Dopamine is a neurotransmitter which plays an important role in both central and peripheral nervous system. There are at least five different dopamine receptor subtypes involved in dopaminergic signal transduction across cellular membranes via coupling to G proteins (1'3). D1-like dopamine receptors D1A (or D1) and D1B (or D5) couple to the activation of adenylyl cyclase (3). These receptors are also coupled to other signalling systems such as stimulation of phospholipase C (4), translocation of protein kinase C (5), activation of arachidonic acid cascade system (6), inhibition of Na/K-ATPase activity (7) and stimulation of K⁺ ion efflux (8). Some of these effects are mediated via coupling of D1 receptors to G proteins especially Ga₁ and Ga₅ (9, 10), but also to Ga₉ protein (11). Interactions of D1 dopamine receptors with G protein subunits were studied by several authors. Some authors (12, 13) indicated that amino acid sequence of ICL2 and ICL3 of the D1 receptor mediate the interaction with Ga proteins. Studies with synthetic peptides (14) suggested that the peptides with amino acid sequence corresponding to the parts of the second and the third intracellular loop, as well as with C-terminal part of rat D1 receptor play a role in coupling of this receptor to Ga₅ protein.

**INTERACTION OF INTRACELLULAR LOOPS OF DOPAMINE D₁ RECEPTOR WITH G PROTEIN SUBUNITS**

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Summary: A simple and rapid method for qualitative and quantitative estimation of Ga₅ subunit interactions with the second and the third intracellular loop, as well as with C-terminal part of human D₁ dopamine receptor has been developed. For this purpose, D₁-ICL₂ and D₁-ICL₃ were cloned in pGEX-2T vector and expressed in E. coli BL21 as fusion proteins with glutathione-S-transferase (D₁-ICL₂-GST and D₁-ICL₃-GST). C-terminal part was cleaved into two fragments which were cloned in pGEX-2T and expressed in E. coli BL21 DE3 as fusion proteins with glutathione-S-transferase (D₁-CTSF-GST and D₁-CTLF-GST). The resulting soluble constructs were purified by affinity chromatography on glutathione-Sepharose. Ga₅ subunits were expressed and purified as His-tagged proteins (Ga₀ and Ga₁ in E. coli BL21 DE3 and Ga₅ in E. coli JM 109). For quantitative assay, varying concentrations of pure His-tagged Ga₅ subunits were immobilized on His-Bind resin and titrated with fusion proteins and the interactions were estimated by a colorimetric assay for GST activity determination. Similar assay was employed to qualitatively demonstrate the interactions. For this purpose pure fusion proteins were immobilized on glutathione-Sepharose in known concentrations and treated with known concentrations of pure His-tagged Ga₅ subunits. Thus created complexes were eluted from glutathione-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It was shown that D₁-CTSF interacts specifically with Ga₅ subunit, and D₁-CTLF with Ga₀. No other interactions were observed. Based on saturation binding analyses, Kᵰ values in nanomolar range of concentrations demonstrated the highest binding affinity of His-Ga₅ for D₁-CTSF-GST and of His-Ga₀ for D₁-CTLF-GST.

Key words: human D₁ dopamine receptor, intracellular loops, G proteins, Ga₅ subunits, interactions.
Divergence in the third cytoplasmic loop and also in C-terminal part between D1 and D5 dopamine receptors (15), studies on other GPC-receptors (16–18) and the effect of these receptors on multiple signalling systems led to the conclusion that they are able to couple differentially to G proteins.

These facts prompted us to examine which part(s) of human D1 receptor is responsible for interactions with Ga subunits. In this study we report the results on qualitative and quantitative estimation of interactions between the parts of the D1 receptor and Ga1, Go2 and Go1i proteins.

Materials and methods

Materials

Plasmid DNA encoding human D1 dopamine receptor was a generous gift of Dr. H.H.M. Van Tol (Dept. Psychiat. Pharmacol., Univ. Toronto). Plasmids NPT7-5, encoding Ga1-His and Go2-His, were kindly provided by Dr. M. Linder (Washington Univ. Sch. Med., St. Louis, MO, USA).

