SH3 Binding Domains in the Dopamine D₃ Receptor

By

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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A. Andrew Ray
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Abstract

The dopamine D₃ receptor is a G protein-coupled receptor (GPCR) and belongs to the family of D₂-like receptors. Like many other catecholaminergic receptors, the dopamine D₃ receptor contains putative SH3 binding motifs, which are characterized by PXXP motifs, within the N- and C-terminal regions of its third cytoplasmic loop. Here it is demonstrated that the D₃ receptor is capable of interacting with SH3 domain-containing proteins in vitro including the SH2-SH3 adapter protein Grb2 (Kₛₐ ≈ 8 μM). Furthermore, the region of interaction is located in the third cytoplasmic loop. Disruption of the PXXP motifs does not significantly diminish SH3 binding, suggesting that its SH3 interactions may not occur through classic PXXP containing sequences.


Here, we also report the binding of the β₁-adrenergic and M₄ muscarinic acetylcholine receptors to several SH3 domains. This suggests that SH3-protein interactions may form an integral part of GPCR functioning.
ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Hubert Van Tol for providing me with the tremendous opportunity to study in his lab. Without his guidance and support, none of this work would have been possible. Much of what I accomplish will be a testament to his help. I would also like to thank John “the vacuum” Oldenhof for sharing part of his project with me and for helping to smooth out some of the rough spots (Is it possible to mutagenize D4? John and I don’t think so!). Of course, James Oak helped a lot as well. From him, I learned too many techniques to mention in this space. I will miss the political debates. I also have to thank Vera Jovanovic for relinquishing her bench for the better part of 2 1/2 years.

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For technical assistance, I have to thank Dr. H.-C. Guan for some of the binding assays presented in this project. I also have to thank Dr. M. Caron for the β1 and βARK cDNAs used and Dr. T. Bonner for the M4 cDNA. Obviously, their contributions made much of this work possible.

Last, to my family “out east”. I’m finally done. Thanks for the support when I needed it. I can’t wait to embark on my new career.
ABBREVIATIONS

Amp  Ampicillin
AR   Adrenergic receptor
ATP  Adenosine 5'-triphosphate
βARK1 β adrenergic receptor kinase 1 (see also GRK2)
βARK2 β adrenergic receptor kinase 2 (see also GRK3)
bp   base pairs
Bmax Maximal binding density
cDNA Complementary deoxyribonucleic acid
CHO  Chinese hamster ovary
Ci   Curies
cpm  Counts per minute
dpm  Disintegrations per minute
Cys  Cysteine
ddH2O Deionized water
DNA  Deoxyribonucleic acid
dNTP Deoxyribonucleotide 5'-triphosphate
DTT  Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
FBS  Fetal bovine serum
fM   Femtomolar
FP   Fusion protein
G418 Geneticin™
GPCR G protein-coupled receptor
Gpp[NH]p 5'-guanylyl imododiphosphate
G protein Guanine nucleotide-binding regulatory protein (subtype: i=inhibitory, s=stimulatory, o=olfactory)
Grb2 Growth-factor-receptor binding protein 2
GRK2 G protein-coupled receptor kinase 2 (see also βARK1)
GRK3 G protein-coupled receptor kinase 3 (see also βARK2)
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>K_d</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Daltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani (medium containing bacto tryptone, yeast extract and NaCl)</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propane-sulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl and 8 mM Na_2HPO_4 (pH 7.4))</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computerized tomography</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris buffer containing 45 mM Tris, 100 mM Boric Acid, 2mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TE</td>
<td>Tris buffer containing 10 mM Tris, 1mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indoyl β galactoside</td>
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1.0 INTRODUCTION

1.1 G Protein-Coupled Receptors

The G protein coupled receptors (GPCRs) are an important class of integral membrane proteins. In the cell, the GPCRs transduce extracellular signals to the interior and in so doing modulate a wide variety of biological processes including neurotransmission, chemoattraction, olfaction, vision as well as the actions of a variety of hormones. The GPCR class of receptors is named for their ability to undergo functional coupling to heterotrimeric G proteins. The G proteins in turn affect various second messenger systems such as phosphatidyl inositol (PI) turnover, and cAMP.

The family of GPCRs is large, encompassing more than 1000 different members in mammals alone (Wess, 1997). This group may be divided into three major subfamilies based on specific key sequences. The three subtypes are the rhodopsin-related receptors (type A), receptors related to calcitonin (type B) and receptors related to the metabotropic glutamate receptor (type C) (Gether and Kobika, 1998). Of these, the rhodopsin family is by far the largest and includes the dopamine, muscarinic and adrenergic receptors, which are the focus of this study. GPCRs may be further distinguished by ligand binding, sequence homology and receptor function in the cell.

Although definitive biochemical data on the structure of dopamine receptors has yet to be generated, primary structure analysis (including hydrophobicity plots) suggests that these receptors belong to the superfamily of seven transmembrane domain G protein-coupled receptors (see Fig.1). At present, high-resolution structures are not available for any GPCRs. This is primarily due to the inherent difficulty in crystallizing integral membrane proteins. However, low-resolution structures have been generated for rhodopsin (Schertler et al., 1993; Schertler and Hargrave, 1995; Unger and Schertler, 1995), confirming the model of 7 hydrophobic transmembrane α-helices arranged in counterclockwise fashion when viewed from the extracellular surface (see Missale et al., 1998 for review). The transmembrane (TM) segments are the regions most highly conserved between GPCRs. In addition, hydrophobicity analysis, site directed mutagenesis and immunohistochemical data suggests that there is an N-terminal tail that projects above the membrane surface and a C-terminal tail that is cytosolic. These regions, and the intracellular loops, are the source of the majority of variation within GPCR subgroups.
Intracellular regions of the receptor are important for functional coupling to G proteins and are the site of protein modifications (such as phosphorylation) that modulate receptor activity.

Two Dimensional Model of the D3 Receptor

![Two dimensional model of the dopamine D3 receptor. Conserved aspartic acid (D) and serine (S) residues implicated in catecholamine binding are shown as potential SH3 binding motifs in the third cytoplasmic loop.](image)

(a) The Allosteric Ternary Complex Model

The ternary complex model is the most widely accepted model used to describe agonist activation of GPCRs (De Lean et al., 1980) (see Fig. 2). This model has been revised to account for different classes of ligands (partial agonists, neutral agonists, inverse agonists, etc.) and alternate states of the receptor. However, such aspects of the model are beyond the scope of this discussion. Under the simple model, a receptor is in equilibrium between its active (R*) and inactive (R) states. The efficacy of a ligand is its intrinsic ability to alter the equilibrium in favour of either the active or inactive state of the receptor. Upon agonist activation, the receptor undergoes a change in conformation that promotes association with an inactive (GDP-bound) heterotrimeric G protein which consist of α, β and γ subunits. Interaction with the receptor promotes GTP exchange for GDP and results in dissociation of the G protein into α and βγ subunits. In turn, dissociation of the G protein from the receptor results in loss of the high affinity state of the receptor (R*→R). The α subunit and βγ dimer are then free to interact with effector systems. Reassembly of the complex requires hydrolysis of GTP by the α subunit. This subunit contains intrinsic GTPase activity that may be enhanced by association with an effector
such as phospholipase Cβ (Biddlecome et al., 1996) or RGS proteins ("regulators of G protein signaling") depending on the subtype of Go (see Dohlman and Thorner, 1997; Arshavsky and Pugh, 1998; Berman and Gilman, 1998) for review. Once bound to GDP, the α subunit reassociates with βγ and the cycle repeats (for review, see Lefkowitz, 1998; Pitcher et al., 1998). The cycle is further complicated by the presence of several different isoforms for each of the G protein subunits. At present, there are at least 17 distinct Go (Wilkie and Yokoyama, 1994), 5 Gβ and 6 Gy isoforms that are known (Neer, 1995; Dohlman and Thorner, 1997). In turn, these may to give rise to many different heterotrimeric G proteins as each of these G proteins may modulate one or more different effector systems.

