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Expression and characterization of human D4 dopamine receptors in baculovirus-infected insect cells

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The human D4 dopamine receptor has been genetically engineered for expression in insect cells using the baculovirus system. A D4 cDNA gene fusion construct [(1991) Nature 350, 610–614] was synthetically modified to remove two introns from the coding region, and expressed in *S frugiperda* (Sf9) cells as a fusion with a short sequence from the polyhedrin protein. Binding assays with [^{3}H]spiperone indicated high levels of D4 receptor binding 90 h after infection and a pharmacological profile identical to that reported for D4 receptors expressed in COS-7 cells using the cDNA gene hybrid. We also show that the agonist binding affinity of D4 receptors expressed in Sf9 cells can be shifted by GTP-γ-S, indicating coupling to G-proteins.

D4 dopamine receptor; Schizophrenia; Baculovirus expression; G-protein

1. INTRODUCTION

Dopamine receptors are the site of action for many of the drugs used currently in the treatment of schizophrenia and Parkinson's disease. Five human dopamine receptors (D1–D5) have been cloned [1–5], and all belong to the family of seven transmembrane domain, G-protein-coupled receptors. Clozapine, a drug used in the treatment of schizophrenia [6], has an affinity at D4 receptors which is significantly higher than that observed for other cloned dopamine receptors [4], suggesting that the D4 receptor may be an important therapeutic target. A preliminary pharmacological characterization of this receptor has been performed using monkey COS-7 cells transiently transfected with a D4 cDNA gene hybrid [4], but the use of this technique is limited by cell number and low levels of receptor expression. Therefore, an alternative expression system is required if sufficient receptor is to be produced for more detailed biochemical and pharmacological studies.

The human D4 construct used for expression of the receptor in COS-7 cells is a cDNA genomic hybrid which includes two introns [4]. A number of the seven transmembrane receptors have been expressed at high levels in insect cells using the baculovirus expression system [7–10], however, these receptors have all been derived from cDNAs. By removing the introns from the D4 sequence we have been able to use a recombinant baculovirus to generate D4 receptors in *Spodoptera frugiperda* (Sf9) cells. Furthermore, we show that agonist binding of D4 receptors in Sf9 cell membranes can be shifted by a GTP analogue, suggesting that the receptor is coupled to G-proteins.

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Abbreviations: GTP-γ-S, guanosine-5′-γ-thiotriphosphate; MOI, multiplicity of infection; Sf9 cells, *Spodoptera frugiperda* cells.

2. MATERIALS AND METHODS

2.1. *Construction of modified D4 gene vector*

A 303 bp synthetic oligonucleotide fragment (Table I) was made from eight overlapping oligonucleotides synthesized on an Applied Biosystems 380A DNA synthesizer. The oligonucleotides were phosphorylated using T4 polynucleotide kinase, mixed, and ligated using T4 DNA ligase [11]. An expression vector, pCD-PS-hD4, containing the human D4 receptor gene, was obtained from Olivier Civelli (Vollum Institute, Portland, OR) as a hybrid construction of genomic and cDNA D4 sequences [4]. The 2.6 kbp *Not*I fragment was replaced with the synthetic 303 bp fragment (Fig. 1), and the DNA sequence of the insertion was confirmed by dideoxy DNA sequencing [11]. Both the original hybrid D4 (pCD-PS-hD4) and modified D4 (pCD-PS-hD4mod) expression vectors were transiently transfected into COS-M6 cells [12] and membranes tested for [^{3}H]spiperone binding.

2.2. *Construction and isolation of recombinant baculovirus*

The *Spodoptera frugiperda* insect cell line, Sf9, was obtained from ATCC (Accession Number CRL 1711). Transfer vector, pAC 373, and wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were a gift from Max Summers (Texas A&M, College, TX). pAC 373 was engineered for expression with the modified D4 cDNA as described in Fig. 2. Procedures for cell culture, co-transfection, viral infection and purification of recombinant baculovirus, were as described [13].

