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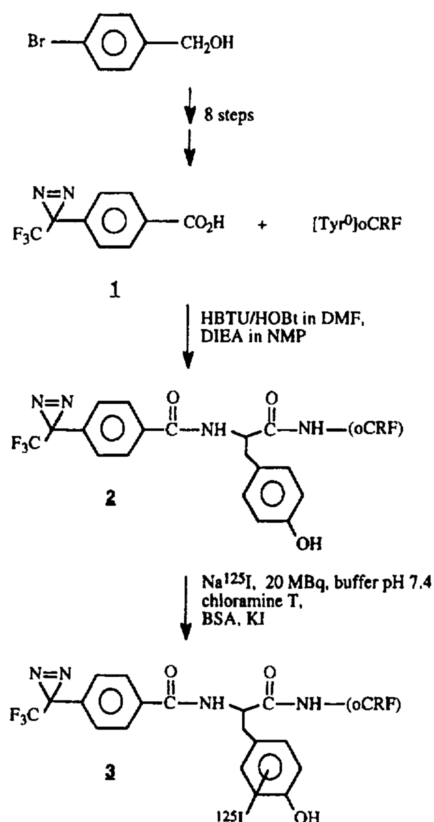
(57) Abstract

The present invention refers to CRF or analogs thereof bearing a photoactivatable moiety and a label and their use in detecting CRF receptor and binding proteins and identifying their binding site.



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CRF Analogs and their Use in Photoaffinity Labeling of CRF Receptors

The present invention refers to CRF or analogs thereof bearing a photoactivatable moiety and a label and their use in detecting CRF receptors and binding proteins and identifying and characterizing their binding site.

Corticotropin-releasing factor (CRF) appears to integrate the endocrine, autonomic, immunologic and behavioral responses to stress in the CNS. The 41 residue polypeptide (1) was originally characterized on the basis of its hypophysiotropic activity stimulating the release of adrenocorticotropic hormone (ACTH) (2), which is known to stimulate the secretion of glucocorticoids from the adrenal cortex. It is generally accepted that CRF is the main regulator of the hypothalamus-pituitary-adrenal (HPA) axis leading to the release of glucocorticoids after exposure to stress.

The various functions of CRF in the endocrine, autonomic and immunologic system are mediated by a variety of receptor proteins. Many of these receptors have been studied and investigations concerning the binding affinity and biopotency of several homologues of the CRF family have been performed.

CRF exhibits its activity through G protein-coupled receptors. CRF receptor, type 1 (CRFR1), mainly found in pituitary and brain was independently cloned from human, mouse and rat brain, and a human Cushing's corticotropic cell tumor (3-6). cDNAs coding for two splice variants of

CRF receptor, type 2 (CRFR2a and CRFR2b), were cloned from brain, heart, and skeletal muscle (7-10). Recently, it has been proposed that urocortin (Ucn), a naturally occurring CRF analog, is the endogenous ligand to CRFR2 (11).

Besides the CRF receptor, a 37 kDa CRF binding protein has been characterized. This protein which is not homologous to any known splice variant of CRFR1 or CRFR2 was demonstrated to bind human/rat CRF (h/rCRF) but not ovine CRF (oCRF) with high affinity (12). The very potent CRF antagonist astressin, cyclo(30-33)[D-Phe^{12}, $Nle^{21,38}$, Glu^{30}, Lys^{33}]h/rCRF-(12-41), with its amino acid sequence based on h/rCRF exhibited similar binding affinity to CRFR1 as found for h/rCRF but did not bind to the CRF binding protein (13). The biopotency of astressin to inhibit CRF mediated ACTH release in an in vitro pituitary cell culture assay was attributed to the built-in lactam bridge at the end of the presumable α-helical part of the peptide stretching from amino acid 5-36 in h/rCRF (13, 14). Surprisingly, the potency of h/rCRF to stimulate ACTH secretion in pituitary cells was not significantly increased when the same lactam bridge motif was introduced into the peptide. It was therefore assumed that the N-terminus of CRF is responsible for CRF receptor activation and induction of α-helicity along the whole molecule (13). Recently, in binding studies with COSM6 cells transiently expressing chimeric receptors of rCRFR1 and the rat growth hormone releasing factor receptor (rGRFR), it was shown high affinity binding of astressin to the N-terminus of rCRFR1 ($rCRFR_N$). The CRF peptide agonists h/rCRF and urocortin still produced cAMP production when bound to $rCRFR_N$/rGRFR but to a lower extent than CRF stimulation of rCRFR1 (15). A study on the characterization of another seven transmembrane spanning G protein-coupled receptor clearly indicated different binding sites for agonist and antagonist binding of Gonadotropin-releasing hormone by site-directed mutagenesis (16).

Considering the numerous important functions of CRF and in order to further investigate agonist and antagonist binding of CRF to its receptors, it would be helpful to identify the amino acid sequence directly involved in CRF binding and to investigate the cell biological fate of the CRF receptor and the binding protein after ligand linkage by means of a CRF analog serving as a label covalently linked to proteins binding CRF with high affinity.

Chemical cross-linking with $[^{125}I]Tyr^{0}$ oCRF has been proved not to be suitable to characterize the actual binding site since the cross-linking efficiency is very low and subsequent chemical and enzymatic cleavages result in the removal of the label from the cross-linked CRF receptor.

Several CRF receptor cross-links with molecular weights in the range of 58,000-75,000 have been characterized applying bifunctional reagents to membranes of bovine anterior pituitary membranes (17), AtT-20 mouse pituitary tumor cells (18), rat brain, and anterior pituitary (19,20). However, all CRF cross-links reported to date were obtained with an extremely low yield (<1%).

Labeling through monofunctional photoaffinity probes is expected to provide higher yields than labeling with chemical cross-linking methods using bifunctional reagents. Additionally, photoactivation is assumed to be superior over thermal activation, because highly reactive species such as carbenes and nitrenes can be selectively formed after irradiation uder mild conditions. The carbenes or nitrenes formed can insert into X-H bonds and thereby attack groups that are normally inert to chemical affinity labeling (21).

A prerequisite for all experiments using a photoaffinity labeling (PAL) technique is that the photoactivatable ligand binds with high affinity to the receptor and that the receptor is not destroyed or

deactivated by the light used to activate the label (21,22). Recently, a new class of photoactivatable compounds, the aryldiazirines, has been introduced, which allows photochemical decomposition under mild conditions (23).

Thus, the technical problem underlying the present invention is to provide CRF or analogs thereof which bind efficiently and with high affinity to the receptor resulting in an irreversible labeling of the receptor.

The solution to said technical problem is provided by the embodiments characterized in the claims.

Accordingly, the present invention provides CRF or analogs thereof bearing a photoactivatable moiety and a label.

In this context, the term "analog" encompasses any variant or fragment of CRF which retains CRF ligand binding activity.

In a specific embodiment the photoactivatable moiety and the label are adjacent to each other.

The photoactivatable moiety should preferably be of such quality that the photoaffinity labeling can be performed under mild conditions at a suitable wavelength. Examples of the photoactivatable moiety are the 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl residue or the phenylalanine analog thereof.

