is important for DNA binding.

EXPERIMENTAL PROCEDURES

Synthesis of Peptides and Oligonucleotides. All peptides were prepared by solid-phase synthesis on an Applied Biosystems 430A automated peptide synthesizer (22). Completed peptides were released from the resin and deprotected by using hydrogen fluoride cleavage methods. The crude peptides were purified by gel filtration (Sephadex G-25, 10% HOAc) and or by semipreparative reverse-phase liquid chromatography (H_2O/CF_3COOH to acetonitrile/CF_3COOH gradient). Identity of the synthetic peptides were confirmed by amino acid analysis following hydrolysis with HCl (24 hr at 110°C under reduced pressure). The number of cysteine residues present in the peptides was confirmed by reaction with 5-5′-dithiobis(2-nitrobenzoic acid) [DTNB; Ellman's reagent].

Synthesis of Oligonucleotides. All oligonucleotides were prepared on an Applied Biosystems 380A DNA synthesizer and purified according to the manufacturer's instructions. Oligonucleotides were labeled with [γ-^{32}P]ATP (New England Nuclear; 6000 Ci/mmol; 1 Ci = 37 GBq) by using T4 polynucleotide kinase as described (23). Oligonucleotides were labeled to a specific activity of 1–4 × 10^4 cpm/fmol.

Atomic Absorption Spectrophotometry. Zinc bound by the peptides was measured by atomic absorption spectrophotometry with a Perkin–Elmer atomic absorption spectropho-

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Abbreviations: GRE, glucocorticoid response element; ERE, estrogen response element; NFI, nuclear factor 1; MMTV, mouse mammary tumor virus.

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tometer model 4000. Peptides (0.015 mM) were dissolved in 50 mM Tris buffer (pH 7.8) containing 5 mM $ZnCl_2$ in a final volume of 20 ml, allowed to come to equilibrium, and then dialyzed 48 hr against 8 liters of zinc-free buffer with or without 10 mM EDTA (24).

Circular Dichroism Spectroscopy. Spectra were recorded from 260 to 190 nm on a Jasco J500 spectropolarimeter, in a 0.1-mm-path-length circular cuvette at room temperature. Peptides were reconstituted to 0.3 mM in 50 mM Tris buffer (pH 7.8) with or without 3 mM $ZnCl_2$ or 10 mM EDTA.

Nitrocellulose Filter Binding Assay. Synthetic peptides (100 pmol) were incubated at room temperature with 10 fmol of ^{32}P-labeled GRE oligonucleotide in 10 mM Tris, pH 8.0/100 mM NaCl/10 mM $MgCl_2$/50 μg of poly(dI·dC) per ml/1 mg of bovine serum albumin per ml with or without 5 mM $ZnCl_2$ in a final volume of 20 μl. Binding was allowed to proceed for 20 min at room temperature. The reaction mixture was then filtered through a nitrocellulose membrane and washed twice with binding buffer lacking $ZnCl_2$, and the radioactivity retained on the filter was assayed. In experiments using multiple oligonucleotides, the cpm retained were normalized relative to the specific activity of the GRE oligonucleotide prior to analysis.

Gel Retardation Competition Assay. The DNA-binding domain of the rat glucocorticoid receptor (T7 440-525) purified from bacteria, synthetic peptides, and GRE oligonucleotide were incubated as described for the nitrocellulose binding assay except that the amount of oligonucleotide was reduced to 2.5 fmol per reaction. At the end of the incubation the reactions were then analyzed on nondenaturing polyacrylamide gels (40:1 acrylamide/methylenebisacrylamide) in a buffer containing (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). Gels were then dried and subjected to autoradiography. For these experiments, 50 ng of receptor double-finger protein (2.5 μg/ml) was incubated with various quantities of peptides (0–50 μg/ml) as indicated in the legend to Fig. 8.

