Identification of Trp-371 as the Main Site of Specific Photoaffinity Labeling of Corticosteroid Binding Globulin Using Δ^6 Derivatives of Cortisol, Corticosterone, and Progesterone as Unsubstituted Photoreagents†

Catherine Grenot, Thierry Blachère, Marc Rolland de Ravel, Elisabeth Mappus, and Claude Y. Cuilleron*

Institut National de la Santé et de la Recherche Médicale, Unité INSERM U 329, Pathologie Hormonale Moléculaire, Hôpital Debrousse, 69322 Lyon, France

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ABSTRACT: Immunopurified human corticosteroid binding globulin (CBG) was photolabeled with Δ^6-[^{3}H]-cortisol, Δ^6-[4-^{14}C]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone. The maximal levels of specific incorporation, as estimated with tritiated photoreagents, were 0.21, 0.14, and 0.08 mol of label/mol of CBG, respectively. Tryptic cleavage of photolabeled CBG gave in all cases a major radioactive peptide that was no longer detectable when a 100-fold molar excess of cortisol was added to the photoreagents. Edman sequencing of tryptic peptides photolabeled with Δ^6-[^{3}H]cortisol or Δ^6-[^{3}H]corticosterone showed that these peptides correspond to residues 357–378 of the human CBG sequence. The major peak of radioactivity of these peptides was eluted at the 15th cycle (Trp-371). The radioactive tryptic peptides photolabeled with the four steroid photoreagents were subcleaved with α-chymotrypsin. The major part of radioactivity was recovered in the T-[*X]-S-S-L-F hexapeptide 370–375 (major peptide) and in the D-H-F-T-[*X]-S-S-L-F nonapeptide 367–375, at the second and fifth Edman cycles, respectively, whereas no PTH derivative could be identified at these cycles, thus suggesting Trp-371 as the main site of photolabeling for all tested photoreagents. Mass spectrometry of tryptic peptides photolabeled with Δ^6-[^{3}H]cortisol and Δ^6-[^{3}H]-corticosterone and of chymotryptic peptides photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone showed molecular masses corresponding to the addition of Δ^6-steroid photoreagents to the peptide.

Human corticosteroid binding globulin (CBG)[1] is a transport glycoprotein that binds about 90% of serum cortisol and thus contributes predominantly to the regulation of the bioavailability of this steroid. Several recent studies have suggested that CBG may also influence the selective delivery of bound steroid to cells, undergo intracellular internalization, or activate a second messenger system (Hammond et al., 1990, Rosner, 1990; Strel'chyonok & Avvakumov, 1990, 1991).

The structure, function, and molecular properties of CBG have been the subject of detailed studies summarized in several recent reviews (Hammond, 1990, Rosner, 1990; Strel'chyonok & Avvakumov, 1990; Hammond et al., 1991).

Structural and binding analyses have established that human CBG is a monomeric glycoprotein of ca. 50–55 kDa, as estimated by polyacrylamide gel electrophoresis under denaturing conditions, containing about 25% carbohydrates (Strel'chyonok & Avvakumov, 1990), with one steroid binding site per mole of protein. Recent studies have pointed up that glycosylation plays an essential role for the acquisition of steroid binding activity of recombinant human CBG (Ghose-Dastidar et al. 1991; Avvakumov et al., 1993).

The primary structure of human CBG, composed of a single polypeptide of 383 residues, was determined by sequencing the hepatic and pulmonary cDNAs and showed a homology with members of the SERPIN (serine protease inhibitors) superfamily such as α_1-proteinase inhibitor, α_1-antichymotrypsin, and plasma transport thyroxine binding globulin (Hammond et al., 1987) whereas the chromosomal localization of CBG was found near the genes of the first two proteins (Seralini et al., 1990a; Seralini, 1991). Primary structures from rat CBG (Smith & Hammond, 1988, 1989), rabbit CBG (Seralini et al., 1990b), and sheep CBG (Berdusco et al., 1993) were also deduced from the corresponding cDNAs, whereas a partial sequence of murine CBG was established by Edman degradation (Nyberg et al., 1990). Amino acid substitutions responsible for the reduced steroid-binding affinity of natural mutant forms of CBG were found to be a Leu-93 → His-93 mutation for human CBG (Smith et al., 1992; Van Baelen et al., 1993) and a Met-276 → Ile-276 mutation for rat CBG (Smith & Hammond, 1991).

Several attempts to identify amino acids present in the binding site of purified human CBG have been reported. Ligand-inactivable specific chemical modifications established the roles of a tryptophan (Akhrem et al., 1981) and of a tyrosine residue (Le Gaillard et al., 1982) as suggested previously by the quenching effects of Δ^4-3-oxosteroid ligands on CBG fluorescence (Marver et al., 1976; Stroupe et al., 1978; Akhrem et al., 1978). Affinity labeling experiments have also shown that one of the two cysteine residues of human CBG was specifically alkylated with 6β-bromoprogesterone (Khan & Rosner, 1977) while a methionine and a histidine were alkylated with 11α-(bromoacetoxy)progesterone and 16α-(bromoacetoxy)progesterone derivatives, respectively, whereas 17β-(bromoacetoxy)testosterone was found to react with two

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* Author to whom correspondence should be addressed [Telephone: (33) 78 25 18 08. FAX: (33) 78 25 61 68].

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[1] Abbreviations: CBG, corticosteroid binding globulin; SHBG, sex hormone binding globulin; Δ^6-cortisol, 11β,17α,21-trihydroxypregna-4,6-dien-3-one; Δ^6-corticosterone, 11β,21-dihydroxypregna-4,6-dien-3-one; Δ^6-progesterone, 3,20-dioxopregna-4,6-diene; Δ^6-testosterone, 17β-hydroxyandrost-4,6-dien-3-one; DCC, dextran-coated charcoal; DEAE, (diethylamino)ethyl; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PTH, phenylthiohydantoin; RIA, radioimmunoassay; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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histidine and methionine residues (Le Gaillard & Dautrevaux, 1977). Specific photolabeling of human CBG with tritiated 21-diazo-21-deoxycorticosterone (Marver et al., 1976) and of hamster CBG with tritiated Δ^6-testosterone (Gray et al., 1987) have been reported, but without identification of the labeled amino acids.

The steroid binding site of human CBG was also studied by electron spin resonance with nitroxide free-radical spin-labels introduced on the C-17 side chains of corticosteroid ligands and *N*-ethylmaleimide–nitroxide conjugates, using different intercalating chain ligands (Defaye et al., 1980). These studies have demonstrated depths of about 25 Å for the cortisol binding site and of 15 Å for the cysteine residues.

The present work was undertaken to characterize unambiguously the site(s) of specific covalent photoaffinity labeling of purified human CBG by parallel experiments with unsubstituted $\Delta^{4,6}$-3-oxosteroid photoreagents, obtained by Δ^6-dehydrogenation of cortisol, corticosterone, and progesterone, three known ligands of CBG.

EXPERIMENTAL PROCEDURES

Chemicals. Cortisol, corticosterone, and progesterone were from Roussel-UCLAF (Paris, France). L-1-*p*-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin) was from Worthington and α-chymotrypsin from Boehringer (Manheim, Germany). Trisacryl GF 05 was from Sepracor (Villeneuve-la-Garenne, France).

Buffers and Reagents. PBS (10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl), PBS–gelatin (PBS containing 0.1% gelatin and 0.1% NaN_3), and DCC (5 g of Norit A, 0.5 g of Dextran T-70 in 1 L of PBS) were used.

Synthesis of Radioactive Photoaffinity Labeling Reagents. Δ^6-[^{3}H]Cortisol (~1.5 Ci/mmol) was prepared from [1,2,6,7-$^3H_4$]cortisol (85 Ci/mmol, purchased from NEN) by acid-catalyzed dehydrogenation of the corresponding 21-acetate derivative with dichlorodicyanobenzoquinone (Turner & Ringold, 1967). A mixture of [1,2,6,7-$^3H_4$]cortisol (1 mCi, ~4.3 μg, 0.012×10^{-6} mol) and of the radioinert steroid (200 μg cortisol, 0.55×10^{-6} mol) was acetylated by a 3:1 mixture of pyridine and acetic anhydride (100 μL) for 2 h at room temperature. The reaction product was purified by TLC on fluorescent silica gel (Merck 60 F_{254}, chloroform–ethyl acetate 3:1). The purified acetylated product (0.5×10^{-6} mol) was dissolved in anhydrous dioxane (40 μL) containing a slight excess of dichlorodicyanobenzoquinone (125 μg, 0.55×10^{-6} mol). Anhydrous hydrogen chloride was then bubbled slowly (5 min) through a glass capillary into the reaction mixture at 22 °C. The reaction mixture was neutralized by addition of a saturated aqueous solution of $NaHCO_3$ (50 μL) and purified immediately, without extraction, by TLC using the solvents described above and by reverse-phase HPLC on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) with an aqueous acetonitrile gradient (30-100% CH_3CN in 45 min). The protecting 21-acetate group was removed by saponification with 50 μL of a mixture of 10% aqueous solution of $KHCO_3$ in methanol for 18 h at room temperature, in the dark, under a nitrogen atmosphere. The saponified product was purified by reverse-phase HPLC, as above, to give tritiated Δ^6-cortisol in about 50% overall yield, with a radiochemical purity of >98%.

Δ^6-[^{3}H]Corticosterone (~1.5 Ci/mmol) was prepared, as described above, from [1,2-$^3H_2$]corticosterone (41 Ci/mmol, purchased from NEN) after acetylation of a mixture of [1,2-$^3H_2$]corticosterone (1 mCi, ~8.4 μg, 0.024×10^{-6} mol) and radioinert corticosterone (200 μg, 0.58×10^{-6} mol).

Δ^6-[4-^{14}C]Cortisol (~13 mCi/mmol) was also prepared as described above from [4-^{14}C]cortisol (51.8 mCi/mmol, purchased from Dositek, Orsay, France) after acetylation of a mixture of [4-^{14}C]cortisol (10 μCi, ~50 μg, 0.14×10^{-6} mol) and radioinert cortisol (150 μg, 0.41×10^{-6} mol).

Δ^6-[^{3}H]Progesterone (~1.4 Ci/mmol) was prepared from [1,2-$^3H_2$]progesterone (48.8 Ci/mmol, purchased from NEN) by acid-catalyzed dehydrogenation of a mixture of [1,2-$^3H_2$]-progesterone (1 mCi, ~6.4 μg, 0.02×10^{-6} mol) and radioinert progesterone (200 μg, 0.64×10^{-6} mol) with dichlorodicyanobenzoquinone (145 μg, 0.64×10^{-6} mol), followed by TLC on silica gel (Merck 60 F_{254}, pentane–ethyl acetate 1:1) and reverse-phase HPLC on the C_{18} column described above with an aqueous acetonitrile gradient (40–100% CH_3CN in 45 min) to give Δ^6-[^{3}H]progesterone in about 60% yield with a radiochemical purity of >98%.

The structures of the radioactive Δ^6-steroid photoreagents were confirmed by the identity of their chromatographic properties with those of the corresponding unlabeled derivatives prepared by the same methods. The unlabeled derivatives were characterized by their UV absorbance in ethanol at 284 nm [Δ^6-cortisol, ϵ = 24 500 M^{-1} cm^{-1}; Δ^6-corticosterone, ϵ = 24 000 M^{-1} cm^{-1}; Δ^6-progesterone, ϵ = 25 000 M^{-1} cm^{-1}, lit. ϵ = 25 000 M^{-1} cm^{-1} at 283 nm for Δ^6-progesterone (Agnello & Laubach, 1960)] and by proton nuclear magnetic resonance [Δ^6-cortisol, (C_5D_5N) δ ppm 1.36 (3H, s, CH_3-18), 1.59 (3H, s, CH_3-19), 4.13 (1H, s, broad, H-11), 4.87 and 5.32, (2H, 2d, J = 19 Hz, CH_2-21), 5.88 (1H, s, H-4), 6.15 and 6.19 (2H, 2d, J = 10 Hz, H-6 and H-7)]; Δ^6-corticosterone, δ ppm 1.24 (3H, s, CH_3-18), 1.56 (3H, s, CH_3-19), 4.46 and 4.55, (2H, 2d, J = 19 Hz, CH_2-21), 4.48 (1H, s broad superimposed on the CH_2-21 signal, H-11), 5.90 (1H, s, H-4), 6.15 and 6.19 (2H, 2d, J = 10 Hz, H-6 and H-7); Δ^6-progesterone, ($CDCl_3$) δ ppm 0.72 (3H, s, CH_3-18), 1.12 (3H, s, CH_3-19), 2.14 (1H, s, CH_3-21), 5.68 (1H, s, H-4), 6.12 (2H, s, H-6 and H-7)].