The label can be a radioactive marker, e.g. ^{125}I, or a fluorescent marker, e.g. fluorescein, or via biotin which interacts with avidin carrying a fluorescent group.

Preferred embodiments of the invention are a CRF agonist, 4-(1-azi-2,2,2-trifluoroethyl)benzoyl-[^{125}I]-tyrosine^{0}oCRF (compound **3**), and CRF antagonists based on the amino acid sequence of astressin carrying the 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl (ATB) residue and a histidine or tyrosine by choice for specific radiolabeling, e.g. ATB-cyclo(30-33)[^{125}I-His^{13}, Nle^{21,38}, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41) (compound **6**) and ATB-cyclo(30-

33)[$Nle^{21,38}$, Glu^{30}, ^{125}I-Tyr^{32}, Lys^{33}]h/rCRF-(13-41) (compound **7**).

The synthesis of the compounds of the invention can be performed by linking the photoactivatable moiety, e.g. ATB, to the CRF or CRF analog and subsequent labeling, e.g. iodination.

For example, the synthesis of compounds **4** and **5** is performed by linking 4-(1-Azi-2,2,2-trifluoroethyl)benzoic acid to cyclo(30-33)[$Nle^{21,38}$, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41) and cyclo(30-33)[$Nle^{21,38}$, Glu^{30}, Tyr^{32}, Lys^{33}]h/rCRF-(13-41). Cyclization of the peptides on the resin prior to coupling of the phenyldiazirine to the N-terminus of the peptides is chosen because of the probable sensitivity of the diazirine group towards tetrakistriphenylphosphine palladium (0) (23). Subsequent iodination with ^{125}I at $histidine^{13}$ or $tyrosine^{32}$ furnishes compounds **6** and **7** with a specific activity of 82 TBq/mmol, respectively.

In a preferred embodiment of the invention the [^{125}I]Tyr^{0}oCRF analog bears the 4-(1-azi-2,2,2-trifluoroethyl)benzoyl residue at its N-terminus, where the disturbance of ligand binding is supposed to be minimal (2, 24, 25). The immediate proximity of the photoactivatable part to the radioactive tracer in the molecule facilitates the identification and purification of peptide fragments after photoaffinity labeling experiments. CRF-R1 with a molecular weight of approximately 75kDa was detected with the new CRF analog in HEK 293 cells, permanently transfected with the CRFR1 gene.

The compounds of the invention can be used for the detection of CRF receptors and binding proteins and for the identification of the binding site of these proteins. The photoaffinity labeling technique of the present invention is

advantageous towards chemical cross-linking methods when identifying the ligand binding site within a receptor molecule as on irradiation of the photoactivatable ligand, a highly reactive short living species is formed, which then irreversibly binds with high yield to its receptor. The affinity tagged receptor polypeptide identified by the label is stable so that it can be further purified, e.g. by HPLC. It can then be cleaved into fragments, and the binding site can be identified by amino acid sequence analysis.

Brief description of the figures

FIG. 1. Synthetic route for the photoactivatable diazirine **1** according to document (26) and its linkage to Tyr^0oCRF 1-41 to generate **2** and its ^{125}iodinated analog **3**.

FIG. 2. (A.) Displacement of $[^{125}I\text{-}Tyr^0]$oCRF bound to membranes from transfected HEK 293 cells by oCRF (●) or ovine photoCRF **2** (□). Data are the mean of triplicates of a representative experiment. (Inset) Scatchard plots of the binding of oCRF (●) and ovine photoCRF **2** (□).

(B). Stimulation of intracellular cAMP accumulation in transfected HEK 293 cells by oCRF (●) and ovine photoCRF **2** (□). Data are the mean from duplicate of a representative experiment. The error bars represent the SEM and are not shown when smaller than the symbol size.

Fig. 3. Stimulation of intracellular cAMP accumulation in Y79 cells by oCRF (●), ovine photoCRF **2** (□), and ovine photoCRF **2** (x) in the presence of 100 nM recombinant human [D-Phe^{12}, $Nle^{21,38}$]CRF-(12-41). Data is the mean ± SEM values (bars) of duplicates of a representative experiment.

FIG. 4. Photoaffinity cross-linking of ovine ^{125}I-photoCRF **3** to HEK 293 cell membrane homogenates. Lanes: 1-5, extracts of cells stably transfected with cDNA coding for rCRFR1; 6 and 7, extracts of nontransfected HEK 293 cells.

Radioactive ovine photoCRF was bound in the absence of oCRF (lanes 1, 5, and 6) or in the presence of 100 nM (lane 2), 1 μM (lane 3), 10 μM (lanes 4 and 7) oCRF or 1 μM vasoactive intestinal peptide (lane 5). Fifty micrograms of total membrane protein was labeled with approximately 100,000 cpm of ovine ^{125}I-photoCRF and incubated (37°C, 30 min.) in the presence (lane 9) or absence (lane 8) of 2000 units of PNGase.

FIG 5. (A) Plot of radioactivity of membrane components covalently labeled with ovine ^{125}I-photo CRF **3** and purified with RPHPLC. (B) Pooled fractions were subjected to SDS/PAGE in 7.5% gels.

Agonist binding studies using compounds **2** and **3**

A. Preliminary experiments

Preliminary experiments with the diazirine function of **1** were performed in order to optimize the photo-affinity labeling experiments with **3** on CRFR1 transfected HEK 293 cell membranes. The photolysis proceeded with a half-life of 100 s, and after 12 min all diazirine was converted to its carbene or diazo valence isomer (80% carbene, 20% diazo valence isomer (26)). The photolysis was performed at a wavelength of 360 nm using a UV Stratalinker (Stratagene) equipped with five 15 watts lamps and monitored with a UV spectrophotometer (Beckman DU650 spectrometer, Fullerton). At a distance of 14 cm from the lamps, **1** was photolyzed (c = 1 mM in ethanol, V = 380 μl, 1 ml quartz cuvette) with first order kinetic and a half-life of 100 s at 4°C. The same results were obtained when photolyzing **3** after incubation with membranes in different time intervals and analyzing the photoproduct with SDS/PAGE.

