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A cDNA CONSTRUCT ALLOWING THE EXPRESSION OF RAT HEPATIC GLUCAGON RECEPTORS[1]

Michal Svoboda, Ernesto Ciccarelli, Michèle Tastenoy,
Patrick Robberecht and Jean Christophe*

Department of Biochemistry and Nutrition, Medical School,
Université Libre de Bruxelles, Building G/E-CP 611,
808 Route de Lennik, B-1070 Brussels, Belgium

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SUMMARY : We constructed a full open reading frame (ORF) for the rat hepatic glucagon receptor using two non functional pCDM8 clones of a putative glucagon receptor. Clone I was complete but contained, in addition, two small introns interrupting the frame, while clone II lacked 104 bp at the 5´ end. We isolated Hind III/Dra III and BamH I/Not I fragments from clone I and a Dra III/BamH I fragment from clone II then ligated the three fragments with pBluescript SK(+) digested with Hind III/Not I. Following this 4 fragment ligation procedure, we obtained one correct insert in a clone we subcloned in the Hind III/Not I sites of pCDM8. This plasmid was used to transiently transfect COSGs1 cells with the lipofectin method. In COSGs1 membranes transfected with this correct plasmid (but not with a truncated plasmid) [^{125}I]iodoglucagon binding was selectively displaced with glucagon (IC_{50} = 4 nM) and adenylate cyclase was stimulated with glucagon (K_{act} = 10 nM) while related peptides were inefficient at 1 µM. This demonstrated that the receptor previously described is indeed the rat hepatic glucagon receptor.

We described recently the sequence of a 485-amino acid rat hepatic G-protein coupled receptor with seven putative transmembrane segments (1). The full-length sequence of the corresponding coding domain was deduced from the overlapping alignment of three reading sequences. This orphan receptor was obviously related to a family that includes receptors for GLP-1 (2), secretin (3), VIP (4), CT (5) and PTH (6). Its highest homology was with GLP-1 receptors and Northern blot analysis revealed an overexpression in liver. A

[1] The sequence reported in this paper has been deposited in the GenBank and EMBL Data Libraries (accession nos. L04796 and X68692).

* To whom correspondence should be addressed.

Abbreviations : CT, calcitonin; GLP-1, glucagon-like peptide 1; PACAP, pituitary adenylate cyclase activating peptide; PTH, parathormone; VIP, vasoactive intestinal peptide; ORF, open reading frame; IC_{50}, median inhibitory concentration; K_{act}, concentration giving half-maximal adenylate cyclase activation.

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direct demonstration that this receptor could be the glucagon receptor was not achieved, however, as the three clones obtained after screening a liver cDNA library were not functional in transfection assays. Clone I was complete but contained, in addition, two small introns of 95 bp and 89 bp that interrupted the ORF at positions 396 and 503 (numbered from the ORF starting point). Clones II and III were incomplete at their 5´ end. In this paper, we describe the construction, with clones I and II, of a pBluescript SK(+) plasmid with full length ORF. This construct was subcloned in a functional pCDM8 expression vector. Subsequent transfection of COSGs1 cells revealed the presence, in their membranes, of a receptor that retained the binding properties of the rat liver glucagon receptor and stimulated adenylate cyclase.

MATERIALS AND METHODS

Construction of an expression vector with full ORF cDNA.