2.3. *Expression of D4 receptors*

Sf9 cells were propagated in agitated spinners (50 rpm), of 500 ml to 3 litre working volume at 27°C, in TC100 medium (Gibco, Switzerland), supplemented with 10% fetal bovine serum (Seromed, Switzer-

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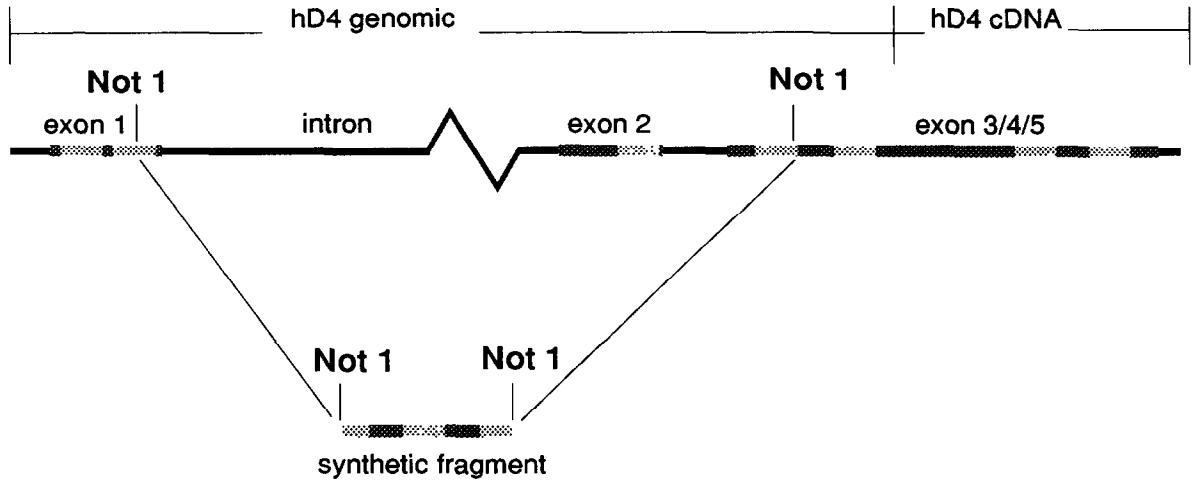


Fig. 1. Modification of the D4 cDNA gene hybrid. The original D4 cDNA gene construction is a hybrid of a 3.0 kbp genomic DNA fragment and 0.65 kbp of cDNA [4]. The internal 2.6 kbp *Not*I fragment containing the two introns, exon II and parts of exons I and III, was excised from pCD-PS-hD4 with *Not*I. A 303 bp fragment containing the D4 receptor coding regions on the 2.6 kbp *Not*I fragment was synthesized and then inserted into the *Not*I site in the pCD-PS-D4 vector.

land). These cultures were used to inoculate a stirred tank reactor (Chemap, Switzerland) with 15 l working volume. The conditions in the reactor were the same as for the spinners except the medium was supplemented with 0.1% (w/v) Pluronic F68 (BASF, Germany) and there was an additional control of the dissolved oxygen level at 50% air saturation achieved by pure oxygen sparging upon demand. A large virus stock (15 l) was made by infecting cells in the reactor at a density of 1×10^6/ml at a multiplicity of infection (MOI) of 0.1. After 7 days, the lysate was separated from cell debris by centrifugation ($300 \times g$, 15 min), stored at 4°C, and the virus titre estimated by plaque assay [13] to be 1×10^8 pfu/ml. This virus stock was used for all subsequent large-scale experiments. For receptor expression, cells were grown in the same conditions to a density of $1\text{–}1.5 \times 10^6$/ml and infected at a MOI of 10.

