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Alanine scanning mutagenesis of the second extracellular loop of type 1 corticotropin releasing factor receptor revealed residues critical for peptide binding[#]

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Running title: Peptide binding determinants in type 1 CRF receptor

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Abbreviations:

CRF; corticotropin releasing factor; CRF_1, type 1 receptor for the corticotropin releasing factor; TM, membrane-spanning segment; EL2, second extracellular loop; WT wild type CRF_1; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

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ABSTRACT

Upon binding of the corticotropin releasing factor (CRF) analog, sauvagine, to the type 1 CRF receptor (CRF_1), the amino-terminal portion of the peptide has been shown to lie in close proximity to Lys257 in the receptor's second extracellular loop (EL2). To test the hypothesis that EL2 residues play a role in the binding of sauvagine to CRF_1 we carried out an alanine-scanning mutagenesis study to determine the functional role of EL2 residues (Leu251 to Val266). Only the W259A, F260A and W259A/F260A mutations reduced the binding affinity and potency of sauvagine. In contrast, these mutations did not appear to significantly alter the overall receptor conformation, as they left unchanged the affinities of the ligands astressin and antalarmin that have been suggested to bind to different regions of CRF_1. The W259A, F260A and W259A/F260A mutations also decreased the affinity of the endogenous ligand, CRF, implying that these residues may play a common important role in the binding of different peptides belonging to CRF family. Parallel amino acid deletions of the two peptides produced ligands with various affinities for wild type CRF_1 compared to the W259A, F260A and W259A/F260A mutants, supporting the interaction between the amino-terminal residues 8-10 of sauvagine and the corresponding region in CRF, with EL2 of CRF_1. This is the first time that a specific region of CRF_1 has been implicated in detailed interactions between the receptor and the amino-terminal portion of peptides belonging to the CRF family.

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INTRODUCTION

Corticotropin releasing factor (CRF), originally isolated from the mammalian hypothalamus, is a 41 amino acid peptide that regulates the activity of the hypothalamic-pituitary-adrenal axis by stimulating the secretion of hypophyseal corticotropin (ACTH) (Vale et al., 1981). In addition to its major role in the regulation of the hypothalamic-pituitary-adrenal axis, CRF also functions as a neurotransmitter within the central nervous system and it is involved in the control of the gastrointestinal, behavioral, immune and reproductive systems (Gravanis and Margioris, 2005; Hillhouse and Grammatopoulos, 2006; Martinez et al., 2004; Venihaki and Majzoub, 2002).

The CRF and its related peptides, including the 40-amino acid sauvagine, exert their actions by interacting with two types of CRF receptor, type 1 (CRF_1) and type 2 (CRF_2), which belong to subfamily B G-protein coupled receptors (GPCRs) (Chen et al., 1993; Lovenberg et al., 1995). CRF_1, like the other GPCRs, consist of an amino-terminal extracellular region, a carboxyl-terminal intracellular tail and seven, mostly hydrophobic, membrane-spanning segments (TMs), connected by alternating intracellular (IL) and extracellular loops (EL) (Fig. 1) (Grigoriadis et al., 2001).

Previous structure-function studies using chimeric receptors, or mutants created by substituting, simultaneously, two or more wild type residues, have shown that the first, second and third extracellular loops of CRF_1 (EL1, EL2 and EL3, respectively) are involved in sauvagine and CRF binding (Liaw et al., 1997a; Liaw et al., 1997b; Sydow et al., 1999). However, these studies did not identify individual residues that are important for ligand/receptor interaction. In addition, the extracellular loops along with the membrane-spanning domains of CRF_1 have been suggested to interact with the amino-terminal portion of peptides belonging to the CRF family (Hoare et al., 2003; Nielsen et al., 2000). This is in agreement with the important

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functional role of the amino-terminal portion of CRF family peptides, as suggested in previous studies. Thus, substitution of most of the twenty amino-terminal residues of CRF by alanine, or by other amino acids, has been shown to produce significant decrease of peptide binding and biological potency (Beyermann et al., 1996; Kornreich et al., 1992; Rivier et al., 1993). Furthermore, deletion of the first nine or eleven amino-terminal residues from CRF and the corresponding eight or ten amino acids from sauvagine, resulted in a decrease in binding affinity and/or biological potency of peptides (Gulyas et al., 1995; Rivier et al., 2002; Rivier et al., 1984; Ruhmann et al., 1998). However the amino acids of CRF_1 and the amino-terminal residues of sauvagine and CRF that interact with each other have so far been elusive.

Some information about the specific interactions emerged recently from a cross-linking study showing that the amino-terminal Lys16 of sauvagine bound to CRF_1 is in close proximity to Lys257, which is located in the second extracellular loop of receptor (EL2) (Assil-Kishawi and Abou-Samra, 2002); but the residues involved in the interaction were not identified.

Based on the available information we reasoned that one or more residues located in the EL2 of CRF_1 possibly play a role in binding, most likely, to the amino-terminal portion of sauvagine and other CRF family peptides. This hypothesis considers as well the demonstrated role of EL2 of different subfamily B GPCRs in ligand binding and receptor function (Bergwitz et al., 1997; Vertongen et al., 2001). To test this hypothesis, we determined the binding of sauvagine, CRF and analogs created by various truncations of the amino-terminal portions of these peptides, to wild type CRF_1 and EL2 mutants generated by alanine substitution (one at a time). Using these constructs, we find that Trp259 and Phe260 in the EL2 of CRF_1 play an important role in ligand-receptor interaction, interrelated with that of the amino-terminal portion of peptides of CRF family.

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MATERIALS AND METHODS

Synthesis of peptides. Sauvagine analogues, Sauvagine(11-40) and Sauvagine(8-40), were synthesized by Fmoc/tBu methodology using "linker" (Rink Bernatowitz)-resin (2-chlorotrityl chloride/CLTR). L-amino acids were used with the appropriate protection groups at side chains as follows: Fmoc-Ile-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Met-OH, Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Pyr-OH. The coupling of each amino acid was achieved in the presence of *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazol (HOBt) in DMF solution. In each case the Fmoc protecting group was removed by treatment with piperidine solution (20% in DMF, 2 x 15 min). The Kaiser test and thin layer chromatography (TLC), n-butanol/acetic acid/water (4:1:1) (BAW) as eluent, verified the completeness of each coupling or Fmoc deprotection. Subsequently, the protected peptides-resin were treated with the splitting mixture DCM/TFE (7/3) for 2 h at room temperature in order to remove peptides from the resin followed by treatment with the solution of DCM/TFA/Ethanedithiol/Anisole/H_2O (32/65/1/1/1), for 4 h at room temperature for complete removal of protecting groups. The resulting solutions were concentrated under vacuum to a small volume and the final free linear peptides after their precipitation (by adding diethyl ether) as a light-yellow amorphous solid, were filtered and dried in vacuo for 12 h (purity >60%). The final crude products were further purified with semi-preparative RP-HPLC (column: Lichrosorb, RP-18, 250X10 mm). Separations were achieved with a stepped linear gradient of acetonitrile (AcN) (0.08%TFA) in water (0.08% TFA) for 50 min at a flow rate of 3 ml/min (gradient separation: 10%AcN to 70%AcN in 45 min). Peptides purity was assessed by analytical RP-HPLC (column: Nucleosil-120 C18, 250X4.0 mm, gradient

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separation: 10%AcN to 100%AcN in 27 min), TLC and Electron Spray Mass Spectrometry (ESI-MS).

Plasmids and site-directed mutagenesis. The cDNA sequence encoding the type 1 human corticotropin releasing factor receptor (CRF_1) was a gift from Dr. D. Grammatopoulos (Warwick University, UK). This cDNA was subcloned into the bicistronic expression vector pcin4 (Liapakis et al., 2000), thereby creating the vector pcin4-CRF_1. Alanine mutations were generated by the polymerase chain reaction (PCR)-mediated mutagenesis, using Pfu polymerase (Fermentas USA) and mutagenic oligonucleotides encoding the desired amino acid substitution, a gift from Dr. D. Grammatopoulos. The PCR-generated DNA fragments containing the alanine mutations were subcloned into the pcin4-CRF_1 plasmid and the mutations were confirmed by DNA sequencing. Alanine mutants are named as (wild-type residue)(residue number)(alanine), where the residues are given in the single-letter code.