The Allosteric Ternary Complex Model

![Fig. 2 The allosteric ternary complex model and G protein cycling. Key: A denotes agonist, R denotes GPCR protein, * denotes activated form of the receptor.](image-url)

(b) Conformational Changes of Receptor Activation

Most of the knowledge of GPCR conformation came from studies with the rhodopsin and β-adrenergic receptors. There is ample experimental evidence suggesting that "ligand-induced changes in the relative orientation of individual transmembrane helices affect the conformation of the intracellular receptor surface, thus allowing productive coupling to G-proteins" (Wess, 1997). Site-directed spin labeling (SDSL) study of the rhodopsin receptor has shown that upon agonist activation there is a small outward movement of the cytoplasmic portion of TM3 with
concomitant changes in the structure of the second intracellular loop (Farahbakhsh et al., 1993; Farahbakhsh et al., 1995). Another study indicated that TM6 of the rhodopsin receptor experienced a similar outward motion upon agonist exposure, coupled with a significant rotation of the TM helix by 30° (Farrens et al., 1996). Because all GPCRs share a similar topology, it is likely that such conformational changes are not limited to the rhodopsin receptor alone. Again, there is experimental support for this idea. For instance, stimulation of the yeast α-factor pheromone receptor with agonist increased accessibility to the third cytoplasmic loop as measured by trypsin cleavage (Bukusoglu, 1996), whereas binding of antagonist inhibited proteolytic cleavage. This result suggests that both agonists and antagonists are capable of mediating conformational changes to intracellular regions of the receptor. Furthermore, there is direct evidence for ligand-induced conformational changes in the catecholamine receptors as well. A study of the β2-adrenergic receptor, that had been modified by a cysteine-specific fluorescent marker, revealed decreases in fluorescence from baseline upon agonist exposure and increases in fluorescence upon antagonist exposure (Gether et al., 1995), further adding to evidence for ligand-induced conformational change.

In the case of bioamine receptors (e.g. the dopamine receptors), the ligand binding pocket is largely formed by the TM helices. In analogy to adrenergic receptors, residues that have been implicated in catecholamine binding have been conserved in the dopamine receptors. This includes two serine residues in TM5 and an aspartic acid residue in TM3 (see Ji et al., 1998 for review). The TM domains of GPCRs are thought to form a hydrophilic binding pocket through which agonists are able to gain access and activate the receptor (Mizobe et al., 1996). Specifically, the meta- and para-hydroxyl groups of the catechol ring appear to undergo hydrogen bonding with the conserved serines (S) while the amine group of catecholamines pairs with the carboxyl group of aspartic acid (D) (Strader et al., 1994) (see fig. 1). This results in a constrained TM3/TM5 conformation and would be expected to alter GPCR packing.

Although there is no published data pertaining to conformational changes in the dopamine receptors, such a possibility would be consistent with other GPCRs. Beyond implications for G-protein coupling, ligand-induced conformational changes in accessibility to intracellular regions could be important for previously unappreciated effector systems. For example, the present study has noted the presence of potential SH3 binding domains within the third cytoplasmic loop of several GPCRs including the D2-like receptors, the β1 adrenergic
receptor and the M₄ muscarinic receptor. Thus, binding sites for protein-protein interactions could be affected by any changes to the intracellular regions of these GPCRs.

(e) Desensitization of GPCRs

A common feature of signal transduction systems mediated by GPCRs is that following prolonged stimulation, signaling becomes attenuated through a process known as desensitization (Lefkowitz, 1993). Several distinct mechanisms seem to be important for this process. In particular, the importance of receptor phosphorylation by serine/threonine kinases has become increasingly apparent (Lefkowitz et al., 1990; Lefkowitz, 1998). In fact, rapid phosphorylation of many GPCRs accompanies stimulus driven desensitization. Two distinct types of kinases mediate this effect: The G protein-coupled receptor kinases (GRKs) and the second messenger kinases PKA and PKC.

The second messenger kinases, protein kinase A (PKA) which is activated by Gₛ, and protein kinase C (PKC) which is activated by Gₒ, form one of the most well-established paradigms of receptor desensitization by feedback inhibition. Both kinases phosphorylate serine residues at consensus sites in the third cytoplasmic loop or C-terminal tail (Bouvier et al., 1988; Hausdorff et al., 1989). This modification impairs coupling to G proteins by altering the conformation of the receptor. Because any receptor that contains PKA or PKC consensus sites may be targeted by the activated kinases, this type of desensitization is not receptor specific and is thus termed "heterologous desensitization" (see (Lefkowitz, 1998) for a brief review).

Study of the β₂-adrenergic receptor has further led to a general model for highly specific GPCR desensitization. After dissociation of the activated heterotrimeric G protein from the receptor (Fig. 3), the receptor becomes a target for G protein-coupled receptor kinases (GRKs). As GRKs target agonist occupied receptors, this mode of inactivation is termed "homologous desensitization" (see (Lefkowitz, 1998) for review). Currently, the family of GRKs includes six members (GRKs 1-6) of which the most thoroughly investigated are rhodopsin kinase (GRK1) and βARK1 (GRK2). A third GRK used in this study, GRK3, is alternately known in the literature as βARK2.

It is known that binding of GRK to the receptor greatly enhances affinity of arrestin for the receptor complex. The binding of arrestin physically occludes the G protein from binding to the receptor and thus inhibits signaling. The association of arrestin also promotes receptor
internalization through coated pits, involving dynamin (Koenig and Edwardson, 1997). The SH2/SH3 adapter protein Grb2 has been implicated in this pathway (Gout et al., 1993), possibly recruiting component proteins to the membrane. Once internalized, receptors may be either dephosphorylated and recycled to the membrane or downregulated by destruction in lysosomes (see (Lefkowitz, 1998) for review).

**Homologous Desensitization of the β2-AR**

*Fig. 3* Homologous desensitization of the β2-adrenergic receptor (adopted from (Pitcher et al., 1998)). Key: A denotes agonist, R denotes GPCR protein, * denotes the activated form of the receptor or Gα subunit and E denotes primary effector of Gα while E2 denotes effector(s) of Gβγ and GRK2.

Little is known about inactivation and internalization mechanisms in the dopamine receptors; however, there is data indicating that they are also subject to downregulation and
desensitization (Barnett and Kuczenski, 1986; Chneiweiss et al., 1990; Chen et al., 1993; Engber et al., 1993; Lewis et al., 1998).

(d) Association of Proteins with GPCRs

Until recently, signaling mechanisms mediated by G protein-coupled receptors have been primarily limited to pathways effected through G-proteins. Recently, it was been reported that the Grb2 SH2/S3 adapter protein is capable of specifically associating with the β2-adrenergic receptor in insulin or serum treated cells (Malbon and Karoor, 1998; Shih and Malbon, 1998). Specifically, this interaction occurred via the Grb2 SH2 domain with a phosphorylated tyrosine residue in the C-terminal tail of the β2 receptor. This finding was interesting as it was the first evidence of direct physical contact between GPCRs and soluble adapter proteins. This interaction of β2 with Grb2 has been shown to be important for internalization of the receptor (Karoor, 1998). Furthermore, this interaction with Grb2 could provide a direct link between GPCRs and the Ras pathway of MAP kinases (see section 1.2 (e)).

There is increasing recognition that other GPCRs are also capable of signaling through alternate pathways such as Jak2 kinase (Ali et al., 1997) and phospholipase Cy (Venema et al., 1998). Both of these enzymes are recruited to the C-terminal tail of the angiotensin (AT1A) receptor by the SHP-2 adapter protein. Protein-protein interactions are also formed through SH2 domain signalling complexes at the plasma membrane. In addition, G protein-coupled receptor kinases (GRKs), arrestin (Lefkowitz, 1998), calmodulin and protein kinase C (PKC) (Minakami et al., 1997) have also been shown to activate signaling cascades. Calmodulin and PKC are interesting because their association with the metabotropic glutamate receptor subtype 5 (mGlur5) are transient and independent. PKC is able to inhibit calmodulin binding by phosphorylating the binding site for calmodulin on the receptor. In this example, PKC not only plays a role in G protein signaling (heterologous desensitization), but in regulating alternate signaling cascades as well. Thus the variety in GPCR signaling is testament to the diversity inherent to this receptor superfamily.

This study investigates a protein-protein interaction that is new to GPCRs. Recently, our group reported that many GPCRs, and the dopamine D4 receptor in particular, are capable of making high-affinity contacts with a modular protein domain known as SH3 (Oldenhof et al.,
The focus of the current study was to investigate possible protein-protein interactions of the dopamine D3 receptor, including one with Grb2.