Table I

Synthetic oligonucleotides used in the construction of the modified D4 receptor sequence

name	bases	sequence
D4SEG-1	66	GGCCGCTGATCTCCTCCTCGCTCTCCTGGTACTTCCACTATTCGTC TATTCAGAGGTCCAAGGTGG
D4SEG-2	81	TGGGCTAAGCAGCCATGCGCCACCTTGGACCTCTGAATAGACGAA TAGTGGAAGTACCAGGAGAGCGAGGAGGAGATCAGC
D4SEG-3	84	CGCATGGCTGCTTAGCCCACGACTGTGTGATGCACTCATGGCTAT GGACGTCATGCTGTGTACTGCATCCATCTTCAACCTATG
D4SEG-4	91	CCACGAATCTATCTACGCTGATAGCACATAGGTTGAAGATGGATGC AGTACACAGCATGACGTCCATAGCCATGAGTGCATCACACAGT CG
D4SEG-5	79	TGCTATCAGCGTAGATAGATTCGTGGCAGTAGCTGTTCCTCTGCGA TACAACCGTCAAGGTGGAAGCCGCCGTCAACTG
D4SEG-6	77	TAGCCACGTAGCACCAATGAGCAGCAGTTGACGGCGGCTTCCACC TTGACGGTTGTATCGCAGAGGAACAGCTACTG
D4SEG-7	75	CTGCTCATTGGTGCTACGTGGCTACTGTCAGCAGCTGTTGCCGCA CCTGTACTGTGTGGACTCAACGACGTGCGC
D4SEG-8	55	GGCCGCGCACGTCGTTGAGTCCACACAGTACAGGTGCGGCAACA GCTGCTGACAG

These oligonucleotides comprise the synthetic 303 bp fragment corresponding to base pairs 231–533 of the human D4 coding sequence [4].

2.4. *Preparation of membranes*

Sf9 cells were harvested by centrifugation ($300 \times g$, 10 min) 72–96 h after infection and resuspended at approximately 3×10^7 cells/ml in 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA and 1.5 mM $CaCl_2$ (assay buffer) containing 0.1 mg/ml bacitracin, 10 μg/ml leupeptin and 0.1 mM PMSF. All subsequent operations were performed at 4°C. Following homogenization using an Ultra Turrax homogenizer (setting 8 for 10 s), unbroken cells and nuclei were pelleted at $2{,}000 \times g$ for 20 min. The supernatant was centrifuged at $48{,}000 \times g$ for 30 min and the pellet resuspended at 1–2 mg protein/ml in the same buffer. Membranes could be stored at −80°C for 1 month without significant loss of binding activity. Preparation of COS cell membranes for binding assays was as described [4].

2.5. *Radioligand binding assays*

[^{3}H]Spiperone (Amersham, UK) binding assays were performed in assay buffer using 100 μl of Sf9 (10–20 μg protein) or COS-M6 membranes (25–75 μg protein) in a total volume of 500 μl. For saturation binding assays [^{3}H]spiperone concentrations of 0.1–1.0 nM were used while competition assays were carried out at a concentration of 0.3 nM with unlabelled ligands at 10^{-12}–10^{-4} M. Incubation was for 120 min at 25°C and the reaction stopped by filtration through Whatman GF/B filters using a Brandel cell harvester. Filters were rapidly washed with 3×4 ml of ice-cold 50 mM Tris-HCl (pH 7.4), dried and radioactivity was measured by liquid scintillation counting. Non-specific binding was defined as that not displaceable by 10 μM haloperidol. Binding data were analyzed using the computer program LIGAND [14].