B. Binding and cAMP assay

For the determination of the binding affinity and the biological potency of ovine photoCRF **2**, a permanent cell line was established from HEK 293 cells stably transfected with cDNA coding for rCRFR1. A pool of HEK cell clones was employed in the following experiments. Binding results obtained with individual HEK cell clones did not differ significantly from the results of binding experiments with the cell clone pool. Scatchard analysis indicated that oCRF was bound with a K_d value of 7.8 ± 6.3 nM at a high-affinity site and a K_d value of 137 ± 90 nM at a low-affintiy site. The B_{max} values of 30 fmol/μg and 347 fmol/μg of protein, respectively, indicated a high efficiency of expression. A similar K_d value of 5.6 ± 2.6 nM (B_{max} = 12 fmol/μg of protein) was found for ovine photoCRF **2** displacing [^{125}I-Tyr^0]oCRF. Scatchard analysis indicated only binding of ovine photoCRF to the high-affinity site (Fig. 2A). Application of oCRF or ovine photoCRF to the transfected HEK 293 cells stimulated the accumulation of cAMP in a dose-dependent manner. EC_{50} values of 0.5 ± 0.2 nM and 0.4 ± 0.1 nM were determined for oCRF and photoCRF, respectively (Fig. 2B). Non-transfected cells did not show significant binding or cAMP accumulation. This observation was also confirmed by photoaffinity-labeling experiments. Ovine ^{125}I-photoCRF **3** did not bind to membranes of non-transfected HEK 293 cells (see Fig. 4). In experiments with membrane preparations from human Y79 retinoblastoma cells, known to carry an endogenous functional CRF receptor (27), a K_d value of 2 nM (B_{max} = 0.19 fmol/μg of protein) was found for oCRF or ovine photoCRF **2**. In Y79 cells, only a high-affinity site was detected for either CRF analog. Ovine photoCRF and oCRF stimulated cAMP accumulation in Y79 cells with EC_{50} values of 2.3 ± 0.5 nM and 1.3 ± 0.6 nM, respectively (Fig. 3). Statistical analysis of the described binding and cAMP data with the program ANOVA revealed no significant differences

between the K_d and EC_{50} values for oCRF and photoCRF. The specificity of the stimulatory action of ovine photoCRF was further demonstrated by the observation that this peptide exhibited lower stimulatory potencies in the presence of the specific CRF antagonist recombinant human [D-Phe^{12}, $Nle^{21,38}$]CRF-(12-41). For the antagonist an apparent inhibitory constant (K_i) of 10.3 ± 5.0 nM was found (Fig. 3).

C. Photoaffinity-Labeling Experiments

Since it had been found (17,28) that BSA interferes with the labeling of the receptor, freshly prepared tracer **3** was stored free of any carrier protein, and photoaffinity-labeling experiments were performed in buffer solutions in the absence of BSA. A 75 kDa cross-link was identified with SDS/PAGE after irradiation at 360 nm of a mixture of ovine ^{125}I-photoCRF **3** and membranes of HEK 293 cells permanently transfected with rCRFR1 (Fig. 4). No cross-link could be identified without light activation at 360 nm. Using commercially available [^{125}I-Tyr^0]oCRF and disuccinimidyltartrate, a 75 kDa protein was labeled in chemical cross-linking experiments. Binding of ovine ^{125}I-photoCRF to the receptor could be efficiently inhibited by addition of 1 μM oCRF but not 1 μM vasoactive intestinal peptide, in agreement with the assumed specificity of this photoprobe. As mentioned above, no photoaffinity cross-linking of ovine ^{125}I-photoCRF to nontransfected HEK 293 membranes was detected. Deglycosylation of the 75 kDa protein cross-link with PNGase generated a 46 kDa protein detected by SDS/PAGE (Fig. 4).

In a preparative photoaffinity-labeling experiment, membrane proteins cross-linked to ovine ^{125}I-photoCRF were purified by RPHPLC. It was found by SDS/PAGE analysis that the radioactive fractions that were eluted after the void volume contained the 75 kDa CRFR1 protein cross-link (Fig. 5). To calculate the yield of the cross-linking procedure,

labeled receptor was divided by the radioactivity of ovine ^{125}I-photoCRF specifically bound to the HEK cell membranes that served as starting material. On this basis, a yield of at least 20-30% was estimated.

Antagonist binding studies using compounds **4** to **7**

A. Binding and cAMP assay

For the determination of the binding affinity and the biological potency of the photoactivatable CRF antagonists **4** and **5**, a HEK 293 cell line, stably transfected with cDNA coding for rCRFR1, and the human Y79 retinoblastoma cell line, expressing an endogenous CRF receptor (CRFR1), were used. The results are shown in Table I. Scatchard analysis indicated high and low affinity binding of oCRF (K_{d1} = 1.1 ± 0.7 nM; K_{d2} = 1.1 ± 1.3 μM) and astressin (K_{d1} = 0.9 ± 1.0 nM; K_{d2} = 1.6 ± 1.6 μM) to membrane homogenates of Y79 cells. Compound **4** exhibited similar binding characteristics as astressin (K_{d1} = 0.6 ± 0.5 nM; K_{d2} = 3.4 ± 2.2 μM). Compound **5** showed decreased binding affinity to CRFR1 in this cell line (K_{d1} = 26 ± 23 nM). Similar results were obtained when oCRF, astressin and compounds **4** and **5** were bound to membrane homogenates of transfected HEK 293 cells with a K_d value of 3.3 ± 0.5 nM, 7.7 ± 2.6 nM, 3.2 ± 2.7 nM and 12 ± 3.6 nM, respectively. Only oCRF showed binding to a low affinity site with a K_d value of 147 ± 78 nM in this cell line. Application of oCRF to the Y79 cells and HEK 293 cells stimulated the accumulation of cAMP in a dose dependent manner with EC_{50} values of 3.8 ± 2.6 nM and 0.4 ± 0.1 nM, respectively. Ovine CRF stimulated cAMP production could be efficiently inhibited in the presence of 5 nM antagonist in Y79 cells. An inhibitory constant (K_i) of 0.5 ± 0.3 nM, 1.0 ± 0.3 nM and 6.0 ± 2.8 nM was determined for astressin and compound **4** and **5**, respectively. Similar results were obtained when oCRF stimulated cAMP accumulation in transfected HEK 293 cells

was inhibited in the presence of 100 nM CRF antagonist. A K_i value of 101 ± 92 nM, 51 ± 52 nM and 497 ± 72 nM for astressin and compounds **4** and **5** were obtained. Application of a higher dosis of CRF antagonist to observe significant reduction of oCRF stimulated cAMP production in HEK 293 was necessary because of a fifty times higher expression of high affinity receptors in transfected HEK 293 cells (oCRF: B_{max1} = 16 ± 6 fmol/µg; B_{max2} = 197 ± 15 fmol/µg) when compared with the Y79 cells (oCRF: B_{max1} = 0.3 ± 0.3 fmol/µg; B_{max2} = 35 ± 57 fmol/µg). Non-transfected cells did not show significant binding or cAMP accumulation. This observation was also confirmed by photoaffinity labeling experiments. Compound **7** did not bind to membranes of non-transfected HEK 293 cells. Statistical analysis of the described binding and cAMP data with the program ANOVA revealed no significant differences between the K_d and K_i values for astressin and compound **4**. Both peptides exhibited high potency to reduce the stimulatory potency of oCRF to produce cAMP in transfected HEK 293 cells and Y79 cells. Compound **5**, however, revealed 5-10 times lower potency to inhibit cAMP production in both cell lines when compared to astressin or compound **4** which was consistent with its decreased binding affintiy to CRFR1.