Cell culture and transfection. Human embryonic kidney cells (HEK 293) were grown in DMEM/F12 (1:1) containing 3.15 g/L glucose and 10% bovine calf serum at 37 ^{o}C and 5% CO_2. Sixty mm dishes of HEK 293 cells at 80-90% confluence were transfected with 2-3 mg of wild type (WT) or mutant pcin4-CRF_1 using 9 μl of Lipofectamine (Invitrogen, USA) and 2 ml of OPTIMEM (Invitrogen, USA). To generate stably transfected pools of cells expressing the receptors five to twelve hours after transfection, the medium was replaced by DMEM/F12 (1:1) containing 3.15 g/L glucose, 10% bovine calf serum and 700 μg/ml of the antibiotic, Geneticin (Invitrogen, USA). The antibiotic was added to select a stably transfected pool of cells.

Harvesting cells and membrane preparation. Cells stably expressing WT or CRF_1 mutants were washed with phosphate-buffered saline (PBS) (4.3 mM $Na_2HPO_4.7H_20$, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2-7.3 at R.T), briefly treated with PBS containing 2 mM

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EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cells suspensions were centrifuged at 1000 x g for 5 min at room temperature, and the pellets were homogenized in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM $MgCl_2$, 2 mM EGTA, 0.2 mg/ml bacitracin and 0.93 μg/ml aprotinin pH 7.2 at 4^{o}C) using a Janke & Kunkel IKA Ultra Turrax T25 homogenizer, at setting ~20, for 10-15 s, at 4 ^{o}C. The homogenates were centrifuged at 16000 x g, for 10 min, at 4 ^{o}C. The membrane pellets were resuspended by homogenization, as described above, in 1 ml buffer B (buffer H containing 0.1% BSA, pH 7.2 at 20^{o}C). The membrane suspensions were diluted in buffer B and used for radioligand binding studies.

[^{125}I]-Tyr^{0}-sauvagine binding. [^{125}I]-Tyr^{0}-sauvagine (NEN Life Science Products, USA) competition binding was performed as described previously (Rominger et al., 1998) with several modifications. Aliquots of diluted membrane suspension (50 μl) were added into low retention tubes (Kisker, Germany), containing buffer B and 25-45 pM [^{125}I]-Tyr^{0}sauvagine (depending on radioligand affinity for the mutant) with or without increasing concentrations of Tyr^{0}-sauvagine (American Peptide Co. Inc, USA) (homologous competition binding), or other CRF analogs (heterelogous competition binding), in a final volume of 0.2 ml. The CRF analogs were peptides (synthesized as described above, or purchased from Bachem, Germany or American Peptide Co. Inc., USA) or the non-peptidic analog, antalarmin (a gift from Dr. G. Chrousos, University of Athens). The mixtures were incubated at 20-21 °C for 120 min and then filtered using a Brandel cell harvester through Whatman 934AH glass fiber filters, presoaked for 1 hr in 0.3% polyethylenimine at 4 °C. The filters were washed 3 times with 0.5 ml of ice-cold PBS, pH 7.1 containing 0.01% Triton X-100. Filters were assessed for radioactivity in a gamma counter (LKB Wallac 1275 minigamma, 80% efficiency). The amount of membrane used was adjusted to insure that the specific binding was always equal to or less than 10% of the total concentration of

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the added radioligand. Specific [^{125}I]-Tyr^0-sauvagine binding was defined as total binding less nonspecific binding in the presence of 500-1000 nM h/r CRF. Data for competition binding were analyzed by nonlinear regression analysis, using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). IC_{50} values were obtained by fitting the data from competition studies to a one-site competition model. The K_i values were determined from heterelogous competition data using GraphPad Prism 4.0 and the equation, $K_i = IC_{50} / (1 + L / K_D)$, where L is the concentration of radioligand (Cheng and Prusoff, 1973). The K_D values for [^{125}I]-Tyr^0-sauvagine binding were determined from homologous competition data, using GraphPad Prism 4.0 and the following equation: $Y = \{(B_{max} * [hot]) / ([hot] + [cold] + K_D)\} + NSB$ (Motulsky and Christopoulos, 2003), where Y is the total binding of [^{125}I]-Tyr^0-sauvagine, NSB is the non-specific binding of the radioligand, B_{max} is the total receptor number, [hot] is the concentration of the [^{125}I]-Tyr^0-sauvagine and [cold] is the concentration of the Tyr^0-sauvagine.

cAMP Accumulation Assays. HEK 293 cells stably expressing WT or CRF_1 mutants were plated in 96-well cell culture plates (pretreated with poly-L-lysine, 0.1 mg/ml). After incubation overnight at 37 °C in 5% CO2, the cells were 95–100% confluent. The medium was removed, and 100 µl of assay buffer (25 mM HEPES, pH 7.4, 2 mM choline, 288 mM sucrose, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM 3-isobutyl-1-methylxanthine) was added. After 1 h incubation at 37 °C, more assay buffer without (basal levels) or with increasing concentrations of Tyr^0-sauvagine was added to a total volume of 200 µl, and the incubation was continued for 20 min at 37 °C. At the end of the incubation the assay buffer was removed. The cells were placed on ice and lysed with 3% trichloroacetic acid. Lysates were incubated on ice for 30–60 min and stored at -20 °C. After 1–5 days, frozen lysates were thawed and centrifuged at 1800 x g for 10 min at 4°C, and the supernatants were neutralized with 2 N NaOH. Quantification of cAMP in the

neutralized supernatants was performed using a competitive binding assay as described previously by Liapakis et al. (Liapakis et al., 2000). Briefly, supernatants were transferred to polypropylene mini-tubes (20 µl /tube) containing buffer A (100 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM EDTA) with 1–1.5 nM [2,8-^{3}H] adenosine 3', 5'-cyclic phosphate (Amersham Pharmacia Biotech, USA). Subsequently, cAMP-binding protein (~100 mg of crude bovine adrenal cortex extract in 500 ml of buffer A) was added to each tube. After incubation on ice for 3 h, the mixtures were filtered through Whatman 934AH glass fiber filters as described for radioligand binding assays and using buffer C (120 mM NaCl, 10 mM Tris-HCl, pH 7.4 at 4 °C) as washing buffer. The amount of cAMP in each sample (one-tenth of a well) was determined by comparison with a standard curve of known concentrations of unlabeled cAMP (1–100 pmol/tube). EC_{50} values were obtained by fitting the data to a one-site sigmoidal model using nonlinear regression analysis (GraphPad Prism 4.0).

Determination of cell surface expression of CRF_1 receptors by flow cytometry. HEK 293 cells stably expressing WT CRF_1 or mutant receptors were harvested, washed twice with PBS and incubated ($5x10^5$ cells) with anti-CRF_1 antibody (sc-12381, Santa Cruz, USA) (1/50) for 30 min on ice. Subsequently, the cells were washed three times with PBS and stained with anti-goat-fluorescein conjugated (AP163F, Chemicon, USA) (1/100 dilution) for 30 min on ice. At the end of the incubation the cells were washed twice with PBS and resuspended in 500 µl of PBS. The staining of cells was analyzed by flow cytometry, using a Beckton-Dickinson FACS Array apparatus and the CELL Quest software (Beckton-Dickinson, Franklin Lakes, NJ).

