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Original paper

INTERACTION OF DIFFERENT THIRD INTRACELLULAR LOOP FRAGMENTS OF HUMAN DOPAMINE D_{2L} RECEPTOR WITH α-SUBUNIT OF G_{i1} PROTEIN – PROSPECTIVE THERAPEUTIC APPLICATION

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Summary: In order to find the essential structural motif of the D_{2L} dopamine receptor necessary for the interaction with α-subunit of G_{i1} protein, four fragments of the third cytoplasmic loop (CPL3) of this receptor were cloned, expressed in *E. coli* and purified. After that, fusion proteins with glutathione-S-transferase (GST) were prepared and the interactions quantified by a colorimetric assay for GST activity determination. The presence of D_{2L}-CPL3 fragment-$G_{i\alpha 1}$ complexes was detected by SDS-polyacrylamide gel electrophoresis (PAGE). Kd values for the interaction of the three fragments with $G_{i\alpha 1}$ were similar and in nmol/L range of concentrations, while the peptide representing the insert in the long form of the dopamine D_2 receptor expressed about 10-fold lower binding affinity. These results could serve to design new therapeutic agents that might act at the level of receptor/G protein interaction rather than at the level of ligand-receptor binding.

Key words: Dopamine D_{2L} receptor, third intracellular loop, synthetic peptide, receptor/G protein coupling.

Introduction

Dopamine receptors are members of a large superfamily of receptor proteins coupled to heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). They are involved in motor control, neuroendocrine regulation, cognition and emotion. These receptors are crucial targets in the therapy of schizophrenia, Parkinson's disease, Tourette's syndrome, tardive dyskinesia and Huntington's disease (1).

On the basis of their biochemical and pharmacological properties five distinct dopamine receptors (DA-Rs) have been isolated, characterized and subdivided into two subfamilies, D_1- and D_2-like. The former comprises D_1 and D_5 receptors, while the latter includes D_2, D_3, and D_4 subtypes (2). Further diversity in the human dopamine receptors can be achieved by alternative RNA splicing and by the existence of expressed polymorphic sequences.

Two isoforms of the dopamine D_2 receptor have been characterized, D_{2L} (long) and D_{2s} (short), gene-

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* *Abbreviations:* G_i and G_o-guanine nucleotide-binding proteins as classified by Gilman (3); Gα-α-subunit of denoted protein; D_{2L}, D_{2S}-dopamine receptor, long and short variant, subfamily 2; D_{2L}-CPL3-the third intracellular loop of dopamine D_{2L} receptor; D_{2L}-CPL3 fragment-GST-the D_{2L}-CPL3 fragment and GST fusion protein.

rated by alternative splicing from the same gene. They differ by an in-frame insert of 29 amino acids specific to D_{2L} within the third cytoplasmic loop of the receptor (4). Expression of two isoforms is tissue-specific (5), D_{2L} appearing to be the major isoform (6, 7). Analysis with subtype-specific antibodies against both the D_{2S} and long isoforms have shown a unique compartmentalization between these two isoforms in the primate brain. Splice variants of the dopamine D_2 receptor are differentially distributed and possess distinct functions. The strategic localization of the D_{2S} isoform in dopaminergic cell bodies and axons strongly suggests that this isoform is the likely dopamine autoreceptor, whereas the D_{2L} isoform is primarily a postsynaptic receptor (8).