B. Photoaffinity labeling experiments

As described above, the freshly prepared tracer **7** was stored free of any carrier protein, and the photoaffinity labeling experiments were performed in buffer solutions in the absence of BSA. A 66 kDa cross-link was identified with SDS PAGE after irradiation at 360 nm of a mixture of compound **7** and membranes of HEK 293 cells permanently transfected with rCRFR1. No cross-link could be identified without light activation at 360 nm. Binding of compound **7** to the receptor could be efficiently inhibited by addition of 1 µM ATB-cyclo(30-33)[$Nle^{21,38}$, Glu^{30}, Tyr^{32}, Lys^{33}]h/rCRF-(31-41) (compound **5**) but not 1 µM vasoactive

intestinal peptide (VIP) in agreement with the assumed specificity of this photoprobe. As mentioned above, no photoaffinity cross-linking of compound 7 to non-transfected HEK 293 membranes was detected. Deglycosylation of the 66 kDa protein cross-link with PNGase generated a 38 kDa protein detected by SDS PAGE.

Thus, the compounds of the invention can be used for the specific irreversible labeling and tracking of receptors in various tissue membranes, of CRF binding proteins, as well as in cytological investigations using a fluorescent analog of **2**, **4** or **5**, e.g. on cell sorting, receptor internalization, trafficking.

The invention is illustrated by the following examples.

Example 1

Synthesis of 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid (1).

In the dark, 420 mg of 4-(1-azi-2,2,2-trifluoroethyl)benzyl alcohol (1.9 mmol; 44 % overall yield starting with 4-bromobenzyl alcohol in a seven step synthesis) (26) was dissolved in 1.4 ml of dioxane and 12 ml of 0.2 N aqueous KOH. Then, $KMnO_4$ (462 mg; 2.9 mmol) was added in portions and the mixture was stirred for 2 hr at ambient temperature. The precipitated MnO_2 was removed by filtration, washed several times with methanol and the combined filtrates were concentrated under reduced pressure. The residual alkaline solution was extracted with ether, acidified to pH 2-3 with 1N aqueous H_2SO_4 and extracted again with ether. The organic layer was washed neutral with water, dried with anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. The product was crystallized from hexane and yielded 230 mg of **1** (1.0 mmol; 53%) :m.p. 123-125°C, decomp. with foam (N_2); ^{1}H-NMR ($CDCl_3$, TMS) 7.72

(AABB, 4H, Ar-H); ^{13}C-NMR ($CDCl_3$, TMS) 28.46 (m, J = 41 Hz), 121.85 (m, J = 274 Hz), 126.49 (m, J = 1.3 Hz), 130.32 (m, J = 2.9 Hz), 130.54 (s), 134.78 (s), 170.81 (s); ^{19}F-NMR (DMSO-d_6, $CFCl_3$)-64.00; UV (ethanol) λ (ε)348 nm (248); MS m/z (rel. intensity) 229 (100, $[M-H]^+$), 201 (21, $[M-N_2]^+$), 157 (51), 137 (8); HRMS calcd. for $C_9H_5N_2F_3O_2$ 229.0249, found 229.0228.

Example 2

Synthesis of 4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-tyrosine^{0}oCRF 1-41 (2).

In the dark, 26 mg of **1** (0.11 mmol) in 0.2 ml of NMP were activated by 0.2 ml of 0.45 M HBTU/HOBt in DMF (6 min.) and 0.1 ml of 2 M of DIEA in NMP (2 min.). 83 mg of peptide resin (7.00 µmol side chain protected [Tyr^0]oCRF 1-41 on TentaGel S RAM resin; capacity 0.22 mmol/g) were added and the mixture was reacted for 15 min. The resin was filtered off, washed three times with 0.5 ml of NMP, added to 750 µl of cleavage mixture (75 µg of crystalline phenol, 25 µl of EDT, 50 µl of thioanisole and 50 µl of dH_2O, 1 ml of TFA) and stirred for 1.4 hr. The resin was filtered off and the peptide precipitated in 20 ml of ice cold ether. After filtration, the crude peptide was dissolved in 2 ml of TFA and 50 ml of 20% MeCN in 0.1 % TFA/water and lyophilized. 21 mg of 38 mg crude product was purified by preparative reversed-phase HPLC and yielded 2.7 mg of **2** (0.54 µmol, 14%):ESI MS calcd. 5045.7; found 5045.1. Analytical RP-HPLC was performed on a Vydac C_{18} silica gel column (0.46 x 25 cm, 5 µm particle size, 30 nm pore size) with solvents A:0.1% TFA/water and B: 80% MeCN in 0.1% TFA/water, flow rate: 1 ml/min, 40% B for 5 min, then 40-90% B for 25 min.R_t = 19.62 min).

Example 3

Synthesis of 4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-[^{125}I]-tyrosine^{0}oCRF 1-41 (3).

2 was iodinated with slight modifications according to literature (29). To a tube containing 4 µl of a 100 µM solution of **2** in 0.01N HOAc in dH_2O, the following reagents were added in a certain order: 10 µl of 0.5 M phosphate buffer, pH 7.4, approximately 20 MBq of ^{125}I (IMS 30, Amersham, UK), 12.5 µg of chloramine T in 5 µl of 0.05 M phosphate buffer, 15 s later the reaction was stopped by adding 10 mg of BSA in 100 µl of 0.5 M phosphate buffer and 1 mg of KI in 100 µl of 0.05 M phosphate buffer. The mixture was pipetted onto a Bond Elut C_{18} cartridge (Varian Associates), prewetted with 5 ml of MeOH, then 5 ml of 0.1 % TFA/water. Five milliliters of dH_2O followed by 5 ml of 0.1 % TFA/water were passed through the column in order to separate the iodinated peptide from free iodine and BSA. The iodinated peptide was then eluted from the column by the addition of 5 ml of 80% MeCN in 0.1 % of TFA/H_2O. The volume of the peptide fraction was reduced to approximately 200 µl with a Speed Vac (Christ) and loaded onto a Vydac C_{18} silica gel column (0.46 x 25 cm, 5 µm particle size, 30 nm pore size) and eluted with solvents A (0.1 % TFA/water) and B (80% MeCN in 0.1 % TFA/water) and a flow rate of 1 ml/min. Elution was performed with 45% B for 5 min, then 45-95% B for 25 min. The retention time for **3** was R_t = 17.36 min. A Beckman 171 Radioisotope Detector equipped with a liquid scintillator flow cell was used. The specific activity of the peptide: 82 TBq/mmol. The peak tubes of radioactivity were pooled and β-mercaptoethanol was added to a final concentration of 0.5 M. The iodinated tracer **3** (Fig. 1) was stored in aliquots at -20°C and typically used for binding assays and photoaffinity labeling experiments for 2 months.

Example 4

Synthesis of ovine CRF, cyclo(30-33)[D-Phe^{12}, Nle^{21,38}, Glu^{30}, Lys^{33}]h/rCRF-(12-41) (Astressin), ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41) (Compound 4), ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, Tyr^{32}, Lys^{33}]h/rCRF-(13-41) (Compound 5)

The CRF peptides were synthesized with Fmoc chemistry on TentaGel S RAM resin (0.1 mmole scale, Rapp, Tübingen, F.R.G.) with a model ABI 433A peptide synthesizer (Applied Biosystems). After cleavage of the peptides from the resin, the crude peptides were purified by preparative reverse-phase HPLC (RPHPLC) performed on a Waters Prep Nova-Pak HR C_{18} silica gel column (5 x 30 cm, 6-μm particle size, 6-nm pore size) with a mixture of aqueous 0.1% trifluoroacetic acid (TFA) and MeCN. The mass spectra of the purified peptides were measured with ESI (electrospray ion) MS on a Micromass AutoSpec-T tandem mass spectrometer.