The other chemicals used in this work were from the following sources: expression vector pGEX-2T, restriction endonucleases, T4 DNA ligase and alkaline phosphatase – BioLabs New England; Pfu DNA polymerase – Stratagene; DNA-se – Boehringer; lysosome Serva; ATP, GDP, DNTPs, PMSF, ampicillin – Sigma; glutathione (GSH) and Na-deoxycholate – Merck; His-Bind Resin Qiagen; CDNB – Squib Bristol Labs; GSH-Sepharose Pharmacia LKB; Bacto peptone, yeast extract and bacto agar – Difco.

Cloning of the second and third intracellular loops and C-terminal fragments of the D1 receptor into pGEX-2T. Standard procedures were used for DNA manipulations (19). Fragments of human D1 dopamine receptor were amplified by the polymerase chain reaction (PCR). To obtain these fragments, the following pairs of primers containing plasmid DNA encoding human D1 dopamine receptor were used:

<table>
<thead>
<tr>
<th>Option</th>
<th>N-terminus (amino acids)</th>
<th>C-terminus (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) for D1- ICL2</td>
<td>N-term 5′-CGCGGATCCCGACAGTTGGGCTATCTCCAGGC-3′</td>
<td>C-term 5′-CGCGGAATTCCTGGGTCATCTTTCCTCATAC-3′</td>
</tr>
<tr>
<td>b) for D1- ICL3</td>
<td>N-term 5′-CGCGGATCCCGACAGTTGGGCTATCTCCAGGC-3′</td>
<td>C-term 5′-CGCGGAATTCCTGGGTCATCTTTCCTCATAC-3′</td>
</tr>
<tr>
<td>c) for D1- CTSF</td>
<td>N-term 5′-CGCGGATCCCGACAGTTGGGCTATCTCCAGGC-3′</td>
<td>C-term 5′-CGCGGAATTCCTGGGTCATCTTTCCTCATAC-3′</td>
</tr>
<tr>
<td>d) for D1- CTLF</td>
<td>N-term 5′-CGCGGATCCCGACAGTTGGGCTATCTCCAGGC-3′</td>
<td>C-term 5′-CGCGGAATTCCTGGGTCATCTTTCCTCATAC-3′</td>
</tr>
</tbody>
</table>

To avoid introduction of errors during subcloning, all PCR products were cut with BamHI and EcoRI and cloned into BamHI-EcoRI sites of the prokaryotic expression vector pGEX-2T. The resulting clones were cut with BamHI and EcoRI and tested by DNA agarose gel electrophoresis for length of the fragments.

Expression and purification of fusion proteins

E. coli BL21 and BL21 DE3 cells were maintained and transformed with:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid DNA</th>
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<tbody>
<tr>
<td>BL21</td>
<td>pGEX-2T-D1-ICL2, pGEX-2T-D1-ICL3</td>
</tr>
<tr>
<td>BL21 DE3</td>
<td>pGEX-2T-D1-CTSF, pGEX-2T-D1-CTLF</td>
</tr>
</tbody>
</table>

