Identification of Lysine 134 in the Steroid-binding Site of the Sex Steroid-binding Protein of Human Plasma*

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The sex steroid-binding protein of human plasma SBP (or sex hormone-binding globulin, SHBG) was specifically inhibited with the alkylating affinity label, 17β-[(2-14C)bromoacetyl]oxy]-5α-androstan-3-one. The natural ligand, 5α-dihydrotestosterone, was shown to protect against inactivation and labeling. The steroid-binding activity of the protein was abolished when approximately 1 mol of label was incorporated into 1 mol of dimeric SBP. In order to identify and locate the labeled amino acid in the steroid-binding site, the steroidal portion of the bound label was first removed and the protein was digested with Achromobacter protease and subdigested with trypsin. Seven radioactive peptides were isolated, sequenced, and found to contain the common sequence QVSGLTSXR. Residue X was identified as lysine-134 from the SBP amino acid sequence (Walsh, K. A., Titani, K., Kumar, S., Hayes, R., and Petra, P. H. (1986) Biochemistry 25, 7584–7590). The results indicate that only 1 of the 2 lysine-134 residues in the homodimer was labeled. This suggests that the steroid-binding site is constructed from an association of the two subunits in an AB to BA “sandwich” configuration with lysine-134 residue of one subunit on one surface near the D-ring and the lysine-134 of the other subunit at the opposite end of the steroid, or away from the steroid-binding site. Although the nature of the data does not allow description of a specific role for lysine-134, its proximity to the 17β-OH of the steroid nucleus suggests participation in the binding process through direct or indirect hydrogen bonding.

EXPERIMENTAL PROCEDURES

Materials—Human pregnancy serum was obtained from Dr. Steve Pymate at the Madigan Army Hospital in Tacoma, WA. 5α-Dihydrotestosterone was purchased from Steraloids. Anhydrous methylene chloride was obtained from Aldrich. Bromoacetic acid, 1,3-dicyclohexylcarbodiimide, and crystalline bovine serum albumin were from Sigma. Silica Gel G was purchased from Brinkmann Instruments. Bromo-1,2-C14lactate (66 mCi/mmol) was purchased from Amer sham and [1,2,3H]DHT (58.4 Ci/mmol) from DuPont-New England Nuclear. DEAE-cellulose filter paper discs (DE81, 2.3-cm diameter) were purchased from Beech Angel & Co. Aquasol-2 was purchased from New England Nuclear.

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The abbreviations used are: SBP, plasma sex steroid-binding protein; SHBG, sex hormone-binding globulin; DHT, 5α-dihydrotestosterone; T, testosterone; DHTBr, 17β-[(2-14C)bromoacetyl]oxy]-5α-androstan-3-one; [14C]DHTBr, 17β-[(2-14C)bromoacetyl]oxy]-5α-androstan-3-one; [3H]DHTBr, 17β-[(3H)bromoacetyl]oxy]-11.2-H15α-androstan-3-one; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IAA, iodoacetate; HPLC, high performance liquid chromatography.

The sex steroid-binding protein of SBP, also called SHBG, sex hormone-binding globulin, is a circulating plasma protein that specifically binds 5α-dihydrotestosterone, testosterone, and estradiol with high affinity in the plasma of humans and other primates (for reviews see: Westphal, 1986; Petra et al., 1986a, and references cited therein) and sequenced (Walsh et al., 1986; Griffin et al., 1989). The native proteins are homodimers with monomeric molecular weights of about 44,000 (Petra et al., 1986a). Complementary DNAs have been isolated and sequenced (Gershagen et al., 1987; Que and Petra, 1987; Hammond et al., 1987), and the nucleotide sequence of the human SBP gene has been recently determined (Gershagen et al., 1989; Hammond et al., 1989). The amino acid sequences of human SBP (Walsh et al., 1986) and rat androgen-binding protein (Joseph et al., 1988) indicate that the two proteins are homologous (Petra et al., 1986c). The sex steroid-binding protein is thought to regulate the metabolic clearance rate of testosterone (Vermeulen et al., 1969; Petra et al., 1985) and estradiol (Pymate et al., 1990) and possibly to assist in the transport of sex steroids into target tissues (Bordin and Petra, 1980; Siiteri et al., 1982; Robel et al., 1983; Tardivel-Iacombe et al., 1984; Strel'chyonok et al., 1984; Avvakumov et al., 1986, Rosner et al., 1986; Stanczuk et al., 1986; Hryb et al., 1989, 1990).