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RESULTS

In this study, we used alanine-scanning mutagenesis to investigate the role of the residues in the second extracellular loop (EL2) of CRF_1 in sauvagine binding and receptor function. Specifically, we mutated to alanine the EL2 residues from Leu251 to Val266 (except for Cys258) (Fig. 1), thus creating 15 different CRF_1 mutants. Cys258 was excluded because substitution by alanine abolished the binding of radiolabelled sauvagine (data not shown), in agreement with a previous study (Qi et al., 1997), most likely due to its important role in receptor function by forming a disulfide bond with Cys188 in EL1 of CRF_1 (Qi et al., 1997). Alanine scanning mutagenesis has been shown to yield important functional insight about proteins for which there is no complete structural information (Cunningham and Wells, 1989; Lu and Hulme, 1999; Ward et al., 1999)

Effects of alanine mutations on the binding of CRF family peptides. To assess the effect of alanine mutations of EL2 residues on the binding of peptides of CRF family we determined the affinities of [^{125}I]-Tyr^0-sauvagine and CRF for CRF_1 before and after mutations. The binding affinities (K_D) of [^{125}I]-Tyr^0-sauvagine for wild type CRF_1 (WT) and mutant receptors were determined from homologous competition experiments performed under equilibrium conditions in membranes from HEK 293 cells stably expressing the receptors. Substitution of alanine for the residues from Leu251 to Lys257 and from Gly261 to Val266 did not significantly affect the affinity of [^{125}I]-Tyr^0-sauvagine for CRF_1 (Fig. 2). In contrast, mutations of Trp259 (W259A) and Phe260 (F260A) to alanine, significantly decreased [^{125}I]-Tyr^0-sauvagine affinity, 9- and 15-fold, respectively (Table 1, Fig. 2 and 3). Next, we sought to determine the effect of simultaneous mutation of Trp259 and Phe260 to alanine, on [^{125}I]-Tyr^0-sauvagine affinity. As shown in Table 1 and Fig. 2 and 3, the simultaneous mutation of Trp259 and Phe260 to alanine,

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thus creating the W259A/F260A mutant, synergistically decreased the affinity of [^{125}I]-Tyr^{0}-sauvagine (39.5-fold).

To assess the role of Trp259 and Phe260 in the binding of other peptides belonging to CRF family, we determined the effect of W259A and F260A mutations on the affinity of CRF. The binding affinities (K_i) of CRF (and for all peptides described below) were determined from heterelogous competition experiments of [^{125}I]-Tyr^{0}-sauvagine performed under equilibrium conditions in membranes from HEK 293 cells stably expressing WT CRF_1 and mutant receptors. Similar to sauvagine, W259A and F260A mutations largely decreased the affinity of CRF (19-fold and 17-fold, respectively) (Table 1, Fig. 3). Comparable to sauvagine, simultaneous mutation of Trp259 and Phe260 to alanine decreased more than additively the binding affinity of CRF (47.5-fold) (Table 1, Fig. 3).

Effects of alanine mutations on the binding of synthetic peptides and nonpeptide analogs. To test whether W259A, F260A and W259A/F260A mutations alter the overall conformation of CRF_1 we determined their effects on binding of the peptide astressin and the non-peptide small molecule, antalarmin, which, as previously suggested, bind to different regions of CRF_1 (Hoare et al., 2003; Perrin et al., 1998). In contrast to the native peptides CRF and sauvagine, the affinity of astressin was reduced by the W259A, F260A and W259A/F260A mutations only 2.5-, 2.5- and 3.5-fold respectively (Table 1, Fig. 4). Similarly antalarmin affinity was not significantly decreased (1.5-fold) by W259A and F260A mutations (Fig. 4), whereas the simultaneous mutation of Trp259 and Phe260 to alanine did not change the binding affinity of this small non-peptide molecule (Fig. 4).

Previous studies have suggested that the extracellular loops of CRF_1 likely interact with the amino-terminal portion of peptides belonging to the CRF family (Hoare et al., 2003; Nielsen et

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al., 2000). To test if Trp259 and/or Phe260 in the EL2 of CRF_1 are involved in receptor interaction with the amino-terminal portion of CRF and sauvagine we determined the effects of W259A, F260A and W259A/F260A mutations on binding affinities of several analogs of CRF and sauvagine produced by their truncation at the amino-terminal portion. As mentioned above, W259A, F260A and W259A/F260A mutations did not significantly alter the binding of astressin, which is a truncated analogue of CRF, lacking the first 11 amino-terminal residues of CRF (Table 1). Similar to astressin, W259A, F260A and W259A/F260A mutations did not significantly affect the affinity of sauvagine(11-40), which is the truncated analog of sauvagine corresponding to CRF analog astressin (Table 1, Fig. 5).

To further identify which of the first 10 and 11 amino-terminal residues of sauvagine and CRF, respectively, play a role in peptide interaction with Trp259 and Phe260, we determined the effects of W259A, F260A and W259A/F260A mutations on the binding affinities of sauvagine(8-40) and alpha-helical CRF(9-41). Alpha-helical CRF(9-41) is a truncated analog of CRF lacking the first eight amino-terminal residues of CRF (Rivier et al., 1984) and sauvagine(8-40) is the corresponding truncated analog of sauvagine (Table 1). These analogs, therefore, have three amino-terminal residues more than astressin and sauvagine(11-40). Removing the side chains of Trp259 and Phe260, by mutating them to alanine, individually (W259A and F260A) or simultaneously (W259A/F260A), largely decreased the binding affinity of alpha-helical CRF(9-41) (> 6.5-fold) (Table 1, Fig. 5). Comparable to alpha-helical CRF(9-41), mutation of Trp259 to alanine significantly reduced sauvagine(8-40) affinity (4-fold) (Table 1, Fig. 5). In contrast to the effect of the W259A mutation, alanine substitution for Phe260 did not affect the affinity of sauvagine(8-40) (Table 1, Fig. 5). The simultaneous mutation of Trp259 and Phe260 to alanine did not further reduce the affinity of sauvagine(8-40), consistent with the

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lack of effect of F260A mutation on the binding properties of this peptidic analog (Table 1, Fig. 5).

Activation of adenylyl cyclase. The functional properties of CRF_1 before and after alanine mutations of EL2 residues were assessed in cAMP accumulation experiments. We determined the ability of Tyr^0-sauvagine to stimulate cAMP accumulation in HEK 293 cells stably expressing WT CRF_1 or mutant receptors, and found the effects of alanine mutations on the potency of Tyr^0-sauvagine to be similar with those on its binding affinity. Alanine mutation of the residues from Leu251 to Lys257 and from Gly261 to Val266 in the EL2 of CRF_1 did not significantly affect the potency of Tyr^0-sauvagine (Fig. 6). In contrast, W259A and F260A mutations decreased the potency of Tyr^0-sauvagine to stimulate cAMP accumulation, 65.5 and 56.5-fold, respectively (Fig.6 and 7). In agreement with the effects of the simultaneous mutation of Trp259 and Phe260 to alanine on the binding affinity of Tyr^0-sauvagine, 259A/260A mutation decreased more than additively (161.5-fold) its potency to stimulate cAMP accumulation (Fig. 6 and 7). In contrast to potency, the maximal stimulation of cAMP accumulation by Tyr^0-sauvagine was much less affected by W259A and F260A mutations, whereas it was not reduced by the W259A/F260A mutation (Fig. 7). The effect of W259A, F260A and W259A/F260A mutations on Tyr^0-sauvagine potency and maximal ability to stimulate cAMP accumulation was not due to a mutation-associated decrease in cell surface expression of CRF_1 because these mutations did not decrease the number of cell surface receptors as determined in flow cytometry experiments, using a CRF_1 selective antibody (Fig. 8).

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DISCUSSION

Alanine substitution of most of the residues in EL2 of CRF_1 (Leu251 to Lys257, and Gly261 to Val266) did not significantly change the binding affinity for sauvagine, and its potency to stimulate cAMP accumulation. Alanine substitution of a receptor residue that interacts with a ligand has been suggested to disrupt the interaction without affecting the receptor's overall conformation (Cunningham and Wells, 1989). Thus, we conclude that the EL2 residues Leu251-Lys257 and Gly261-Val266 do not interact with sauvagine. Surprisingly, among these residues, Lys257 has been shown to lie in close proximity to CRF_1-bound sauvagine (Assil-Kishawi and Abou-Samra, 2002). This, in conjunction with the results of our study, suggests that Lys257 is possibly located in the interface between sauvagine and receptor, without however contributing significantly to the overall binding energy. This is similar to the conclusion reached in (Clackson and Wells, 1995) based on the X-ray structure of human growth hormone in complex with its receptor, and a thorough mutational analysis of both of these proteins; Clackson and Wells suggested that only a small fraction of the contact residues in the ligand/receptor complex contribute significantly to the overall binding energy.