using CaCl2 method (19). The cells were grown in Luria-Bertani broth supplemented with ampicillin (final conc. 100 mg/mL) at 37 °C until A600 reached 0.5, then the expression was induced with isopropylthiogalactoside (IPTG; final conc. 0.1 mmol/L) and glucose was added (final conc. 20 mmol/L). Temperature was decreased to 25 °C, for D1- ICL2 and D1- ICL3, and to 26 °C for D1- CTSF and D1- CTLF. The cells were harvested after 6 h (3000×g, 10 min, Sorvall SS-1 centrifuge), resuspended in the solution containing (in mmol/L): NaCl 140, KCl 2.7, Na2HPO4 10, KH2PO4 18, pH 7.30 (PBS; 50 mL buffer per mL culture) and incubated (15 min, 25 °C) with the solution consisting of lysosome (final conc. 0.2 mg/mL), Triton X-100 (final conc. 0.1%) and phenylmethylsulfonylfluoride (PMSF; final conc. 17 mg/mL). After that, Na-deoxycholate (final conc. 1.6 mg/mL), MgCl2 (final conc. 10 mmol/L) and DNA-se (final conc. 20 mg/mL) were introduced and the incubation continued (15 min, 25 °C). The lysates were centrifuged (15000×g, 20 min, Sorvall SS-1 centrifuge) and supernatants loaded onto GSH-Sepharose (1.0 mL gel per 1000 mL culture) and incubated (15 min, 25 °C) with the solution consisting of lysosome (final conc. 0.2 mg/mL), Triton X-100 (final conc. 0.1%) and phenylmethylsulfonylfluoride (PMSF; final conc. 17 mg/mL). After that, Na-deoxycholate (final conc. 1.6 mg/mL), MgCl2 (final conc. 10 mmol/L) and DNA-se (final conc. 20 mg/mL) were introduced and the incubation continued (15 min, 25 °C). The lysates were centrifuged (15000×g, 20 min, Sorvall SS-1) and supernatants loaded onto GSH-Sepharose (1.0 mL gel per 1000 mL culture) equilibrated with 10 vol. of ice-cold PBS containing 1.0 mmol/L EDTA. Proteins were eluted with 10 mmol/L GSH, 50 mmol/L Tris, pH 8.0. The fractions containing fusion proteins were pooled and dialyzed overnight against 1.0 mmol/L EDTA, 10 mmol/L Tris, pH 8.0, at 4 °C. Purified proteins were concentrated by PEG-20 000 and stored at 20 °C in 40% (v/v) glycerol until used.

Expression and purification of His-Ga proteins

This was done exactly as described by Lee et al. (20).
1-Chloro-2,4-dinitrobenzene (CDNB) assay for GST-fusion protein activity determination

This was performed as suggested by Pabst et al. (21) and Simonović et al. (22).

Assay for quantitative estimation of His-Gα subunit interactions with fusion proteins

His-Bind Resin was equilibrated with the solution of 0.1% ovalbumin and 10 mmol/L Tris, pH 7.4, by repeated centrifugations (2000 x g, 1 min, Fisher Sci. microfuge). Varying concentrations of His-Gαs, His-Gαo, or His-Gαi (0.019 14.286 mmol/L, 0.013 13.636 mmol/L and 0.012 14.634 mmol/L, respectively) prepared in the same solution and preincubated with 1.0 mmol/L GDP and 5 mmol/L MgCl2, were mixed with 40 mL of His-Bind Resin. The mixtures (final vol. 90 mL) were incubated (24 °C, 60 min, constant shaking) and after that, unbound His-proteins were removed by double washing with the above solution. Fusion proteins (D1-ICL2-GST 14 nmol; D1-ICL3-GST 10 nmol; D1-CTSF-GST 21 nmol; D1-CTLF-GST 16 nmol) were added and incubated (24 °C, 60 min, constant shaking). Unbound fusion proteins were removed by double washing with ice-cold 10 mmol/L Tris-HCl, pH 7.4. The samples were subjected to CDNB assay. GST reaction was terminated after 40 min with 90 mL of 2 mol/L HCl. After that, the mixtures were centrifuged (2000xg, 2 min, Fisher Sci. microfuge) and absorbancy (340 nm) in the resulting supernatants was recorded.

Assay for qualitative estimation of His-Gα subunits interaction with fusion proteins

Glutathione-Sepharose was equilibrated with binding buffer (0.3% BSA, 10 mmol/L Tris pH 7.4, 0.1% Triton X-100, 360 mmol/L NaCl). Fusion proteins (12 μg) were mixed with 40 μL of Glutathione-Sepharose and incubated for 45 min (25 °C, constant shaking). His-Gα subunits (20 μg) were added and the incubation continued for 60 min. Unbound proteins were removed by 3 x 1.0 mL washing with ice cold binding buffer and centrifugation. Electrophoretic sample buffer (40 μL) was added and the samples were prepared for SDS-PAGE electrophoresis (23).