1.2 Dopamine Receptors

The neurotransmitter dopamine is involved in the control of numerous physiological functions such as movement, cognition, emotion, hormonal release, motivation and reward (Hornykiewicz, 1966; Caron et al., 1978; Lee et al., 1978; Seeman, 1987; Carlsson, 1988). Thus, it is not surprising that for more than three decades there has been tremendous interest in understanding the dopamine system.

Dopamine have been implicated in schizophrenia and other psychiatric disorders for some time (Seeman, 1987). It had been shown that dopamine mimetics could produce schizophrenia-like psychosis (Randrup and Munkvad, 1965; Snyder, 1973; Lieberman et al., 1987) and that these symptoms could be relieved by D2 antagonists (Seeman and Lee, 1975). The clinical potency of different classes of antipsychotic drugs varies directly with their affinity for the D2 receptor (Seeman and Lee, 1975; Creese et al., 1976; Seeman et al., 1976; Seeman, 1992). In addition, it was observed that D2-like receptor densities were elevated in post-mortem schizophrenic brain (Seeman, 1987). These data led to the formulation of the "dopamine hypothesis of schizophrenia" which holds that certain dopaminergic pathways are overactive in the disease state (Seeman, 1987). Positron emission tomography (PET) and single photon emission computerized tomography (SPECT) studies of D2 receptor density in schizophrenic patients have been unable to conclusively link dopamine upregulation with schizophrenia due to conflicting results. Some studies have shown increased D2 density (Wong et al., 1986; Tune et al., 1993) while others have not observed any changes in receptor density (Farde et al., 1990; Martinot et al., 1991; Hietala et al., 1994; Nordstrom et al., 1995). Recently, there has been new evidence supporting the role of dopamine in schizophrenia. New PET techniques have been developed that measure synaptic dopamine levels indirectly by displacement of the radioligand [11C]-raclopride by endogenous dopamine. Increased synaptic dopamine levels upon amphetamine stimulation have been shown to occur in many schizophrenic patients as compared to controls (Laruelle et al., 1996; Breier et al., 1997). Findings of increased dopamine levels can now be linked with earlier work showing that psychotomimetic compounds such as amphetamine not only lead to worsening symptoms in one-third of schizophrenic patients (Lieberman et al.,
1987), but also the induction of schizophrenic symptoms in healthy individuals (Angrist, 1994). As such, at least a subgroup of schizophrenia may be associated with dopaminergic hyperactivity.

Until 1990, it was believed that the dopamine receptor population consisted of two subtypes, D₁ and D₂ (Seeman and Grigoriadis, 1987). Today, there are five known dopamine receptor subtypes that may be grouped into the aforementioned classes. The D₁-like receptors (D₁ and D₅) functionally couple to Gₛ, stimulating cyclic AMP (cAMP) production through adenylate cyclase. The D₂-like receptors (D₂, D₃ and D₄) couple the pertussis toxin sensitive Gᵢₒᵢ₉₂ subset of proteins and inhibit cAMP production. Interestingly, the D₁-like and D₂-like receptors are also structurally distinct, the D₁-like being intron-less in their coding sequence, the D₂-like possessing introns. In addition, the D₂-like receptors have long third intracellular loops relative to the D₁-like receptors. This is a common feature of aminergic receptors that couple to Gᵢ (Civelli et al., 1993; Gingrich and Caron, 1993). In turn, D₁-like receptors possess much longer carboxyl tails. Again, this feature appears to be common to receptors that couple to Gₛ.

(a) Cloning of the Dopamine Receptors

Of the dopamine receptors, the dopamine D₂ receptor was the first to be cloned. This was accomplished using degenerate probes based on the β₂-adrenoreceptor sequence (Bunzow et al., 1988). Once the D₂ cDNA had been obtained, a similar homology based approach was employed to subsequently obtain cDNAs for D₃ (Sokoloff et al., 1990) and D₄ (Van Tol et al., 1991). The D₁ receptor was cloned independently by several groups using either low stringency screening or PCR based approaches (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). D₅ was later cloned based on its homology to D₁ (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991).

Because D₂-like receptors contain introns, receptor variants may also be generated for these genes. The D₂ receptor has two main variants, D₂S and D₂L, which differ by 29 amino acids in the third cytoplasmic loop (Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989). Similarly, splice variants also exist for D₃. In mice, there are two forms, D₃L and D₃S, which differ by 21 amino acids in the third cytoplasmic loop (Fishburn et al., 1993). As yet, no splice variants for the third loop have been reported in humans; however, truncated forms do exist (Giros et al., 1991; Snyder et al., 1991; Nagai et al., 1993; Pagliusi et al., 1993; Schmauss et al.,
1993; Griffon et al., 1996b). None of the reported truncated forms of the D3 receptor appears to have function in the cell. The D4 receptor is particularly striking because of the number of polymorphic variants that occur within its third cytoplasmic loop. This loop contains a variable number of tandem repeats (VNTRs), ranging from 2 to 10, which consist of 16 amino acids each (Van Tol et al., 1992). Of these, four repeats (hence D4a) is the most common, occurring in up to 60% of the population depending on ethnicity (see (Seeman and Van Tol, 1994) for review).

There is considerable homology between dopamine receptors, especially within sub-families. The D1 and D5 receptors share 80% of the residues within their TM domains. Similarly, D2 exhibits 75% homology with D3 and 53% homology with D4 in the TM regions. Overall identity of D2 with D3 and D4 has been shown to be 41% and 39% respectively (Missale et al., 1998). The primary focus of this work is with members of the D2-like family. Thus, my discussion will henceforth be limited to these receptors.

(b) Pharmacology of D2-like Receptors

The D2-like family of dopamine receptors possesses easily distinguishable pharmacological profiles. Specifically, D3 displays a 20-fold greater affinity for dopamine compared to D2 (Sokoloff et al., 1990). As determined from studies with chimeric receptors, much of this selectivity has been attributed to differences in their third cytoplasmic loops (Robinson et al., 1994). Similarly, other agonists also display higher affinity for D3 sites than for D2. These include TL-99, pergolide, quinpirole, and 7-hydroxy-dipropylaminotetralin (7-OH-DPAT) (see (Missale et al., 1998) for review). Most neuroleptics bind with nanomolar affinity to both receptor subtypes. However, haloperidol and spiperone favour D2 with 10-20 fold higher affinity. Other commonly used drugs such as (-)-sulpride, clozapine and raclopride do not discriminate between these receptor subtypes (Missale et al., 1998).

It must be noted that binding studies should be interpreted with caution as results may vary depending on the expression system, the radio-ligand used and the in vitro assay conditions (Tang et al., 1994; Burris et al., 1995; Levent, 1995; Seeman and Van Tol, 1995). Discrepancies in agonist binding have been reported. Specifically, quinpirole has been found to favour D3 over D2 by 100-fold higher affinity in some systems (Sokoloff et al., 1990; Levesque et al., 1992; Burris et al., 1995), but to have equal affinity in others (Levant and DeSouza, 1993; Tang et al., 1994). Similar discrepancies have also been seen with other agonists such as dopamine,
quinerolane, 7-OH-DPAT, 7-trans-OH-PIPAT and pramipexole (Sokoloff et al., 1990; Levant and DeSouza, 1993; Freedman et al., 1994; Tang et al., 1994; Burris et al., 1995; Sautel et al., 1995a). This is likely because the high affinity state of the D₂ receptor appears to share similar affinity for agonists as the D₃ receptor (Burris et al., 1995). Thus, reported D₃-selectivity for some agonists may have been caused by assay conditions that disfavour the high affinity state of D₂. Such conditions include the use of Na⁺ in the assay when testing benzamide ligands (Grigoriadis and Seeman, 1985; Burris et al., 1995; Levant et al., 1995). In contrast, results for antagonists are much more consistent between studies.

(c) Distribution of D₃

The D₃ receptor is of particular interest because of its relatively restricted distribution in the brain. Unlike D₂, very low levels of expression have been detected in the caudate putamen and in the pituitary. These regions have been associated with many of the undesirable neurologic and endocrine side effects produced by most common antipsychotics. As such, there has been speculation that D₃ may be a target for novel antipsychotics free of extrapyramidal or endocrine effects (Sokoloff et al., 1990).