The specific activities mentioned above for the four radioactive photoreagents were estimated from the measurements of the radioactivity (dpm) and of the UV absorbance at 284 nm of an aliquot of the purified tracers. The values measured for tritiated tracers were slightly lower than the values calculated from the dilution of the corresponding radioactive precursors by unlabeled steroids (~1.8 Ci/mmol for Δ^6-[^{3}H]cortisol, ~1.7 Ci/mmol for Δ^6-[^{3}H]corticosterone and ~1.5 Ci/mmol for Δ^6-[^{3}H]progesterone), owing probably to experimental errors as well as to a possible partial loss of the tritium atoms at position C-2 of corticosterone and at positions C-2, C-6, and C-7 of cortisol.

Purification of Human CBG. Purification of CBG was performed on a pool of human sera constituted of daily residual samples remaining after diagnostic tests (Hôpital Edouard Herriot, Lyon, France). The concentration of CBG in the initial pool of sera (30 mg/L) was measured by RIA (Pugeat et al., 1989). CBG was isolated by immunopurification with a monoclonal antibody against human CBG obtained in our laboratory, according to a protocol similar to that previously employed for purification of SHBG (Grenot et al., 1992), with some modifications. Pooled sera (1.5 L) were incubated batchwise overnight at 4 °C with 200 mL of affinity resin and the unretained fraction was filtered. The gel was washed several times in batch at 4 °C with 500 mL of 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.2% Tween 20, until the protein content could not be detected by UV absorbance at 220 nm. After equilibration of the gel with 1 L of 50 mM NH_4HCO_3, pH 7.5, CBG was eluted by 700 mL of 1 M NH_4OH, at 4 °C. The NH_4OH eluate was immediately frozen and lyophilized in the presence of 0.5% glycerol. The lyophilized residue was purified by anion-exchange HPLC on a DEAE column (0.75 × 7.5 cm HEMA-

IEC 1000 DEAE, Alltech) using a linear gradient of 1 M NaCl in 10 mM sodium phosphate buffer, pH 7.5 (0–30% in 30 min). These two purification steps gave purified CBG in about 70% overall yield (estimated by RIA). The concentrations of CBG in the eluate of the DEAE column varied from one purification to another, ranging from 1.8 to 2.3 mg/mL (34–43 × 10^{-9} M), determined either by RIA or by UV absorption at 278 nm using an absorbance value $E_{1cm}^{1\%}$, of 6.5 (Mickelson et al., 1982) and an average molecular weight of 53 000 estimated from that established from the peptide sequence: MW = 42 646 (Hammond et al., 1987), assuming the presence of 25% carbohydrate chains (Strel'chyonok & Avvakumov, 1990). Purified CBG, if not employed immediately, was stored at –20 °C.

Binding Measurements. The number of binding sites and association constants of serum CBG and of immunopurified CBG with cortisol, corticosterone, progesterone, Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone were determined by equilibrium dialysis at 4 °C, using Scatchard plots (Scatchard, 1949) analyzed by a computer program with least-squares fit of the data. Serum was depleted from endogenous steroids by stripping with a DCC suspension (Hammond et al., 1983). Aliquots of the supernatant of serum depleted from endogenous steroids (1 mL, containing to 30 μL of serum and 970 μL of DCC, incubated for 30 min at 20 °C, and centrifuged at 3000 rpm for 20 min at 4 °C) or of immunopurified CBG (18 nM) in PBS–gelatin buffer were placed in dialysis bags and dialyzed for 48 h at 4 °C against eight concentrations (ranging from 4 to 80 nM) of ^{3}H-labeled steroids (the specific activity of tritiated steroid tracers was adjusted to 1.4 Ci/mmol with radioinert steroid).

Fluorescence Measurements. Fluorescence measurements were performed with a SLM 8000 C spectrofluorimeter equipped with a 450-W xenon lamp (emission power automatically corrected in the reference channel), according to a reported procedure (Sontag et al., 1993). Excitation was at 295 nm and emission was measured in the wavelength range of 310–380 nm with the corresponding bandpaths set at 0.5 and 4 nm, respectively. The samples (1 mL) were prepared by incubation for 1 h at 4 °C of purified CBG (0.2 × 10^{-6} M solution in 50 mM phosphate, pH 7.4) with different concentrations of cortisol, corticosterone, and progesterone (0.1 to 2 × 10^{-6} M in the same buffer containing less than 1% of ethanol). The incubated samples were thermostated for 5 min at 20.0 ± 0.1 °C before fluorescence measurements. The percentages of fluorescence quenching were estimated by comparing the intensities of the emission peaks, at their maximal values, using the maximum fluorescence of a similar sample of CBG incubated with a 10-fold molar excess of 5β-dihydropregnane-3,20-dione, as reference value (Stroupe et al., 1978). A correction was made for the fluorescence of identical samples without CBG.

Photoaffinity Labeling. The conditions of photoaffinity labeling were essentially those previously reported (Grenot et al., 1992). Immunopurified CBG was first incubated in the dark for 2 h at 4 °C (the last 15 min being under an argon atmosphere) with the Δ^6-steroid photolabeling reagent (steroid protein ratios are mentioned below) and irradiated with a high-pressure mercury lamp (Hanovia, 450 W), under an argon atmosphere at $\lambda > 300$ nm using a 2-mm-thick Pyrex filter (Taylor et al., 1980). After irradiation, the dissociation of noncovalently bound steroid was performed by exclusion chromatography of the irradiated mixture on columns of Trisacryl GF 05 equilibrated with 0.25 M Tris-HCl, pH 8.5, and 6 M guanidinium chloride, as described for SHBG (Grenot et al., 1992).

The following stoichiometries were employed in the incubation step prior to irradiation. (1) Photoinactivation experiments: CBG (1.5 × 10^{-11} mol) was incubated with Δ^6-cortisol, Δ^6-corticosterone, or Δ^6-progesterone (1.5 × 10^{-10} mol) in 100 μL of PBS-gelatin buffer. (2) Kinetics of covalent attachment: CBG (1.5 × 10^{-11} mol) was incubated with Δ^6-cortisol, Δ^6-corticosterone, or Δ^6-progesterone (3 × 10^{-11} mol) containing the corresponding tritiated photoreagents (10^5 cpm) in 100 μL of PBS–gelatin buffer in the absence or in the presence of 100-fold excess of cortisol. (3) Determination of HPLC profile of tryptic digest: CBG (4.5 × 10^{-9} mol) was incubated with Δ^6-cortisol, Δ^6-corticosterone, or Δ^6-progesterone (9 × 10^{-9} mol) containing the corresponding tritiated photoreagents (10^6 cpm) in 200 μL of PBS buffer in the absence or in the presence of 100-fold excess of cortisol. (4) Sequence analysis: (a) tryptic peptides, (i) CBG (0.18 × 10^{-6} mol) was incubated with Δ^6-cortisol (0.27 × 10^{-6} mol) containing Δ^6-[^{3}H]cortisol (12.4 × 10^7 cpm, 9 × 10^{-8} mol) in 5 mL of PBS buffer (sample containing 20% contaminant after purification); (ii) CBG (0.18 × 10^{-6} mol) was incubated with Δ^6-cortisol (0.295 × 10^{-6} mol) containing Δ^6-[^{3}H]cortisol (9.2 × 10^7 cpm, 6.5 × 10^{-8} mol) in 5 mL of PBS buffer (sample totally purified); (iii) CBG (0.18 × 10^{-6} mol) was incubated with Δ^6-[^{3}H]corticosterone (12.4 × 10^7 cpm, 0.09 × 10^{-6} mol) in 5 mL of PBS buffer; (b) chymotryptic peptides (i) CBG (0.36 × 10^{-6} mol) was incubated with Δ^6-cortisol (0.72 × 10^{-6} mol) containing Δ^6-[^{3}H]cortisol (1.9 × 10^7 cpm, 1.4 × 10^{-8} mol) in 10 mL of PBS buffer; (ii) CBG (0.72 × 10^{-6} mol) was incubated with Δ^6-corticosterone (1.44 × 10^{-6} mol) containing Δ^6-[^{3}H]corticosterone (12.2 × 10^7 cpm, 8.8 × 10^{-8} mol) in 20 mL of PBS buffer; (iii) CBG (0.72 × 10^{-6} mol) was incubated with Δ^6-progesterone (1.44 × 10^{-6} mol) containing Δ^6-[^{3}H]progesterone (4.6 × 10^7 cpm, 3.3 × 10^{-8} mol) in 20 mL of PBS buffer; (iv) CBG (0.25 × 10^{-6} mol) was incubated with Δ^6-cortisol (0.17 × 10^{-6} mol) containing Δ^6-[4-^{14}C]-cortisol (6.8 × 10^6 cpm, 0.24 × 10^{-6} mol) in 10 mL of PBS buffer. (5) Molecular mass determinations: (a) CBG (0.72 × 10^{-6} mol) was incubated with Δ^6-cortisol (1.44 × 10^{-6} mol) containing 10^7 cpm of Δ^6-[^{3}H]cortisol in 20 mL of PBS buffer; (b) CBG (1.44 × 10^{-6} mol) was incubated with Δ^6-corticosterone or Δ^6-progesterone (2.88 × 10^{-6} mol) containing 2 × 10^7 cpm of Δ^6-[^{3}H]corticosterone or Δ^6-[^{3}H]progesterone in 40 mL of PBS buffer.

Gel Electrophoresis. Immunopurified unlabeled CBG was analyzed by native polyacrylamide gel electrophoresis (Davis, 1964) whereas both unlabeled and photolabeled CBGs were characterized by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (Laemmli, 1970). These electrophoreses were performed in a vertical slab-gel apparatus. Unlabeled CBG was stained with silver nitrate (Heukeshoven & Dernick, 1985), whereas photoaffinity-labeled CBG was detected by fluorography, after treatment of the gel with 1 M sodium salicylate, using a Kodak X-OMAT film. Molecular weight markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme) were purchased from Amersham.

Tryptic Cleavage of Photolabeled CBG and Purification of Peptidic Fragments. Photolabeled CBG was reduced with dithioerythritol, carboxymethylated by iodoacetic acid, and transferred in 0.5 M Tris-HCl buffer, pH 8.2, by exclusion chromatography on Trisacryl GF 05. This solution of photolabeled CBG was incubated with TPCK-trypsin (1% enzyme/CBG ratio, w/w) for 24 h at 37 °C. Trypsin hydrolyzates were separated by reverse-phase HPLC using an aqueous acetonitrile gradient in the presence of 0.1% TFA. The preparative separation of photolabeled tryptic peptides

was performed by successive HPLC separations, using an aqueous acetonitrile gradient. The first step was performed in acidic medium (0.1% TFA), the second step in the presence of 20 mM NH_4HCO_3, pH 7.5. The final purification was performed by successive chromatographies in acidic medium, as in the first step.

Chymotryptic Subcleavage. The eluate of the second HPLC step containing the partially purified radioactive tryptic peptides was concentrated under a nitrogen stream in order to eliminate acetonitrile. Then, $CaCl_2$ was added to reach a final concentration of 20 mM. The peptide mixture was treated with chymotrypsin (5% w/w) for 18 h at 37 °C. Radioactive chymotryptic peptides were purified by reverse-phase HPLC on a C_{18} column using an aqueous acetonitrile gradient in the presence of 0.1% TFA.