Results

Expression and purification of fusion proteins

D1-ICL2, D1-ICL3, D1-CTSF and D1-CTLF were cloned into BamHI-EcoRI sites of the prokaryotic expression plasmid pGEX-2T. E. coli BL21 strain was used as a host for pGEX-2T-D1-ICL2 and pGEX2T-D1-ICL3 and E. coli BL21 DH3 strain for pGEX2T-D1-CTLF. To avoid extensive proteolysis of fusion proteins, the expression proceeded at 25 °C for D1-ICL2-GST and D1-ICL3-GST and at 26 °C for D1-CTSF-GST and D1-CTLF-GST. Under these conditions the fusion proteins were successfully expressed and purified (Figure 1) in soluble form yielding 1.04 mg D1-ICL2-GST per liter bacterial culture (final conc. 0.23 mg/mL in 4.5 mL), 0.36 mg D1-ICL3-GST per mL bacterial culture (final conc. 0.52 mg/mL in 4.0 mL) and 0.68 mg D1-CTLF-GST per mL bacterial culture (final conc. 0.17 mg/mL in 4.0 mL).

Expression of Gα subunits

Gα subunits were expressed as His-tagged proteins all being tagged at C-terminus and purified in soluble form to provide 2.04 mg Gαs/L bacterial culture (0.51 mg/mL in 4.0 mL); 2.15 mg Gαo/L bacterial culture (0.53 mg/mL in 4.0 mL) and 1.88 mg Gαi1/L bacterial culture (0.47 mg/mL in 4.0 mL).

Quantification of His-Gα subunit interactions with fusion proteins

Interactions of soluble form of fusion proteins with Gα subunits were measured using His-Bind Resin. GST activity was determined by CDNB assay (21, Jugoslov. Med. Biohem. 2002.; 21 (3) 257 Figure 1 SDS-PAGE of purified ICL2-GST and ICL3-GST C-terminal parts of GST-fragments of human D1 dopamine receptor.

a. Lanes: 1-ICL2-GST eluate; 2-GibcoBRL marker; 3-GibcoBRL marker; 4-ICL3-GST eluate, b. Lanes: 1-GibcoBRL marker; 2-GST-CTLF-D1 eluate; 3-GST-CTSF-D1 eluate; 4-glutathione-S-transferase.
Various concentrations of His-\( \Gamma \)\( \alpha \) proteins were immobilized on His-Bind Resin and titrated with fusion proteins. The results presented as saturation binding curves are shown in Figure 2, graphs a-e. Kd values for the interaction of D\( \Gamma \)-CTSF-GST with His-\( \Gamma \)\( \alpha \)s and D\( \Gamma \)-CTLF-GST with His-\( \Gamma \)\( \alpha \)o, calculated from saturation binding curves are listed in Table I. No other interactions were recorded (Figure 2, graphs c-e).

It can be seen (Figure 2, graphs a and b; Table I) that His-\( \Gamma \)\( \alpha \)s and His-\( \Gamma \)\( \alpha \)o have the highest binding affinity at D\( \Gamma \)-CTSF-GST and D\( \Gamma \)-CTLF-GST, while expressing no affinity to bind at \( \Gamma \)\( \alpha \)i. Also, D\( \Gamma \)-ICL2-GST and D\( \Gamma \)-ICL3-GST did not interact with either of \( \Gamma \)\( \alpha \) subunits.

Qualitative estimation of His-\( \Gamma \)\( \alpha \) subunit interaction with fusion proteins

Interaction of fusion proteins D\( \Gamma \)-CTSF-GST and D\( \Gamma \)-CTLF-GST with \( \Gamma \)\( \alpha \) subunits were checked using GSH-Sepharose. Known concentrations of fusion proteins were immobilized on GSH-Sepharose and treated with known concentrations of \( \Gamma \)\( \alpha \)s subunits. The results obtained by SDS-PAGE are shown in Figure 3. As demonstrated by the above assay, D\( \Gamma \)-CTSF-GST

Table I Kd values of GST-CTSF-D\( \Gamma \) interaction with His-\( \Gamma \)\( \alpha \)s and GST-CTLF-D\( \Gamma \) interaction with His-\( \Gamma \)\( \alpha \)o