The most abundant expression of the dopamine D₃ receptor has been found in the nucleus accumbens and the Islands of Calleja (Landwehrmeyer et al., 1993; Murray et al., 1994; Suzuki et al., 1998). As well, D₃ receptor mRNA has been detected at high levels in the granular cell layer of the dentate nucleus (Meador-Woodruff et al., 1994). Low to moderate expression has also been detected in all cortical regions, putamen, anterior and medial thalamic nuclei, mamillary body, amygdala, hippocampal CA region, lateral geniculate body, substantia nigra, locus coeruleus, and raphe nuclei (Herroelen et al., 1994; Murray et al., 1994; Lahti et al., 1995). This pattern of expression suggests a role for the receptor in limbic related functions such as emotion and cognition as well as non-limbic functions such as the processing of motor and sensory information.

(d) D₃ Function

The function most widely ascribed to D₃ is the modulation of locomotor activity. In contrast to D₂, D₃ appears to inhibit locomotor activity. This effect has been demonstrated using the D₃ preferring drug 7-OH-DPAT. Upon administration to rats, this drug elicits a biphasic
effect on locomotion. At low doses, locomotion is inhibited, presumably due to occupation of D₃ sites. At higher doses, locomotion is stimulated, due to occupation of D₂ sites (Daly and Waddington, 1993; Ahlenius and Salmi, 1994; Svensson et al., 1994). In addition, inhibition of locomotion can be achieved using doses of 7-OH-DPAT that do not significantly occupy the D₂ receptor in vivo (Levant et al., 1996). Consistent with this effect, the D₃ preferring antagonist nafadotride can produce a similar biphasic effect on locomotion, stimulating it at low doses (Sautel et al., 1995b). Last, increased locomotor activity has been observed in one study with knock-out mice (Accili et al., 1996). When considered together, a role for the dopamine D₃ receptor in locomotion seems likely.

Another role that has been attributed to D₃ is in reinforcement and reward. In particular, 7-OH-DPAT has been shown by one group to decrease self-administration of cocaine (Caine and Koob, 1993; Parsons et al., 1996; Caine et al., 1997). Similarly, stimulation of D₃ sites is implicated in blocking the reinforcing effects of both cocaine and d-amphetamine (Kling-Petersen et al., 1994). In addition, D₃ sites have been found to be elevated in the caudate, nucleus accumbens and substantia nigra of post-mortem brains of chronic cocaine abusers (Staley and Mash, 1996). However, much work remains to be done before the specific role of D₃ or other dopamine receptor subtypes in reinforcement and reward can be stated with certainty.

At present, there is no direct genetic linkage between D₃ and schizophrenia. One study however, did note the loss of D₃ mRNA in specific brain regions and the appearance of a splice variant (D₃a) in their place (Schmauss et al., 1993; Schmauss, 1996). The D₃a splice variant contains a deletion in the region encoding the third cytoplasmic loop that when translated results in a truncated, non-functional form of the receptor. Increased incidence of D₃a in schizophrenic brain is a potential contributor to the disease. Also of interest is the finding that the Bal I or Msc I polymorphism in the first exon of D₃ may increase susceptibility to the disease. This finding has been reported by several groups (Crocq et al., 1992; Mant et al., 1994; Kennedy et al., 1995; Griffon et al., 1996a).

In Parkinson’s disease, no alterations in D₃ expression in the brain have been observed thus far (Hurley et al., 1996) compared to normal brain. However, decreased D₃ mRNA expression in the lymphocytes of Parkinson’s patients has been observed (Nagai et al., 1996). Interestingly, the magnitude of loss of D₃ mRNA correlated with the progress of the disease. This suggests that D₃ may be useful as a marker to monitor the progression of the disease.
However, as this work could not be replicated by others (Vile and Strange, 1996), it remains controversial.

(e) Signal transduction of D₃

The D₃ receptor was originally reported to be insensitive to guanine nucleotides upon agonist binding. It did not exhibit decreased affinity for agonist in the presence of the GTP analog Gpp[NH]p (Sokoloff et al., 1990), suggesting that the receptor may not couple to G proteins at all. Some groups have duplicated this result (Freedman et al., 1994; Tang et al., 1994; Woodcock et al., 1995) while others have discovered a G-shift (Seabrook et al., 1992; Sokoloff et al., 1992; Chio et al., 1994; MacKenzie et al., 1994). Clearly, this is still a point of some contention. In addition, D₃ is relatively ineffective at regulating cAMP when expressed in CHO cells (Sokoloff et al., 1990). Many different cell lines have been used to try to demonstrate D₃-mediated adenylate cyclase inhibition, but effects are minimal compared to D₂ (Seabrook et al., 1992; Castro and Strange, 1993). One difficulty in these studies is that cell lines come from many sources and may not express the appropriate G proteins or effectors necessary for true D₃ function (see (Kenakin, 1996) for review). However, regulation of dopamine release, neurite extension and mitogenesis are all events that have been linked to D₃ control (Pilon et al., 1994; Swarzenski et al., 1994; Tang et al., 1994). Recently, it has also been found that D₃ may couple directly to the modulation of both Ca²⁺ and K⁺ currents as well as extracellular acidification in some cell lines (Chio et al., 1994; Pilon et al., 1994; Potenza et al., 1994; Seabrook et al., 1994; Liu et al., 1996; Werner et al., 1996). These results were shown to be sensitive to the effects of pertussis toxin, suggesting that the effects were mediated by coupling of D₃ to a Gᵢ or G₀ isoform. However, another study found that increases in extracellular acidification were not pertussis toxin sensitive (Cox et al., 1995). Thus, the cellular signaling pathways affected may depend on the host cell line and may not reflect the functional response in the brain (see (Shafer and Levant, 1998) for review).

It has been known for some time that GPCRs that act through the pertussis toxin (PTX) sensitive G proteins, including the D₂-like receptors, are able to activate the mitogen-activated protein kinase (MAP kinase) cascade (see (Luo et al., 1998; Welsh et al., 1998) and see (van Biesen et al., 1995; Post and Brown, 1996) for review). It is currently thought that non-receptor tyrosine kinase is stimulated as a result of receptor activation and the subsequent release of Gₓᵧ.
Receptor tyrosine kinase activation results in the phosphorylation of the Shc adapter protein, which then associates with the Grb2 adapter protein via SH2. The end result is the recruitment of both adapters to the membrane. Another protein, Sos, also plays an important role. Sos is a cytoplasmic guanine nucleotide exchange factor that is constitutively associated with the Grb2 adapter. Once targeted to the membrane by Grb2, Sos promotes guanine nucleotide exchange thereby activating Ras and the MAP kinase pathway (see (Lopez-Ilasaca, 1998) for review). Recent findings that adapter proteins such as Grb2 may also associate directly with membrane-bound receptors could imply that the receptors are able to activate MAP kinase in a G protein independent manner.

1.3 SH3 Domains

Src-homology (SH) domains were originally recognized in the viral oncogene product, v-Src, but have now been characterized in many additional proteins. Src and its cellular homologue, c-Src, were the first proto-oncogenes described and two of the first proteins discovered to possess tyrosine kinase activity (Stehelin et al., 1976). As a result, src has become the prototype for understanding signal transduction that is mediated by tyrosine phosphorylation.

It has been recognized for some time that Src contains two distinct protein domains capable of mediating protein-protein interactions. The first of these, the SH2 domains, is small (approximately 100 amino acids) and binds phospho-tyrosine residues within the context of a larger recognition sequence (Koch et al., 1991; Songyang et al., 1993). The second type of domain in Src, SH3, was first localized to the amino terminal of SH2 (Mayer et al., 1988), and has been widely recognized to bind ligands within the context of PXXP motifs (Ren et al., 1993). The current study focuses on SH3 interactions with the dopamine D3 receptor. These interactions have only recently been reported by our group (Oldenhof et al., 1998).