Sequence Determinations. Automated Edman degradation of purified peptides was performed in a gas-phase sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer (Applied Biosystems, Model 470 A sequencer).

Amino Acid Analysis. Hydrolysis was performed in the vapor phase on dry samples of purified peptide (1 nmol), including norleucine as internal standard, exposed to 6 N HCl, under nitrogen atmosphere, for 24 h at 110 °C, in the presence of 1% phenol. Amino acids were identified as phenyl isothiocyanate (PTC) derivatives by reverse-phase HPLC on a C_{18} column (Waters Picotag), at 50 °C, using a multistep gradient from 0 to 100% of solvent B. (Solvent A was 0.14 M sodium acetate, 0.05% triethylamine, pH 6.4, 6% CH_3CN; solvent B was 60% CH_3CN in water.) The PTC derivatives were quantified by reference to phenylalanine, as the mean value of three separate measurements, with a lower limit of detection of PTC residues of 5 pmol for standard experiments.

Mass Spectrometry. Liquid secondary ion mass spectrometry experiments were performed on a VG ZAB SEQ mass spectrometer equipped with a Cs^+ gun operating at 40 keV. The accelerating voltage was 8 kV and the instrumental resolution was 800 (10% valley). Calibrations were performed with $(CsI)_nCs^+$ cluster ions. The tryptic peptide photolabeled with Δ^6-[^{3}H]cortisol (100 pmol), the unlabeled tryptic peptide (500 pmol), and the sample of tryptic peptide partially photolabeled with Δ^6-[^{3}H]corticosterone (100 pmol), obtained from CBG irradiated in the presence of a 2-fold molar excess of photoreagent, were dissolved in 2 μL of 5% aqueous acetic acid and added to 2 μL of matrix (1-thioglycerol acidified with 1% TFA) introduced on a stainless-steel probe tip. Spectra were recorded in the mass range 500–4000. Data were acquired using data system control in the multichannel analyzer mode (MCA) and two to three scans were summed to give the final spectrum. Exact masses were measured for the two $[M + H]^+$ peaks of the tryptic peptide partially photolabeled with Δ^6-[^{3}H]corticosterone.

The electrospray mass spectra of the tryptic peptide photolabeled with Δ^6-[^{3}H]corticosterone (100 pmol) and of the hexapeptide photolabeled with Δ^6-[^{3}H]cortisol (300 pmol) were recorded with a Hewlett-Packard 5989 mass spectrometer equipped with an atmospheric pressure electrospray ion source at a resolution of 1000 (50% valley). The peptides were injected in 60 μL of 50% aqueous methanol containing 1% acetic acid at a flow rate of 2 μL/min. The capillary exit voltage was 300 V. The quadrupole was scanning from m/z 200 to 1200 for the hexapeptide photolabeled with Δ^6-[^{3}H]-cortisol and from m/z 500 to 1650 for the tryptic peptide photolabeled with Δ^6-[^{3}H]corticosterone. The electrospray mass spectra of the hexapeptides photolabeled with Δ^6-[^{3}H]-corticosterone (50 pmol), and Δ^6-[^{3}H]progesterone (50 pmol) were recorded with a VG BioTech BIO-Q mass spectrometer using reported conditions (Van Dorsselaer et al., 1990). The samples were dissolved in aqueous methanol containing 2% acetic acid and placed in the spectrometer. The quadrupole was scanning from m/z 500 to 2000 at a resolution of 800.

RESULTS

Purification and Characterization of CBG. Human CBG was purified from fresh human blood pools by immunoabsorption with an immobilized monoclonal anti-human CBG antibody, followed by elution with a 1 M NH_4OH solution and by anion-exchange chromatography. The overall yield of this purification was 70% (estimated by RIA).

The concentration of steroid binding sites in the freshly purified solution of CBG (15.0 nmol/mL), estimated from the abcissa intercept of Scatchard plots (Scatchard, 1949) established after equilibrium dialysis experiments with tritiated cortisol was about 20% lower than the corresponding CBG concentration as measured by radioimmunoassay (18.0 nmol/mL) or as estimated from UV absorption at 280 nm (18.2 nmol/mL). These measurements indicate that the immunopurification protocol dissociates most bound steroids. No significant losses of binding activity of immunopurified CBG could be observed after 8 days of continuous storage at 4 °C, after a single thawing of the CBG solution frozen at −20 °C. However, in order to limit denaturation, experiments involving incubations of steroid ligands for the characterization and for the photoaffinity labeling of CBG were all performed immediately after the last step of purification. The association constant for cortisol binding to immunopurified CBG at 4 °C (1.0×10^9 M^{-1}), estimated by equilibrium dialysis, was only slightly higher than the association constant measured for corticosterone (0.9×10^9 M^{-1}) but significantly higher than for progesterone (0.6×10^9 M^{-1}). These values were identical to those measured in parallel for the binding of these three steroids to CBG in the initial pool of human sera, after depletion of endogenous steroids with DCC, thus suggesting that the binding affinity of immunopurified CBG had not been significantly altered. These association constants agree in magnitude with those previously reported for the binding of cortisol, corticosterone, or progesterone to purified human CBG, either in the absence or in the presence of reducing agents (Stroupe et al., 1978; Mickelson et al., 1981, 1982) and for the binding of these same steroids to unpurified CBG from human plasma (Westphal, 1971). However, it has been reported that the number of binding sites and the affinity of CBG in human plasma may be underestimated, as compared with those of purified CBG, owing to differences in environment effects (Mueller & Potter, 1981, 1984).

Immunopurified CBG was also characterized by Edman sequencing of its N-terminal part (13 amino acid residues), of three fragments obtained by treatment with human neutrophil elastase of partially deglycosylated CBG (16 amino acid residues of the N-terminal end, 16 amino acid residues from Asn-9 to Phe-24 with the first amino acid transformed in aspartic acid by deglycosylation with *N*-glycosidase, and 16 amino acid residues from Thr-345 to Pro-360) (unpublished experiments from this laboratory), and of the N-terminal end (23 amino acid residues) of the tryptic fragment between Ala-327 and Lys-351. Edman degradation of these five fragments showed no difference with the corresponding sequences established for human CBG (Hammond et al., 1987).

Characterization of the Binding of Δ^6-Cortisol, Δ^6-Corticosterone, and Δ^6-Progesterone to Immunopurified CBG. The association constants for Δ^6-cortisol binding to CBG (1.0×10^9 M^{-1}) as well as those for Δ^6-corticosterone (1.0×10^9 M^{-1}) and Δ^6-progesterone (0.6×10^9 M^{-1}), determined by

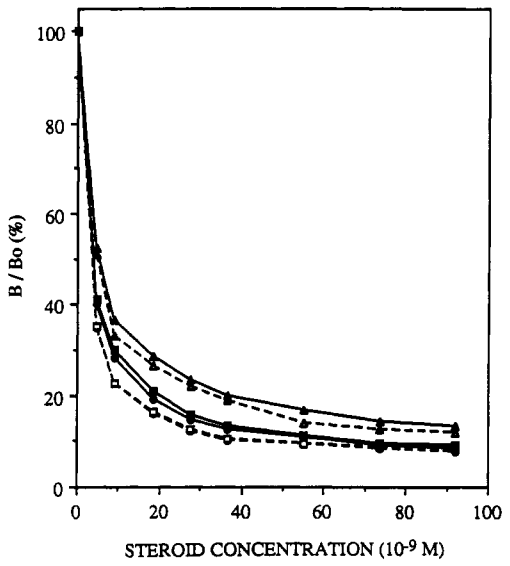

FIGURE 1: Competitive binding assay for cortisol, corticosterone, progesterone, and the corresponding Δ^6-steroid photoreagents with immunopurified CBG. Aliquots (100 μL) of purified CBG (2.0 nM in PBS–gelatin) were incubated with [^{3}H]cortisol (100 μL, 50 000 cpm, 6.8×10^{-13} mol) and seven concentrations (ranging from 5 to 90 nM) of cortisol and of each of the radioinert competitors in 500 μL of PBS–gelatin for 2 h at 4 °C. Free and bound steroids were separated by DCC. For each competitor concentration, the radioactivity of bound [^{3}H]cortisol (B) is expressed as percent of radioactivity bound in the absence of competitor (B_0). Cortisol (○), corticosterone (□), progesterone (△), Δ^6-cortisol (●), Δ^6-corticosterone (■), and Δ^6-progesterone (▲).

equilibrium dialysis at 4 °C with the corresponding tritiated tracers, were not significantly different from the association constants estimated above for cortisol, corticosterone, and progesterone. The abcisssa intercepts of the linear Scatchard plots for the binding of these three Δ^6-derivatives and of cortisol, corticosterone, and progesterone to immunopurified CBG were the same within experimental error, thus suggesting the binding of all these derivatives at the same site.

However, experiments of displacement of [^{3}H]cortisol bound to purified CBG (Figure 1) by Δ^6-cortisol, Δ^6-corticosterone, or Δ^6-progesterone showed that the presence of a Δ^6-double bond leads to a slightly decreased cross-reactivity when compared with the corresponding cortisol, corticosterone, and progesterone ligands. Moreoever, the displacement curves for Δ^6-cortisol and Δ^6-corticosterone were almost superimposable whereas Δ^6-progesterone was significantly less cross-reactive. Conversely, displacement of the binding activity of CBG for Δ^6-[^{3}H]cortisol, Δ^6-[4-^{14}C]cortisol, Δ^6-[^{3}H]corticosterone, or Δ^6-[^{3}H]progesterone by a 100-fold molar excess of either cortisol or of the three corresponding nonradioactive analogs led to a decrease of more than 90% of bound radioactivity (data not shown).

Fluorescence Measurements. The interaction of purified CBG with the three cortisol, corticosterone, and progesterone ligands was analyzed by fluorescence quenching experiments similar to those reported for purified human CBG (Marver et al., 1976; Stroupe et al., 1978; Akhrem et al., 1978) or guinea pig CBG (Mickelson & Westphal, 1979). In the present study, the use of a 295-nm wavelength for the selective excitation of tryptophan residues (Werber et al., 1972; Sontag et al., 1993), resulted in a broad peak of emission culminating between 325 and 330 nm. The higher emission wavelength at 330 nm was observed in the absence of added steroid ligand while a shift to the lower wavelength at 325 nm occurred in the presence of 5β-dihydropregnane-3,20-dione, corresponding presumably to a hydrophobic effect of this steroid (Stroupe et al., 1978). The incubation of CBG with increasing concentrations of the three enone ligands was found to cause similar maximal quenching of ca. 37% of the CBG fluorescence, which was almost totally inhibited in the presence of a 100-fold molar excess of 5β-dihydropregnane-3,20-dione, thus suggesting a specific interaction of all these steroid ligands with a tryptophan residue in the binding site.

Time Course of Photolysis of Δ^6-Cortisol, Δ^6-Corticosterone, and Δ^6-Progesterone Photoreagents. Photolysis of Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone at $\lambda > 300$ nm, using a 2-mm-thick Pyrex filter (Taylor et al., 1980), was followed by recording the UV absorption spectra of the photolyzed samples as a function of irradiation time. Photolysis was complete by about 30 min for Δ^6-progesterone, as observed previously for Δ^6-testosterone (Grenot et al., 1992), whereas photolyses of Δ^6-cortisol and Δ^6-corticosterone were still incomplete after 90 min (Figure 2).

Kinetics of Photoinactivation. Irradiation, for 45 min at $\lambda > 300$ nm, of CBG incubated in the presence of a 10-fold molar excess of Δ^6-cortisol or Δ^6-corticosterone caused the inactivation of about 20% of the initial binding capacity of CBG for cortisol, whereas only 10% inactivation was observed, under similar conditions, after irradiation of CBG incubated in the presence of Δ^6-progesterone (Figure 3). A much slower photoinactivation process, culminating at 3% of the initial binding capacity, after 45-min irradiation, was observed for both unbound (data not shown) and cortisol-bound CBGs, and corresponded probably, for its major part, to photodenaturation of CBG binding sites.