Knowledge of the amino acid sequence of SBP allows characterization of the steroid-binding site. We have therefore undertaken affinity labeling of the human protein with the goal of identifying amino acid residues at the binding site (Petra et al., 1988). In this report, we show that 17β-[(bromoacetyl)oxy]-5α-androstan-3-one, DHTBr, is an affinity label of human SBP that specifically and irreversibly alkylates the steroid-binding site at lysine-134. The reagent contains a bromomethyl ketone functional group in the steroid structure and is therefore a pseudostuctural analog of the natural ligand, DHT. This functional group was first used by Shaw and co-workers (Shaw, 1967) for designing affinity labels for trypsin and chymotrypsin. The bromomethyl ketone functional group has been incorporated into various steroids to specifically label steroid-binding proteins and steroid-metabolizing enzymes (Sweet and Murdock, 1987). In particular, DHTBr has been reported to label yeast 3α,17β-hydroxysteroid dehydrogenase (Ganguly and Warren, 1971; Sweet and Samant, 1980), rat liver 3α-hydroxysteroid dehydrogenase (Penning et al., 1987), and the androgen receptor (Chang et al., 1984; Kovacks and Turney, 1988).

Experimental Procedures

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from DuPont-New England Nuclear Research Products. All other chemicals used in previous publications from this laboratory and were reagent grade.

**Purification of Human SBP**—SBP was purified to homogeneity as previously described (Petra and Lewis, 1980; Bordin and Petra, 1980; Griffin et al., 1989) except that DHT was omitted from all buffers used after the affinity chromatography and antitransferrin immunoaffinity chromatography. The concentrate of the antitransferrin column, was dialyzed overnight at 4 °C against 1 liter of 10 mM Tris-Cl, 10% glycerol (v/v), pH 7.4, and then purified by preparative PAGE in gel lacking DHT. The purpose was to minimize the interference of DHT (at least unbound DHT) in the affinity-labeling reaction. The pure protein was concentrated to 1–2 mg/ml using a membrane filter grade PM 10 and stored at −20 °C. The levels of DHT remaining were found to be very low as estimated by radioimmunoassay. Pure human SBP is stable in the absence of DHT and glycerol and can be stored for months at −20 °C without loss of activity. The concentration of SBP was determined spectrophotometrically using εmax = 1.14 × 10³ cm−¹ M−¹ (Petra et al., 1986a) and M = 95,400 (Petra et al., 1986b).

**Assay of SBP Activity**—The DEAE-cellulose filter assay previously described for measuring steroid-binding proteins in plasma (Michelson and Petra, 1974; Schiller and Petra, 1975) was used with some modification. Aliquots from the DHTBr reaction vessel (20 μl containing about 2 μg of SBP) were added to 500 μl of 1% bovine serum albumin solution followed by a 30-min incubation at 25 °C. These were then added to 2 ml of buffer with 0.5% charcoal, 0.05% dextran (w/v), 0.17% gelatin, 10 mM Tris-Cl, pH 7.4. The suspensions were shaken gently for 15 min at room temperature and centrifuged at 4 °C for 5 min at 15,000 × g to remove charcoal-containing steroid. Aliquots were further diluted 10-fold with 10 mM Tris-Cl, pH 7.4, and incubated for 20 min at room temperature with a 3-fold molar excess of [1,2-14C]DHTBr over SBP, in the presence or absence of 100 times molar excess of radioinert DHT over [1,2-14C]DHTBr. The pure protein was concentrated to 1–2 mg/ml and stored at −20 °C.

**Removal of Covalently Bound Steroid from Labeled SBP**—The protein was labeled in two different reactions carried out in parallel, one with [2-14C]DHTBr and the other with [1,2-3H]DHTBr. After 180 min, the reactions were treated with charcoal to remove excess unreacted DHT, pH was adjusted to 11 with 0.5 M NaOH, and incubation continued at 37 °C for an additional hour. The solutions were then neutralized with 0.5 M HCl, frozen, lyophilized, and electrophoresed, and the gels were sliced and counted as described above.