D_1 receptors activate adenylyl cyclase and are coupled to Gs regulatory protein. In contrast, activation of D_2 receptors results in various responses including inhibition of adenylyl cyclase and phosphatidylinositol turnover, increase in K^+ channel activity and inhibition of Ca^{2+} mobilization (6). All these different signalling pathways involve the activation of the Gi/Go family of G proteins (7, 9). First, G_{i2} appeared to couple selectively to the D_2 receptor with ~10-fold higher affinity than any other tested Gi subtype (10). Later, the same authors have shown that alternative splice forms of D_2 dopamine receptor are able to signal to different Gi proteins (11). Specifically, it seems that 29 amino acid insert of D_{2L} confers interaction selectivity for G_{i2} (4). Preferential interaction of the D_{2S} isoform with G_{i1} than G_{i2} was demonstrated (7) that was consistent with previous results. Using different experimental approach other authors demonstrated an essential difference in coupling of dopamine D_{2L} and D_{2S} receptors to G proteins (12, 13). As the two forms of dopamine D_2 receptors differ within the third cytoplasmic loop, the data presented support the data on the importance of this domain in receptor coupling to G proteins, as well as the functional significance of the 29-amino acid insert in the third loop of D_{2L} (4). Comparison of the D_2 receptor gene in humans and mice demonstrates that its sequence and splicing events have been highly conserved through the evolution. This might indicate that the presence of the two isoforms and their selective interaction with different G proteins represent an essential feature of the dopamine D_2 receptor function *in vivo* (4).

Cerebral D_2 dopamine receptor is the target for pharmaceuticals used to alleviate the main symptoms of schizophrenia and seems to play an essential role in the dopaminergic control of physiological functions (14). All currently used antipsychotic agents have antidopaminergic properties, but the side effects associated with their use suggest that therapeutic alternatives are necessary (15). Compounds that interact directly with G proteins can mimic the receptor/G protein interaction or can block the activation of G proteins by receptors (16). To date, virtually all known antagonists of these receptors share the common property of blocking access of the ligand to the receptor. Alternative strategy for designing receptor antagonists might be to block the interaction of the receptor with the G protein (17). The third intracellular domain (3i) of G protein coupled receptors plays a major role in the activation of G proteins. Alterations in this region of the receptor can affect receptor/G protein coupling efficiency and specificity. Coexpression of the 3i loop with its homologous receptor resulted in specific inhibition of agonist-stimulated second messenger generation mediated via homologous receptor (17). This suggests that peptides derived from a G protein-coupled receptor might serve as antagonists of receptor/G protein interactions (18). Inhibition could occur by several mechanisms, such as competition for Gα subunits of a hormone-receptor complex and the free 3i loop of the receptor, or interaction between the 3i loop peptide and the intracellular domains of the parent receptor thus disrupting receptor conformation and keeping it inactive (17). The finding that: a) the 3i domain peptide provoked inhibition of homologous receptor signalling was surmountable at high receptor density and b) that weak enhancement of basal second messenger generation in cells expressing the 3i domain alone is detected (18) led to conclusion that 3i domain peptides act as weak partial agonists at the level of the G protein. Partial DA-receptor agonists should be eminently suitable for stabilizing neurotransmitter function. Such agents can also be described as mixed agonist-antagonists, implying that they serve as an agonist when the level of endogenous full agonist is low at receptor sites and as an antagonist when this level is high (15). The specificity with which the 3i domain peptides inhibit receptor-mediated signalling suggests the feasibility of developing drugs that might exert inhibition at the level of receptor/G protein interaction rather than at the level of ligand-receptor binding (18). Therefore, finding domains essential for this interaction could be of great significance in future design of pharmacologically active compounds.

Materials and methods

Plasmid DNA encoding hD_{2L} CPL3 (pCD3hD-2lPDX) was kindly donated by Dr. O. Civelli (Oregon Health Sci. Univ., Portland, OR, U.S.A.). Plasmid pQE-60, encoding $G_{i\alpha1}$, was a generous gift of Dr Christiane Kleuss (Freie Unive. Berlin, Inst. Pharmacol., Berlin, Germany). Plasmids encoding fragments of D_{2L}-CPL3 cloned into pGEX-2T (fr1: 211-241. a.a., fr3-211-277. a.a. and fr4-271-373. a.a.) were previously constructed in our laboratory by Ms Marija Backović and Ms Bosiljka Tasić (data not published).