However, we find for two residues in EL2, Trp259 and Phe260, that alanine substitution reduced both binding affinity and functional potency of sauvagine. In contrast, these mutations did not significantly affect the affinities of ligands considered to interact with receptor regions other than EL2 (Hoare et al., 2003; Perrin et al., 1998), i.e., astressin and antalarmin. These results suggest that Trp259 and Phe260 play an important, most likely direct, role in sauvagine binding, without significantly altering the overall conformation of the CRF_1 protein. Notably, Trp259 and Phe260 are located only two amino acids away from Lys257, which has been shown to be positioned near CRF_1-bound sauvagine (Assil-Kishawi and Abou-Samra, 2002).

Furthermore, previous studies have suggested that only few of the interactions between receptor and ligand, may be important for binding and these are predominantly interactions between hydrophobic residues, such as typtophan and phenylalanine (Clackson and Wells, 1995; Young et al., 1994). The results of our study are in agreement with previous studies on different GPCRs, which have shown that the EL2 plays an important role in ligand binding (Audoly and Breyer, 1997; Bergwitz et al., 1997; Holtmann et al., 1996; Moro et al., 1999; Runge et al., 2003; Shi and Javitch, 2004).

Similar to our findings for sauvagine, the same two residues mutated to alanine reduced CRF affinity as well, suggesting a role for Trp259 and Phe260 in the binding of various peptides belonging to CRF family, possibly being common contact sites for them.

All the effects we measured for these two mutant constructs (W259A and F260A) on the affinities of amino-terminal truncated peptides (astressin, a CRF analog lacking the first eleven amino-terminal residues (Gulyas et al., 1995); sauvagine(11-40), a truncated analog of sauvagine corresponding to astressin; alpha-helical CRF(9-41), a truncated analog of CRF lacking the first eight amino-terminal residues; sauvagine(8-40), a truncated analog of sauvagine corresponding to alpha-helical CRF(9-41)), suggest that Trp259 and/or Phe260 interact with the amino-terminal region of peptides, which corresponds to the amino acids 8-10 and 9-11 of sauvagine and CRF, respectively and it is highly conserved among the peptides of CRF family. At the very least, even if these amino-terminal regions of CRF and sauvagine interact with receptor sites other than Trp259 and Phe260, they are likely to be important for the positioning of other portions of the peptide for proper interaction with Trp259 and/or Phe260. Detailed structure-function studies are now in progress to elucidate the mode of interaction of sauvagine and CRF with Trp259 and Phe260 of CRF_1.

We note that the F260A mutation decreased the affinity of the truncated CRF analog, alpha helical CRF (9-41), without however having any effect on the affinity of the corresponding to alpha helical CRF (9-41), truncated analog of sauvagine, sauvagine(8-40). A possible explanation for this discrepancy could be that these truncated analogs of CRF and sauvagine are likely to bind in different ways to CRF1. The different affinities between the alpha helical CRF (9-41), the three residues shorter CRF-truncated analog astressin and the full-length CRF could also be attributed to a possible different mode of their binding to CRF_1. Previous studies have shown that the high affinity binding of CRF (full agonist), is due to two sets of interactions (Hoare et al., 2003; Nielsen et al., 2000); a first one between the amino-terminal portion of CRF and the J-domain (membrane-spanning domains and extracellular loops) of receptor and a second one between the carboxyl-terminal portion of peptide and the N-domain (amino-terminal extracellular region) of CRF_1. Deletion of the first 11 amino-terminal residues of CRF (thus creating the antagonist astressin) likely abolished the first set of interactions (or the most important ones for CRF function) and repositioned the peptide into the receptor such as to conserve its high affinity binding but losing its ability to activate the CRF_1. In contrast, deletion of some, but not all, of the first 11 amino-terminal residues of CRF (thus creating the partial agonist, alpha helical CRF (9-41)) likely abolished part of the first set of interactions with the remaining ones to position the truncated peptide into CRF_1 in such a way (possibly different than that of CRF) as to bind with lower affinity to receptor and having partial agonist properties. The concept that different peptides belonging to CRF family bind differentially to CRF_1 has also been suggested in a previous study, in which the pharmacological properties of different peptides were differentially affected by several mutations of receptor (Assil et al., 2001).

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The larger than additive effect of simultaneous substitution of Trp259 and Phe260 with alanine on the affinity of sauvagine and CRF suggests a combined role in binding for these adjacently positioned residues. This finding is similar to the observation of the super-additive effect of simultaneous alanine substitution for Ser203 and Ser204 in the beta2 adrenergic receptor on epinephrine binding (Liapakis et al., 2000). These two serines have been shown to participate in a network of hydrogen-bond interactions with the meta-OH of catecholamines, as well as with other receptor residues (Liapakis et al., 2000).

Conclusively, this study, for first time revealed that Trp259 and Phe260 in the EL2 of CRF_1 play an important role in ligand-receptor interaction, interrelated with that of the amino-terminal portion of peptides of CRF family. In addition, the interplay between the amino-terminal portion of peptides and Trp259 and Phe260 seems to be critical for CRF_1 activation and the subsequent appearance of a biological effect (truncation of the amino-terminal portion of peptides resulted in the creation of antagonists, such as astressin, which are insensitive to alanine mutations of Trp259 and Phe260). This conclusion substantiates the key role of EL2 of CRF_1 in the translation of agonist binding to receptor activation, a role that is likely related both to its position in the receptor structure, near the key (for receptor function) TMs 4, 5 and 6, and to its own conformational properties that we are now exploring.

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LEGENDS FOR FIGURES

Fig. 1. Snake plot representation of CRF_1 showing the residues of its second extracellular loop. The residues, Leu251 – Val266, of the second extracellular loop (EL2) of CRF_1 are displayed as circles. The thicker circles indicate the residues (Trp259 and Phe260), which play an important role in peptide binding. The amino-terminal extracellular region, the carboxy-terminal intracellular tail, the three intracellular loops, and the first (EL1) and third (EL3) extracellular loops are drawn as lines. The membrane is shaded and the membrane spanning domains (TM1-TM7) are shown as cylinders.

Fig. 2. Effect of alanine mutations on [^{125}I]-Tyr^0-sauvagine binding affinity. Competition binding studies (using as competitor Tyr^0-sauvagine) were performed, as described under "Materials and Methods", on membrane preparations from HEK 293 cells stably expressing wild type (WT) CRF_1 or mutants, which were created by alanine substitution of residues from Leu251 to Val266 in the second extracellular loop of CRF_1, as well as by the simultaneous mutation of Trp259 and Phe260 to alanine. The data were fit to a one-site competition model by nonlinear regression and the $\log K_D$ values were determined according to the method of Motulsky and Christopoulos (Motulsky and Christopoulos, 2003). The mean ± S.E. values are from 3–5 independent experiments. The bars represent the change in [^{125}I]-Tyr^0-sauvagine affinity caused by alanine mutation ($\log K_D$ value for mutant - $\log K_D$ value for WT). Statistically significant differences between the $\log K_D$ values of WT and mutant receptors were evaluated using one-way ANOVA followed by Bonferoni's post hoc test (*$p < 0.05$).

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Fig. 3. Competition binding isotherms of Tyr^0-sauvagine and CRF to wild type CRF$_1$, W259A, F260A and W259A/F260A receptors. Competition of [^{125}I]-Tyr^0-sauvagine specific binding by Tyr^0-sauvagine (A) or CRF (B) was performed, as described under "Materials and Methods", on membranes from HEK 293 cells stably expressing wild type (WT) CRF$_1$, W259A, F260A, or W259A/F260A receptors. The means and S.E. (duplicate determination) are shown from a representative experiment performed 3-5 times with similar results. The data were fit to a one-site competition model by nonlinear regression. The logK_D and logK_i values for Tyr^0-sauvagine and CRF, respectively, determined as described under "Materials and Methods", are given in Table 1.

Fig. 4. Competition binding isotherms of astressin and antalarmin to wild type CRF$_1$, W259A, F260A and W259A/F260A receptors. Competition of [^{125}I]-Tyr^0-sauvagine specific binding by astressin (A) and antalarmin (B) was performed, as described under "Materials and Methods", on membranes from HEK 293 cells stably expressing wild type (WT) CRF$_1$, W259A, F260A, or W259A/F260A receptors. The means and S.E. (duplicate determination) are shown from a representative experiment performed 3-5 times with similar results. The data were fit to a one-site competition model by nonlinear regression. The logK_i values were determined from the resulting logIC_{50} as described under "Materials and Methods". The -logK_i ± S.E. values of antalarmin are 7.81 ± 0.06, 7.57 ± 0.09, 7.61 ± 0.17 and 7.86 ± 0.12 for WT, W259A, F260A and W259A/F260A respectively. The logK_i values of astressin are given in the Table 1.