**Protein Digestion and Fractionation of Peptides**—Twenty nmol of dimeric SBP (40 nmol of polypeptide chains, M, = 46,700) was labeled with 10 μM [2-14C]DHTBr in 1 ml of buffer as described above for 180 min. The reaction mixture was placed on ice, made 1 mM in IAA, and dialyzed overnight at 4 °C against 2 liters of 1 mM IAA, 10 mM Tris-Cl, pH 7.4. The purpose of this step is to prevent radiolabeling of any cysteine residues while removing [2-14C]DHTBr. Dialysis against H2O was continued for an additional 4 h to reduce the IAA concentration. The solution was adjusted to pH 11 and incubated at 37 °C for 1 h, adjusted to pH 7.4, dialyzed overnight at 4 °C against 10 mM Tris-Cl, pH 7.4, and lyophilized. The protein was then reduced and S-carboxymethylated in 1 ml of 6 M guanidine HCl as described by Takio et al. (1983). After dialysis against 0.1 M NH4HCO3, a sample corresponding to about 15 nmol (estimated by amino acid analysis) of monomeric protein, M, = 46,700, was digested in 1 ml with 2 μg of Achromobacter protease I for 18 h at 37 °C. The volume was reduced to 0.5 ml on a Speed-Vac concentrator, and peptides were separated by size on a tandem series of one TSK-SW3000 and two TSK-SW2000 columns, using 6 M guanidine HCl, 10 mM phosphate, pH 6, at 1 ml/min. Fractions were monitored at 226 nm, and 5% of each were analyzed for 14C. Radioactive peptide fractions were pooled separately and fractionated by reverse phase HPLC on a Beckman Ultrapure RPSC column (7-μ C8, 4.6 × 75 mm) using an acetonitrile/water gradient at 0.08% trifluoroacetic acid. Tryptic subdigests were done in 1 mM NH4HCO3 and fractionated on a column of RP300/102 (7-μ C8, 2.1 × 100 mm). Peptide purity was checked by amino acid analysis.

**Gel Electrophoresis—SDS-gel electrophoresis was carried out according to Petr et al. (1983).** Native gel electrophoresis was carried out in tubes according to Petra et al. (1985). Amino Acid Analysis and Peptide Sequence Determination—Amino acid analyses were carried out as previously published (Bidlingmeyer et al., 1984). Peptides were sequenced with an Applied Biosystems model 470 Sequencer with on-line phenylthiohydantoin analysis using published programs (Honkapiller et al., 1983). Mass Spectrometric Analysis—Time-of-flight measurements were carried out by Dr. Pat Griffin at Genetech (San Francisco, CA) according to published procedures (Griffin et al., 1989).

**RESULTS**

**Kinetics of DHTBr Labeling**—The kinetics of SBP inactivation with DHTBr and incorporation of the label are shown in Fig. 1, A and B. In the presence of 10 mM excess of label over SBP, 50% inactivation occurs in approximately 2 h at 37 °C. During that time, about 0.5 mol of label is covalently incorporated into 1 mol of dimeric SBP. Labeling occurs in the steroid-binding site because loss of activity and label incorporation are both retarded in the presence of a 10-fold molar excess of DHTBr over SBP. Reaction of native SBP with [14C]iodoacetic acid results in no incorporation of radioactivity into the protein (data not shown) indicating that the halogen on the methyl ketone functional group must be attached to the natural ligand, as in the case of DHTBr, in order to react specifically in the steroid-binding site. Figure 1, A and B also indicate specifically that about 30% of the activity is lost after 4 h in the presence of DHT with a concomitant incorporation of about 30% of label into SBP.