Kinetics of Covalent Photoattachment of Δ^6-[^{3}H]Cortisol, Δ^6-[^{3}H]Corticosterone, and Δ^6-[^{3}H]Progesterone. The time course of photoaffinity labeling was studied at $\lambda > 300$ nm, using CBG samples incubated with a 2-fold molar excess of Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone photoreagents (Figure 4). In all cases, the maximal levels of specific radioactivity covalently attached to CBG were reached after 30-min irradiation. Under these conditions the specific attachment of the three tritiated photoreagents corresponded respectively to 0.21, 0.14, and 0.08 mol of label/mol of CBG, whereas only low and nonspecific labeling occurred under similar conditions with tritiated cortisol, corticosterone, and progesterone ligands. Control experiments, without irradiation, showed a total absence of irreversibly bound radioactivity, thus indicating that photoactivation is necessary for covalent labeling. Increasing the steroid–protein ratio up to a 10/1 molar excess did not augment significantly the amount of specific labeling, but increased the difficulties for purification of photolabeled peptides as observed previously for SHBG (Grenot et al., 1992). In all our experiments the level of specific attachment of Δ^6-[^{3}H]corticosterone has always been found to be lower than that of Δ^6-[^{3}H]cortisol, despite the similarity of photoinactivation curves.

Gel Electrophoresis of Photolabeled CBG. The CBG samples photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, or Δ^6-[^{3}H]progesterone ligands were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. In all cases, the radioactivity was concentrated at the level of the characteristic bands of pure unlabeled CBG, while some radioactivity was also observed at the top of the gel, corresponding probably to the formation of small amounts of irreversible aggregates (Figure 5). On the other hand, the analysis of purified unlabeled CBG by native polyacrylamide gel electrophoresis confirmed the absence of polymeric forms in the CBG employed for photolabeling experiments.

Tryptic Cleavage of Photolabeled CBG. The covalently labeled fractions of CBG were reduced, carboxymethylated,

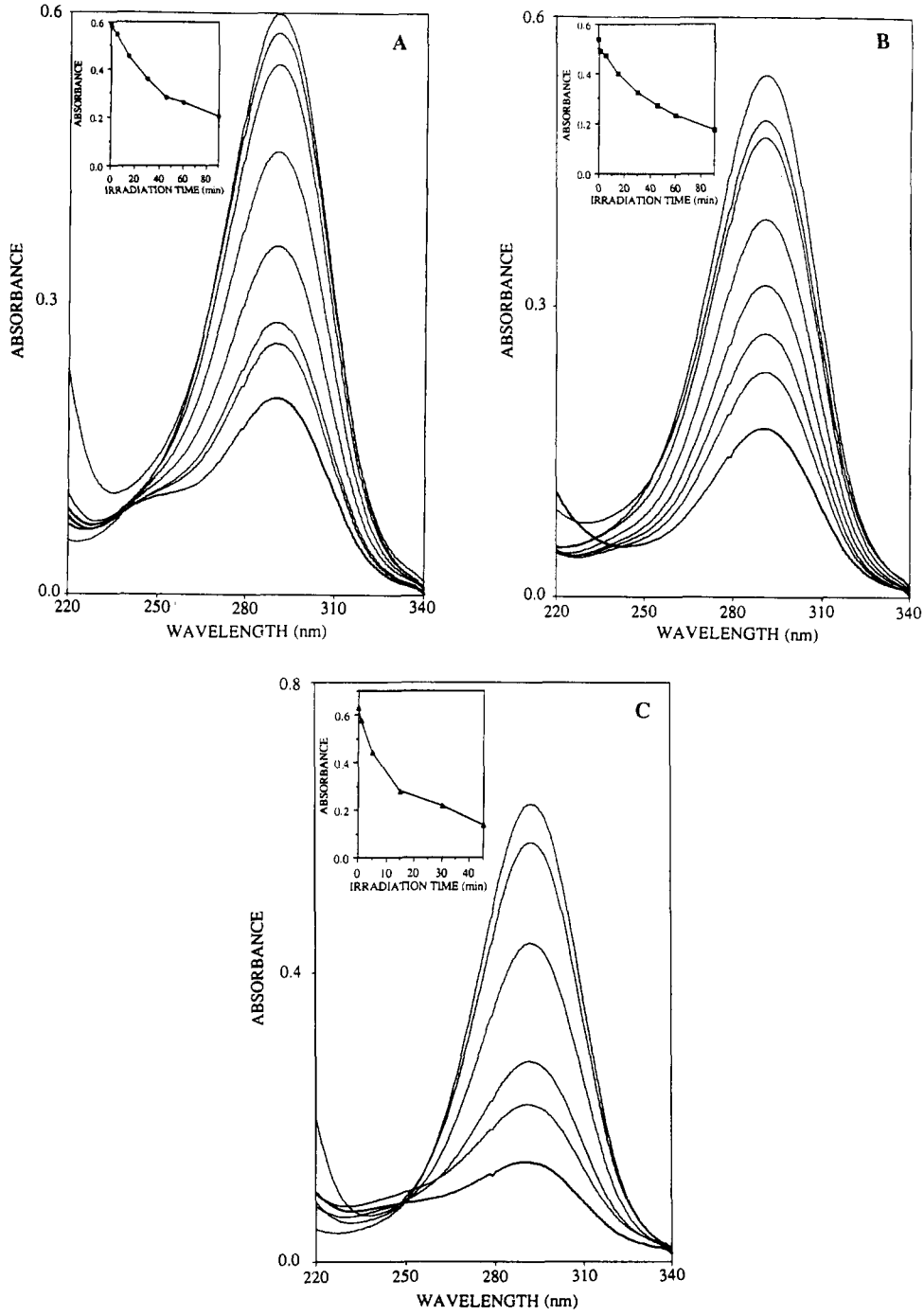

FIGURE 2: Time course of photolysis of Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone. Aliquots (1 mL) of aqueous solutions (PBS containing 0.5% ethanol) of Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone (10 μg/mL) were irradiated at 4 °C, under an argon atmosphere, as described in Experimental Procedures. UV absorbance spectra were recorded between 220 and 340 nm immediately after irradiation, following exposure at $\lambda > 300$ nm for 0, 1, 5, 15, 30, 45, 60, and 90 min [(A) Δ^6-cortisol, (B) Δ^6-corticosterone] and for 0, 1, 5, 15, 30, and 45 min [(C) Δ^6-progesterone].

and digested with trypsin. The radioactivity profiles of tryptic digests of CBG samples photolabeled with Δ^6-[^{3}H]cortisol or Δ^6-[^{3}H]corticosterone were established by reverse-phase HPLC on a C_{18} column using an aqueous acetonitrile gradient in acidic conditions (0.1% TFA). In both cases a major single peak at similar retention times was present and was no longer detectable when a 100-fold molar excess of cortisol was added to the Δ^6-photoreagent (Figure 6). The radioactivity profile of the tryptic digest of CBG photolabeled with Δ^6-[^{3}H]-progesterone showed two peaks. The first one, observed at a retention time similar to that of the major radioactive peaks found above after photolabeling with tritiated Δ^6-cortisol and Δ^6-corticosterone, was completely abolished when a 100-fold molar excess of cortisol was added to the photoreagent, whereas the second one remained unchanged and was therefore not further studied. For the three photoreagents, the total disappearance of radioactivity when the sample was irradiated in the presence of an excess of radioinert cortisol indicates that the corresponding radioactive peaks represent specific photolabeling, as confirmed by a control experiment performed on heat-denatured CBG (30 min at 60 °C) irradiated in the presence of Δ^6-[^{3}H]cortisol photoreagent which gave no radioactive peak at the expected HPLC retention time in the radioactivity profile of the tryptic digest.

Purification of Photolabeled Tryptic Peptides. The purification of the major radioactive peptide from the tryptic digest of CBG photolabeled with Δ^6-[^{3}H]cortisol by three successive chromatographies on a reverse-phase C_{18} HPLC

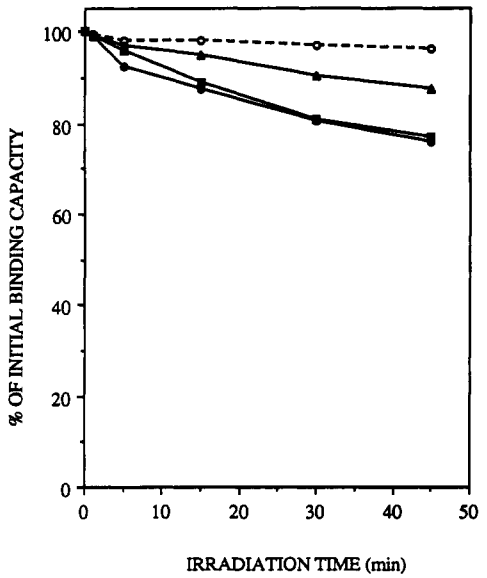

FIGURE 3: Photoinactivation of immunopurified CBG by Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone. Irradiation of CBG incubated with Δ^6-cortisol (●), Δ^6-corticosterone (■), Δ^6-progesterone (▲), and cortisol (○) was performed at $\lambda > 300$ nm for 0, 1, 5, 15, 30, and 45 min, as described in Experimental Procedures. Noncovalently bound steroids were removed by treatment with DCC (1 mL, 1 h at 20 °C). CBG binding activity was measured on aliquots of supernatants (50 μL) incubated with [^{3}H]cortisol (50 000 cpm) for 2 h at 4 °C. Free and bound steroids were separated by DCC.

column using acidic, neutral, and acidic conditions, respectively, led to the separation of the photolabeled peptide from the corresponding unlabeled peptide which was also isolated at a slightly higher retention time. However, this photolabeled peptide, when purified in three steps, was found to contain ca. 20% (estimated from the results of Edman sequencing) of a peptidic contaminant which could be totally eliminated by repeating the third chromatographic step, thus resulting in a pure sample totally photolabeled with Δ^6-[^{3}H]cortisol. The overall yield of this purification is illustrated by the preparative separation of the radioactive tryptic peptide obtained after irradiation of 180 nmol of CBG in the presence of 2-fold molar excess of Δ^6-[^{3}H]cortisol which led to ca. 2 nmol of pure steroid–peptide conjugate. The purification by the same protocol of two samples of tryptic digests photolabeled with Δ^6-[^{3}H]corticosterone, obtained from CBG irradiated in the presence of either 0.5 or 2 mol equiv of photoreagent, showed that the radioactive photolabeled peptide and the unlabeled peptide had identical retention times under all the tested chromatographic conditions, thus resulting in a partially photolabeled peptide. However, the tryptic peptide partially photolabeled with Δ^6-corticosterone contained no peptide contaminant after the third step of the purification protocol. On the other hand, the tryptic peptide photolabeled with Δ^6-[^{3}H]progesterone was easily separated from the unlabeled peptide but could not be totally purified with an acceptable yield, owing to contaminations by radioinert peptides, which proved to be very difficult to eliminate.