Like SH2, SH3 domains are small — only 60-80 amino acids in length, and capable of structural and functional independence. The first structural data of SH3 domains was achieved in 1992 with the publishing of c-Src SH3 structure that had been resolved by NMR [Yu, 1992 #106] and α-spectrin structure by X-ray crystallography (Musacchio et al., 1992). Since then, numerous other SH3 structures have been determined in both their ligand-bound and unbound forms. Although their primary sequences may vary considerably, tertiary structure appears to be
well conserved in these domains. In fact, SH3 domains share a common arrangement of five β-strands folded into a β-sandwich (Koch et al., 1991; Musacchio et al., 1994a). Slight variations on this theme do occur due to differences in primary amino acid sequences, but the basic pattern is retained (Dalgarno et al., 1997). An additional feature found in SH3 domains, first noticed in Src, is the RT (for arginine-threonine) loop. The RT loop forms the junction between strands βa and βb of the SH3 domain. Its constrained configuration makes contact with its partner, the SH3 binding domain, thereby conferring ligand specificity. Although the exact sequence may vary, the RT loop is extremely important for high affinity interactions. Reflecting this importance, in v-Src the RT loop is a site of transforming mutations (Dalgarno et al., 1997).

(a) The Role of SH3

More than 50 SH3 domains are currently recognized in many diverse proteins. These include kinases, lipases, small GTP binding proteins, cytoskeletal proteins, viral regulatory proteins, adapter proteins and proteins involved in assembly of the phagocyte NADPH oxidase system (see (Dalgarno et al., 1997) for review). Further adding to the complexity, SH3 domains are often found in conjunction with other domains that mediate protein-protein interactions such as SH2 and pleckstrin-homology (PH) domains. One group of proteins known as adapters is common in the cell, and are characterized by having no catalytic activity of their own. Instead, they contain one or more of the above-mentioned modular domains and serve to "nucleate the formation of protein complexes" (Pawson, 1995). In such a manner, signaling complexes can be brought into close contact with the proteins that regulate them, in effect creating a "protein scaffold". Not surprisingly, the protein scaffold itself may be highly regulated. Several SH3 binding sites contain consensus sequences for proline-directed kinases such as MAP kinase. Phosphorylation on these residues may affect interactions with SH3 domains (Cherniack et al., 1994).

Much work still remains to be done in defining the precise roles of SH3-ligand interactions within the cell. However, it is becoming increasingly apparent that it is not simply the mediator of protein association interactions that it was once believed. Deleting or mutating SH3 domains can lead to oncogenic transformation of non-receptor tyrosine kinases such as c-Src and c-Abl, suggesting that these domains may be negative regulators of such transformation.
(Jackson and Baltimore, 1989; Seidel-Dugan et al., 1992). Also, its association with the cytoskeleton implies a role in regulation or in organizing cell polarity.

In phagocytes, SH3 domains are responsible for functional assembly of the NADPH oxidase system (Leto et al., 1994). In this multi-component assembly, SH3 domains mediate recruitment and arrangement of components at the membrane.

Endocytosis is another membrane-associated action that involves SH3 domains. Dynamin, a GTPase, is an important player in endocytosis as it regulates the scission of clathrin-coated and non-clathrin-coated pits from the plasma membrane (Shpetner et al., 1996). Dynamin contains SH3 binding motifs in its C-terminal (Herskovits et al., 1993; van der Bliek et al., 1993), and removal of these motifs has been shown to block both GTPase activity and targeting of dynamin to clathrin-coated pits (Herskovits et al., 1993; Shpetner et al., 1996). These studies suggest a role for SH3 in endocytosis.

Another important SH3 containing protein is the adapter protein Grb2. This protein was initially identified while investigating the mechanisms by which receptor tyrosine kinases activate the Ras pathway to stimulate cell growth and differentiation (Lowenstein et al., 1992). Grb2 contains one SH2 domain flanked by two SH3 domains (Lowenstein et al., 1992). Its SH3 domains bind to proline-rich regions in Sos, a guanine nucleotide exchange factor for Ras (Li et al., 1993), and recruit Sos to the membrane where its nucleotide exchange activity activates membrane bound Ras. Grb2 has also been implicated in recruitment of other proteins to the membrane through its SH3 domains including dynamin (again suggesting a role in endocytosis), c-Cbl, p62, PTP1 and Vav (Gout et al., 1993; Ye and Baltimore, 1994; Khwaja et al., 1996; Wong and Johnson, 1996).

1.4 SH3 Binding Domains

SH3 binding domains (SH3 ligands) are found in an even larger variety of proteins than the SH3 domains themselves. Indeed, this is not surprising as the minimum secondary structure required for SH3 binding is the poly-proline (PPII) helix (Musacchio et al., 1994a; Yu et al., 1994). Also, there is evidence that PPII helix formation is not the rare structure it was once believed to be. Not only do PPII helices commonly occur in proteins, but they also tend to occur on the protein surface, enhancing accessibility to this site (Adzhubei and Sternberg, 1993).
The first real study into SH3 binding domains was carried out by screening a pre-B cell expression library with the Abl SH3 domain (Cicchetti et al., 1992) in search of binding partners. The two clones that were discovered, 3BP-1 and 3BP-2, cross-reacted with both the Abl and Src kinases as well as other SH3 domains with variable affinity. It was not long before the residues responsible for SH3 binding in these two proteins had been narrowed down to short proline-rich fragments with a consensus of “XPXXPPψXP” (in which P represents non-variant proline residues that directly contact the SH3 domain, X denotes any amino acid and ψ represents any hydrophobic amino acid) (Ren et al., 1993). At the time, it was noted that this consensus was also present in other proteins, including formin (proteins implicated in mouse limb deformities) and the rat M₄ muscarinic acetylcholine receptor. Proline-rich regions had officially become the target to look for when investigating SH3 ligands (Musacchio et al., 1994a; Musacchio et al., 1994b; Yu et al., 1994). This sequence, now characterized by a more common XPpXP motif has become the focus of the majority of studies into SH3 binding domains (see (Dalgarno et al., 1997) for review).

To date, the majority of studies into SH3 ligands have been made using combinatorial peptide libraries of 10-12 amino acids or less (Cheadle et al., 1994; Rickles et al., 1994; Sparks et al., 1994; Yu et al., 1994; Alexandropoulos et al., 1995; Feng et al., 1995; Rickles et al., 1995; Grabs et al., 1997). These studies demonstrated that SH3 ligands commonly contain PXXP motifs, and thus reinforced the notion of “core sequences”. From this work arose a number of different SH3-ligand co-structures using both NMR and X-ray crystallography. It is now known that the interactions between an SH3 domain and its ligand are mainly hydrophobic in nature and that conserved prolines in a PXXP motif constitute direct contact points (see Dalgarno et al., 1997 for review). In turn, the interaction is further strengthened by hydrogen bonding from side chains. Interestingly, the conformation of the ligand-bound form of Src-SH3 is essentially unchanged from its unbound form (Feng et al., 1994).

The analysis of core ligands led to two important observations. First, SH3 binding domains are pseudo-symmetrical within the context of their proline-rich motifs (Feng et al., 1994). In other words, they may exist in either an N- to C- or a C- to N- orientation, providing that structural constraints (such as residues involved in scaffolding) are retained (see Fig. 4). Second, in each case, crystallographic or solution structure analysis revealed that proline-rich motifs bound in a left-handed helical conformation – now known as a polyproline type II helix.
Put together, this data led to a model under which SH3 binding domains could be described (see Fig. 4):

**SH3 Ligands Can Bind in Either Orientation**

<table>
<thead>
<tr>
<th>Class I (+ orientation)</th>
<th>H₂N-</th>
<th>P₃</th>
<th>P₂</th>
<th>P₁</th>
<th>P₀</th>
<th>P</th>
<th>P₂</th>
<th>P₃</th>
<th>-COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class II (- orientation)</td>
<td>COOH-</td>
<td>R</td>
<td>X</td>
<td>X</td>
<td>P</td>
<td>P</td>
<td>X</td>
<td>P</td>
<td>H₂N</td>
</tr>
</tbody>
</table>

![Fig. 4 SH3 binding peptides](Adopted from Lim et al, 1994). Boldface prolines represent the PXXP consensus. Boxed regions represent the three contact points with the SH3 domain. Class I refers to ligands in the N to C orientation, while class II refers to ligands in the C to N orientation.

First, it is currently thought that SH3 domains may be subdivided into 3 binding pockets (Lim et al., 1994). Since PPII helices contain three residues per turn, residues at positions \( i \) and \( i+3 \) lie on the same side of the helix. Thus, in the case of the class I ligand depicted above, amino acids at positions \( P₃, P₁/P₀ \) and \( P₂/P₃ \) all constitute contact points with the SH3 domain as they are on the same side (see also Fig. 5). Both a PXXP motif and a terminal arginine that binds in the RT loop of SH3 are heavily conserved (Feng et al., 1994). This model takes into account the discovery that ligands may bind in either orientation (Yu et al., 1994) and led to the notion of class I (+ orientation) and class II (- orientation) ligands (Feng et al., 1994). As such, it is possible that secondary structure alone may be more important for ligand recognition than the actual protein sequence. Ligand orientation will determine the orientation of the resulting protein scaffold, with possible implications for signaling (Pawson, 1995).