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Kd (nmol/L)</th>
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<tbody>
<tr>
<td>His-( \Gamma )( \alpha )s/GST-CTSF</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>His-( \Gamma )( \alpha )o/GST-CTLF</td>
<td>0.10 ± 0.03</td>
</tr>
</tbody>
</table>

![Figure 3](image-url) SDS-PAGE showing interactions of \( \Gamma \)\( \alpha \) subunits with intracellular fragments of C-terminal of human D\( \Gamma \) dopamine receptor. Lines: 1 - GST-CTSF-D\( \Gamma \); 2 - GST-CTSF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)s-His; 3 - GST-CTSF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)o-His; 4 - GST-CTSF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)1-His; 5 - GST-CTLF-D\( \Gamma \); 6 - GST-CTLF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)s-His; 7 - GST-CTLF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)o-His; 8 - GST-CTLF-D\( \Gamma \) + \( \Gamma \)\( \alpha \)1-His; 9 - Glutathione-S-transferase. GST-fusion proteins (12 mg) were mixed with 40 mL of Glutathione-Sepharose equilibrated in binding buffer (0.3% BSA, 10 mmol/L Tris pH 7.4, 0.1% Triton X-100, 360 mmol/L NaCl) and incubated for 45 min at 25 °C with constant agitation. His-\( \Gamma \)\( \alpha \) subunits (20 mg) were added and the incubation continued for 60 min. Unbound proteins were removed by 3 x 1 mL washing by centrifugation with ice-cold binding buffer. Glutathione-Sepharose pellets were treated with 40 µL Laemmli sample buffer (23) and the supernatants were run on 12% SDS-PAGE.
and D₁-CTLF-GST expressed the highest affinity for the binding at Gₐ₆s and Gₐ₀ subunits.

**Discussion**

Sidhu et al. (25) and Kimura et al. (9) showed that D₁ dopamine receptor couples to Gₛ and Gₒ proteins. Several studies were concentrated on the examinations of which parts of the D₁ receptor interact with G proteins (12, 14). All these authors pointed out the importance of the second and third intracellular loops, as well as of C-terminal part of this receptor in coupling to G proteins. However, procedures employed were rather expensive and time consuming.

In this work the method of Simonović et al. (22) was applied, previously shown to be both rapid and simple for qualitative and quantitative analyses of the interactions of the third intracellular domain of the D₂₅ receptor with Gᵦ proteins. Using this method and bearing in mind the results of the above authors, we attempted to show which part(s) of the human D₁ dopamine receptor plays a role in coupling to G proteins.

All fragments of the D₁ receptor were cloned in pGEX-2T plasmid and in this way fusion proteins with glutathione-S-transferase (GST) were created. Such constructs facilitated purification of the D₁ receptor fragments and enabled to estimate the interactions by measuring the activity of GST, as an active enzyme. All Gₛ subunits were expressed as His-tagged proteins and purified on His-Bind Resin which was used for immobilization of these proteins in quantitative assay.

Our results show that just C-terminal part of the human D₁ dopamine receptor, interacts specifically with Gₛₛ and Gₓ₀ proteins as suggested earlier by König and Gratzel (14) and quite recently by Jackson et al. (26). König and Gratzel (14) demonstrated that synthetic peptides, with amino acid sequence of the second and third intracellular loop and C-terminal part of rat D₁ dopamine receptor interact with Gₛₛ protein. The data of Jackson et al. (26) who pointed to the role cytoplasmic tails of human D₁ and D₅ dopamine receptors play in coupling to G proteins are in the accordance with our results reported in the present study.

However, although several authors (12, 13) suggested that the second and third intracellular loop of the D₁ dopamine receptor are important for the coupling to G proteins, our results did not support such an opinion. It is very interesting to note that two different fragments of C-terminal part of the human D₁ dopamine receptor (D₁-CTSF and D₁-CTLF) take part in coupling to two different Gₛ subunits (Gₛₛ and Gₓ₀). This leads to a hypothesis that different Gₛ subunits are coupled to different sequences within the same receptor, but to prove this assumption further studies are necessary.

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References


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