Fig. 5. Competition binding isotherms of analogs of CRF and sauvagine to wild type CRF$_1$, W259A, F260A and W259A/F260A receptors. Competition of [^{125}I]-Tyr^0-sauvagine specific

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binding by sauvagine(8-40) (A), sauvagine(11-40) (B) and alpha-helical CRF(9-41) (C) was performed, as described under "Materials and Methods", on membranes from HEK 293 cells stably expressing wild type (WT) CRF_1, W259A, F260A, or W259A/F260A receptors. The means and S.E. (duplicate determination) are shown from a representative experiment performed 3-8 times with similar results. The data were fit to a one-site competition model by nonlinear regression. The $logK_i$ values, determined from the resulting $logIC_{50}$ as described under "Materials and Methods", are given in the Table 1.

Fig. 6. Effect of alanine mutations on Tyr^0-sauvagine potency. Stimulation of cAMP accumulation by increasing concentrations of Tyr^0-sauvagine was performed, as described under "Materials and Methods", in intact HEK 293 cells stably expressing wild type (WT) CRF_1 or mutants, which were created by alanine substitution of residues from Leu251 to Val266 in the second extracellular loop of CRF_1, as well as by the simultaneous mutation of Trp259 and Phe260 to alanine. The $logEC_{50}$ values were obtained by fitting the data to a one-site sigmoidal dose-response model by nonlinear regression analysis. The mean ± S.E. values are from 3-22 independent experiments. The bars represent the change in Tyr^0-sauvagine potency caused by alanine mutation ($logEC_{50}$ value for mutant - $logEC_{50}$ value for WT). Statistically significant differences between the $logEC_{50}$ values of wild type and mutant receptors were evaluated using one-way ANOVA followed by Bonferoni's post hoc test (*$p < 0.05$).

Fig. 7. Agonist-stimulation of cAMP accumulation in cells expressing wild type CRF_1, W259A, F260A and W259A/F260A receptors. Stimulation of cAMP accumulation by the indicated concentrations of Tyr^0-sauvagine was performed as described under "Materials and

Methods" in intact HEK 293 cells stably expressing wild type (WT) CRF_1, W259A, F260A, or W259A/F260A receptors. The means and S.E. (duplicate determination) are shown from a representative experiment repeated 5-22 times with similar results. The data were fit to a one-site sigmoidal dose-response model by nonlinear regression and $logEC_{50}$ values were calculated and given in Fig. 6.

Fig. 8. Cell surface expression of wild type CRF_1 and mutant receptors. HEK 293 cells stably transfected with wild type CRF_1 (WT) or mutant receptors (W259A, F260A, or W259A/F260A), as well as, untransfected cells (HEK 293) were harvested, washed twice with PBS and incubated ($5x10^5$ cells) with anti-CRF_1 antibody for 30 min on ice. Subsequently, the cells were washed three times with PBS and stained with anti-goat-fluorescein conjugated (1/100 dilution) for 30 min on ice. At the end of the incubation the cells were washed twice with PBS and resuspended in 500 µl of PBS. The staining of cells was analyzed by flow cytometry, using a Beckton-Dickinson FACS Array apparatus and the CELL Quest software (Beckton-Dickinson, Franklin Lakes, NJ).

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TABLE 1

Peptide binding to wild type (WT) CRF_1 and mutants. Competition binding studies were performed on membrane preparations from HEK 293 cells stably expressing wild type (WT) CRF_1 or mutant receptors, as described in "Materials and Methods". The -logIC_{50} values were obtained by fitting the data to a one-site competition model by nonlinear regression. The -logK_i values were determined from the -logIC_{50} values, according to the method of Cheng and Prusoff (Cheng and Prusoff, 1973). The -logK_D values (italics) were taken from Fig. 2. The mean ± S.E. values are from 3-8 independent experiments. Values in parentheses are K_i or K_D values of the mutants for each agonist divided by the K_i or K_D value of WT, respectively. These values represent the decrease (↓) in the affinity after the modification of CRF_1.

Peptide	Amino terminal amino residues of peptides																				-log K_D or -log K_i			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	WT	W259A	F260A	W259A/ F260A
Tyr^0-Sauvagine[a]	Y	Q	G	P	P	I	S	I	D	L	S	L	E	L	L	R	K	M	I	E	*8.90 ± 0.07*	*7.94*[c] *± 0.10* (9.0 ↓)	*7.71*[c] *± 0.14* (15.0 ↓)	*7.30*[c] *± 0.22* (39.5 ↓)
Sauvagine (8-40)									D	L	S	L	E	L	L	R	K	M	I	E	5.46 ± 0.12	4.86[c] ± 0.07 (4.0 ↓)	5.37 ± 0.04 (1.0)	4.85[c] ± 0.06 (4.0 ↓)
Sauvagine (11-40)												f[b]	H	L	L	R	K	M	I	E	5.76 ± 0.11	5.36 ± 0.03 (2.5 ↓)	5.71 ± 0.17 (1.0)	5.36 ± 0.10 (2.5 ↓)
CRF	S	E	E	P	P	I	S	L	D	L	T	F	H	L	L	R	E	V	L	E	9.14 ± 0.07	7.85[c] ± 0.07 (19.0 ↓)	7.90[c] ± 0.07 (17.0 ↓)	7.46[c] ± 0.22 (47.5 ↓)
Alpha-helical CRF(9-41)									D	L	T	F	H	L	L	R	E	M	L	E	6.80 ± 0.08	**<6.00 (>6.5 ↓)**	**<6.00 (>6.5 ↓)**	**<6.00 (>6.5 ↓)**
Astressin												f[b]	H	L	L	R	E	V	L	E	8.00 ± 0.08	7.60 ± 0.05 (2.5 ↓)	7.64 ± 0.09 (2.5 ↓)	7.46 ± 0.16 (3.5 ↓)

[a]Tyr^0-sauvagine is a sauvagine analog having an extra Tyr (Tyr^0), added before the amino-terminal Gln of sauvagine; [b]f denotes the D-Phe; [c]$P < 0.05$ compared with the WT CRF_1 (one-way ANOVA followed by Bonferoni's post hoc test).