Src kinase contains the protoypical SH3 domain, and as such is the best model to illustrate an actual SH3-ligand interaction (see Fig. 5) in which ligands may bind in either orientation. Class I ligands of Src have a consensus of RXLPPLP, while class II ligands have a consensus of XPPLPXR (i.e. they are pseudo-symmetrical). Again, contact is made in three distinct binding pockets \( P₃, P₁/P₀ \) and \( P₂/P₃ \) (Lim et al., 1994). In the case of Src, the second (\( P₁ \) and \( P₀ \)) and third (\( P₂ \) and \( P₃ \)) pockets bind leucine-proline pairs through hydrophobic interactions with the aromatic amino acid tyrosine (Y) as well as tryptophan (W) (Feng et al., 1994).
arginine residue in the first binding pocket (P₃) contacts amino acids associated with the RT loop of Src SH3.

**SH3 Ligand Binding to c-Src**

![Diagram of SH3 ligand binding](image)

*Fig. 5 SH3 ligands may bind in either orientation. This diagram is a composite, depicting core binding of proline-rich peptides to the SH3 domain of c-Src [adapted from Dalgarno et al., 1998]. Residues that make contact with the SH3 domain are labeled P₃, P₁, P₀, P₂ and P₃ (Feng et al., 1994). Important contact residues in c-Src are shown in ovals.*

Some progress has been made in describing ligand selectivity for some well-characterized SH3 domains. Using peptides and an *in vitro* system, it has been predicted that Src, Abl and Grb2(N) favour peptides in the class I orientation, while PLCγ and Crk(N) SH3 all favour the class II orientation (Sparks et al., 1996). However, even though Grb2(N) and Src appear to favour class I peptides, their reported endogenous targets contain SH3 binding motifs that match the class II consensus (Li et al., 1993). The fact that endogenous and *in vitro* targets do not bind in the same orientation reinforces the idea that factors other than core regions may contribute to SH3-mediated protein-protein interactions *in vivo*. Such factors could include steric limitations, subcellular localization of SH3 domains and their ligands as well as intra/inter-molecular cooperativity of different binding domains.

The results of the peptide-ligand experiments have repeatedly demonstrated that the same "core" polyproline motifs are capable of binding to many different targets with similar affinities. Typically, core binding is relatively weak (ranging from 5-100 μM) and displays little selectivity.
Such cross-reactivity between SH3 binding domains would make it difficult for any high degree of specificity to be achieved within the cell. As such, it is now being realized that flanking regions not only contribute to binding, but also play an important role in determining ligand selectivity. Some more recent studies have attempted to take this into account and have included flanking sequences within the context of the core when searching for high affinity ligands (e.g. $X_6PXXP_6$ (Sparks et al., 1996)). Specifically, it has been discovered that when additional non-core residues are included, binding affinity may be enhanced by up to 20 fold, progressing into the high nanomolar range (Dalgarno et al., 1997). Additionally, in many proteins, there is not one binding site, but several. This arrangement may function to enhance affinity relative to monovalent ligands (Mayer and Gupta, 1998). As dissociation constants are in the range of the concentrations of potential targets in the cell, it is important to note that binding may be highly susceptible to local concentrations of ligand (Mayer and Gupta, 1998). For example, proteins with restricted distribution, such as membrane-bound receptors, may display proportionately more binding.

Recently, there has been an increasing body of evidence suggesting that although the "core" SH3 binding domains generally contain PXXP motifs which favour PPII helix formation, this no longer seems to be an absolute prerequisite. SH3 binding that does not involve proline-rich sequences, has been reported. The first hint of this was the discovery that both Src and another family member, Hck, adopt the PPII conformation without PXXP motifs (Xu et al., 1997) (Sicheri et al., 1997).

![Structure of c-Src](image)

**Fig. 6** Structure of c-Src (adapted from Schwartzberg 1998). Intramolecular binding between Y$^{227}$ and SH2 partially inactivate the kinase domain. Complete inactivation also requires intramolecular binding of SH3 to the linker region (residues 249-253) of c-Src. The illustration is not drawn to scale.
Early theories about Src kinases held that regulation of the catalytic domain occurred through inter-domain contacts within the protein. Specifically, it was believed that the Src SH2 domain sterically blocked the kinase active site by binding to a phosphorylated tyrosine (Y527) residue in its tail. However, this is only partially true. In fact, the kinase active site remains exposed even while in the SH2 mediated “closed” conformation. Instead, an SH3 interaction is required to completely inactivate the kinase (see Schwartzberg, 1998 #96 for review). Crystal structure analysis has revealed that although the Src kinase linker region does not contain traditional PXXP motifs, it is capable of adopting a PPII conformation and binding Src SH3 in a minus (type II ligand) orientation (Xu et al., 1997). Instead, additional contacts nearby may strengthen the interaction. Because Src can be activated by co-transfection with the Sin (Src interacting protein) SH3 binding domain and tyrosine phosphorylation (Alexandropoulos and Baltimore, 1996), the newly proposed model is that Src recognizes Sin through its SH3 binding site and then phosphorylates it. Next, Src binds phosphorylated Sin via SH2, thereby tying up SH2 and fully activating its kinase domain (Schwartzberg, 1998). The modest affinities that have been reported for SH3 domain interactions thus far could mean that interactions are short lived and susceptible to remodeling in response to availability of local partners (Mayer and Gupta, 1998); thus, explaining the SH3 mediated activation of Src kinase by its binding partner, Sin. The necessity of SH3 for the phosphorylation of some Src substrates has also been demonstrated (Weng et al., 1994).

There are still other examples of non-proline mediated interactions. Another group has reported the preferential association of Bruton’s Tyrosine Kinase (Btk) with a small (30 amino acid) region of a novel protein termed Sab (SH3 domain-binding protein which preferentially associates with Btk) (Matsushita et al., 1998). Notably, the region of interaction contains only a single proline residue. It is likely that this region is also able to adopt the PPII conformation without a proline-rich sequence.

One last example that has recently been reported is particularly striking. The protein p53 is the most frequently mutated gene observed in human cancers. Gorina and Pavletich recently were able to crystallize the p53 tumour suppressor in a form bound to 53BP2. What made this find so interesting was that binding occurred through SH3, but involved neither PXXP motifs nor PPII helices (Gorina and Pavletich, 1996). In this particular case, the L3 loop of p53 forms a rigid structure that is capable of making several contacts with the SH3 domain of 53BP2. The
amino terminal segment of L3 occupies a position similar to P₀/P₁ region of a PXXP ligand, and methionine (M²⁴³) side chains occupy the hydrophobic pocket corresponding to P₀. The structure is further stabilized by intermolecular hydrogen bonding. Additional contacts, not seen in other SH3-ligand interactions, are also made. Perhaps even more interesting is the Arg²⁴⁸ contact of p53 with Asp⁴⁷⁵ and Glu⁴⁷⁶ of 53BP2 (corresponding to the RT loop of the SH3 domain). The significance of this interaction is made clear when it is noted that the most common point mutation seen in p53 is this Arg²⁴⁸ residue (Gorina and Pavletich, 1996). It is possible that p53 also loses its anti-oncogenic effect when its SH3 binding specificity is reduced. Despite this novel binding, p53BP2 retains its ability to isolate traditional ligands with PXXP motifs from a phage display library (Sparks et al., 1996).

Clearly, the role that SH3-SH3BD play in the cell is much more diverse than was once thought. SH3 is involved not only in the establishment of signaling networks, but in the regulation of such networks as well. In addition, in the future, it will be necessary to expand our search for binding partners beyond ligands containing traditional PXXP motifs to other proteins where the requirements of conformation and proximity to binding partners are also met. Inevitably, this will lead to a much better understanding of the mechanisms that regulate protein-protein interactions.