3. RESULTS AND DISCUSSION

The majority of genes recombinantly expressed at high levels using baculovirus have been derived from cDNAs or intronless genomic DNAs [15]. To facilitate the expression of human dopamine D4 receptors in Sf9 cells using the baculovirus system, the introns were removed from the original D4 cDNA gene hybrid construction (Fig. 1). Additionally, the coding sequence for the D4 gene contains an unusually high G/C content

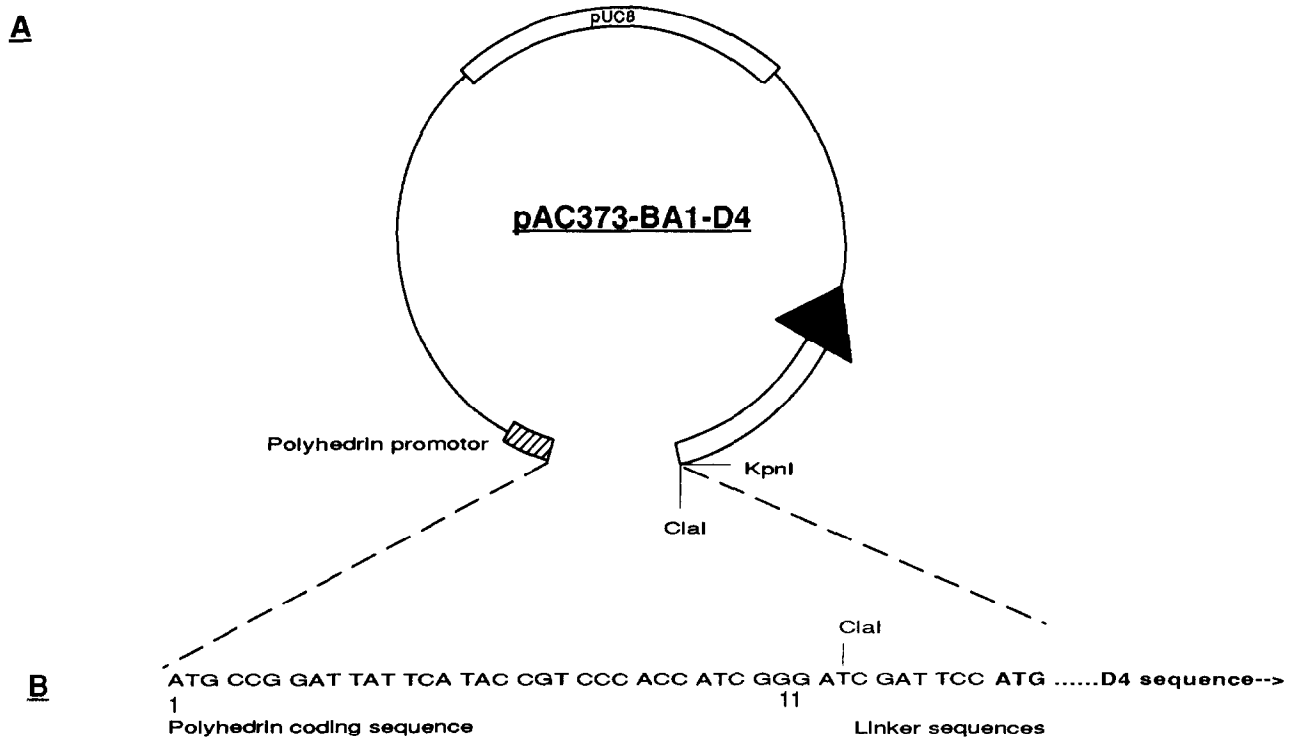


Fig. 2. Structure of recombinant baculovirus transfer vector. (A) Plasmid pAC 373 BA1 was derived from pAC 373 as follows; the *Cla*I sites were eliminated by fill-in with the Klenow DNA polymerase and the unique *Eco*RV site was replaced by a unique *Xba*I site. A 135 bp synthetic *Xba*I–*Kpn*I fragment, comprising the exact polyhedrin promotor sequence followed by polyhedrin sequences encoding the first 11 amino acids and a *Cla*I–*Kpn*I linker sequence was used to replace the corresponding region of pAC 373 yielding plasmid, pAC 373 BA1. The modified D4 cDNA was subcloned into the unique site in pAC 373 BA1 using synthetic *Cla*I linkers. (B) Sequence of the polyhedrin–D4 fusion polypeptide.