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Fig.1

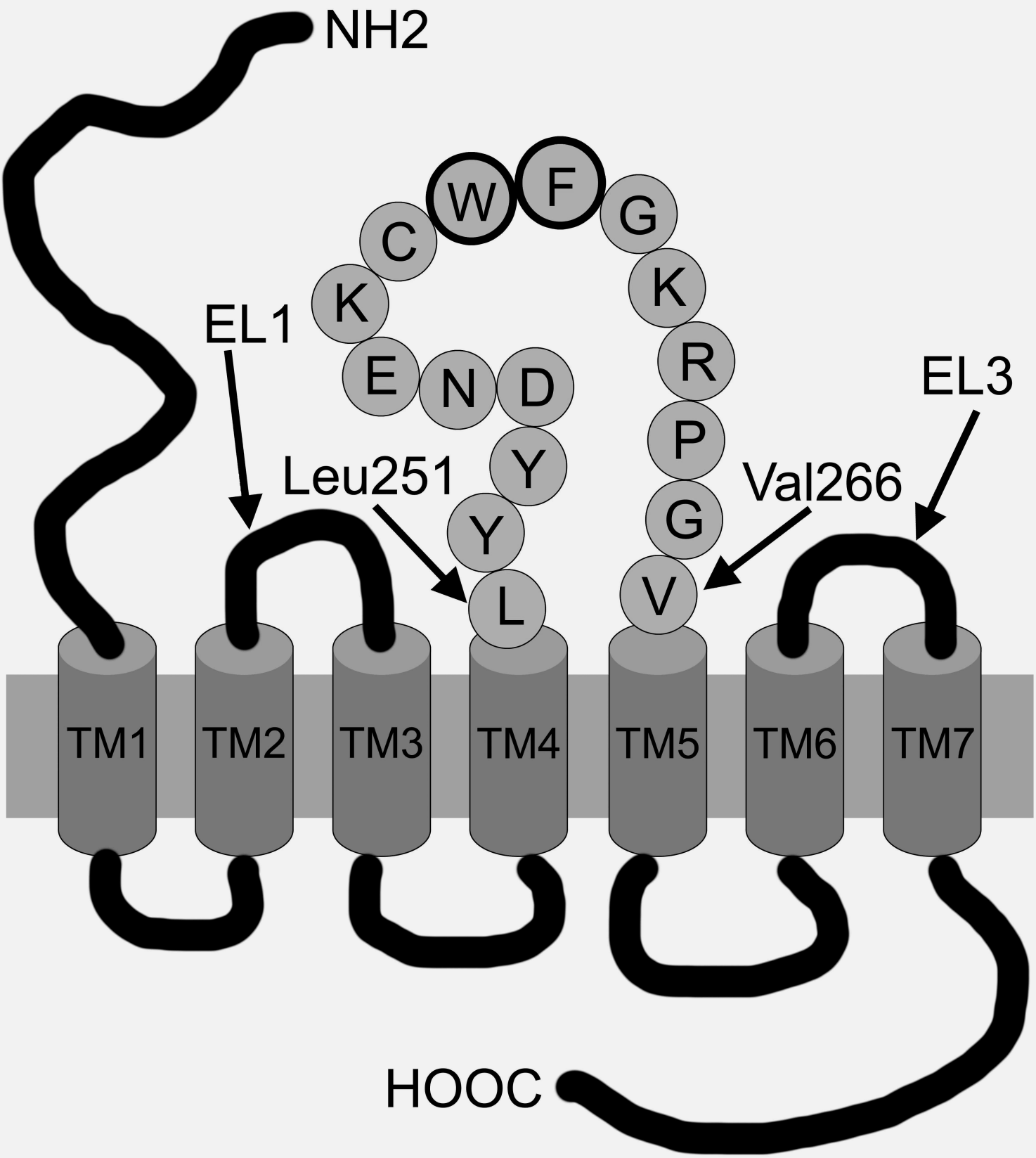
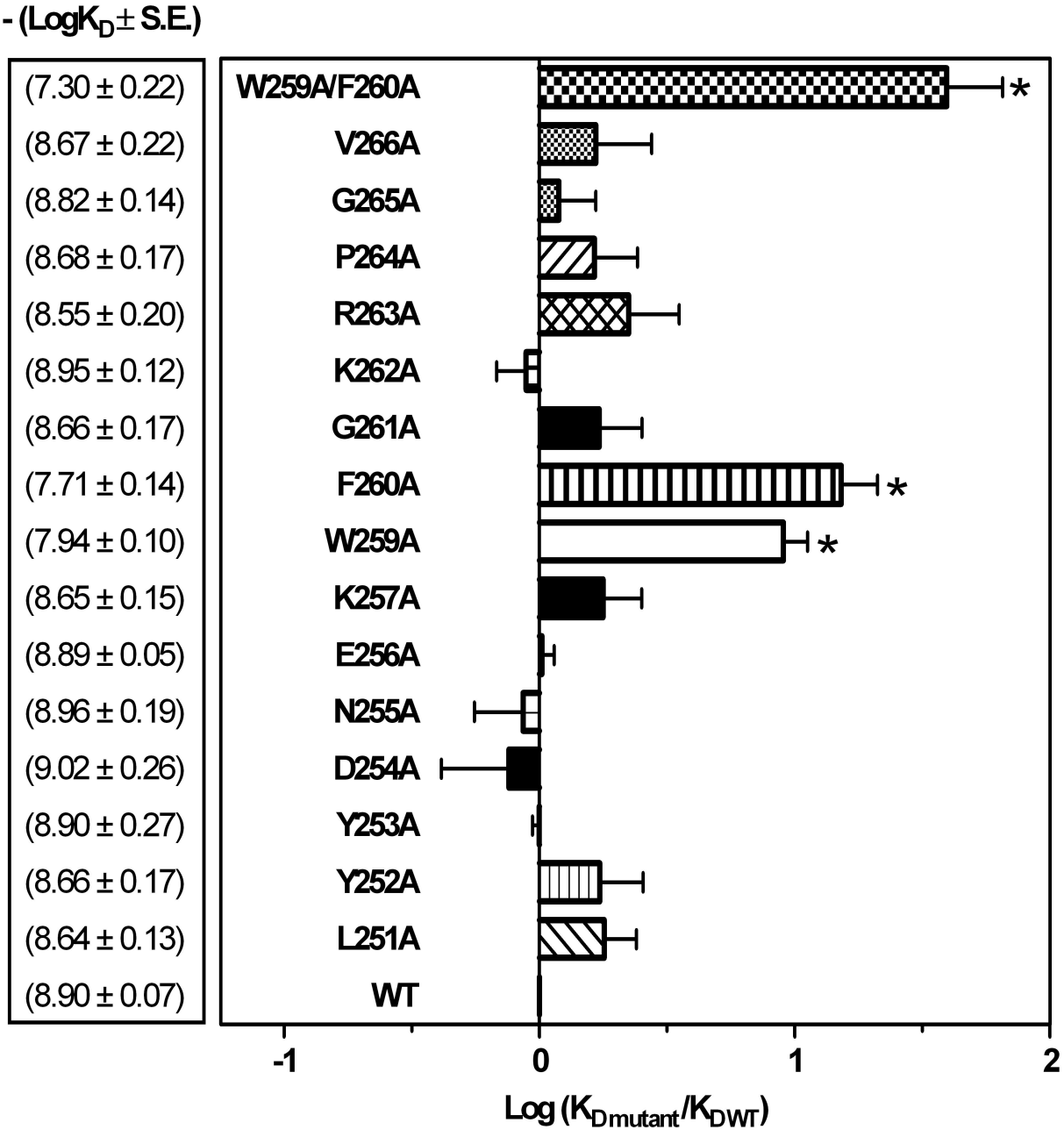


Fig. 2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(8.67 ± 0.22)
(8.82 ± 0.14)
(8.68 ± 0.17)
(8.55 ± 0.20)
(8.95 ± 0.12)
(8.66 ± 0.17)
(7.71 ± 0.14)
(7.94 ± 0.10)
(8.65 ± 0.15)
(8.89 ± 0.05)
(8.96 ± 0.19)
(9.02 ± 0.26)
(8.90 ± 0.27)
(8.66 ± 0.17)
(8.64 ± 0.13)
(8.90 ± 0.07)
W259A/F260A
V266A
G265A
P264A
R263A
K262A
G261A
F260A
W259A
K257A
E256A
N255A
D254A
Y253A
Y252A
L251A
WT
*
*
*
-1
0
1
2
Log (K$_{Dmutant}$/K$_{DWT}$)