**Stoichiometry of Label Incorporation**—The distribution of radioactivity in SDS-PAGE after a 180-min reaction is shown in Fig. 2. Two radioactive peaks are detected, peak I (major) at M, = 44,000 where the denatured human SBP monomer...
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FIG. 1. Kinetics of SBP inhibition and label incorporation with DHTBr at pH 7.4 and 37 °C. [SBP] = 1 μM; [DHTBr] = 10 μM; [DHT] = 100 μM. A, (Δ--Δ), control, SBP activity in the absence of DHT and DHTBr. Percent loss of SBP activity in the absence (●—●) or presence (○—○) of DHT. B, label incorporation into peak I of Fig. 2 as measured by SDS-PAGE, in the absence (■) or presence (●) of DHT. The star in the chemical structure of DHTBr indicates position of the [14C] isotope. Mole of label incorporated per mol of dimeric SBP is calculated from the specific radioactivity of [14C]DHTBr (1.23 μCi/μmol) and spectrophotometric determination of SBP concentrations using ε_280 = 1.14 × 10^3 cm^-1 M^-1.

FIG. 2. Analysis of affinity-labeled SBP by SDS-PAGE. Left panel shows radioactivity distribution in a SDS gel (2-mm slices) derived from a 180-min reaction using the conditions described under “Experimental Procedures” and in the legend of Fig. 1. Affinity labeling is carried out in the absence (●—●) or in the presence (○—○) of DHT. The right panel displays a SDS slab-gel stained with Coomassie Blue. Lane I, 10 μg of standard proteins (Bio-Rad); lane 2, 20 μg of SBP; lane 3, 40 μg of affinity-labeled SBP. The roman numerals next to the M_1 = 44,000 (44K) and 28,000 (28K) bands correspond to their position in the pattern shown in the left panel.

Electrophoresis of Labeled SBP in Native Gels—In order to determine whether or not labeling disrupts the dimeric structure of SBP, protein was reacted with DHTBr as described above and electrophoresed in native gels along with native SBP. Fig. 3 shows that the labeled protein migrates at the same place as native protein indicating that the native dimeric structure is maintained.

Removal of Steroid from Labeled SBP—To gain further insight into the labeling reaction, SBP was inactivated with [14C]DHTBr as described above, and also with [1,2-3H]DHTBr, which contains the label in the steroid nucleus instead of the 17β side chain. As described above, both reactions were exhaustively dialyzed against IAA, and against water to remove IAA. Both reactions were then incubated at pH 11 for 1 h at 37 °C to cleave the covalently bound steroid through hydrolysis of the ester linkage in the acetoxy side chain. The freeze-dried products were electrophoresed in SDS. The gel pattern shown in Fig. 4A demonstrates that the 3H-labeled steroid was removed since neither peak I nor II was radioactive after base treatment, whereas SBP retained label from [3H]DHTBr (Fig. 4B). The data further suggest that the label is not esterified to aspartic or glutamic acid residues because the 3H label remains with the protein at high pH. These experiments show that cleavage of the acetoxy ester bond by base removes the steroidal moiety of the label, thereby reducing the hydrophobicity of labeled peptides and facilitating their isolation.

Identification of the Site of Affinity Labeling—Fifteen nmol of the affinity-labeled SBP polypeptide (calculated as the monomer, M_0 = 46,700), was dialyzed as described above, incubated at pH 11 to remove the steroidal moiety, and reduced and S-carboxymethylated. After dialysis against 0.1 M NH_4HCO_3, the sample contained 50,000 cpm. Since 15 nmol (monomeric) of labeled SBP should contain about 18,000 cpm based on label incorporation of 1.1 mol/mol of dimeric SBP (combined peaks I and II of Fig. 2), the data indicated that about 32,000 cpm of unbound label was still present after dialysis. To reduce protein losses, the labeled protein was not dialyzed further but digested at lysyl residues and fractionated by size (see “Experimental Procedures”). Two peptide-bound radioactive fractions were obtained, and each was fractionated on reverse phase HPLC C3 columns to yield four radioactive fractions containing a total of 5,275 cpm (2.1 nmol of label, 25% yield). Preliminary analyses of small aliquots suggested that these fractions contained overlapping products of incomplete digestion. The four labeled fractions were pooled and

FIG. 3. Electrophoresis of labeled and native SBP in native polyacrylamide gels. Methods as described in Petra et al., 1983, except that electrophoresis was carried out in tubes. One nmol of SBP was reacted with a 10-fold molar excess of DHTBr as described under “Experimental Procedures” in the absence of DHT (●—●) or the presence of 10-fold molar excess over DHTBr (○—○). The gels were sliced and counted. The arrow represents the position where native SBP migrates as detected by Coomassie Blue staining.