1.5 WW Domains and Proline-Rich Motifs

Perhaps not surprisingly, there is degeneracy among domains that mediate protein-protein interactions (Sudol, 1996b). In particular, poly-proline motifs are recognized by another distinct protein module, the WW domain. This module is smaller than SH3 (less than 40 amino acids in length) and is so named because of the presence of two highly conserved tryptophan (W) residues that are spaced 20-22 amino acids apart (see (Sudol, 1996a) for review). The WW domain is relatively new to the group of modules that are known to bind proline-rich ligands. It was originally identified in the Yap (Yes-associated protein) (Sudol, 1994); however, two other groups independently isolated the same domain under different names: the WWP motif (Andre and Springael, 1994) and the Rsp5-repeat (Hofmann and Bucher, 1995). The WW domain is not merely smaller than SH3, but it appears to be evolutionarily older (Sudol et al., 1995) and structurally distinct (Macias et al., 1996).
By using functional screens, the WW binding region of Yap was isolated to a proline-rich region containing the consensus (xPPxY) (Chen and Sudol, 1995). More recent work has recognized that there are two distinct classes of WW domains. Class I domains bind the consensus outlined above, whereas class II domains bind an identical core to SH3 domains: PXXP (Bedford et al., 1997). Some WW and SH3 domains may even share overlapping targets. This was suggested by a group studying formins. It was already known that formins contained poly-proline sequences and were capable of interacting with SH3 (Ren et al., 1993). Screening of an expression library with the formin poly-proline SH3 binding motif revealed two sets of formin binding proteins (FBPs). As expected, one set contained SH3 domains, but the other set contained WW domains (Chan et al., 1996). The implication is clear: different classes of protein domains may compete for the same ligands. In such a manner, WW domains could regulate SH3 binding by directly competing for the same proline-rich sequences (Sudol, 1996b). However, much of this work is still in an early phase and still needs to be further explored.

1.6 SH3 Binding Domains in GPCRs

Close examination of the third cytoplasmic loop in the dopamine D₄ receptor, revealed that the region is extremely proline-rich, and contains many overlapping regions with the minimal PXXP motif for SH3 binding. The identification of several putative SH3 binding domains in D₄ led to a scan of other GPCRs, where it was noted that many of these receptors also contained potential SH3 binding motifs in either their third cytoplasmic loop or C-terminal tail or both. In fact, PXXP motifs appear in several types of GPCRs including the α- and β-adrenergic, muscarinic and dopamine receptors. The incidence of these sequences in either the third cytoplasmic loop, or C-terminal tail (where they could make functional intracellular contacts), is ten-fold higher than would be expected from random amino acid distribution. The idea that at least select GPCRs could serve as targets for SH3 binding has been proposed at least once before (Ren et al., 1993), but the idea has never been fully explored experimentally.

Our group was the first to characterize SH3 binding in the dopamine D₄ receptor. In particular, it was discovered that this receptor can bind to a number of SH3 containing proteins including the adapter proteins Grb2 and Nck through its third loop (Oldenhof et al., 1998). Not all of the PXXP motifs are necessary for this interaction. In fact, the variable number of tandem repeats (VNTR) region of the loop does not seem to be involved in binding. Interestingly, by
itself the loop binds Grb2 with an affinity of 10 μM. This is in a range consistent with other known high affinity ligands (Pawson, 1995). The fact that the loop by itself binds with higher affinity than the full-length receptor is potentially of interest. As mentioned earlier, agonist induced conformational changes may enhance accessibility to the third cytoplasmic loop.

**SH3 Interactions with the Dopamine D4 Receptor**

![SH3 Interactions with the Dopamine D4 Receptor](image)

**Fig. 7** *In vitro* SH3 Interactions in the dopamine D₄ receptor. The top panel shows the relative amounts of GST fusion protein (Brilliant blue stain) used to precipitate the *in vitro* labeled GPCRs. Molecular masses are given in kilodaltons. Data on D₄.4 (220-236) used with permission.
1.7 Research Objectives

Close analysis of the human dopamine D_{3} receptor reveals that there are two potential SH3 binding domains (PXXP motifs) contained in the third cytoplasmic loop (see Fig. 8). The characterization of SH3 binding domains in the dopamine receptors could have profound implications for other catecholamine binding receptors and GPCRs in general. Specifically, SH3 may serve an important, albeit unappreciated, role in vivo through signaling cascades, recruitment of proteins to the receptor complex, regulation of G protein coupling, desensitization, receptor processing in the endoplasmic reticulum or some other process. The primary goal of this study was thus to establish paradigms for SH3 binding in the dopamine D_{3} receptor in vitro.

Hypothesis: There are functionally important SH3 binding sites in the dopamine D_{3} receptor.

(1) The first objective of this thesis was to determine whether the dopamine D_{3} receptor is capable of interacting with SH3 domain containing proteins in vitro. Over the past two years, several new signaling pathways for GPCRs have begun to emerge. Among the most recent was the discovery that the dopamine D_{4} receptor contains SH3 binding domains (Oldenhof et al., 1998). Establishing that SH3 binding can occur in the D_{3} receptor would help to focus research toward another novel effector system for these receptors.

(2) The second objective of this thesis was to investigate other GPCRs that contain PXXP motifs (specifically, the β_{1} adrenergic and the M_{4} muscarinic receptors) to determine whether binding to SH3 domains is limited to the D_{2}-like family or a phenomenon of GPCRs in general.

(3) The third objective of this study was to investigate which regions of the D_{3} receptor are important for binding, including the role of PXXP motifs in the third cytoplasmic loop. Traditional SH3 binding has demonstrated that high affinity contact often occurs through proline-rich regions. Some recent work has demonstrated that endogenous SH3 ligands may not be proline-rich at all. However, SH3 binding in the D_{4} receptor was narrowed down to two proline-rich regions in the N- and C-terminal regions of the third cytoplasmic loop (Oldenhof et al., 1998).
### GPCR Alignment

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<th>D3</th>
<th>D4.4</th>
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</table>

**Fig. 8.** Primary amino acid alignment of the third cytoplasmic loop and C-terminal tails of the dopamine receptors and β-adrenergic and M₄ muscarinic acetylcholine receptors. Their potential SH3 binding motifs are boxed. The VNTR region of D₄₄ is also highlighted. The PXXP motifs in the VNTR region of D₄₄ have been ruled out as targets for SH3 binding (see Oldenhof et al., 1998).
2.0 MATERIALS

2.1 Chemicals and reagents

All chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO) or B.D.H. Chemicals Ltd. (Toronto, ON). Bacto Tryptone, agar and yeast extract were purchased from DIFCO (Detroit, MI). Agarose was purchased from FMC Bioproducts (Rockland, ME). Chloroform, and potassium phosphate were obtained from Mallinckrodt Chemicals Ltd. (St. Louis, MO). Tris-HCl and sodium dodecyl sulphate (SDS) were purchased from ICN (Aurora, OH). Adenosine triphosphate was purchased from Boehringer Mannheim (Laval, PQ), while dNTPs (dTTP, dCTP, dGTP and dTTP) were purchased from Pharmacia (Baie d'Urfe, PQ). Ethidium bromide and Hepes were purchased from Gibco (Burlington, ON). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Molecular Probes, Inc. (Eugene, OR). Potassium acetate and magnesium acetate were purchased from J.T. Baker Chemicals (Toronto, ON). Ethanol and methanol were purchased from Commercial Alcohols, Inc. (Brampton, ON). Anhydrous acetone was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI).

Materials required for small scale (mini) and large-scale ( maxi) plasmid preparation were obtained from Qiagen (Chatsworth, CA). Reagents for protein quantitation were purchased from Bio-Rad (Mississauga, ON). Oligonucleotide primers used for PCR and site-directed mutagenesis were synthesized by Gibco BRL (Burlington, ON). Glutathione Sepharose 4B beads were purchased from Pharmacia Biotech (Baie d'Urfe, PQ). Novex 8-16% Tris-glycine gels were purchased from Helixx Technologies (Scarborough, ON). Genistein was purchased from RBI (Sigma-Aldrich, Oakville, ON). Drugs for pharmacological or functional assays were obtained as follows: haloperidol from Janssen Pharmaceutical (Beerse, Belgium), YM-09151-2 nemonapride from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Guanilylimidophosphate (Gpp[NH]p) and dopamine were purchased from Sigma Chemical Co. (St. Louis, MO). LY171555 [(-)-quinpirole] was purchased from Research Biochemicals Inc. (Natick, MA). Raclopride was purchased from Astra Arcus AB (Sodertalje, Sweden).