Fig. 3

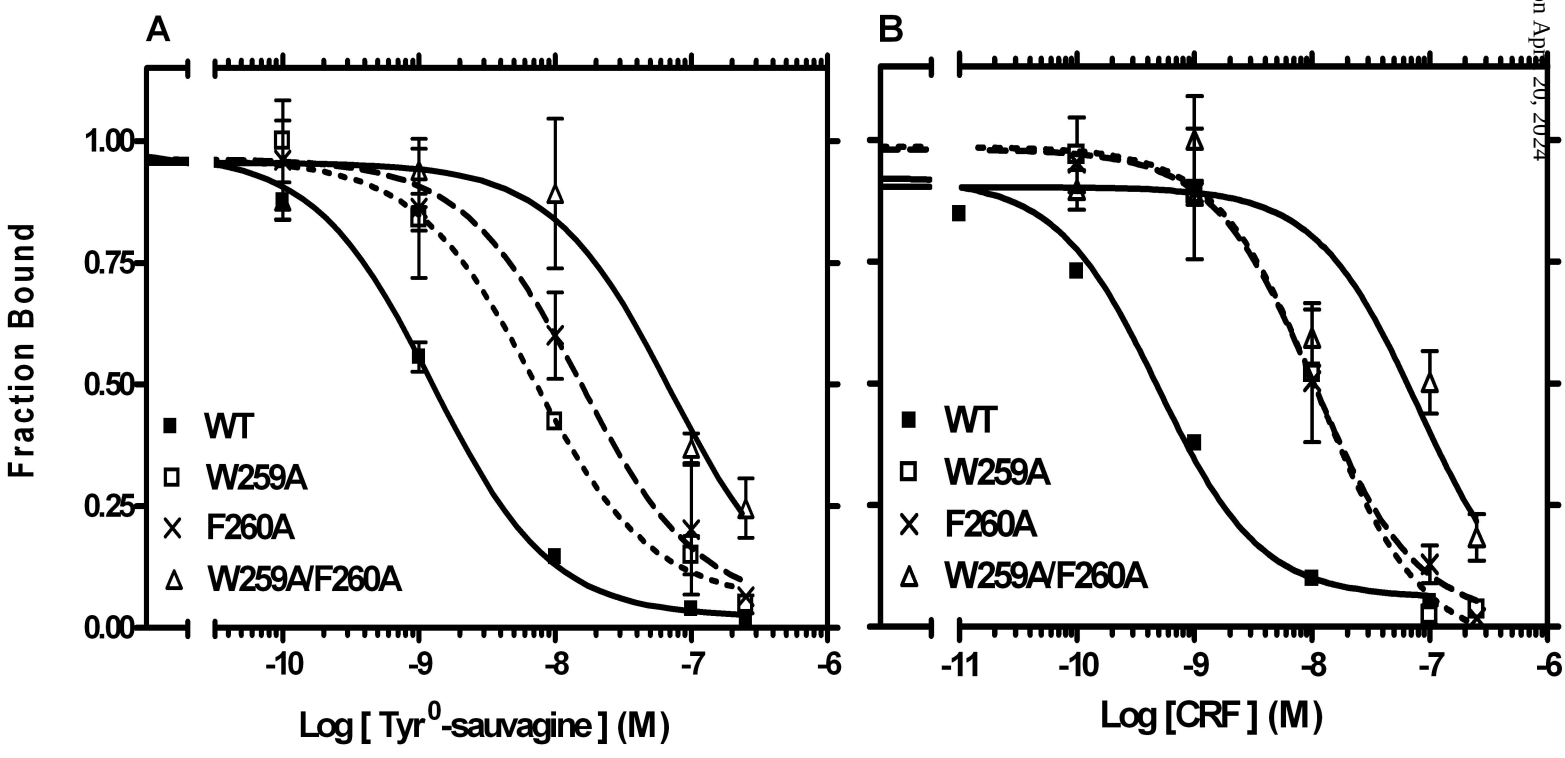
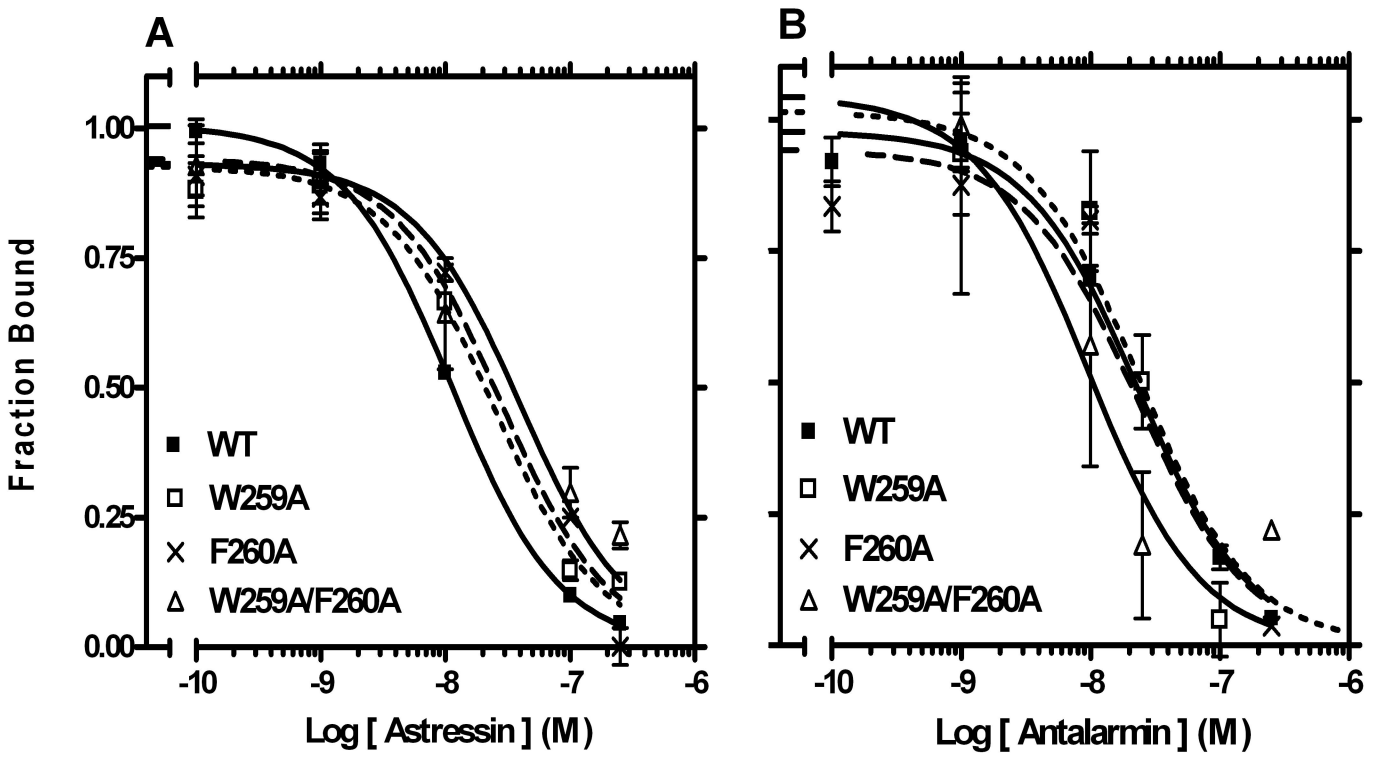


Fig. 4


A
B
Fraction Bound
1.00
0.75
0.50
0.25
0.00
-10
-9
-8
-7
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WT
W259A
F260A
W259A/F260A
Log [Astressin] (M)
Log [Antalarmin] (M)