For the synthesis of the cyclized CRF analogs, amino acid derivatives Fmoc-Glu(OAl)-OH and Fmoc-Lys(Aloc)-OH (PerSeptive Biosystems GmbH, Hamburg, F.R.G.) were used. The side-chain protected peptides were reacted with $Pd^0[PPh_3]_4$ in HOAc/N-methylaniline/dichloromethane (v/v; 2:1:40) for three hours and then cyclized with HOBt/HBTU in DMF and DIEA in NMP for eight hours. After removal of the N-terminal Fmoc group with piperidine in NMP, 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid was linked to the N-terminus of the peptide resin with HOBt/HBTU in DMF and DIEA in NMP in the dark. The peptides were then cleaved from the resin and purified by preparative RPHPLC. The purified CRF peptides were subjected to analytical RPHPLC on a Vydac C_{18} silica gel column (0.46 x 25 cm, 5-μm particle size, 30-nm pore size) with solvents A (0.1% TFA in water) and B (80% MeCN in 0.1% TFA in water) at a flow rate of 1 ml/min. The samples were eluted with 5% B for 5 min. and then with a linear gradient of 5-95% B in 30 min. (oCRF: ESI MS calcd

4670.4, found 4669.2, R_t = 25.9 min; astressin: ESI MS calcd 3565.1, found 3563.1, R_t = 24.8 min; **4**: ESI MS calcd 3562.1, found 3561.1, R_t = 30.2 min; **5**: ESI MS calcd 3654.2, found 3653.7, R_t = 29.6 min).

ATB-cyclo(30-33)[^{125}I-His^{13}, Nle^{21,38}, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41) (Compound 6) and ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, ^{125}I-Tyr^{32}, Lys^{33}]h/rCRF-(13-41) (Compound 7)

Compounds **6** and **7** were iodinated as described (29,30). The peptides were partially purified with a Bond Elut C_{18} cartridge (Analytichem, Harbor City, CA, USA) and subsequently with RPHPLC performed on a Vydac C_{18} silica gel column (0.46 x 25 cm, 5 μm particle size, 30 nm pore size) with solvents A (0.1% TFA in water) and B (80% MeCN in 0.1% TFA in water) at a flow rate of 1 ml/min. The samples were eluted with 45% B for 5 min. and then with a linear gradient of 45-95% B in 25 min (**6**: R_t = 21.9 min; **7**:R_t = 20.4 min). A Beckman 171 Radioisotope Detector equipped with a liquid scintillation flow cell (Beckman, Fullerton, CA, USA) was used to monitor radioactivity. The specific activity of the peptides was 82 TBq/mmol.

Example 5

Transfection of HEK 293 cells

Human embryonic kidney cells 293 (Graham, Smiley, Russell & Naim, 1977) (supplied by Dr. C. Stevens and G. Sharma, The Salk Institute, La Jolla) were grown in Dulbecco's modified eagle medium (GIBCO BRL, Gaitherburg, MD, USA, cat. no.: 041-01885M) supplemented with 10% fetal calf serum (Sigma, St. Louise, MO, USA, cat. no.: F-7524) and brought to a final concentration of 4 mM L-glutamine (GIBCO BRL, cat. no.: 043-05030), 0.45% glucose. They were maintained as described (31). The rat CRFR1 gene fragment

(1284 bp, BamHI, EcII26II fragment) was subcloned into the vector pcDNA3 (Invitrogen, San Diego, Ca, USA). The recombinant plasmid ($pCDNA3-rCRF_1$) was isolated, and purified with the Qiagen plasmid preparation system (Qiagen, Hilden, Germany). The ligation sites were verified by DNA sequence analysis.

HEK 293 cells were transfected with pCDNA3-rCRF-R1 utilizing the calcium/BBS transfection method (32). Sixteen hours after transfection, the medium was removed and replaced by selection medium (600 μg/ml Geneticin in medium). Cells were grown until confluent and split 1:2 with further selection. Following one to two weeks of growing under selection conditions, all cells were geneticin-resistant and grew normally.

Example 6

Preparation of Crude Membranes

The cells obtained according to Example 5 were dislodged from the cell culture flasks with a cell scraper into ice cold PBS buffer. The cells were precipitated at 150 g for 10 min. at 4°C, resuspended in 1 x PBS buffer and recentrifuged. The supernatant was entirely removed and the wet weight of the cell pellet was determined. The cells were suspended in 3 ml/g cells of CRF membrane buffer (50 mM Tris/Cl, 5 mM $MgCl_2$, 2 mM EGTA, 500 μl Trasylol (FBA, New York, USA), 1 mM DTT, pH 7.4) and treated for 10 strokes (each 2 s) with the medium sized polytron tool at power level 5. The nuclei were precipitated for 5 min at 600 g in the cold. The supernatant was carefully removed with a Pasteur pipette and collected on ice. The pellet was reextracted with the same amount of membrane buffer using some strokes of the polytron. The nuclei were again precipitated from this suspension as described. The combined supernatants were centrifuged at 10,000 g for 15 min to precipitate the membranes. The pellet was resuspended with 3

ml/g of cells in storage buffer (membrane buffer containing 20% glycerol) with 10 strokes of a glass Teflon homogenizer. A micro BCA assay (Pierce, Rockford, USA) was performed with 2 µl and 4 µl of the suspension to estimate the total protein concentration (about 2.5 µg/µl). The membranes were frozen in liquid nitrogen and stored at -70°C until use.

Example 7

Binding assays with oCRF, astressin and compounds 2, 4 and 5

To a tube containing the peptides (c = 0-1 µM) and 100,000 or 200,000 cpm, respectively, of [^{125}I-Tyr^{0}]oCRF in 200 µl incubation buffer (membrane buffer supplemented with BSA to 1 mg/ml), 100 µl of membrane suspension containing 25 µg of protein (HEK 293 cells) or 100 µg of protein (Y79 cells) was added. After incubation (1 hr, 23°C), membrane buffer (1 ml) was added. After centrifugation at 14,000 x g (4°C, 5 min), the pellet was washed twice with 1 ml of membrane buffer. Radioactivity was measured with a 1470 WIZARD automatic gamma counter (Berthold, Hannover). Data analysis was achieved with the non-linear curve fitting program LIGAND.