RESULTS AND DISCUSSION

We have begun to examine the role of the individual zinc finger domains of the glucocorticoid receptor by chemically synthesizing the CI and CII fingers as individual peptides. The sequence of the peptides are shown in Fig. 1 and correspond to amino acids 438–464 (CI) and 474–504 (CII) of the rat glucocorticoid receptor (14). The first series of experiments were designed to ascertain if an isolated "finger peptide" could bind zinc and to determine what, if any, effect this would have on the conformation of the peptide. The

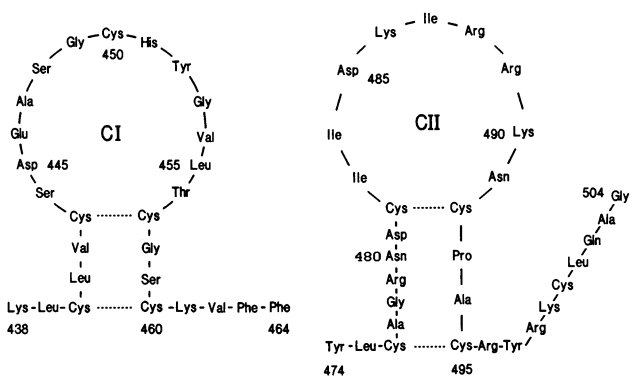

FIG. 1. Sequences of synthetic peptides CI and CII. The amino acid sequences of the amino-terminal CI finger and carboxyl-terminal CII finger of the rat glucocorticoid receptor correspond to amino acids 438–464 and 474–504, respectively (14). The sequence of CI_M, a mutated CI finger, is identical to the wild type except that Cys-440 was replaced by alanine.

Table 1. Zinc binding by synthetic "finger peptides"

Peptide	Zinc bound, mol per mol of peptide	
	Without EDTA	With EDTA (10 mM)
CI	1.18	0.11
CI_M	0.96	0.11
CII	1.04	0.05

The data are representative of several determinations with an accuracy of ±0.1 mol.

binding of zinc to the synthetic finger was assessed by mixing the peptide with a 10-fold excess of metal ion followed by extensive dialysis. The quantity of bound zinc was ascertained by atomic absorption, and the results of this analysis indicated that ≈1 mol of zinc was bound by each mole of peptide (Table 1). Subsequent dialysis of the peptide–zinc complex against a buffer containing the metal chelator EDTA demonstrated that >90% of the metal associated with the peptide could be removed by the EDTA. This result is consistent with the suggestion that four cysteines are bonded to a single metal ion (14). Previous studies have not distinguished between the possibilities that metal ion may be shared between the two proposed finger protein motifs rather than a single ion per finger protein. The data presented here show that the proposed zinc fingers can act as independent units in the chelation of zinc (one zinc ion per finger), but it does not unequivocally establish this binding mode for the native protein.

Subsequent studies have used circular dichroism (CD) spectroscopy to monitor changes in secondary structure of the peptides that may accompany metal binding. Our initial structural studies have focused on the CII peptide, for which we have been able to obtain a set of preliminary spectra. (The extremely hydrophobic nature of peptide CI has prevented the execution of similar studies on that peptide.) A comparison of the CD spectra obtained in the absence and presence of exogenous zinc indicates that the peptide–zinc complex (Fig. 2*B*) adopts an altered secondary structure relative to the apopeptide (Fig. 2*A*). The spectrum of the peptide–zinc complex, when compared to free peptide or apopeptide, shows an increase in molar ellipticity at 190 nm and a corresponding decrease at 222 nm. This metal-dependent change in conformation is reversed upon the addition of the chelating agent EDTA, producing a spectrum that is identical to that seen with the apopeptide alone (Fig. 2*C*). The spectra presented here are similar to those obtained recently for a biologically produced finger peptide from transcription factor

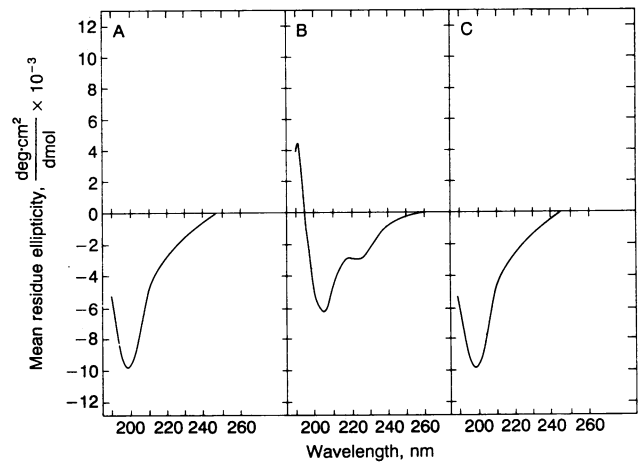

FIG. 2. Circular dichroism spectra of CII finger peptide. Peptides were reconstituted at 0.3 mM in 50 mM Tris buffer (pH 7.8) (*A*), 50 mM Tris/3 mM $ZnCl_2$ (*B*), and 50 mM Tris/3 mM $ZnCl_2$/10 mM EDTA (*C*).