Unless otherwise stated we used standard cloning methods (7). Five µg of plasmid DNA of clone I and clone II (1) were digested separately with pairs of restriction enzymes generating fragments of interest, as follows (Fig. 1) : 1/ Clone I with Hind III (cleaving in the pCDM8 polycloning site at the 5´ end of the insert) and Dra III (cleaving at base 374 of the ORF); 2/ Clone II with Dra III (at 374) and BamH I(at 1021); and 3/ Clone I with BamH I (at 1021) and Not I (cleaving in the pCDM8 polycloning site at the 3´ end of the insert). One µg of each digested cDNA was separated on 1.2 % agarose gel and slices with cDNA of, respectively, 410 bp, 650 bp and 940 bp (approximately) were excised then extracted in 10 µl water with the Geneclean kit (Bio 101). Each selected fragment (in 2 µl) was ligated overnight at 12 °C, in a 10 µl final volume, with 0.1 µg pBluescript SK(+) vector (Stratagene) digested with Hind III and Not I and separated from the stuffer fragment on agarose gel as above. The ligation mix was used for transforming competent XL-1 Blue cells (Stratagene). Colonies carrying a recombinant plasmid were selected by hybridization with a synthetic 34 mer oligodeoxynucleotide complementary to ORF nucleotides 681 to 714 in clone I (1).

The presence and correct orientation of all fragments in recombinant plasmids was tested by multiple digestion with restriction endonucleases. The clone carrying an uninterrupted candidate glucagon receptor ORF was digested with Hind III and Not I, and the insert subcloned into the pCDM8 plasmid digested with Hind III and Not I, as described above. High amounts (1 - 2 mg) of recombinant pCDM8 were isolated by centrifugation on CsCl gradient.

Transient transfection of COSGs1 cells by the lipofectin method.

Monkey COSGs1 cells were grown in Dulbecco´s modified Eagle´s medium supplemented with 10 % fetal calf serum in 5 % CO_2 at 37°C. About 30 µg of recombinant pCDM8 with a probable full ORF construct for the glucagon receptor (1) were preincubated for 15 min with 120 µl of lipofectin reagent (Gibco-BRL) in a 400 µl final volume, then added in 7 ml of Optimem medium (Gibco) to a 175 cm^2-culture flask containing COSGs1 cells at 80 % confluency. Cells were incubated for 7 h before addition of the complete culture medium (see above) and collected 56 h after transfection. We used, as control, cells transfected with 30 µg of the pCDM8 plasmid with truncated clone III (1).

Fresh membrane preparations from COSGs1 cells and rat liver.

Transfected cells were scraped with a rubber policeman, centrifuged at 100 x g for 5 min at 20°C and lyzed in hypotonic 1 mM $NaHCO_3$ then quickly frozen in liquid nitrogen. After thawing, the lyzate was centrifuged at 400 x g for 10 min at 4°C. The resulting supernatant was centrifuged at

10,000 x g for 15 min and the pellet, resuspended and rehomogenized in 1 mM $NaHCO_3$ to a final concentration of 1 mg protein/ml, was immediately used for binding assay with [^{125}I]iodoglucagon and for adenylate cyclase assay.

Crude rat liver membranes were prepared as described in (8). Protein concentrations were determined using the Lowry et al. method.

Binding of [^{125}I]iodoglucagon.

[^{125}I]iodoglucagon ((3-[^{125}I]iodotyrosyl^{10})glucagon, ≈ 74 TBq/mmol) was purchased from Amersham. Tracer binding was carried out in 120 µl of a medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 10 mg/ml bovine serum albumin, 20 - 30 pM [^{125}I]iodoglucagon, increasing concentrations of unlabelled peptide, and 20 µg membrane protein. The mixture was incubated at 37 °C for 20 min to allow binding equilibrium then suspended in 2 ml ice-cold 50 mM sodium phosphate (pH 7.4) and rapidly filtered through a glass-fiber filter (GF/C Whatman) presoaked for 24 h in 0.1 % polyethyleneimine. Each filter was rinsed three times with 2 ml of the same buffer and its radioactivity was measured. To determine nonspecific binding 1 µM unlabeled glucagon was included in the assay mixture, and subtracted from the total binding to yield the specific binding.

Adenylate cyclase assay.

Adenylate cyclase activity was determined in the presence of 10 µM GTP according to the procedure of Salomon et al. (9) and as previously described (8).

RESULTS

ORF construct of a putative glucagon receptor.