Example 8

a) Photoaffinity labeling experiments with 3

Photoaffinity labeling experiments were in principle performed in the same manner as mentioned above except that the incubation buffer used was without BSA. To a concentration series of either oCRF (0, 100 nM, 1 µM, 10 µM) or VIP (1 µM) and 180,000 cpm of **3** per tube, HEK 293 membrane homogenates of either transfected or non-transfected cells (75 µg of protein/tube) were added and incubated for the indicated time. Before photolysis, the pellets were washed three times, resuspended in 300 µl of

buffer and irradiated at 360 nm for 30 min (4°C, 8 cm distance from the lamps). After photolysis, 1 ml of buffer was added and the pellets were spinned out at 15,000 rpm for 5 min. The pellet was resuspended in 15 µl of dH_2O and 15 µl of 2xSDS sample buffer and heated at 100°C for 5 min. The samples were subjected to electrophoresis in a 7.5% SDS gel and autoradiography developed on a BAS-IP NP 2040P imaging plate with a Fujix BAS 2000 scanner (Raytest). Apparent molecular masses were estimated from gel mobilities relative to those of commercial markers (SDS-PAGE high range markers, BioRad). Gel documentation was performed with the programs TINA (Straubenhardt) and WINCAM (Cybertech).

b) Photoaffinity labeling experiments with compound 7

The photoaffintiy labeling experiments were carried out like the binding assay except that no BSA was use. Samples (25 µg of protein/tube) were irradiated at 360 nm for 30 min (4°C, 8 cm distance from the lamps) after incubation with ligand (1 hr, 23°C). In some experiments the photolabeled receptor was deglycosylated with PNGase (New England Biolabs, Schwalbach). Samples were then heated (100°C, 5 min) and subjected to SDS PAGE. Autoradiography was carried out on a BAS-IP NP 2040P imaging plate. Radioactivity was monitored with a Fujix BAS 2000 scanner (Raytest, Straubenhardt). Gel documentation was accomplished with the program TINA (Raytest).

Example 9

cAMP stimulation

HEK 293 and human Y79 retinoblastoma cells (American Type Cell Culture, Rockville) were incubated with different CRF analogs in the presence of 1 or 5 mM 3-isobutyl-1-methylxanthine (37°C, 30 min), respectively. The incubation medium of the Y79 cells contained additionally 1 mg/ml BSA

and 0.05 mg/ml ascorbic acid. When compound **2** or the photoactivatable astressin analogs were used, all experiments were performed in the dark. After removal of the medium, cells were lyzed with aqueous 6% trichloroacetic acid (100°C, 5 min). The cell lysates were stored at -70°C until assayed with a RIA kit (Amersham, Little Chalfont). Data analysis was achieved with the sigmoidal dose-response curve fitting programs ALLFIT. Statistical significance was determined across groups by one-way ANOVA.

Example 10

Purification and characterization of the 75 kDa Protein Cross-Link

Membrane protein (250 μg) was labeled with 1.1×10^7 cpm of **3** (2.82 pmol). One-tenth of the sample was dissolved in 50% ethanolic formic acid (100 μl) and subjected to RPHPLC using a Vydac C_4 silica gel column (0.46 x 25 cm, 5 μm particle size, 30 nm pore size.) Elution was accomplished with a mixture of aqueous 0.5% trifluoroacetic acid and EtOH.

Table 1. Biological characterization of CRF analogs

No.	Structure	K_{d1}, nM		EC_{50}/K_i, nM	
		Y79	HEK (rCRFR1)	Y79	HEK (rCRFR1)
	oCRF-(1-41)	1.1 ± 0.7	3.3 ± 0.5	3.8 ± 2.6	0.4 ± 0.1
	cyclo(30-33)[D-Phe^{12}, Nle^{21,38}, Glu^{30}, Lys^{33}]h/rCRF-(12-41)*	0.9 ± 1.0	7.7 ± 2.6	0.5 ± 0.3	101 ± 92
4	ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41)	0.6 ± 0.5	3.2 ± 2.7	1.0 ± 0.3	51 ± 52
5	ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, Tyr^{32}, Lys^{33}]h/rCRF-(13-41)	26 ± 23	12 ± 3.6	6.0 ± 2.8	497 ± 72

*Compound 2 is astressin

ATB = 4-(1-azi-2,2,2-trifluoroethyl)benzoyl

	1 10 20 30 40
oCRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA
h/r CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII

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CLAIMS:

1. A CRF or an analog thereof bearing a photoactivatable moiety and a label.

2. The CRF or analog of claim 1 wherein the photoactivatable moiety and the label are adjacent to each other.

3. The CRF or analog of claim 1 or 2 wherein the photoactivatable moiety is a 4-(1-azi-2,2,2-trifluoroethyl)-benzoyl residue.

4. The CRF or analog of any one of claims 1 to 3 wherein the label is a radioactive marker.

5. The CRF or analog of claim 4 wherein the label is ^{125}I.

6. The CRF or analog of any one of claims 1 to 3 wherein the label is a fluorescent marker.

7. The CRF or analog of any one of claims 1 to 5 which is 4-(1-azi-2,2,2-trifluoroethyl)benzoyl-[^{125}I]-tyrosine^{0}oCRF.

8. The CRF or analog of any one of claims 1 to 5 which is ATB-cyclo(30-33)[^{125}I-His^{13}, Nle^{21,38}, Glu^{30}, Ala^{32}, Lys^{33}]h/rCRF-(13-41).

9. The CRF or analog of any one of claims 1 to 5 which is ATB-cyclo(30-33)[Nle^{21,38}, Glu^{30}, ^{125}I-Tyr^{32}, Lys^{33}]h/rCFR-(13-41).

10. Use of CRF or an analog thereof of any one of claims 1 to 9 for detecting CRF receptors and binding proteins.

11. The use of CRF or an analog thereof of any one of claims 1 to 9 for the identification of the binding site of a CRF receptor or binding protein.

12. The use of claim 10 or 11 wherein the receptor protein is detected in a tissue membrane.

13. The use of any one of claims 10 to 12 wherein the receptor protein is detected in the membrane of HEK 293 cells.

14. The use of any one of claims 10 to 13 wherein the CRF receptor protein has a molecular weight of 66 or 75 kDa.

15. A process for the purification of a CRF receptor protein which comprises reacting a membrane preparation containing the protein with CRF or an analog thereof of any one of claims 1 to 9, performing photolysis and purifying the resultant product by HPLC.

16. A process for the characterization of the binding site of a CRF receptor or binding protein which comprises purifying the CRF binding protein or the CRF receptor according to the process of claim 15, fragmenting the purified product and determining the amino acid sequence of the relevant fragment.

17. A CRF receptor or binding protein the binding site of which has been identified according to the process of claim 16.