Purification of Photolabeled Chymotryptic Peptides. In order to obtain pure radioactive steroid–peptide conjugates with the three Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone photoreagents, the corresponding radioactive tryptic peptides were subcleaved with chymotrypsin. The chymotryptic hydrolysates were chromatographied in one step on a reverse-phase C_{18} HPLC column using an aqueous acetonitrile gradient under acidic conditions (0.1% TFA). The recovery of applied radioactivity varied from one separation to another and ranged from 40% for the low amounts of peptides photolabeled with Δ^6-[^{3}H]corticosterone or Δ^6-[^{3}H]progesterone to 80% for the larger amounts of peptides photolabeled with Δ^6-[^{3}H]cortisol. In all cases, the recovered radioactivity was localized exclusively in two adjacent radioactive peptides, which could be separated, the less retained one corresponding to 75% of the recovered radioactivity. These double peaks of radioactivity appeared at three different retention times (34–35 min, for both Δ^6-[^{3}H]- or Δ^6-[^{14}C]cortisol–peptides, 37–38 min for the Δ^6-[^{3}H]corticosterone–peptides, and 43–44 min for the Δ^6-[^{3}H]progesterone–peptides), thus showing that distinct pairs of steroid–peptide conjugates were obtained with retention times corresponding to the decreasing order of polarity of the three steroids. The use of a much larger excess of chymotrypsin was found to eliminate the minor peak, thus indicating that this peak resulted from an incomplete cleavage. However, this procedure was not employed for a preparative purpose, since under these conditions the radioactive peptide could not be totally purified as above by a single chromatographic step, while further chromatographic separations were found to decrease sharply the recovery of pure peptides, owing probably to strong absorption during the concentration steps,

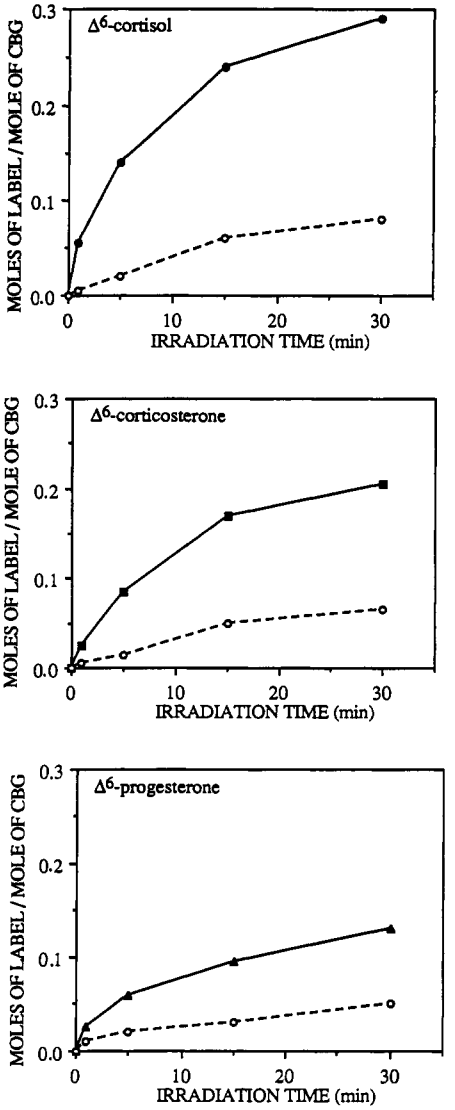

FIGURE 4: Kinetics of covalent attachment of Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone to immunopurified CBG. Irradiation of CBG incubated with Δ^6-[^{3}H]cortisol (●), Δ^6-[^{3}H]corticosterone (■), and Δ^6-[^{3}H]progesterone (▲) in the absence (total labeling) or in the presence (nonspecific labeling) of radioinert cortisol (○) was performed at $\lambda > 300$ nm for 1, 5, 15, and 30 min, as described in Experimental Procedures. The stoichiometry of labeling is expressed as moles of label incorporated per mole of CBG. The specific labeling corresponds to the difference between total labeling and nonspecific labeling.

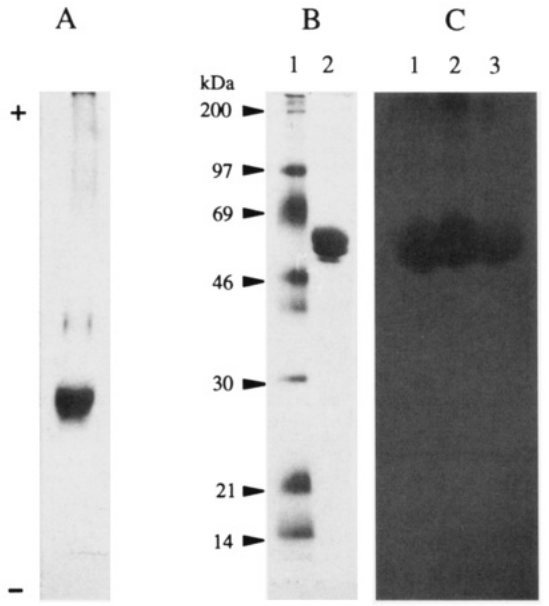

FIGURE 5: Polyacrylamide gel electrophoresis of photolabeled and unlabeled samples of immunopurified CBG: native polyacrylamide (7.5% acrylamide, 2.5% cross-linking) gel electrophoresis of (A) immunopurified CBG stained with $AgNO_3$, and $NaDodSO_4$–polyacrylamide (10% acrylamide) gel electrophoresis of (B) molecular weight markers (lane 1) and unlabeled CBG (lane 2) stained by $AgNO_3$ and (C) CBG photolabeled with Δ^6-[^{3}H]cortisol (lane 1), Δ^6-[^{3}H]corticosterone (lane 2), and Δ^6-[^{3}H]progesterone (lane 3), revealed by fluorography.

especially for peptides photolabeled with Δ^6-[^{3}H]corticosterone and Δ^6-[^{3}H]progesterone.

Edman Sequencing of Photolabeled Peptides. Edman sequencing was performed on two samples of the radioactive tryptic peptide photolabeled with Δ^6-[^{3}H]cortisol (one totally purified and one contaminated with ca. 20% of a single peptide identified as the fragment between Ala-327 and Lys-351 of human CBG sequence) and on the sample of radioactive tryptic peptide photolabeled with Δ^6-[^{3}H]corticosterone, prepared from CBG irradiated in the presence of 0.5 mol equiv of photoreagent (Table 1). For the two samples of the peptide photolabeled with Δ^6-[^{3}H]cortisol, usual yields of PTH derivatives were obtained up to the 13th Edman cycle, whereas a much lower yield was observed at the 14th cycle corresponding to a threonine residue. Furthermore, no PTH derivatives could be detected in the subsequent cycles. The first 14 PTH derivatives were found to correspond to the N-terminal sequence of a tryptic peptide located between the Phe-357 and Arg-378 residues of human CBG. Edman degradation of the tryptic peptide photolabeled with Δ^6-[^{3}H]-corticosterone revealed the same sequence as above, but this peptide could be sequenced up to the last Arg-378 residue, owing to the presence of a major amount of unlabeled peptide, as shown by comparison between the picomoles of covalently bound radioactive tracer and the picomoles of PTH derivatives of the first amino acid (see Table 1), which indicates the presence of ca. 10% of radiolabeled peptide. However, sequence analysis of this peptide showed a very low yield for the tryptophan residue as compared with adjacent amino acids. Such a low yield cannot be explained by the covalent attachment of the photoreagent, which corresponds to only 10% of the peptide, but may be due to partial degradation. On the other hand, the Edman sequencing of this peptide showed no decrease of the yield at the level of threonine, in contrast with the low yield of this residue observed for the two peptide samples totally photolabeled with Δ^6-[^{3}H]cortisol.

The total amount of radioactivity recovered in all the eluates of Edman degradation cycles of the three photolabeled tryptic

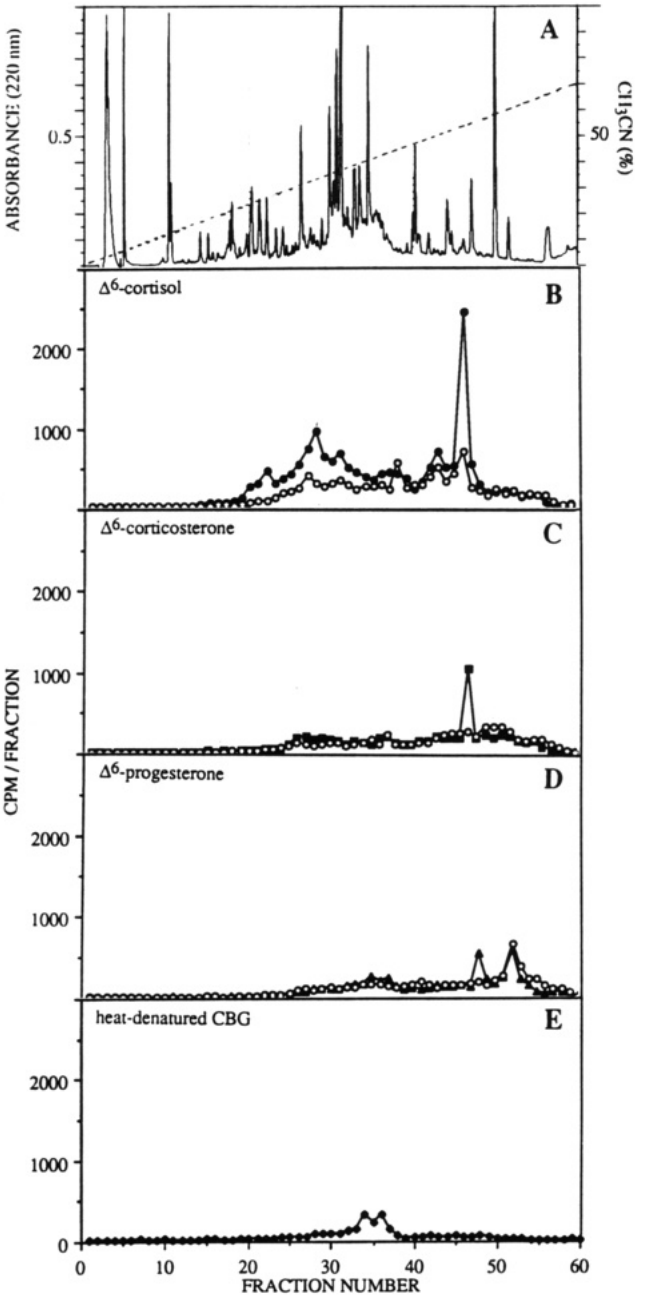

FIGURE 6: Reverse-phase HPLC separation of tryptic peptides of CBG photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone: the tryptic digests were applied on a C_{18} column (Nucleosil 5 mm, 0.46 × 15 cm) and eluted at 1 mL/min using a linear aqueous acetonitrile gradient in the presence of 0.1% TFA (0–70% CH_3CN in 60 min). Peptide elution was monitored at 220 nm. The radioactivity was measured using aliquots (100 μL) of each of the 1-mL fractions; (A) UV absorbance detection of tryptic digest of CBG photolabeled with Δ^6-[^{3}H]cortisol, as described in Experimental Procedure; (B) radioactivity detection of tryptic digest of CBG photolabeled with Δ^6-[^{3}H]cortisol, (●) alone and of CBG photolabeled with Δ^6-[^{3}H]cortisol in the presence of 100-fold molar excess of radioinert cortisol (○); (C) radioactivity detection of tryptic digest of CBG photolabeled with Δ^6-[^{3}H]corticosterone (■) alone and of CBG photolabeled with Δ^6-[^{3}H]corticosterone in the presence of 100-fold molar excess of radioinert cortisol (○); (D) radioactivity detection of tryptic digest of CBG photolabeled with Δ^6-[^{3}H]-progesterone (▲) alone and of CBG photolabeled with Δ^6-[^{3}H]-progesterone in the presence of 100-fold molar excess of radioinert cortisol (○); and (E) radioactivity detection of tryptic digest of heat-denatured CBG (1 h, 60 °C) photolabeled with Δ^6-[^{3}H]cortisol (●).

peptides represented 5–13% of the radioactivity introduced in the sequencer. This percentage range agrees in magnitude with the recovery of radioactivity reported for sequencing experiments on radiolabeled peptides of similar size (Dennis