In preliminary experiments, we tried to construct directly a functional plasmid, with a pCDM8 vector carrying receptor sequences from clones I and II, taking advantage of Dra III sites as follows : 1) one Dra III restriction site present in the ORF sequence at position 374 i.e. after the beginning of clone II (position 104) and before two small introns in clone I (ref. 1 and Fig. 1); 2) the second Dra III site at position 1193 i.e. after both introns; 3) the third Dra III site at position 825 of the pCDM8 vector. We attempted to ligate the three following Dra III/Dra III fragments : the central insert fragment of clone II with two fragments of clone I, each of them containing one remaining insert sequence and one vector portion. We failed to obtain a correct construct by this approach.

We next used the indirect approach illustrated in Fig. 1 : the Hind III/Dra III fragment of clone I, the Dra III/BamH I fragment of clone II and the BamH I/Not I fragment of clone I were ligated with a pBluescript SK(+) vector digested with Hind III/BamH I. We obtained 21 colonies hybridizing with the probe (1) that recognizes the central Dra III/BamH I fragment, and six of them contained a high number of plasmid copies.

The restriction map of one plasmid matched the predicted construction. The pBluescript SK(+) containing this selected insert was digested with Hind III/Not I and the insert was next ligated in a Hind III/Not I digested pCDM8 plasmid. Sequencing of the splice junctions Dra III and BamH I in the

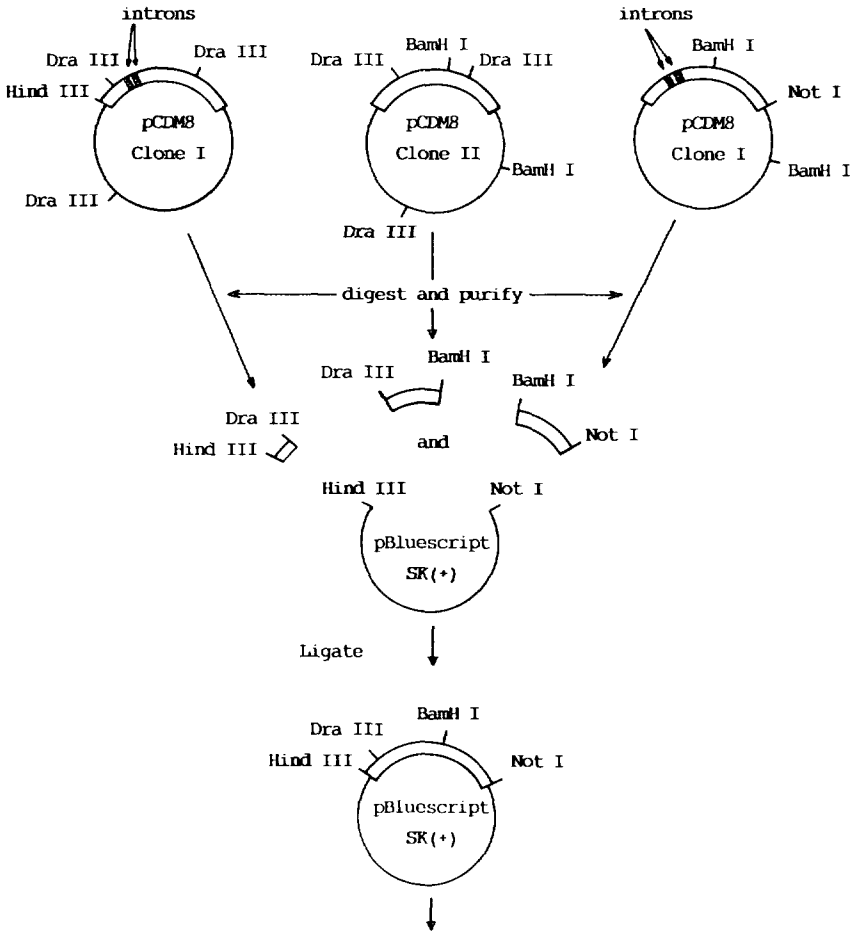

Fig. 1 . Construction scheme of the recombinant plasmid pCDM8. Three restriction fragments derived from clones I and II in pCDM8 vectors (1) were ligated to a pBluescript SK(+) vector digested with Hind III and Not I, as described in Methods. The constructed insert was then subcloned in Hind III/Not I sites of a pCDM8 vector. The boundaries of the fragments are demarcated by the restriction nucleotidases used for isolation.

cloned insert demonstrated the presence of a correct uninterrupted ORF sequence susceptible to code for the putative glucagon receptor, as previously inferred (1).