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GRE   5'-GATCCAAAGTCAGAACACAGTGTTCTGATCAAAGA-3'
        3'-GTTTCAGTCTTGTGTCACAAGACTAGTTTCTCTAG-5'
                  **        **
ERE   5'-GATCCAAAGTCAGGTCACAGTGACCTGATCAAAGA-3'
        3'-GTTTCAGTCCAGTGTCACTGGACTAGTTTCTCTAG-5'

NF1   5'-GATCCGTCTTTTGGAATTTATCCAAATCTTATGT-3'
      3'-CTAGGCAGAAAACCTTAAATAGGTTTAGAATACATCTAGTCC-5'
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FIG. 3. Sequences of synthetic DNA-binding sites. Sequences of oligonucleotides used in DNA binding experiments. The asterisks indicate base differences between the GRE and ERE sequences (27–30). The DNase 1 footprint for NF1 is indicated by the solid line (37).

IIIA of *Xenopus laevis* and a synthetic finger peptide derived from the yeast transcription activator ADR1 of the alcohol dehydrogenase gene (25, 26). In all cases the spectra suggest that the peptide moves from a relatively random structure to a more ordered state when metal is bound. The data obtained here are consistent with an increase in the α-helical content of the peptide upon zinc binding, as reported for the ADR1 peptide (26).

Given that the peptides were able to bind zinc and that the metal ion altered the conformation of the peptide, experiments were performed to examine whether the individual fragments of the DNA-binding domain of the glucocorticoid receptor were sufficient to bind selectively to DNA. Experiments using the nitrocellulose filter binding assay have addressed a series of questions. (*i*) Are the peptides able to bind DNA? (*ii*) Is DNA-binding metal (zinc) dependent? (*iii*) Is the DNA-binding specific for a GRE? In the case of peptide CI, the data show that in the absence of exogenous zinc, the peptide will bind a synthetic DNA oligonucleotide containing a consensus GRE (Fig. 3) at levels at or only slightly above background (Fig. 4). The addition of zinc leads to a significant increase in the quantity of DNA bound with the peptide as monitored by this assay. The metal-dependent binding in this experiment shows a >30-fold increase in binding relative to that seen in the absence of zinc. DNA binding studies with peptide CII yield results that were similar to that seen with peptide CI (Fig. 4). When the concentration of peptide is reduced by a factor of >20, binding in the absence of metal is reduced to background levels. The addition of zinc leads to an ≈10-fold increase in binding of the peptide to DNA relative to that in the absence of the metal. These experiments show that both the CI and CII fingers are able to bind to the GRE as independent units only in the presence of zinc. In other experiments we have established that cadmium is able to substitute for zinc with the peptides (unpublished experiments) as it does for the intact DNA binding domain of the receptor (14).

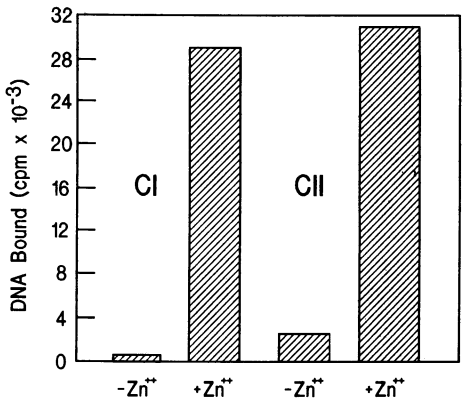

FIG. 4. DNA binding by synthetic finger peptides to GRE oligonucleotide. Peptides were incubated with labeled oligonucleotides and processed as described. The results illustrated here are for peptides CI and CII without (−) and with (+) $ZnCl_2$.