Table 1: Amino Acid Sequence Analysis of Photolabeled Tryptic Peptides

		Δ^6-[^{3}H]cortisol–tryptic peptide				Δ^6-[^{3}H]corticosterone–tryptic peptide:	
		335 000 dpm (400 pmol)[a]		140 000 dpm (229 pmol)[a]		300 000 dpm (90 pmol)[b]	
cycle	amino acid	PTH deriv (pmol)[c]	radioactivity (dpm)	PTH deriv (pmol)	radioactivity (dpm)	PTH deriv (pmol)	radioactivity (dpm)
1	F	329	411	172	117	780	425
2	N	291	459	84	147	680	689
3	Q	376	857	86	264	600	1512
4	P	308	617	56	384	442	1248
5	F	257	689	41	120	364	1162
6	I	202	891	33	387	341	1388
7	I	196	857	*d*	325	374	1285
8	M	137	706	27	199	429	1183
9	I	84	439	21	281	235	1320
10	F	99	329	28	319	277	1340
11	D	*c*	363	25	195	175	1327
12	H	40	309	10	202	86	2256
13	F	53	326	18	123	197	2527
14	T	5.6	377	3	353	189	2345
15	W	(5.0)[e]	4166	(2.7)[e]	1670	21 (+1.5)[e]	5098
16	S		3086		593	105	4166
17	S		2109		610	117	3082
18	L		857		394	30	2790
19	F		411		281	27	1838
20	L		336		206	21	1358
21	A		391		237	29	1145
22	R		363		295	4	1118

[a] Picomoles of labeled peptide applied to the sequencer calculated from the specific activity of the photoreagent. [b] Picomoles of labeled peptide present in the unlabeled peptide calculated from the specific activity of the photoreagent. [c] Peptide partially contaminated with the peptide fragment 327–351 containing a D residue at cycle 11. [d] Residue identified but not quantified. [e] Picomoles of amino acid estimated from the radioactivity eluted at the 15th Edman cycle.

et al., 1988). Moreover, no significant residual radioactivity was found on the filter. The major peak of radioactivity of these peptides contained from 1.2 to 1.7% of the applied radioactivity and was eluted in all cases at the 15th Edman cycle, corresponding to Trp-371, thus indicating that the radioactivity covalently bound to this amino acid is relatively stable under the conditions of Edman sequencing. Smaller peaks of radioactivity were also eluted in decreasing amounts in the following cycles.

Edman sequencing of the major radioactive peak of chymotryptic peptides photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[4-^{14}C]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone respectively showed a single sequence, Thr-(*Xaa)-Ser-Ser-Leu-Phe (Table 2), identified as the hexapeptide fragment 370–375 of human CBG sequence (Hammond et al., 1987). The minor peak, isolated only in the case of the chymotryptic peptide photolabeled with Δ^6-[^{3}H]cortisol, was found to contain the Asp-His-Phe-Thr-(*Xaa)-Ser-Ser-Leu-Phe sequence (Table 3) corresponding to the nonapeptide fragment 367–375, resulting from an incomplete cleavage by chymotrypsin, due possibly to the presence of the photolabeling steroid. The yields of the PTH derivative of threonine for the steroid–hexapeptide conjugates and for the Δ^6-[^{3}H]cortisol–nonapeptide conjugate were relatively low but in much better agreement with the amount of peptide introduced in the sequencer (hexapeptide conjugates) or with the picomoles of the preceding amino acid residue (nonapeptide conjugate) than observed above for the tryptic peptide photolabeled with Δ^6-[^{3}H]cortisol. No trace of PTH derivative of tryptophan could be detected in the eluate of the second Edman cycle for the four steroid–hexapeptide conjugates and in the eluate of the fifth cycle, for the nonapeptide conjugate. On the other hand, in contrast with the tryptic peptides photolabeled with Δ^6-[^{3}H]cortisol, the four last amino acid residues could be unambiguously identified but were recovered in much lower yields for the nonapeptide photolabeled with Δ^6-[^{3}H]cortisol and for the hexapeptides photolabeled with Δ^6-[^{3}H]corticosterone or Δ^6-[^{3}H]progesterone than for the hexapeptide photolabeled with Δ^6-[^{3}H]cortisol.

The major part of the radioactivity of the three hexapeptides photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone was recovered in the PTH eluates (71, 60, and 47% of the total radioactivity added to the sequencer, respectively) and in the different washes (14, 39, and 53%). In all cases, no significant amount of radioactivity was found on the filter. The major peaks of radioactivity (34, 28, and 22% of the total radioactivity) were eluted at the second Edman cycle corresponding to Trp-371 (Table 2) whereas radioactivity was still released in decreasing amounts in the four next cycles representing a cumulated percentage similar to that observed for the major peaks (37, 31, and 20%). A significant amount of radioactivity was also present in the eluate of the PTH derivative of threonine for the hexapeptide photolabeled with Δ^6-[^{3}H]progesterone. The elution of the major peak of radioactivity at the level of Trp-371 residue was also confirmed by sequencing the nonapeptide photolabeled with Δ^6-[^{3}H]-cortisol and the Δ^6-[4-^{14}C]cortisol–hexapeptide conjugate.

Amino Acid Composition Analysis. Amino acid analysis of the acid hydrolyzate of the purified Δ^6-[^{3}H]cortisol steroid–hexapeptide conjugate (1 nmol, estimated from the specific activity of the steroid photoreagent) showed the presence of 0.86, 1.10, and 1.00 nmol of the three T, L, and F amino acids, respectively, and of 2.07 nmol of S amino acids corresponding to the two S residues, whereas no trace of tryptophan could be identified. The absence of any other detectable amino acid residues confirmed the purity of the radioactive hexapeptide. Furthermore, no radioactivity could be detected at the level of the peaks of the phenylthiocarbamyl derivatives of the T, S, L, and F amino acids in the HPLC separation, thus confirming the absence of contamination of these derivatives by products of degradation of the photolabeled amino acid.

Mass Spectrometry. The molecular masses of the steroid–peptide conjugates were determined by liquid secondary ion mass spectrometry and by electrospray mass spectrometry. Liquid secondary ion mass spectrometry of the tryptic peptide 357–378 photolabeled with Δ^6-[^{3}H]cortisol (Figure 7A) showed a $[M + H]^+$ ion at m/z 3092, whereas the $[M + H]^+$ ion of the unlabeled peptide (Figure 7B) was found at m/z 2732. These values are in good agreement with the corresponding calculated masses of 3092.7 and 2732.2, respectively, assuming the addition of 1 mol of photoreagent/mol of peptide. A small amount of the ion at m/z 2732 was also observed in the mass spectrum of the photolabeled peptide and may correspond either to a contamination of the sample with the unlabeled peptide or to a partial loss of the photolabel. The liquid secondary ion mass spectrum (data not shown) of the sample of tryptic peptide partially photolabeled with Δ^6-[^{3}H]corticosterone, isolated from CBG irradiated in the presence of a 2-fold molar excess of photoreagent, exhibited also two $[M + H]^+$ ions at m/z 2732 (major peak) and at m/z 3076 (small signal). The presence of these two molecular ions was confirmed by the measurement of exact masses of 2731.9692 and 3076.0256, in good agreement with calculated masses of 2732.2 and 3076.7 for the $[M + H]^+$ ions of the corresponding unlabeled and photolabeled peptides, and by the observation of two characteristic protonated $[M + 3H]^{3+}$ ions at m/z 911.5 (major peak) and m/z 1026.3 in the electrospray mass spectrum of the same peptide sample which correspond to measured masses of 2731.5 and 3075.9 Da

Table 2: Amino Acid Sequence Analysis of Photolabeled Chymotryptic Hexapeptides

		Δ^6-[^{3}H]cortisol–hexapeptide 25 500 dpm (400 pmol)[a]				Δ^6-[^{3}H]corticosterone–hexapeptide 31 330 dpm (155 pmol)[a]				Δ^6-[^{3}H]progesterone–hexapeptide 14 850 dpm (195 pmol)[a]				Δ^6-[^{14}C]cortisol–hexapeptide 1600 dpm (95 pmol)[a]	
			radioactivity (dpm)				radioactivity (dpm)				radioactivity (dpm)				radioactivity (dpm)
cycle	amino acid	PTH deriv (pmol)	S_1, S_2 wash[b]	PTH elution	S_3 wash[b]	PTH deriv (pmol)	S_1, S_2 wash[b]	PTH elution	S_3 wash[b]	PTH deriv (pmol)	S_1, S_2 wash[b]	PTH elution	S_3 wash[b]	PTH deriv (pmol)	PTH elution
B[c]			305	62	45		1751	142	2167		2246	40	2404		
1	T	257	523	83	118	77.4	1361	202	2137	82	1106	896	588	44	22
2		(135)[d]	1058	8584	54	(43)[d]	1560	8624	896	(42)[d]	543	3200	265	(23)[d]	395
3	S	58	544	4116	41	4.1	801	4763	443	7	272	1336	154	9	135
4	S	55	344	2496	32	3.5	287	2556	167	3.7	113	904	71	*e*	70
5	L	33	266	1700	22	1.8	185	1539	115	2.3	64	512	46	5.6	30
6	F	10.5	166	1104	16	0.5	104	868	129	0.5	38	160	30	2.3	20

[a] Picomoles of labeled peptide applied to the sequencer calculated from the specific activity of the photoreagent. [b] Washing steps performed with *n*-heptane (S_1) and ethyl acetate (S_2), after derivatization with phenyl isothiocyanate, and with 1-chlorobutane (S_3), after elution of the PTH derivative. [c] Blank Edman cycle (B) performed without TFA cleavage and with the S_3 wash before S_1 and S_2 washes. [d] Picomoles of amino acid estimated from the radioactivity eluted at the second Edman cycle. [e] Residue identified but not quantified.

Table 3: Amino Acid Sequence Analysis of Photolabeled Chymotrypic Nonapeptide

Δ^6-[^{3}H]cortisol–nonapeptide 3820 dpm (60 pmol)[a]			
cycle	amino acid	PTH deriv (pmol)	radioactivity (dpm)
1	D	44	33
2	H	18	26
3	F	57	51
4	T	19	51
5		(11.3)[b]	723
6	S	1.6	291
7	S	*c*	121
8	L	0.7	51
9	F		48

[a] Picomoles of labeled peptide applied to the sequencer calculated from the specific activity of the corresponding radioactive photoreagent. [b] Picomoles of amino acid estimated from the radioactivity eluted at the fifth Edman cycle. [c] Residue identified but not quantified.

(Figure 8). Electrospray mass spectrometry of the hexapeptide photolabeled with Δ^6-[^{3}H]cortisol (Figure 9) showed a characteristic protonated $[M + H]^+$ ion at m/z 1101 which corresponded well to the calculated $[M + H]^+$ molecular ion value 1101.3. Under similar conditions, the $[M + H]^+$ ions of the two other hexapeptides photolabeled with Δ^6-[^{3}H]-corticosterone and Δ^6-[^{3}H]progesterone could also be detected at m/z 1085 and 1053 (data not shown), as expected from the calculated $[M + H]^+$ molecular ion values 1085.3 and 1053.3, but the intensities of the corresponding peaks were much lower than for the peptide photolabeled with Δ^6-[^{3}H]cortisol, especially in the case of the peptide photolabeled with Δ^6-[^{3}H]progesterone.

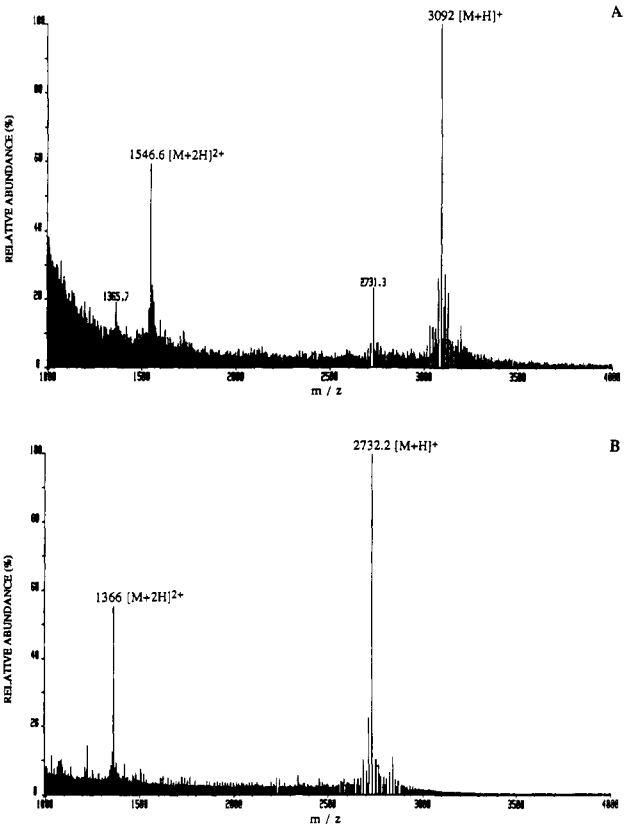

FIGURE 7: Liquid secondary ion mass spectrometry: (A) mass spectrum of the tryptic peptide Phe-357 to Arg-378 photolabeled with Δ^6-cortisol and (B) mass spectrum of unlabeled tryptic peptide Phe-357 to Arg-378.