Expression studies.

Receptor identification was conducted by [^{125}I]iodoglucagon binding and glucagon stimulation of adenylate cyclase in membranes from COSGs1 cells transfected with the constructed plasmid. We used as control COSGs1 cells transfected with a truncated pCDM8 plasmid (clone III, ref. 1). High [^{125}I]iodoglucagon binding (more than 10 % of specific binding for 1 - 2 % of

nonspecific binding) was specific as GLP-1, secretin, VIP, PACAP-27, PACAP-38, and helodermin provoked insignificant displacement at a 1 µM concentration (Fig. 2A).

Membranes from both COSGs1 cells transfected with the constructed plasmid and control COSGs1 cells transfected with the truncated plasmid showed similar basal adenylate cyclase activity (58 and 56 pmol cAMP formed.min^{-1}.mg protein^{-1}) and were both stimulable 4- 5-fold by isoproterenol (maximal cAMP production at 232 and 306 pmol.min^{-1}.mg protein^{-1}, respectively). Glucagon stimulated the enzyme of appropriately transfected cells 2.6-fold and dose-dependently with a Kd at 10 nM (Fig. 2B), while remaining inefficient in membranes from cells transfected with clone III. Neither membranes were stimulated at 1 µM with GLP-1, secretin, VIP, PACAP-27, PACAP-38 or

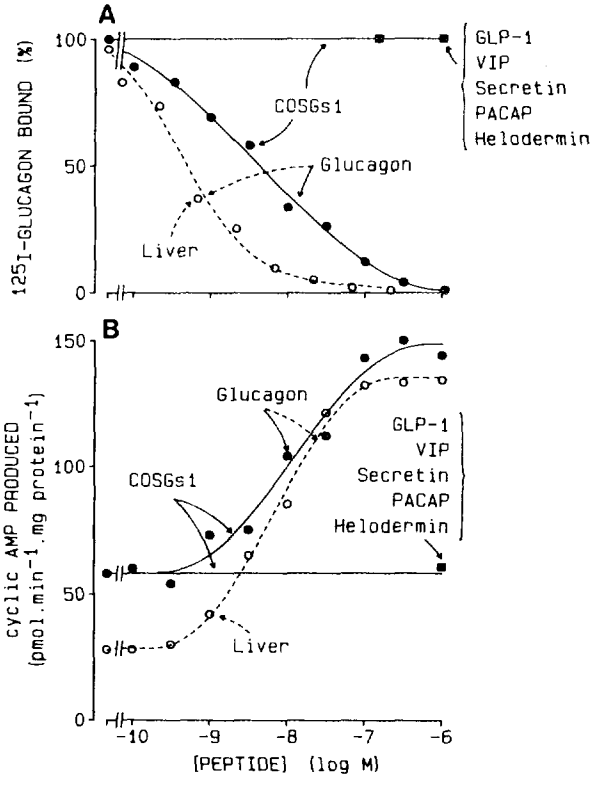

Fig. 2 . (A) Displacement of [^{125}I]iodoglucagon binding by glucagon (●,○), GLP-1, secretin, VIP, PACAP-27, PACAP-38 and helodermin (■) to membranes from : a) COSGs1 cells transiently transfected with a full ORF cDNA construct of the glucagon hepatic receptor (maximum binding in the absence of competitor (100 %) was 12.5 fmol.mg protein^{-1}), and b) rat liver (dashed line).
Each point represents the mean of two experiments.
(B) Adenylate cyclase stimulation in membranes from : a) COSGs1 cells transiently transfected with a full ORF cDNA construct of the glucagon hepatic receptor, and b) rat liver (dashed line).

helodermin. In conclusion, the constructed plasmid produced specific glucagon receptors positively coupled to adenylate cyclase.