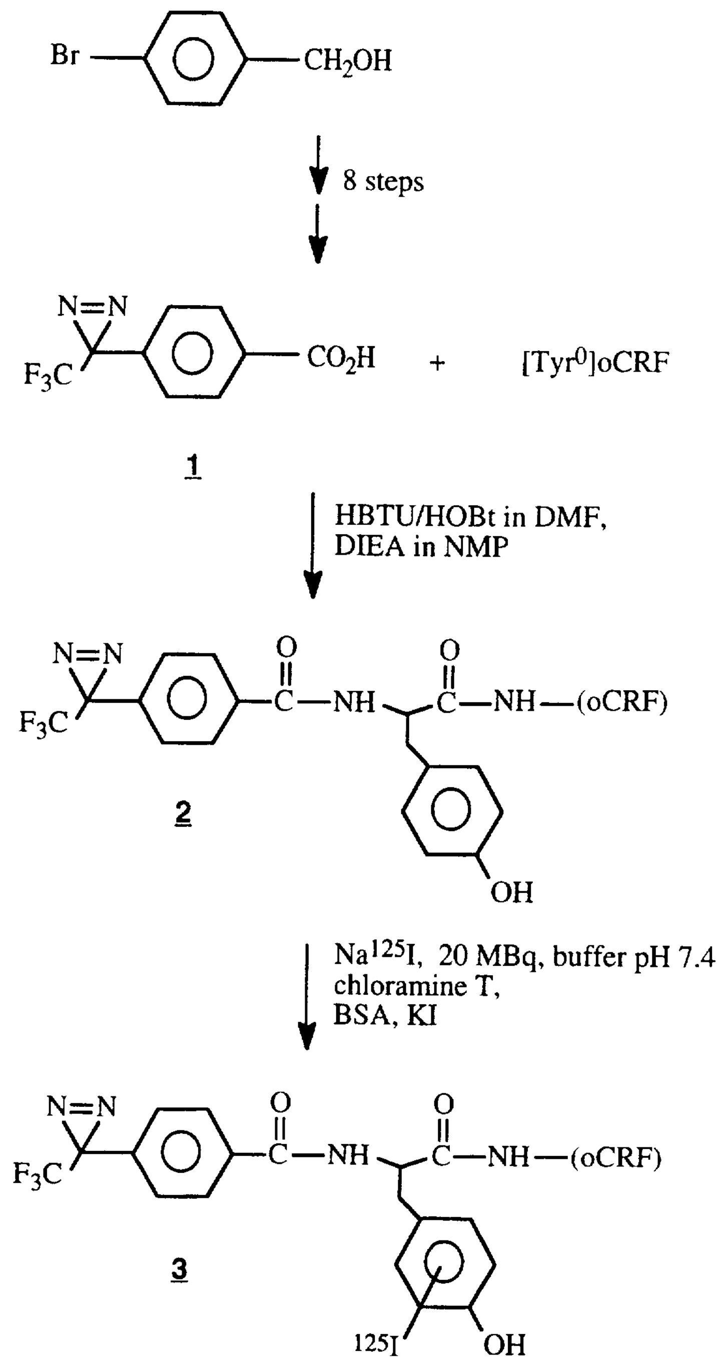

Fig. 1

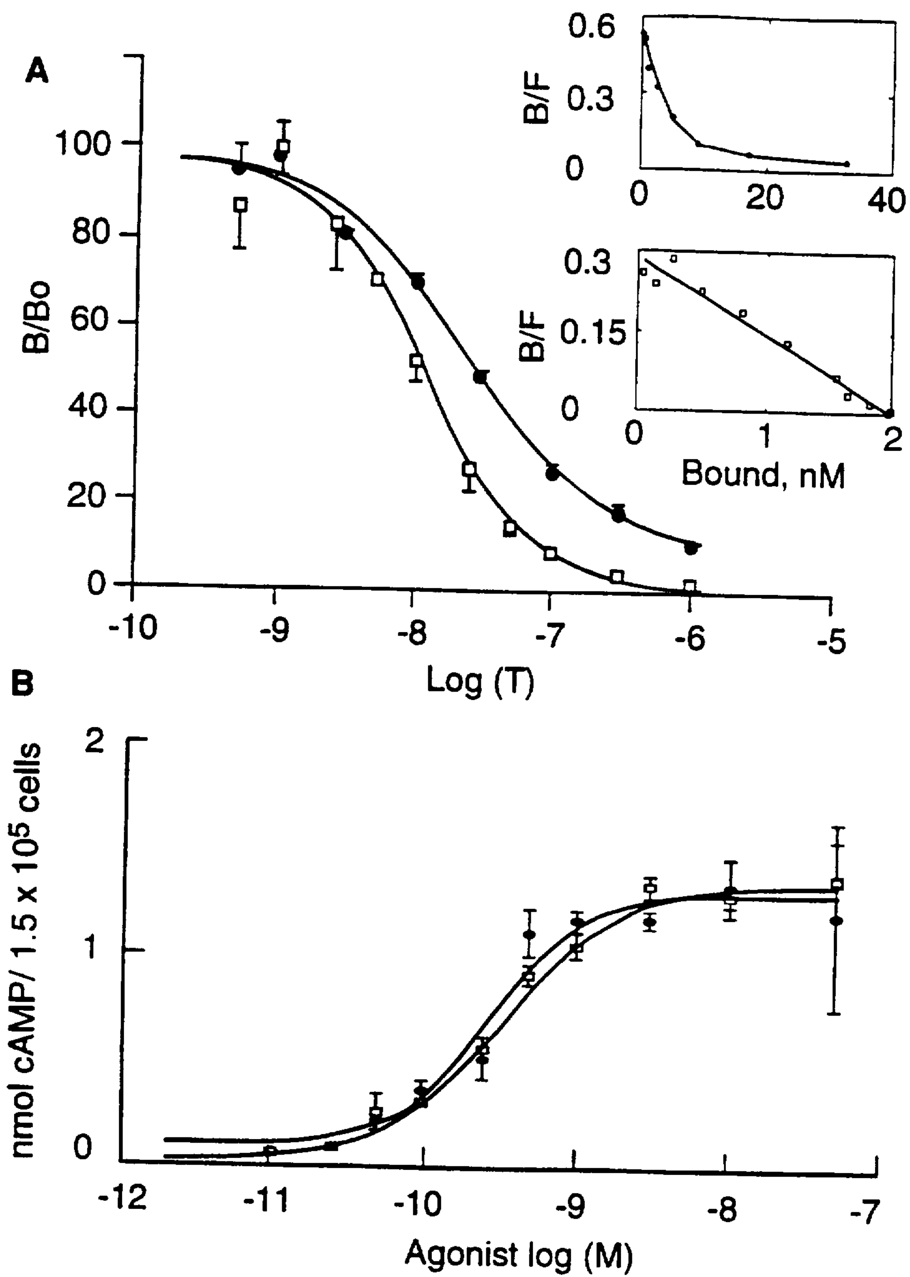

Fig. 2

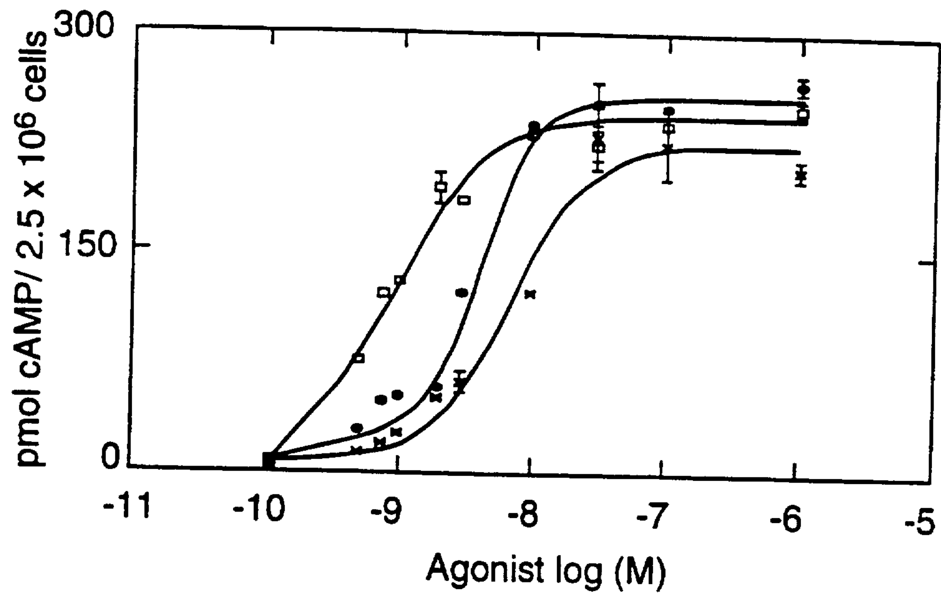

Fig. 3

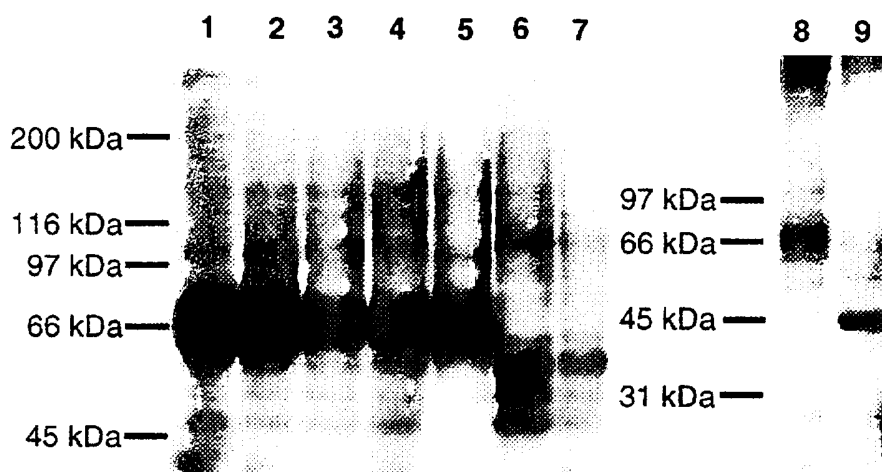

Fig. 4

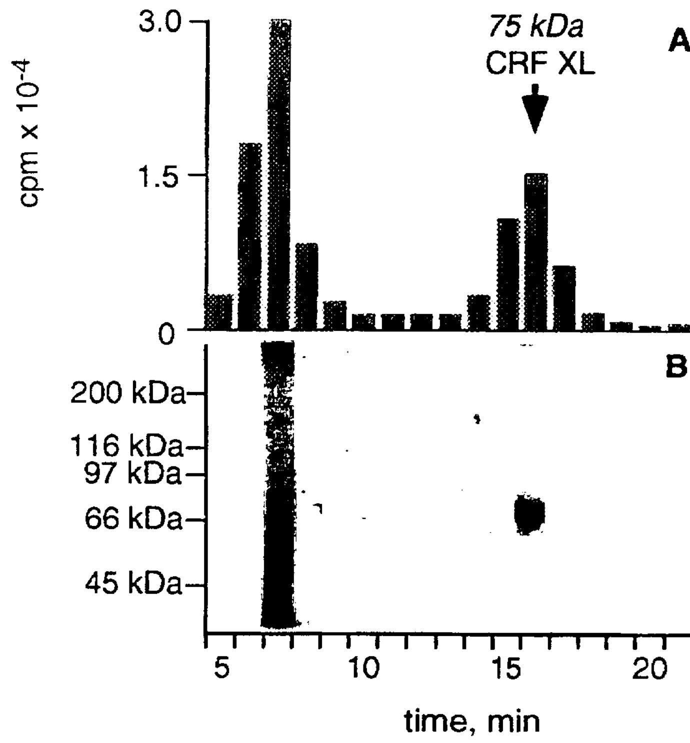

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/05011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/72 C07K14/47 C07K14/575 A61K51/08 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, vol. 34, no. 12, 7 July 1995, pages 1296-1312, XP000510821 KOTZYBA-HIBERT F ET AL: "RECENT TRENDS IN PHOTOAFFINITY LABELING" see abstract see paragraph 2.1.2 see paragraph 2.1.3 see paragraph 2.2.2 see tables 6,7 see paragraph 6 see figures 6,7 ---	1-16
Y	FR 2 628 750 A (PASTEUR INSTITUT) 22 September 1989 see page 22, line 26 - page 23, line 32 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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3

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 1997	29.04.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Dullaart, A

International Application No
PCT/EP 96/05011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INNOVATION PERSPECT. SOLID PHASE SYNTH. COLLECT. PAP., INT. SYMP., 2ND, 1992, PAGES 95-103, XP000646246 EBERLE, ALEX N. ET AL: "Solid phase peptide synthesis and application of photoreactive peptides" see page 96 - page 98 see page 100 - page 102 ---	1-16
Y	J. RECEPT. RES., 1993, VOL. 13, NO. 1-4, PAGE(S) 27-37, XP000647454 EBERLE A N: "Peptides containing multiple photolabels: a new tool for the analysis of ligand-receptor interactions" see abstract see figure 1 ---	1-16
Y	J. ORG. CHEM., 1993, VOL. 58, NO. 26, PAGE(S) 7598-601, XP002029659 RESEK J F ET AL: "A new photo-crosslinking reagent for the study of protein-protein interactions" see page 7598 - page 7599, left-hand column see figure 2 ---	1-16