DISCUSSION

The site-specific character of the photoaffinity labeling of CBG with the Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone reagents is shown by the parallelism of the time course of covalent incorporation of each of these tritiated photoreagents with the time course of photoinactivation by the corresponding radioinert reagents and with the extent of photolysis of the steroid photolabels alone as well as by the almost total inhibition of photolabeling in the presence of an excess of cortisol. The levels of specific incorporation of Δ^6-cortisol and Δ^6-progesterone photoreagents (0.21 and 0.08 mol of steroid/mol of CBG) are in agreement with the decreasing order of association constants and probably reflect differences in dissociation rates, as suggested by the half-times of dissociation at 4 °C of cortisol and of progesterone bound to human CBG (4 and 1 min, respectively) (Westphal, 1983). Differences in dissociation rates, probably similar to those reported for cortisol and corticosterone (Stroupe et al., 1978), may also explain the lower yields of photolabeling with Δ^6-corticosterone as compared with Δ^6-cortisol although these two ligands have the same affinity constants. Attempts to increase the photolabeling yields with Δ^6-cortisol and Δ^6-corticosterone by extending irradiation times from 30 up to 90 min failed, despite the slow rate of photoconversion of these two chromophores in the absence of CBG, owing probably to progressive denaturation effects.

Sequence analysis of the major radioactive tryptic peptides isolated from human CBG photolabeled with Δ^6-[^{3}H]cortisol and Δ^6-[^{3}H]corticosterone showed that these peptides correspond to the fragment 357–378 of human CBG sequence. Edman degradation of the major radioactive chymotryptic peptides obtained after subcleavage of the radioactive tryptic

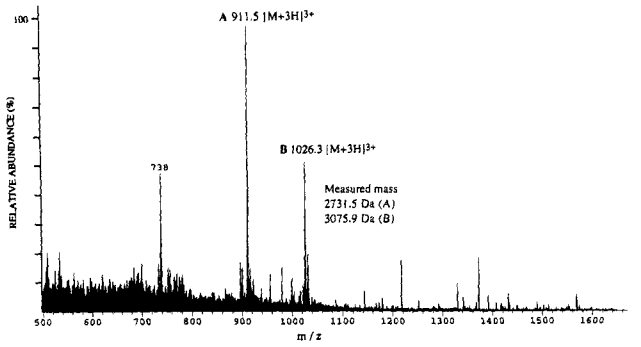

FIGURE 8: Electrospray ionization mass spectrum of tryptic peptide Phe-357 to Arg-378 photolabeled with Δ^6-corticosterone (mixture of unlabeled peptide and of peptide photolabeled with Δ^6-corticosterone).

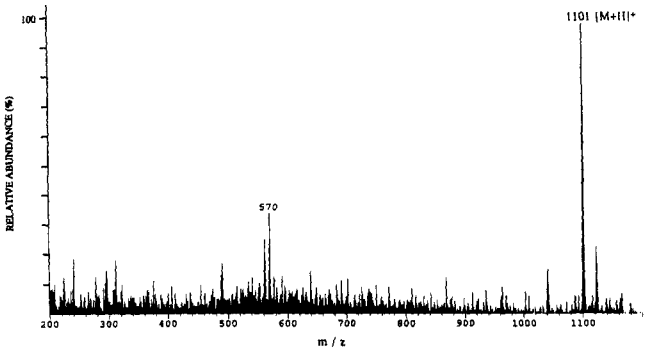

FIGURE 9: Electrospray ionization mass spectrum of the T-(*W)-S-S-L-F hexapeptide photolabeled with Δ^6-cortisol.

peptides photolabeled with Δ^6-[^{3}H]cortisol, Δ^6-[4-^{14}C]cortisol, Δ^6-[^{3}H]corticosterone, and Δ^6-[^{3}H]progesterone showed in all cases a T-(*W)-S-S-L-F sequence corresponding to the hexapeptide fragment encompassing amino acid residues 370–375 of human CBG, whereas the minor chymotryptic peptide photolabeled with Δ^6-[^{3}H]cortisol appeared to represent the nonapeptide fragment 367–375 containing the additional D-H-F peptide sequence at the N-terminal end. In all these sequencing experiments, except for the tryptic peptide obtained from CBG partially photolabeled with Δ^6-[^{3}H]corticosterone which contains predominantly the unlabeled peptide, no identifiable PTH derivative could be found at the Edman cycle corresponding to the Trp-371 residue, but for all peptides, the major peak of radioactivity was eluted at this cycle. Moreover, the elution of the major peak of radioactivity at the second cycle of Edman degradation of the radioactive hexapeptide photolabeled with Δ^6-[4-^{14}C]cortisol confirms that the major peaks of radioactivity observed at the second Edman cycle with the tritiated photolabeling reagents correspond to the presence of a photoreagent and do not represent artifactual labeling due to tritium exchange or to contaminations with tritiated byproducts. The picomoles of the missing photolabeled tryptophan residue could be estimated from the specific activity of the photolabeling steroids and from the radioactivity eluted at the corresponding Edman cycle, assuming that 1 mol of steroid is attached per mole of peptide, as shown by mass spectrometry. In all cases, these calculated values were significantly lower but still agreed in magnitude with the values measured for the PTH derivative of the preceding amino acid residue. Therefore, all these observations argue strongly that Trp-371 is the main site of specific photoaffinity labeling with the radioactive Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone reagents.

On the other hand, the PTH derivatives of the four S, S, L, and F amino acid residues beyond Trp-371, which were undetectable in the case of the tryptic peptides photolabeled with Δ^6-[^{3}H]cortisol, could be unambiguously identified in the case of the smaller photolabeled chymotryptic peptide fragments. The rapidly decreasing yields of Edman sequencing of these steroid–peptide conjugates may be due to the strong hydrophobicity of L and F residues, which could increase markedly the washout of residual peptide remaining on the filter. The additional effect of the higher hydrophobicity of derivatives of corticosterone or progesterone as compared with derivatives of cortisol could explain the presence of increased amounts of radioactivity in the wash steps of Edman degradations of hexapeptides photolabeled with Δ^6-[^{3}H]-corticosterone and Δ^6-[^{3}H]progesterone and the much lower yields of S, L, and F residues for these two peptides. For all the photolabeled peptide conjugates, additional peaks of radioactivity were present in decreasing amounts in the four Edman cycles beyond Trp-371, but their intensities exceeded significantly the usual extent of carry over effects (estimated at 10–20% for the first T residue), thus suggesting the possibility of a partial photolabeling of these residues.

The recovery, in low but usual yields, of the PTH derivatives of the T residue in all photolabeled chymotryptic hexapeptide and nonapeptide conjugates contrasts with the sharp decrease in the sequencing yield which occurred at the level of the same residue in the case of the larger tryptic peptide precursor photolabeled with Δ^6-[^{3}H]cortisol. The explanation for this inhibiting effect remains to be determined. The low fractions of radioactivity eluted at the level of the T residue in all peptide fragments photolabeled with Δ^6-[^{3}H]cortisol demonstrate the absence of any significant amount of photoreagent covalently attached at this site in these peptides. On the other hand, the much lower amount of radioactivity observed at the level of the T residue for the hexapeptide photolabeled with Δ^6-[^{3}H]-corticosterone than for the corresponding partially photolabeled tryptic peptide precursor did not confirm the presence of covalently bound radioactivity at this site while the significant amount of radioactivity eluted at the level of the PTH derivative of the T residue in Edman degradation of the hexapeptide photolabeled with Δ^6-[^{3}H]progesterone suggests the possibility of partial labeling of Thr-370 in this peptide.

Amino acid analysis of the acid hydrolyzate of the purified T-(*W)-S-S-L-F hexapeptide conjugate photolabeled with Δ^6-[^{3}H]cortisol gave an almost quantitative recovery of unmodified T, S, L, and F amino acids whereas no tryptophan could be detected, thus suggesting that this tryptophan might be the unique site of photolabeling of human CBG with Δ^6-[^{3}H]cortisol, since the coupling of only 1 mol of steroid/mol of peptide was established by mass spectrometry before hydrolysis. However, in these experiments, the absence of tryptophan cannot be put forward as an argument for establishing the presence of covalently bound photoreagent exclusively at this site, since the usual acidic conditions employed in this work for peptide hydrolysis are known to alter tryptophan residues while the hypothesis of an acid-labile partial photolabeling of T, S, L, and F amino acids leading, after hydrolysis, to a quantitative formation of the corresponding unmodified residues cannot be excluded. On the other hand, the hexapeptides photolabeled with Δ^6-[^{3}H]-corticosterone and Δ^6-[^{3}H]progesterone could not be purified in amounts sufficient for precise determination of amino acid composition.

The mass spectra of the two tryptic peptides photolabeled with Δ^6-cortisol and Δ^6-corticosterone and of the chymotryptic hexapeptides photolabeled with Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone showed molecular ion values which indicate the photochemical addition of 1 mol of steroid photoreagent/mol of peptide and establish unambiguously the formation of covalent steroid–peptide conjugates.

The mechanism of photoaffinity labeling with the Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone ligands is probably similar to that mentioned for the interpretation of the photoreactivity of Δ^6-testosterone with steroid-binding sites (Grenot et al., 1992). The most plausible hypothesis involves a photochemical conversion of the dienone chromophore to an excited triplet diradical that should react preferentially by hydrogen abstraction. On the other hand, the three $\Delta^{4,6}$-3-oxosteroid photoreagents employed in this work do not meet the criteria required for a mechanism involving an energy transfer with photoexcited tryptophan (Goeldner et al., 1980). Moreover, a control experiment with Δ^6-[^{3}H]cortisol (data not shown), using irradiation conditions at $\lambda > 315$ nm (Payne et al., 1980), which should restrict direct photoexcitation of tryptophan residues, was found to give results similar to other photolabeling experiments performed in this work at $\lambda > 300$ nm. Other possible mechanisms involving photooxidation processes can be ruled out since the experiments performed under inert atmosphere were found to improve the yields of photoaffinity labeling (data not shown), as reported for SHBG (Grenot et al., 1992).

The ability of the steroid binding site of human CBG to accommodate cortisol, corticosterone, and progesterone (Murphy, 1969) as well as 11-, 16-, 17-, and 21-substituted derivatives (Le Gaillard & Dautrevaux, 1977; Defaye et al., 1980; Berko & Pearlman, 1981) suggests a loose interaction of rings C or D with the binding site. Conversely, it has been shown that the structures of rings A and B, which are common to all ligands, are more specifically recognized (Le Gaillard & Dautrevaux, 1977) since neither 2α-hydroxyprogesterone nor dexamethasone could displace bound cortisol, although 6β-bromoprogesterone (Khan & Rosner, 1977) or 6α- or 6β-hydroxyprogesterone, but not 6α- or 6β-(bromoacetoxy)-progesterone, could bind CBG. A more detailed study of the binding specificity of purified human CBG has also shown the importance of a 10β-methyl group and of the Δ^4-double bond for optimal binding (Mickelson et al., 1981). In the present work, the introduction of a Δ^6-double bond on the cortisol ligand did not seem to alter significantly the binding affinity for CBG, in contrast to the reduction of cortisol to 5β-tetrahydrocortisol, which was reported to result in the complete loss of binding affinity (Murphy, 1969).