DISCUSSION

Construction of a functional transfectable plasmid.

A first direct construction in pCDM8, using Dra III sites (CACNNN/GTG) and the ligation of only three fragments was unsuccessful. This was perhaps because the three nucleotides of the Dra III overhangs (AAA, GAA and TAC) were insufficiently sticky to insure correct ligation and/or due to an increased recombination probability as a result of testing a bacterial strain that contains the SupF-carrying pCDM8 plasmid and also a P3 plasmid.

By contrast, the construction of the full ORF was achieved in pBluescript SK(+) by a four fragment ligation procedure, and a functional transfectable plasmid was obtained after subcloning in pCDM8.

Characterization of the cloned glucagon receptor expressed in COSGs1 membranes.

To confirm that the cloned cDNA encoded the glucagon receptor, the pCDM8 cDNA was transiently expressed in a COSGs1 cell line. This cell line is a COS-7 cell line stably transfected with pEF-BOS-Gs that expresses a certain level of Gs (3). Cotransfection of this cell line with the pCDM8 plasmid carrying the secretin receptor is known to increase the proportion of secretin receptors in a high affinity state, in the absence of GTP (3). In the present situation, glucagon was the only agent displacing [^{125}I]iodoglucagon binding. The specific [^{125}I]iodoglucagon displacement curve extended over more than 3 logarithms with an IC_{50} (4 nM) somewhat higher than in hepatic membranes (Fig. 2A and ref. 8). [^{125}I]iodoglucagon binding to rat hepatic membranes revealed a single class (state) of receptors (IC_{50} at 0.5 nM) for homologous competition with unlabeled glucagon.

Parent peptides belonging to the glucagon superfamily including VIP, secretin, PACAP-27, PACAP-38 and helodermin, were unable to inhibit the binding of [^{125}I]iodoglucagon to both COSGs1 and rat liver membranes.

To examine whether the cloned glucagon receptor expressed in membranes from transfected COSGs1 cells could transduce the signal, we studied adenylate cyclase stimulation in the presence of 10 µM GTP. There was a notable activation with a half-maximal response at 10 nM glucagon, a value close to that obtained with rat liver membranes (Fig. 2B and ref. 8). In both cases the monophasic slope of dose-effect curves suggested interaction with one class (state) of glucagon receptors in the presence of 10 µM GTP. GLP-1 was - like other less closely related peptides - unable to operate as an agonist.

Membranes from COSGs1 cells transfected with the truncated clone did not respond to glucagon. These results were consistent with the radioligand affinity.

The status of the intronic sequences.

The presence of introns has not yet been described for other members of the secretin receptor family (2-6) and many genes for the entire group of G-protein-coupled receptors do not contain introns (10). However, receptors for tachikinins contain four large introns (1 kb to 26 kb) (11). D_2, D_3 and D_4 dopamine receptors contain, respectively, six, five and four introns of various length (12) and at least three polymorphic variations are observed in the coding sequence of the human D4 receptor (13).

In the present case, multiple transcripts for glucagon receptors are suggested on the basis of the Northern blot pattern (1). The first three small introns we described previously (1) interrupt the ORF before the end of the seven transmembrane domain and prevent the translation of an active receptor. For instance, the persistence of introns 1 and 2 in clone I with the start codon provokes its inactivity in transfection studies. Accordingly, these first two small introns must be spliced out to generate the mature exonic form. On the other hand, unsplicing of intron 4, that codes for 60 amino acids inframe with ORF (1), could conceivably yield two active forms of glucagon receptors with different C-terminal ends. Such a situation has recently been demonstrated for two forms of somatostatin-14 (SSTR2) receptors with different C-terminals, one of them with an unspliced intron of 300 bp in the C-terminal cytoplasmic tail (14).

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