The other materials were obtained from the following commercial sources: expression vector pGEX-2T, restriction endonucleases, T4 DNA ligase, alkaline phosphatase and T4 polynucleotide kinase Biolabs-New England, U.S.A.; PfuDNA polymerase Stratagene, La Jolla, CA, U.S.A.; DNAse Boehringer, Man-

cheim, Germany; ATP, GDP, phenylmethylsulfonylfluoride (PMSF), ampicillin Sigma Chem.Co., St. Louis, MO, U.S.A.; glutathione (GSH) and Na-deoxycholate Merck, Darmstadt, Germany; Glutathione-Sepharose Pharmacia LKB, Uppsala, Sweden; bactopeptone, yeast extract and bacto agar Difco Laboratories, Detroit, MI, U.S.A.; CDNB Squib-Bristol. All other chemicals were of analytical grade of purity.

Cloning of the insert of the third intracellular loop of the human dopamine D_{2L} receptor into pGEX-2T Standard molecular biology procedures were used for DNA manipulations (19). The insert of the CPL3 of the hD_{2L} receptor was amplified by polymerase chain reaction (PCR). To obtain insert of the CPL3-D_{2L} the following pair of primers was used in PCR containing plasmid DNA encoding hD_{2L} CPL3 as a template: forward-5'-GCGC GGA TCC CAC CTG AGG GCT CCA CTA AAG-3' and reverse-5'-GCCG GAA TTC CTG GGC TCG CCG GGC AGC CTC-3'. The PCR product was cut with BamHI and EcoRI restriction enzymes and cloned into BamHI-EcoRI sites of the prokaryotic expression plasmid pGEX-2T. The validity of the DNA constructs was confirmed by automated fluorescent DNA sequencing using reagents and instruments from Applied Biosystems (California, USA).

Expression and purification of the D_{2L}–CPL3 fragment fusion proteins *E. Coli* BL21 was maintained and transformed with pGEX-2T-D_{2L}-CPL3 fragment using $CaCl_2$ method (19). The cells were grown in LB medium supplemented with ampicillin and expression was induced with iso-propylthiogalactoside (IPTG) as described by Simonović et al. (20, 21). D_{2L}-CPL3 fragment-GST fusion proteins were purified using GSH-Sepharose (20, 21).

Expression and purification of His-Gα protein This was done as described by Lee et al. (22).

Gα-D_{2L}-CPL3 fragments-GST His-Bind Resin Assay This was performed as suggested by Simonović et al. (20).

Assay for detection of D_{2L}-CPL3 fragment-Gα complexes on SDS-PAGE Glutathione-Sepharose Resin was washed with buffer (10 mmol/L Tris, pH-7.4, 0.1% BSA) by repeated centrifugation (1000 rpm, 1 min) and 10 mg of each GST-fusion protein in the same buffer (total volume ~90 μL) was loaded onto ~40 μL of equilibrated 50% resin suspension. The resin was incubated (30 min, 22 °C, constant shaking), double washed with ice-cold buffer, to remove unbound proteins. $G_i\alpha_1$ solution (20 mg, in ~90 mL of buffer containing 10 mmol/L Tris-HCl, pH 7.4, 0.1% BSA, 1.0 mmol/L GDP and 5 mmol/L $MgCl_2$) was added and the incubation continued for 60 min. Unbound protein was removed by successive washing with ice-cold buffer (10 mmol/L Tris-HCl, pH 7.4, 120 mmol/L NaCl; 0.1% Triton X-100). The agarose beads were boiled in sample buffer, subjected to SDS/PAGE and the gel was silver stained (23). The controls containing only Gα without GST-CPL3 fragment and only GST-CPL3 fragment without Gα, served to distinguish binding of Gα to CPL3 fragment from non-specific binding of Gα to the resin.

Data analysis Saturation binding data were analyzed and graphically displayed by one site binding curve fitting using the Micorcal Origin 6.0 program which was also employed to calculate Kd values.

Miscellaneous Proteins were determined after Bradford et al. (24) using bovine serum albumin (BSA) as a reference. Degree of protein purification was checked by SDS-PAGE as described by Laemmli (25).