To address the question of the specificity of the binding by the peptide, we have carried out similar studies with DNA-binding sites for nuclear factor 1 (NF1) and the estrogen receptor (27–30). The results showed that peptide CI binds poorly to both sites for NF1 and the estrogen response element (ERE) in both the presence and absence of zinc (Fig. 5*A*). When the level of binding by the peptide for the NF1 and ERE oligonucleotides are compared to that of the GRE they represent <10% of the metal-dependent binding of the peptide for its cognate binding site (Fig. 5*B*). These data are of particular significance because the GRE and ERE sequences used in the experiment differ by only four nucleotides, two changes per half site (Fig. 3). Despite this subtle difference, the peptide shows a >10-fold difference in binding for the former over the latter in the same assay. Thus, these data are consistent with previous experiments that show that the amino-terminal finger provides the specificity for the selection of receptor binding sites in chimeric receptors (18, 31).

In contrast to the above results with peptide CI, the data with peptide CII show a significant metal-dependent binding to an ERE site that is comparable to that seen with the GRE oligonucleotide (Fig. 5*A*). A comparison of binding of the GRE and ERE for this CII finger peptide reveals that the peptide will bind to either site with nearly equal affinity (Fig. 5*B*). Thus, the finding that the CII finger did not significantly distinguish between an ERE and a GRE by full-length chimeric receptors was maintained with the isolated peptides (18, 19, 21, 31). In addition, peptide CII, as with peptide CI, did not show a significant level of binding with respect to the unrelated NF1 oligonucleotide (Fig. 5), a property shared

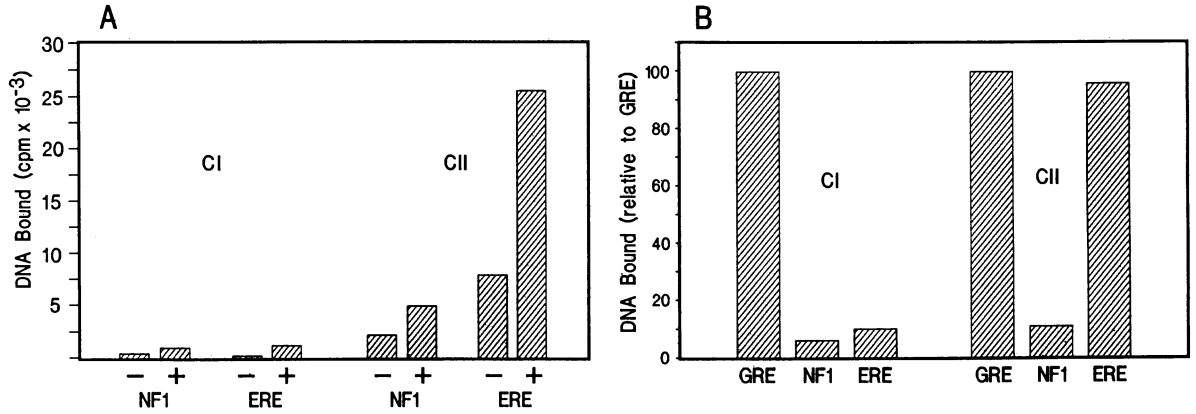

FIG. 5. Specificity of DNA binding by synthetic finger peptides. (*A*) Binding reactions were as described in Fig. 4 except that oligonucleotides NF1 and ERE were used. The presence of $ZnCl_2$ in the reactions is indicated by + and − for fingers CI and CII. (*B*) Binding of the synthetic peptides CI and CII to the GRE, NF1, and ERE oligonucleotides is expressed as a percentage of the binding to the GRE.

with the native receptor. In this case the individual finger behaves as might be predicted from "finger swap" experiments. If the CI finger could be exchanged to alter the specificity of the resulting chimera, then the implicit result is that the CII finger to which the new CI finger is joined would be able to interact with both sequences (18, 19, 21, 31).