Several amino acids which could play a role in the steroid binding properties of human CBG have been previously identified by specific chemical modifications and by labeling experiments. The chemical detection of a single tyrosine residue (Le Gaillard et al., 1982) and of a tryptophan residue (Akhrem et al., 1981) which could correspond to the Trp-371 identified in this work is in keeping with the results of fluorescence quenching studies of the interaction of human CBG with 3-enone ligands (Stroupe et al., 1978). However, it is probable that tryptophan only is involved in the fluorescence quenching effect, as shown in this work by selective excitation of tryptophan at 295 nm (Werber et al., 1972; Sontag et al., 1993), thus confirming the role of tryptophan as fluorophore, as mentioned in earlier reports (Marver et al. 1976; Akhrem et al. 1978). Alkylations with 16α-(bromoacetoxy)progesterone, 11α-(bromoacetoxy)progesterone, and 17β-(bromoacetoxy)testosterone affinity labeling reagents have suggested a model of the steroid binding site of human CBG in which the 11β-hydroxy and the 20-keto groups respectively interact with methionine and histidine residues (Le Gaillard & Dautrevaux, 1977). On the other hand, Cys-228 has been proposed from interspecies sequence correlation (Hammond et al., 1987; Hammond, 1990) as the probable alkylation site of human CBG with 6β-bromoprogesterone (Khan & Rosner, 1977). This assignment concords with the results of the comparison of the primary structure of human CBG with the three-dimensional structure of cleaved α_1-proteinase inhibitor (Löbermann et al., 1984; Huber & Carrell, 1989), a related protein of the same SERPIN superfamily, which showed that Cys-228 in the human CBG sequence was located within a proposed β-barrel that could represent at least a portion of the steroid binding site (Underhill et al., 1989; Seralini et al., 1990; Smith et al., 1991, 1992). However, in a recent report (Ghose-Dastidar et al., 1994), the steroid binding affinity of human CBG was found unchanged after site-directed mutagenesis of Cys-228 to serine or alanine whereas the inactivation effects produced by SH-specific chemical modifications were put forward as an argument supporting a possible role of the other Cys-60 residue.

The results presented in this paper demonstrate that human CBG can be specifically photolabeled with radioactive Δ^6-cortisol, Δ^6-corticosterone, and Δ^6-progesterone ligands and provide indirect evidence that the same Trp-371 is the main site of covalent labeling of human CBG with these three photoreagents. This tryptophan residue is located in the poorly conserved C-terminal hydrophobic segment of human CBG (Smith et al., 1989; Seralini et al., 1990). However, the T-W-S-S-L-F hexapeptide fragment corresponding to the photolabeled Trp-371 is conserved in the sequences established for rat CBG (Smith & Hammond, 1989), rabbit CBG (Seralini et al., 1990), and sheep CBG (Berdusco et al., 1993), thus suggesting that Trp-371 as well as other amino acid residues of this peptide could play a functional role in the steroid-binding properties of CBG, as confirmed by the cleavage of human CBG by human neutrophil elastase between Val-344 and Thr-345, which led to a large decrease of the binding affinity for cortisol (Pemberton et al., 1988; Hammond et al., 1990).

However, the experimental data reported here cannot exclude the possibility of a partial photolabeling of one or several amino acid residues other than the Trp-371 in the hexapeptide 370–375, which may reflect differences in the rigidity of positioning of the steroid ligands in the binding site. The importance of other amino acids in the human CBG sequence, especially those present in the photolabeled hexapeptide or in the vicinity of this peptide, for the specific recognition of the different steroid ligands remains to be established by complementary affinity labeling studies using other photoreagents, by the characterization of the photolabeled amino acid residues, and by experiments of site-directed mutagenesis. Further investigations on the structure of the steroid–peptide covalent conjugates will help to determine the nature of the photochemical processes involved in their formation and may provide more detailed information on the positioning of the photoreagents in the steroid binding site.

ADDED IN PROOF

After this manuscript was completed for publication, Drs. Hammond and Avvakumov kindly informed us that site-directed mutagenesis experiments had been performed in their laboratory on the four tryptophan residues of human CBG (Avvakumov & Hammond, 1994). The results of these studies point out the conclusion that Trp-371 is most likely located in the steroid binding site.

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REFERENCES

Agnello, E. J., & Laubach, G. D. (1960) *J. Am. Chem. Soc. 82*, 4293–4299.

Akhrem, A. A., Avvakumov, G. V., Kukushkina, I. I., Prishchepov, A. S., Sviridov, O. V., & Strel'chyonok, O. A. (1978) *Bioorg. Khim. 4*, 421–423.

Akhrem, A. A., Sviridov, O. V., Strel'chyonok, O. A., & Prishchepov, A. S. (1981) *Bioorg. Khim. 7*, 662–669.

Avvakumov, G. V., & Hammond, G. L. (1994) *J. Steroid Biochem. Mol. Biol.* (in press).

Avvakumov, G. V., Warmels-Rodenhiser, S., & Hammond, G. L. (1993) *J. Biol. Chem. 268*, 862–866.

Berdusco, E. T. M., Hammond, G. L., Jacobs, R. A., Grolla, A., Akagi, K., Langlois, D., & Challis, J. R. G. (1993) *Endocrinology 132*, 2001–2008.

Berko, R. M., & Pearlman, W. H. (1981) *J. Steroid Biochem. 14*, 1035–1039.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Defaye, G., Basset, M., Monnier, N., & Chambaz, E. M. (1980) *Biochim. Biophys. Acta 623*, 280–294.

Dennis, M., Giraudat, J., Kotziba-Hibert, F., Goeldner, M., Hirth, C., Chang, J. Y., Lazure, C., Chrétien, M., & Changeux, J. P. (1988) *Biochemistry 27*, 2346–2357.

Ghose-Dastidar, J., Ross, J. B. A., & Green, R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6408–6412.

Ghose-Dastidar, J., Green, R., & Ross, J. B. A. (1994) *J. Steroid Biochem. Mol. Biol. 48*, 139–144.

Goeldner, M. P., & Hirth, C. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6439–6442.

Gray, G. O., Rundle, S., & Leavitt, W. W. (1987) *Biochim. Biophys. Acta 926*, 40–53.

Grenot, C., de Montard, A., Blachère, T., Rolland de Ravel, M., Mappus, E., & Cuilleron, C. Y. (1992) *Biochemistry 31*, 7609–7621.

Hammond, G. L. (1990) *Endocr. Rev. 11*, 65–79.

Hammond, G. L., & Lähtenmäki, P. L. A. (1983) *Clin. Chim. Acta 132*, 101–110.

Hammond, G. L., Smith, C. L., Goping, I. S., Underhill, D. A., Harley, M. J., Reventos, J., Musto, N. A., Gunsalus, G. L., & Bardin, C. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5153–5157.

Hammond, G. L., Smith, C. L., Paterson, N. A. M., & Sibbald, W. J. (1990) *J. Clin. Endocr. Metab. 71*, 34–39.

Hammond, G. L., Smith, C. L., & Underhill, D. A. (1991) *J. Steroid Biochem. Mol. Biol. 40*, 755–762.

Heukeshoven, J., & Dernick, R. (1985) *Electrophoresis 6*, 103–112.

Khan, M. S., & Rosner, W. (1977) *J. Biol. Chem. 252*, 1895–1900.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Le Gaillard, F., & Dautrevaux, M. (1977) *Biochim. Biophys. Acta 495*, 312–323.

Le Gaillard, F., Racadot, A., Aubert, J. P., & Dautrevaux, M. (1982) *Biochimie 64*, 153–158.

Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951–8966.

Löbermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531–556.

Marver, D., Chiu, W.-H., Wolff, M. E., & Edelman, I. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4462–4466.

Mickelson, K. E., & Westphal, U. (1979) *Biochemistry 18*, 2685–2690.

Mickelson, K. E., Forsthoefel, J., & Westphal, U. (1981) *Biochemistry 20* 6211–6218.

Mickelson, K. E., Harding, G. B., Forsthoefel, M., & Westphal, U. (1982) *Biochemistry 21*, 654–660.

Mueller, U. W., & Potter, J. M. (1981) *Biochem. J. 197*, 645–653.

Mueller, U. W., & Potter, J. M. (1984) *J. Steroid Biochem. 20*, 1261–1266.

Murphy, B. E. P. (1969) *Recent Progr. Hormone Res. 25*, 563–601.

Nyberg, L., Marekov, L. N., Jones, I., Lundquist, G., & Jornvall, H. (1990) *J. Steroid Biochem. 35*, 61–65.

Payne, D. W., Katzenellenbogen, J. A., & Carlson, K. E. (1980) *J. Biol. Chem. 255*, 10359–10367.

Pemberton, P. A., Stein, P. E., Pepys, M. B., Potter, J. M., & Carrell, R. W. (1988) *Nature (London) 336*, 257–258.

Pugeat, M., Bonneton, A., Perrot, D., Rocle-Nicolas, B., Lejeune, H., Grenot, C., Déchaud, H., Brébant, C., Motin, J., & Cuilleron, C. Y. (1989) *Clin. Chem. 35*, 1675–1679.

Rosner, W. (1990) *Endocr. Rev. 11*, 80–91.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Seralini, G.-E. (1991) *C. R. Soc. Biol. 185*, 500–509.

Seralini, G.-E., Bérubé, D., Gagné, R., & Hammond, G. L. (1990a) *Human Genet. 86*, 73–75.

Seralini, G.-E., Smith, C. L., & Hammond, G. L. (1990b) *Mol. Endocrinol. 4*, 1166–1172.

Smith, C. L., & Hammond, G. L. (1988) *Steroids 52*, 331–332.

Smith, C. L., & Hammond, G. L. (1989) *Mol. Endocrinol. 3*, 420–426.

Smith, C. L., & Hammond, G. L. (1991) *J. Biol. Chem. 266*, 18555–18559.

Smith, C. L., Power, S. G. A., & Hammond, G. L. (1992) *J. Steroid Biochem. Mol. Biol. 42*, 671–676.

Sontag, B., Reboud, A. M., Divita, G., Di Pietro, A., Guillot, D., & Reboud, J. P. (1993) *Biochemistry 32*, 1976–1980.

Strel'chyonok, O. A., & Avvakumov, G. V. (1990) *J. Steroid Biochem. 35*, 519–534.

Strel'chyonok, O. A., & Avvakumov, G. V. (1991) *J. Steroid Biochem. Mol. Biol. 40*, 795–803.

Stroupe, S. D., Harding, G. B., Forsthoefel, M. W., & Westphal, U. (1978) *Biochemistry 17*, 177–182.

Underhill, D. A., & Hammond, G. L. (1989) *Mol. Endocrinol. 3*, 1448–1454.

Taylor, C. A., Jr., Smith, H. E., & Danzo, B. J. (1980) *J. Biol. Chem. 255*, 7769–7773.

Turner, A. B., & Ringold, H. J. (1967) *J. Chem. Soc. C*, 1720–1730.

Van Baelen, H., Power, S. G. A., & Hammond, G. L. (1993) *Steroids 58*, 275–277.

Van Dorsselaer, A., Bitsch, F., Green, B., Jarvis, S., Lepage, P., Bischoff, R., Kolbe, H. V. J., & Roitsch, C. (1990) *Biomed. Environ. Mass Spectrom. 19*, 692–704.

Werber, M. M., Szent-Györgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883.

Westphal, U. (1971) In *Steroid Protein Interactions-Monographs on Endocrinology* (Gross, F., Labhart, A., Mann, T., Samuels, L. T., & Zander, J., Eds.) Vol. 4, pp 164–236, Springer, Berlin.

Westphal, U. (1983) *J. Steroid Biochem. 19*, 1–15.