Results

Four fragments of the D_{2L}-CPL3: fr1-211.-241.a.a., fr2-235.-277.a.a.(insert of D_{2L}-CPL3), fr3-211.-277.a.a. and fr4-271.-373.a.a. were successfully cloned and expressed. Upon purification they were obtained in soluble form in the yield of 0.66, 0.86, 1.17, 0.17 mg/L, respectively. Various concentrations of Gα-proteins were immobilized on His-Bind Resin and titrated by D_{2L}-CPL3 fragment-GST fusion protein. The results presented as saturation binding curves are shown in *Figure 1–4*. Concentration of $G_i\alpha_1$-His-GDP ranged from 0.0136 16.26 nmol/L. The interactions were estimated by colorimetric assay for GST activity determination. Representative curves are shown. Kd values were calculated from saturation binding curves. The results are the mean values of SEM from at least 3 experiments done in triplicate. Kd values for the interactions of D_{2L}-CPL3 fragments-GST and His-tagged Gα-GDP proteins calculated from saturation binding curves are inserted in *Figure 1–4*. As seen, all Kd values were in nmol/L range of concentrations being rather close for fragments 1, 3 and 4, while that of fragment 2 (peptide representing insert in the long form of the dopamine D_2 receptor) was about 10-fold higher.

The appearance of the band representing $G_i\alpha_1$ on SDS-PAGE slab gel *(Figure 5)* was an alternative proof for the interaction of the Gα subunit with all four fragments of D_{2L}-CPL3 examined in the present study.

Discussion

Several authors used a strategy of coexpression of peptides from D_{2L}-CPL3 with its homologous receptor *in vivo* (26, 27) and showed that regions flanking each extremity of the loop are fundamental for coupling of the receptor to G proteins. Insert in the long form of the dopamine D_2 receptor doesn't seem to affect functional coupling of the D_{2L} receptor to G protein (26), but plays a critical role in the selectivity of these interactions (4). Since it is located outside the regions responsible for interactions with G protein, it was hypothesized that it might affect coupling specificity indirectly by incorporating novel stretch of

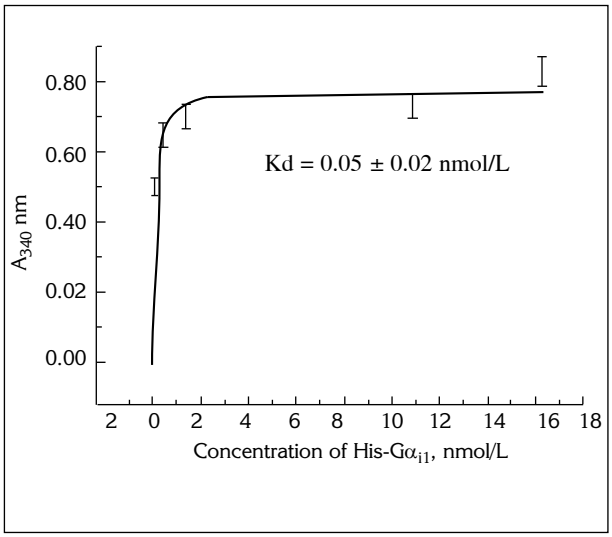


Figure 1. Saturation binding curve of $G_i\alpha_1$-His-GDP form interaction with FR1 (211.-241. a.a.)