normal migrates, and peak II (minor) at about 28,000. The presence of DHT in the mixture lowers the radioactivity content of both peaks. The SDS-PAGE data of Fig. 1B indicate that about 0.8 mol of label is incorporated per mol of dimeric SBP after 4 h; when peak II is added to peak I, the stoichiometry reaches about 1.1 mol/mol. A stained SDS slab-gel containing samples from a 30K-min reaction and from unreacted SBP is also shown in Fig. 2. As expected, there are two stained bands at about 44,000 and 28,000 corresponding to radioactive peaks I and II (lane 3). Native SBP (lane 2) does not contain the M_1 = 28,000 band.

These experiments show that cleavage of the acetoxy ester bond by base removes the steroidal moiety of the label, thereby reducing the hydrophobicity of labeled peptides and facilitating their isolation.
FIG. 4. Removal of covalently bound steroid from affinity-labeled SBP at pH 11 for 1 h at 37 °C as determined by SDS-PAGE. A, SBP labeled with [1,2-3H]DHTBr, untreated (O---O) or treated (O---O) at pH 11. B, SBP labeled with [14C]DHTBr, untreated (O---O) or treated (O---O) at pH 11.

Fig. 5. Separation of tryptic peptides from a mixture of labeled fractions derived by cleavage of affinity-labeled SBP with *Achromobacter* I. HPLC was carried out on a column of RP300/102 (a 7-μ C8 column, 2.1 x 100 mm) using an acetonitrile/water gradient in 0.1% trifluoroacetic acid. Hatch marks indicate pooled fractions containing label.

subdigested with trypsin, and products were separated on a C8 reverse phase HPLC column. As shown in Fig. 5, five labeled peptide fractions were recovered and 40-80% of each was subjected to Edman degradation.

The results in Table I reveal that the common sequence QVSGPLTSXR is present in each fraction, although minor contaminants are seen as well as long versions of the same peptide. The residue denoted X in the major peptides of Table I did not yield an identifiable phenylthiohydantoin but, from the amino acid sequence of human SBP (Walsh et al., 1986), that residue corresponds to lysine 134. Since all the other residues in this sequence were easily recognized, it is concluded that Lys 134 is the site of N'-carboxymethylation by the affinity label, and that the phenylthiohydantoin of N'-carboxymethyllysine does not elute in a readily recognized location in our chromatographic system.

Assignment of Lys-134 as the site of labeling was confirmed by time-of-flight mass spectrometry on fractions T-17 and T-20 of Table I. The major ion in T-17 had a m/z of 1400.6, corresponding to the calculated mass (1400.5) of N'-carboxymethyl-LRQVSGPLTSKR. Fraction T-20 revealed two ions, of m/z 1749 and 1766. The difference in mass between these two ions corresponds to the 17 mass units of lost NH3 when glutamine cyclizes to a pyroglutamyl residue. Therefore, the two ions in T-20 correspond in mass to the N'-carboxymethyl forms of QVSGPLTSKRPHIPMR and its blocked pyroglutamyl derivative. The blocked pyroglutamyl form of that peptide probably accounts for much of the label in T-22, although this was not proven by mass spectrometry.

In summary, all of these results point to Lys-134 as the site of labeling. No other minor peptide was found in more than one labeled fraction. As shown in Table I, the specific activity of the label (2500 cpm/nmol) agrees well with the amount of the major peptide observed in the Sequencer with each of the peptide preparations. The total amount of radioactivity recovered as Lys-134-containing peptides was 15-20%; this is a good recovery considering that the generally accepted peptide recovery yield per HPLC column is 50%.

**DISCUSSION**

Description of the chemical environment of steroid binding sites is needed for understanding the molecular basis of steroid-binding specificity. In this paper we report the presence of lysine 134 in the steroid-binding site of human SBP by virtue of its specific alkylation with the affinity label, 17β-(bromoacetyl)oxy-5α-androstan-3-one. Specificity of the labeling reaction is shown by protection with the natural ligand, 5α-dihydrotestosterone. Since incorporation of approximately 1 mol of label per mol of dimeric SBP abolishes the steroid-binding activity of the protein, inactivation is a direct consequence of Lys-134 labeling.