Y	US 4 986 979 A (MORGAN JR A CHARLES ET AL) 22 January 1991 see examples 23-25 ---	1-16
Y	LIEBIGS ANN. CHEM., 1983,, NO. 9, PAGE(S) 1510-23, XP002029660 NASSAL M: "4-(1-Azi-2,2,2-trifluoroethyl)benzoic acid, a highly photolabile carbene generating label readily fixable to biochemical agents" see the whole document ---	1-16
P,X	PROC. NATL. ACAD. SCI. U. S. A., 1996, VOL. 93, NO. 20, PAGE(S) 10609-10613, XP002029661 RUEHMANN A ET AL: "Synthesis and characterization of a photoactivatable analog of corticotropin-releasing factor for specific receptor labeling" see the whole document ---	1-17
P,Y	WO 96 18649 A (SALK INST FOR BIOLOGICAL STUDI) 20 June 1996 see the whole document ---	1-17

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3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/05011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	WO 95 00640 A (SALK INST FOR BIOLOGICAL STUDI ;PERRIN MARILYN H (US); CHEN RUOPIN) 5 January 1995 see claim 17 see claims 1-3	17
X,Y	WO 92 13074 A (SALK INST FOR BIOLOGICAL STUDI ;UNIV READING (GB)) 6 August 1992 see sequence 1	17

3

International application No.

PCT/EP 96/05011

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-17
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See continuation sheet

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1-16: a CRF bearing a photoactiveable moiety and a label is claimed, its use in detecting CRF receptors and binding proteins.
17 (in part): a CRF receptor protein
17 (in part): a CRF binding protein

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/05011

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