which may pose problems for recombinant expression. Therefore, silent G/C base pairs were replaced in the synthetic sequence with A/T base pairs wherever possible. The original hybrid construction, pCD-PS-hD4, and the modified construction, pCD-PS-hD4mod, were transfected into COS-M6 cells and tested for D4 receptor binding using [^{3}H]spiperone. An affinity for clozapine of 30 nM was observed for both D4 expression constructs, indicating that the deletion of the introns had not altered the pharmacology of the receptor. Replacement of the genomic sequence in this region did, however, increase D4 receptor expression in COS-M6 cells with pCD-PS-hD4mod, giving maximal binding of 2 pmol/mg protein compared to 0.4 pmol/mg for pCD-PS-hD4 (data not shown).

The pCD-PS-hD4mod cDNA gene was then inserted into a recombinant baculovirus vector (Fig. 2A) such that the coding sequence for the first 11 amino acids of viral polyhedrin protein was fused to the N-terminus of the D4 coding sequence via a three amino acid linker sequence (Fig. 2B). It has been shown [16] that this manipulation can result in higher levels of expression of the heterologous protein and initial D4 constructs in which this sequence was omitted failed to produce any detectable D4 receptor binding in infected Sf9 cells.

Infection of Sf9 cells with recombinant polyhedrin–D4 fusion baculovirus resulted in the appearance of saturable [^{3}H]spiperone binding (Fig. 3A). Non-infected cells and cells infected with wild-type virus showed no specific radioligand binding. The appearance of this binding was time-dependent, showing a sharp rise after 24 h and reaching a maximum of 5 pmol/mg 90–96 h after infection (Fig. 3B). A comparable profile of receptor expression has been observed for human β_2-adrenergic receptors in Sf9 cells [8]. The K_d value of 0.3 nM for [^{3}H]spiperone binding did not change during the course of expression. An examination of the affinities

Table II

Affinities of dopamine receptor ligands for human D4 receptor expressed in Sf9 cells: comparison with COS-7 cells

Ligand	Sf9	COS-7
Clozapine	53 ± 4.6	25
Haloperidol	6.96 ± 0.5	5.1
Quinpirole	86 ± 30	50
Raclopride	1,367 ± 133	1,621
Spiperone	0.3 ± 0.05	0.08
Thioridazine	40 ± 3	12
YM-09151-2	0.44 ± 0.07	0.1

Data are expressed as K_i values (nM) ± S.E.M. from 3–8 separate experiments. COS-7 data are taken from [4] and [17].

of a number of dopaminergic compounds, including clozapine, for D4 receptors expressed in Sf9 cells showed K_i values similar to those reported previously using D4 receptor-transfected COS-7 cells (Table II). The appropriate rank order of potency was maintained, indicating that D4 receptors in Sf9 cells display the characteristic pharmacology despite the presence of an additional 14 amino acids at the N-terminal of the receptor. Similar N-terminal modifications of β_2-adrenergic receptors expressed in Sf9 cells were also without effect on the receptor pharmacology [18].

Dopamine receptors in brain have been shown to exist in high and low affinity states for agonist binding [19]. The high affinity state can be converted to low affinity by the addition of guanine nucleotides and is thought to represent receptors coupled to G-proteins [20]. Binding curves for quinpirole displacement of [^{3}H]spiperone binding to D4 receptors expressed in Sf9 cells could best be fitted [14] to a two-site model (Fig. 4), indicating the presence of high and low affinity agonist binding sites. Addition of a non-hydrolyzable GTP analogue, GTP-γ-S, caused a rightward shift of the curve (Fig. 4) where a one-site model was a better description of the data. This suggests that D4 receptors are able to couple to endogenous G-proteins in Sf9 cells. COS-7 cells expressing D4 receptors also showed a high affinity form of the receptor which was guanine nucleotide-sensitive [4].