As shown in Fig. 1, inactivation and label incorporation also occur in the presence of DHT, although at a lower rate. Incomplete protection would occur if DHTBr were to compete efficiently with DHT, thereby displacing it in the steroid-binding site. This would result in a gradual decrease in SBP activity with time as indicated in Fig. 1. Similar observations were reported for DHTBr inactivation of the androgen receptor in prostate cytosols where protection by DHT was also found to be incomplete (Kovacks and Turney, 1988). These authors report that DHTBr binding to the receptor was only 2.3-fold lower than DHT. We have not analyzed peptides obtained from labeled SBP in the presence of DHT, but we strongly suspect that lysine 134 is also alkylated under those conditions. This is supported by the fact that only one site of modification was found when the protein was reacted in the absence of DHT. Although similar data would be expected if some labeling were to occur at nonspecific sites in addition to the steroid-binding site, this seems unlikely because label incorporation and loss of activity occur at the same rate in a 1:1 stoichiometry in the absence of DHT. Therefore, the likely explanation is that DHTBr competes efficiently with DHT for the binding site.

Fig. 1 also indicates that the rate of inhibition and label...
polypeptide chain. In any case, this side reaction does not affect our interpretation of the data since the digested material from which the labeled peptides were isolated contained both M₁ = 44,000 and 28,000 components and all the radioactive peptides that we isolated the hydrophobic steroid facilitates isolation of steroid-binding site peptides in good yield. The results presented here also show that the conditions used for removing the steroidal moiety are mild enough to preserve the primary structure of the protein.

The data also indicate that labeling of only one of the two identical subunits of homodimeric SBP is sufficient to abolish the activity. The radiolabeled peptides contain only one site of modification, carboxymethyllysine 134. Since there are two Lys-134 residues per dimer, the second one must remain unmodified during the inhibition reaction and therefore may not be located near the D-ring of the steroid. This suggests that the steroid-binding site is constructed through an association of the two subunits in an AB to BA “sandwich” configuration. This interpretation lends support to the hypothesis placing the steroid at the interface between the subunits (Petra et al., 1983). This molecular arrangement would provide an explanation for the problem of a homodimeric protein molecule recognizing an asymmetric ligand. The AB/BA configuration would present two different surfaces to the two faces of the steroid. The results presented here support the positioning of the Lys-134 of one subunit on one surface near the D-ring, while the Lys-134 of the other subunit could be either at the opposite end of the steroid or well away from the steroid-binding site.

Analysis of SDS gels reveals that in addition to the M₁ = 44,000 labeled SBP band there is a second radioactive band at about 28,000 (Fig. 2). The origin of this band remains unclear. It does not represent a contaminating protein because it is absent from the SDS-PAGE pattern of the unreacted protein (Fig. 2, lane 2). It appears that labeling at Lys-134 may promote a side reaction involving cleavage of the SBP polypeptide chain. In any case, this side reaction does not affect our interpretation of the data since the digested material from which the labeled peptides were isolated contained both M₁ = 44,000 and 28,000 components and all the radioactive peptides that we isolated contained the sequence encompassing Lys-134.

DHTBr has the characteristics of an ideal affinity label for steroid-binding proteins and points the way to the design of isomeric labels with the reactive group at different positions. Placing the radioactive isotope in an acetoxy side chain is particularly advantageous because it provides an experimental means for removing the steroid while leaving the radioactive tag covalently attached to an amino acid residue. Removal of the steroid-binding proteins and points the way to the design of active peptides that we isolated contained the sequence encompassing Lys-134.
spectrometry measurements, Dr. Dave McCrae for his advice in the synthesis of steroid affinity labels, and Dr. Harry Charbonneau for valuable discussions during the course of this work.

Note Added in Proof—A paper was recently published suggesting that DHTBr alkylates His-235 instead of Lys-134 in human SBP (Khan and Rosner, 1990). Since a N'-carboxymethyllysine standard was not included in their analyses of alkylated protein hydrolysates, alkylation of a lysine residue cannot be ruled out in that work. Furthermore, Edman degradation of their radioactive peptide preparation yields phenylthiohydantoin-histidine at position 235 in relatively good yield for a charged amino acid indicating that His-235 is not alkylated.

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