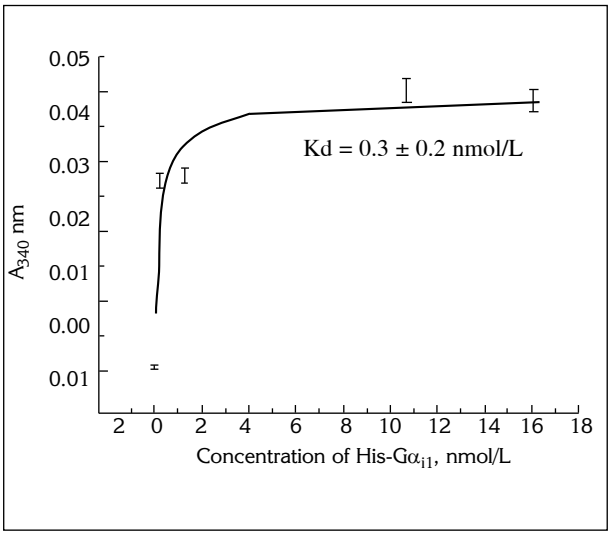


Figure 2. Saturation binding curve of $G_i\alpha_1$-His-GDP form interaction with FR2 (235.-277. a.a.)

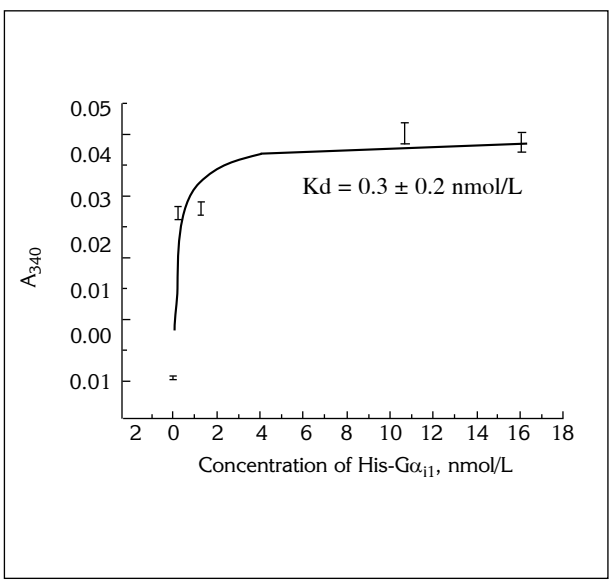


Figure 3. Saturation binding curve of $G_i\alpha_1$-His-GDP form interaction with FR3 (211.-277. a.a.)

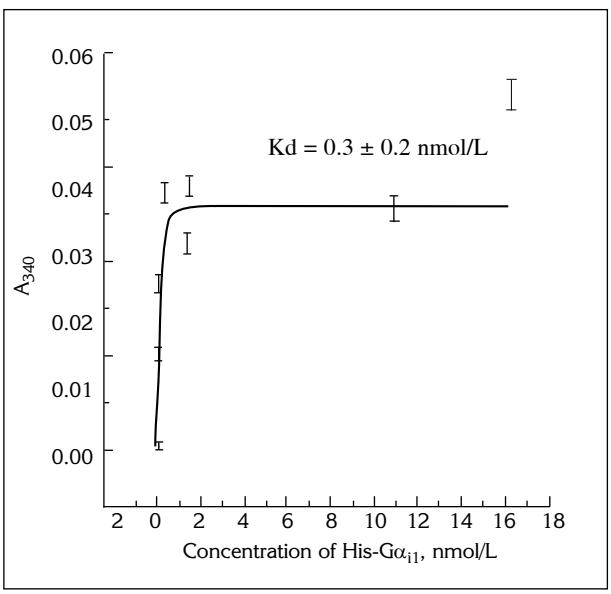
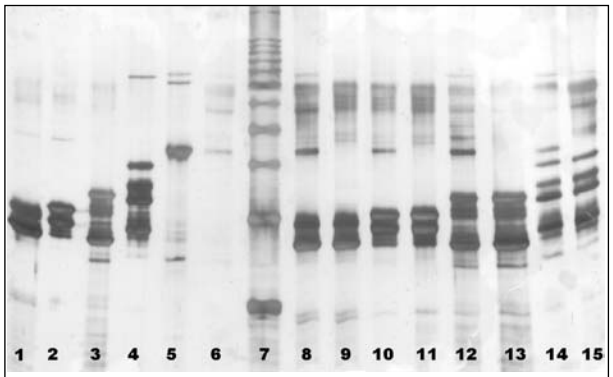


Figure 4. Saturation binding curve of $G_i\alpha_1$-His-GDP form interaction with FR4 (271.-373. a.a.)

residues in CPL3 and thus interrupting a putative α-helical structure, generating a novel structure that confers interaction selectivity for $G_i\alpha_2$ (4). Quantitative estimation of these interactions hasn't been performed previously.