Fig. 5

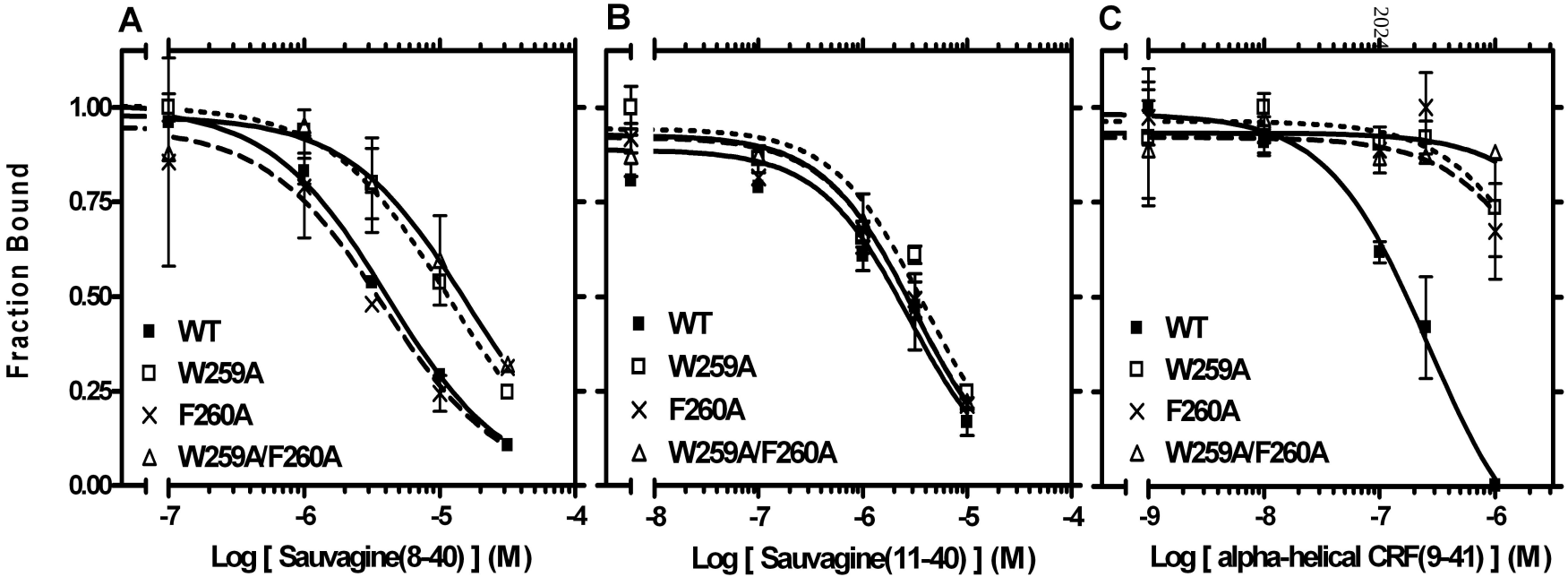

Fig. 6

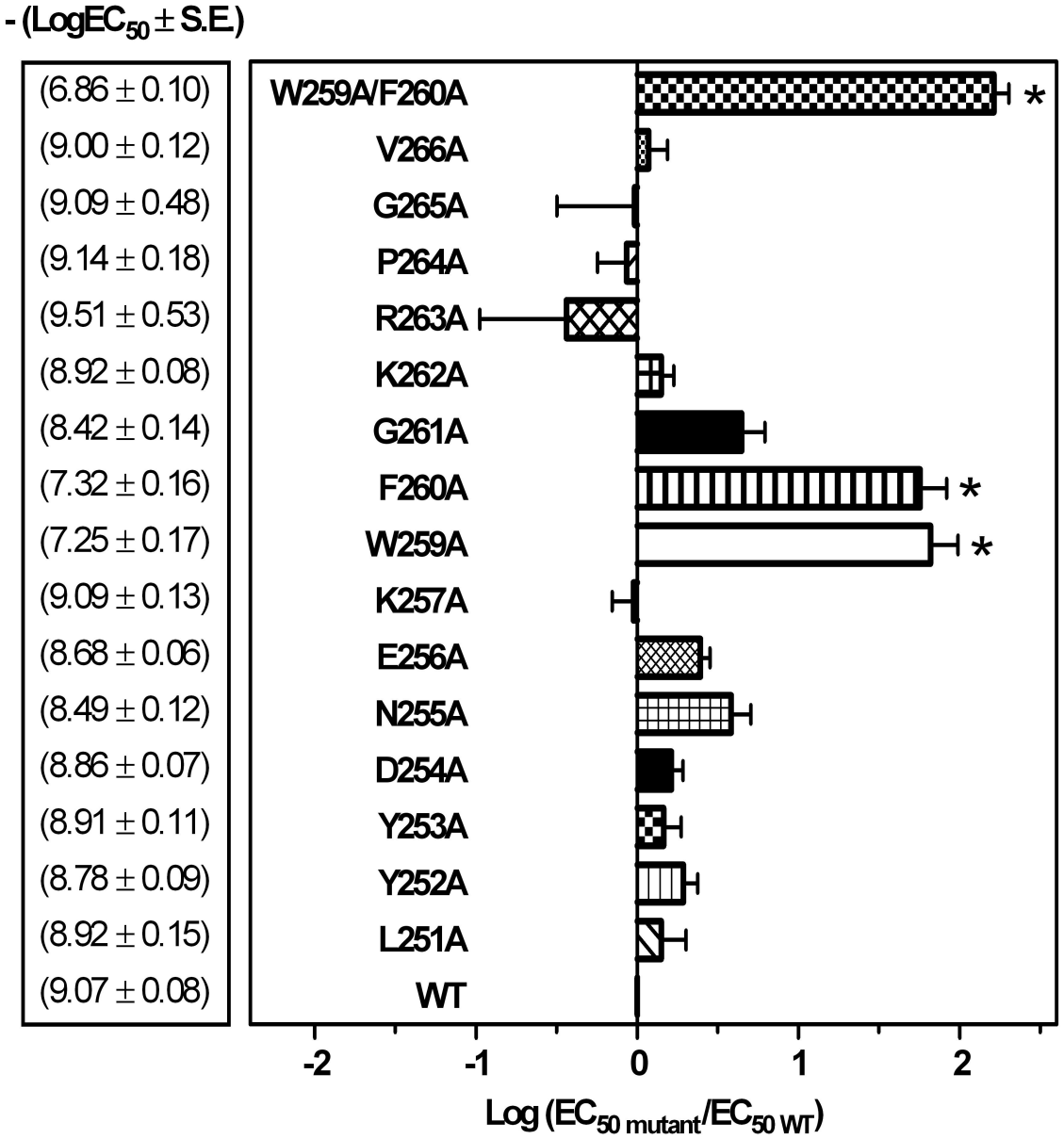
- (LogEC50 ± S.E.)
(6.86 ± 0.10)
(9.00 ± 0.12)
(9.09 ± 0.48)
(9.14 ± 0.18)
(9.51 ± 0.53)
(8.92 ± 0.08)
(8.42 ± 0.14)
(7.32 ± 0.16)
(7.25 ± 0.17)
(9.09 ± 0.13)
(8.68 ± 0.06)
(8.49 ± 0.12)
(8.86 ± 0.07)
(8.91 ± 0.11)
(8.78 ± 0.09)
(8.92 ± 0.15)
(9.07 ± 0.08)
W259A/F260A
V266A
G265A
P264A
R263A
K262A
G261A
F260A
W259A
K257A
E256A
N255A
D254A
Y253A
Y252A
L251A
WT
*
*
*
-2
-1
0
1
2
Log (EC50 mutant/EC50 WT)

Fig. 7

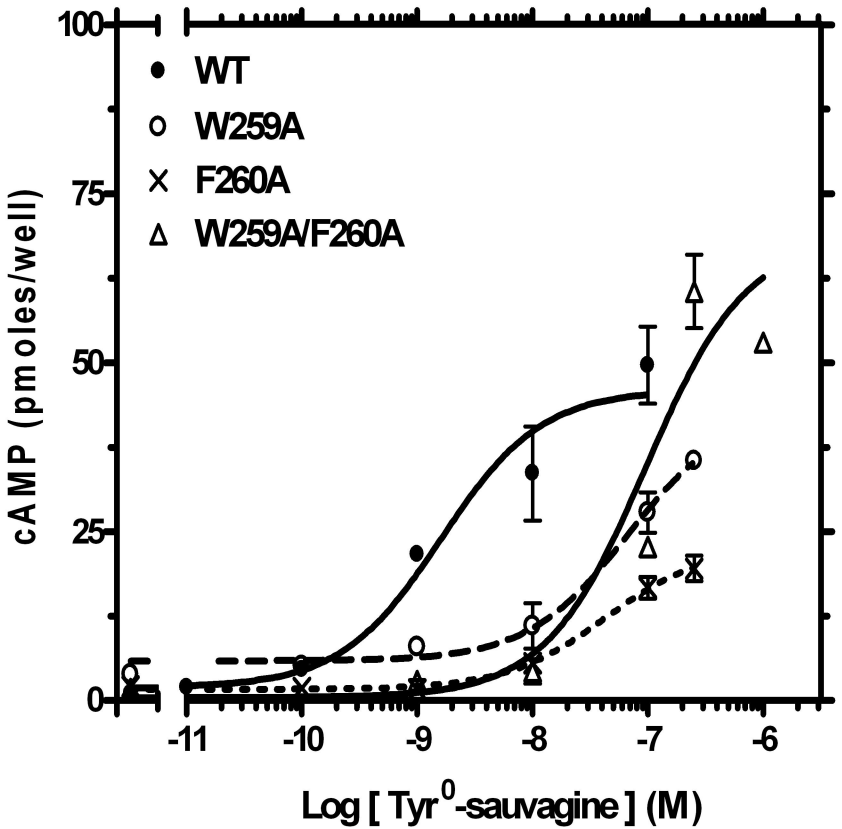
100
75
50
25
0
cAMP (pmoles/well)
WT
W259A
F260A
W259A/F260A
-11
-10
-9
-8
-7
-6
Log [Tyr0-sauvagine] (M)

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Fig. 8