In the native receptor molecule, mutation at a single conserved cysteine residue is sufficient to eliminate DNA binding (2, 3, 8–10). It is not clear if this loss of function results from an inability to chelate metal ion or to chelate it in a "correct" spatial array or from a more extensive alteration in the structure of the molecule. To examine this question, a mutated CI finger peptide (CI_M) was synthesized in which the Cys-440 of the glucocorticoid receptor is replaced by alanine and all other amino acid residues are identical to the wild-type peptide (Fig. 1 *Left*). Atomic absorption studies showed that the CI_M peptide was able to bind zinc at levels roughly equivalent to those of the wild-type CI and CII peptides (Table 1). DNA binding experiments with the CI_M and CI peptides revealed that substitution of cysteine by alanine resulted in a drastic reduction in binding of a GRE oligonucleotide (Fig. 6). The levels of binding by this mutated finger peptide never exceeded 10% of the wild-type finger peptide. Thus, alterations in the isolated finger behave like mutations in the intact receptor in that mutation of a single cysteine abolishes DNA binding (32–35). In addition, the data may be interpreted to suggest that binding zinc *per se* is necessary but not sufficient to allow binding to DNA. This is important, as there are five cysteine residues present in the finger with several chelation schemes possible.

The results presented here differ in at least one significant facet from reports on mutations introduced into cloned receptors (2, 3, 32, 33). Numerous elegant experiments have been performed on a variety of steroid receptors whereby the single mutation of any of the conserved cysteine residues in either CI or CII fingers leads to the loss of DNA binding by the mutant receptor (4–10, 32, 33). These results have been interpreted to suggest that the DNA-binding domain consists of both putative fingers and that the entire structure (both fingers) is required for this function. The data presented here, establish that individual peptide fingers are capable of binding DNA in a sequence-specific manner. These differences may represent the inherent difficulties in comparing a small peptide, with relatively few conformational constraints, to a relatively large protein that must undergo an "activation" process to bind DNA. Alternatively, the divergence of the data may reflect differences in the choice of assay used to monitor DNA-binding activity. In this regard it is interesting to note that experiments using mutant receptors containing only one finger but otherwise complete fail to detect DNA binding in a gel retardation assay (31). However, the same molecules are able to bind DNA when this activity is monitored by an electron microscopy assay (34). This may indicate that, although a single finger is able to bind DNA, two fingers are required to form a complex with sufficient stability for detection in the gel retardation assay.

We have tested this hypothesis directly by comparing the synthetic finger peptides with the entire DNA binding domain of the rat glucocorticoid receptor (expressed in bacteria) by the nitrocellulose filter binding assay (36). The double-finger protein fragment is able to bind to the consensus GRE but does not bind an unrelated NF1 oligonucleotide (Fig. 7). A very interesting result is obtained when the double finger is incubated with a consensus ERE. In the filter binding assay, this protein fragment is able to bind to the ERE. When the interactions of this protein fragment with the various oligonucleotides are examined under the same conditions but the analysis of binding is by gel retardation, then only the GRE will show a DNA protein complex (data not shown). The display of reduced specificity—i.e., binding both GRE and ERE—in this assay is identical to the results seen with the CII finger (Fig. 5*B*). Although both GRE and ERE are bound by the double finger, the level of binding to the GRE is consistently greater than that seen with the ERE in all experiments. Since these experiments are carried out in DNA excess, the protein fragment has an apparent greater affinity for its natural target, as would be predicted. In filter binding experiments that compare the double and single fingers, the affinity of the double finger for a GRE is 30-fold greater than that for the single finger. We have developed a competition gel retardation assay with the intact DNA-binding domain that allows us to compare the relative affinities of the receptor fragment and the peptides directly. Results from these experiments reveal that the CI individual finger peptide, while unable to retard the GRE oligonucleotide, is able to compete for binding to a GRE by the double-finger fragment (Fig. 8*A*, compare lanes 2–4). In agreement with filter binding studies presented in figure 6, CI_M peptide is unable to compete in this assay (Fig. 8*A*, compare lanes 5 and 6 with 3 and 4). Thus, mutations that abolish binding in the filter binding assay fail to block binding by the intact DNA-binding domain in the competition assay. The data presented in Fig. 8*B* provides a more precise assessment of the relative affinities of the CII peptide and the DNA-binding domain. The results indicate that a 60-fold molar excess of the peptide will completely inhibit the formation of DNA-binding domain—GRE com-

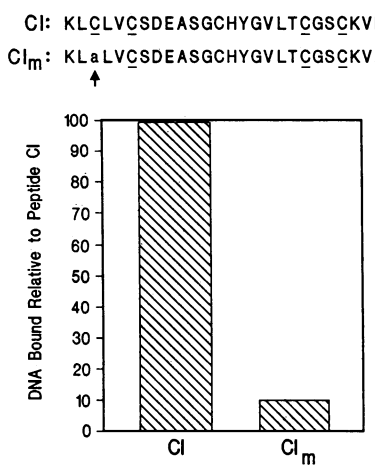

FIG. 6. DNA binding by synthetic finger peptides CI and CI_M. The binding reaction was as described in Fig. 7 with a GRE oligonucleotide. The binding of the synthetic peptides is expressed as a percentage of the binding to the GRE. The results in the figure represent DNA binding by peptides CI and CI_M plus $ZnCl_2$.