Our results are in accordance with the data of several authors (26, 27) demonstrating that the NH_2- and COOH- terminal portions of the third cytoplasmic loop of the dopamine D_2 receptor do interact directly with α subunit of G protein. Further, we showed that there is no particular domain in the D_{2L}-CPL3 responsible for this interaction, as judged by a high affinity binding of all fragments except one, representing the insert in the long form of the D_2 receptor, which also interacts with Gα, but with about 10-fold lower binding affinity. This high binding affinity suggests that selectivity was achieved by direct contact of insert with G protein rather than inducing changes in conformation of other parts of CPL3.

The identification of domains crucial for the in-



1	*FR1–GST*	9	RESIN+FR1
2	*FR2–GST*	10	RESIN+FR2 +$G_i\alpha_1$
3	FR3-GST	11	RESIN+FR2
4	FR4-GST	12	RESIN+FR3+$G_i\alpha_1$
5	$G_i\alpha_1$	13	RESIN+FR3
6	RESIN+$G_i\alpha_1$	14	RESIN+FR4+$G_i\alpha_1$
7	STANDARD	15	RESIN+FR4
8	RESIN+FR1+$G_i\alpha_1$		

Figure 5. SDS-PAGE analyses of interactions of GST-fusion proteins with $G_{i\alpha 1}$-His-GDP. As standard we used 10 kDa Protein Ladder (Gibco BLR). Gel was silver stained.

teraction with G protein, and qualitative and quantitative estimation of these interactions could be useful in developing new therapeutic agents that would behave as weak partial agonists at the level of receptor/G protein interaction. However, peptides themselves are unlikely to be suitable as therapeutics because of problems with peptidases and inability to penetrate through target cell membrane. Solutions to these problems might be found in the synthesis of peptidomimetics non-peptide analogues of similar structure that could mimic peptide activities or introduction of these domains to target cells by gene therapy (28).

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INTERAKCIJA RAZLIČITIH FRAGMENATA TREĆE CITOPLAZMATIČNE PETLJE DOPAMINSKOG D_{2L} RECEPTORA ČOVEKA SA α-PODJEDINICOM G_{i1} PROTEINA MOGUĆA TERAPEUTSKA PRIMENA

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Kratak sadržaj: U cilju pronalaženja bitnih strukturnih motiva potrebnih za interakciju sa α podjedinicom G_{i1} proteina klonirana su, eksprimirana i prečišćena 4 fragmenta treće citoplazmatične petlje (CPL3) dopaminskog D_{2L} receptora koji su dalje pripremljeni kao fuzioni proteini sa glutation-S-transferazom (GST). Interakcije su kvantifikovane bojenom reakcijom za određivanje aktivnosti GST. Postojanje kompleksa D2L-CPL3 fragment-$G_i\alpha_1$ je dokazano elektroforetskom analizom na SDS-poliakrilamidnom gelu (PAGE). Kd vrednosti za tri fragmenta su bile vrlo slične i u nmol/L opsegu koncentracija, dok je peptid koji predstavlja insert u dugom obliku dopaminskog D_2 receptora posedovao oko 10 puta manji afinitet vezivanja za $G_i\alpha_1$. Ovi rezultati mogu biti osnova za sintezu novih terapeutskih agenasa koji bi delovali na nivou interakcije receptora i G proteina umesto na nivou vezivanja liganda za receptor.

Ključne reči: dopaminski D_{2L} receptor, treća intracelularna petlja, sintetski peptid, sprega receptor/G protein.

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