In conclusion, we have used a modified cDNA coding for the human dopamine D4 receptor to express D4 receptors in Sf9 cells which exhibit the correct pharmacology and are able to couple to endogenous G-proteins. The high efficiency of receptor expression attained will allow further pharmacological and structural analysis of this potentially important dopamine receptor.

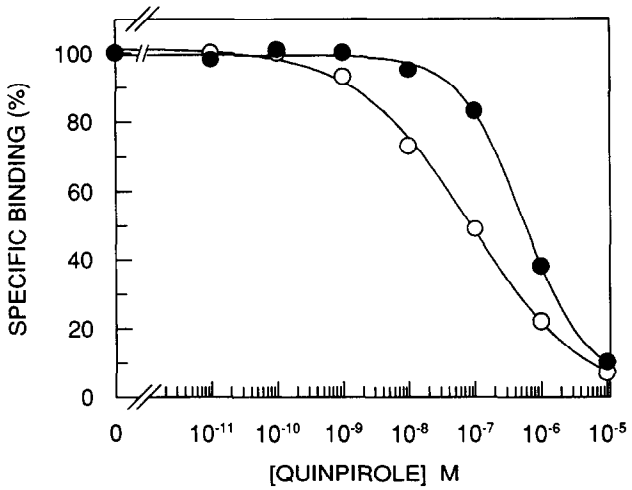
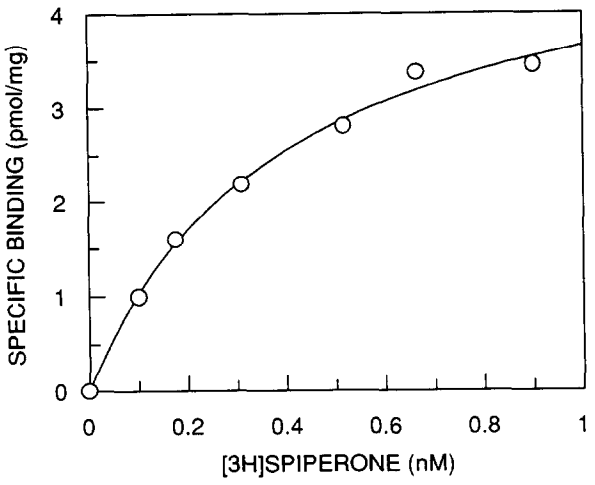


Fig. 4. Displacement of 0.3 nM [^{3}H]spiperone binding by quinpirole in the presence (●) and absence (○) of 100 μM GTP-γ-S. Each point is the mean of triplicates and similar results were obtained in 4 independent experiments.



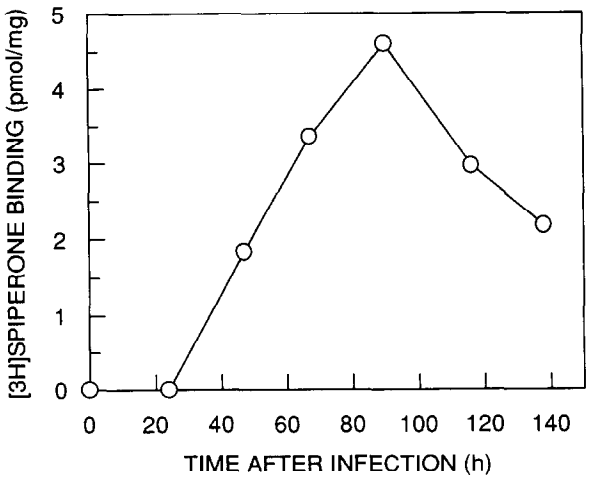


Fig. 3. Expression of human dopamine D4 receptors in Sf9 cells infected with a baculovirus expression construct. Top panel, saturation binding of [^{3}H]spiperone to membranes prepared from Sf9 cells expressing D4 receptors. Bottom panel, time-course of receptor expression. Cells were harvested before (time 0) and at 20–24 h intervals after infection and assayed for [^{3}H]spiperone binding to membranes.

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