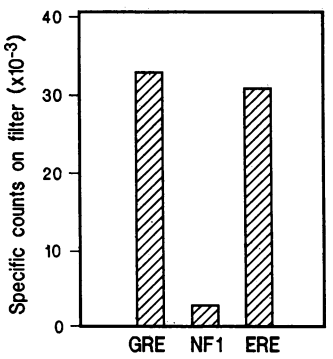

FIG. 7. DNA binding by cloned glucocorticoid receptor double-finger fragment. Nitrocellulose filter binding assay with cloned glucocorticoid receptor DNA binding domain T7 440-525 with GRE, ERE, and NF1 DNA-binding sites.

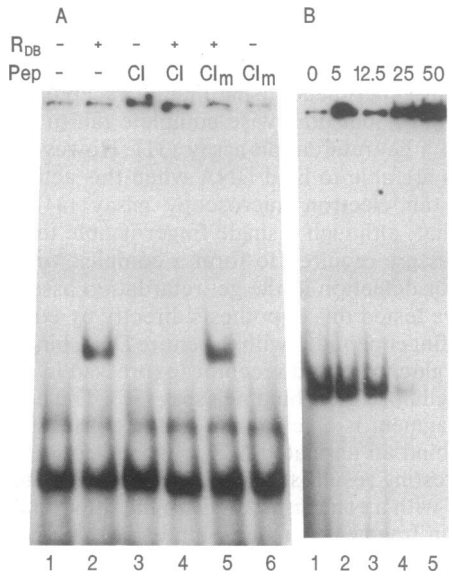

FIG. 8. Gel retardation competition assay with synthetic peptides and cloned glucocorticoid receptor double-finger fragment. (*A*) The CI but not CI_M synthetic peptide can block double-finger DNA binding. Reaction conditions were as described. Lanes: 2, 4, and 5, 50 ng of the double finger protein (R_{D8} +); 1 and 2, no peptides; 3 and 4, 1 μg of CI peptide; 5 and 6, 1 μg of CI_M peptide. All lanes contained 2.5 fmol of ^{32}P-labeled GRE oligonucleotide incubated with protein and/or peptide. (*B*) Titration of CII peptide with the double-finger fragment. Lanes 1–5 each contain 2.5 μg of double finger per ml (50 ng). The concentration of CII peptide varied from 0 to 50 μg/ml (0–1 μg).

plex (Fig. 8*B*, lane 5). While the affinity of the peptides is such that the peptide–DNA complex is unstable to gel retardation analysis, it is sufficient, when present in molar excess, to prevent the appearance of a double-finger–DNA complex as monitored by gel retardation assays.

In summary, we have synthesized peptides corresponding to the putative zinc fingers of the glucocorticoid receptor protein. The peptides bind zinc at molar ratios and interact with a GRE oligonucleotide only when the metal is present. The CI (amino-terminal) peptide displays significant binding only to GRE sequences, while the more promiscuous CII (carboxyl-terminal) peptide will bind an ERE sequence as well as a GRE sequence. The results from experiments with a cloned double-finger receptor fragment indicate that the nitrocellulose assay provides an alternate method of assessing DNA-binding activity. In these experiments the double-finger fragment and the individual CII peptide are qualitatively indistinguishable while being quantitatively distinct. In addition, a mutation in the native receptor that results in loss of function produces the same effect when introduced into the synthetic peptide, indicating that a particular structure is required for DNA binding. These results are consistent with recent studies that demonstrate that the amino terminal finger contains sufficient information to determine target specificity among hormone response elements *in vivo*, suggesting that the individual peptides retain significant DNA-binding characteristics of the native glucocorticoid receptor (1). These findings argue that important features of zinc finger–DNA interactions can be modeled with small, chemically synthesized peptides.

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