The Chameleon™ Double Stranded, Site-Directed Mutagenesis Kit was purchased from
Stratagene (La Jolla, CA). The TNT® Coupled Reticulocyte Lysate System and the pTarget™ Mammalian Expression Vector System were purchased from Promega (Madison, WI). The GST Gene Fusion System was purchased from Pharmacia Biotech (Baie d'Urfe, PQ). The BCA Protein Assay Reagent Kit was purchased from Pierce (Rockford, IL).

2.2 Isotopes

[N-methyl-\textsuperscript{3}H]YM-09151-2 nemonapride (specific activity 85.5 Ci/mmol) and [\textgreek{a}-\textsuperscript{35}S]dATP (specific activity 1250 Ci/mmol) were purchased from NEN(Boston, MA). [\textsuperscript{35}S]L-cysteine (specific activity 1075 Ci/mmol) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA).

2.3 Enzymes

Restriction endonucleases were purchased from New England Biolabs (NEB) (Beverly, MA), Gibco BRL Ltd. (Grand Island, NY), Pharmacia (Baie d'Urfe, PQ) or Boehringer Mannheim (Laval, PQ). Trypsin, RNase, AmpliTaq DNA Polymerase, T4 polynucleotide kinase were purchased from Gibco BRL Ltd. (Grand Island, NY). TNT T7 RNA polymerase and RNasin were purchased from Promega (Madison, WI).

2.4 Bacterial and mammalian cell culture

Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD). The bacterial strain Escherichia Coli XL-1 was purchased from Stratagene (La Jolla, CA). Fetal bovine serum, horse serum, Geneticin™ (G418) and alpha-minimal essential media (\textalpha-MEM) were obtained from Gibco BRL Ltd. (Grand Island, NY) and Central Media Preparation Service (University of Toronto, ON). All tissue culture supplies were obtained from Nunc (Roskilde, Denmark), Dupont (Mississauga, ON), Corning (Corning, NY) or Falcon (Beckton Dickinson Labware, Lincoln Park, NJ)
2.5 Bacterial strains and cloning vectors

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<tr>
<th>Bacterial Strains</th>
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<td>E. Coli XL-1</td>
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<td>JM109</td>
<td>Promega (Madison, WI)</td>
<td>General use</td>
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<td>Growth of GST-FP (protease deficient strain)</td>
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<td>Site-directed mutagenesis (repair deficient strain)</td>
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<td>Pharmacia (Baie d'Urfe, PQ)</td>
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<tr>
<td>pRC/RSV</td>
<td>Invitrogen (Carlsbad, CA)</td>
<td>Mammalian expression</td>
</tr>
</tbody>
</table>

Table 1. Bacterial strains and cloning vectors used in this study.

2.6 cDNAs

The dopamine D₃ receptor was obtained from Dr. J.-C. Schwartz and Dr. P. Sokoloff (Unité de Neurobiologie et Pharmacologie de l’INSERM, Paris, France). The M₄ muscarinic acetylcholine receptor cDNA was obtained from Dr. Tom Bonner (National Institute of Health, Bethesda, MD). The β₁-adrenergic receptor was obtained from Dr. M. Caron (Duke University Medical Centre, Durham, NC). SH3 domain containing cDNAs were a kind gift of Mordechai Anafi and Tony Pawson (Mount Sinai Hospital, Toronto, ON).
3.0 METHODS

3.1 Plasmid DNA Amplification using the *E. Coli* XL-1 strain of bacteria

(a) Competent *E. Coli* XL-1 bacterial cell preparation

A glycerol stock of *E. Coli* XL-1 bacterial cells was scraped with a sterile inoculation needle and used to inoculate 4ml of LB media containing 10µg/ml tetracycline (1% (w/v) yeast extract, 0.5% (w/v) bacto tryptone and 1% NaCl in double-distilled water (ddH2O); sterilized) in a 15ml Falcon tube. After growth overnight at 37°C in a shaking incubator, cells were transferred to 76 ml of LB media in a 250 ml erlenmyer flask and grown for an additional 1.5 hours. The culture was then poured into two 50ml Nunc tubes, chilled on ice for 10 minutes and centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellets were combined and resuspended in 20 ml potassium 2-[N-morpholino] ethanesulfonic acid (KMES) buffer (60 mM CaCl₂, 5 mM MnCl₂ and 20 mM KMES, pH 5.8). The tube was chilled on ice for 1-1.5 hours, then spun again at 2000rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 4 ml KMES buffer and stored at 4°C.

(b) Transformation of *E. Coli* XL-1 bacteria

Several wild type and mutant G protein-coupled receptor (GPCR) constructs were subcloned into the bacterial phagemid expression vector pBluescript SK (+/-) (Stratagene, La Jolla, Ca) and amplified in *E. Coli* XL-1 bacteria. To transform *E. Coli* XL-1 bacteria, competent XL-1 cells (75-100 µl) were combined with plasmid DNA (50 ng) in an eppendorf and chilled on ice for 45 minutes. The mixture was then heat shocked in a 42°C water bath for 1 minute and again chilled on ice for a further 2 minutes. The transformed cells were then pipetted onto 100 mm LB agar plates containing antibiotic for selection (e.g. plates which contain LB media with 1.2% (w/v) agar and 25 µg/ml ampicillin or other antibiotic at the appropriate concentration) and spread evenly with a sterilized glass spreader. The plates were then incubated for 16 hours at 37°C. Only bacterial colonies that had been transformed by the plasmid would grow on the selection medium. Bacterial colonies that contained the recombinant plasmid DNA were identified by performing diagnostic
restriction enzyme analysis on the purified plasmid. Glycerol stocks (850 μl of bacterial culture with 150 μl glycerol) were made of the selected clones and stored at -80°C.

(c) Small-scale plasmid DNA purification (minipreparation)

This procedure was used to obtain up to 20 μg of high-copy plasmid DNA. Colonies were selected from LB agar plates using a sterilized inoculation needle and added to 4 ml of LB with ampicillin or other appropriate antibiotic. This culture was allowed to grow overnight at 37°C with vigorous shaking. The following morning, the bacterial cells were collected by centrifugation for 1 minute at 14,000 rpm and the supernatant discarded. DNA was isolated using the QIAprep Spin Miniprep protocol from QIAGEN (Chatsworth, CA). This technology is based on silica-gel anion exchange resin. The isolated bacterial cell pellet was then resuspended in 250 μl of Buffer P1 (100 μg/ml RNase A, 50mM Tris-HCL, 10mM EDTA (pH 8.0)) to lyse the cells and the cellular proteins denatured by adding 250 μl of Buffer P2 (200mM NaOH, 1% SDS) and mixing gently. Next, 350 μl of Buffer N3 (neutralization buffer with chaotropic salt) was added and the tubes mixed gently. Cellular debris was removed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was then loaded onto a QIAprep column and again centrifuged for 1 minute at 14,000 rpm. The efluent was discarded and the column washed with 500 μl of wash buffer PB. Again, efluent was removed by centrifugation at 14,000 rpm for 1 minute. A final wash was then performed with 750 μl of buffer PE that was again discarded after centrifugation for 1 minute at 14,000 rpm. Finally, the columns were eluted with 50 μl of elution buffer (EB) (10mM Tris-Cl (pH 8.5)). After letting stand for 1 minute to enhance dissociation of the DNA, the columns were eluted by centrifugation at 14,000 rpm for one minute.

(d) Large-scale plasmid DNA purification (maxipreparation)

In order to obtain large quantities of very pure plasmid DNA, large-scale plasmid preparation was performed on constructs using a QIAGEN plasmid preparation kit (Chatsworth, CA). The QIAGEN resin contains an anion-exchange resin that is based on a unique surface modification of

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1 Components of buffer N3 and wash buffer PB are proprietary information of QIAGEN (Chatsworth, CA)

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An inoculation for each construct was made in 500 ml LB-Amp. Flasks were then incubated for 16 hours with vigorous shaking at 37°C. The next day, bacterial cells were harvested by centrifugation at 4°C for 15 minutes at 2,600 x g. The bacterial pellet was resuspended in 10ml of buffer P1 (100μg/ml RNase A, 50mM Tris-HCL, 10mM EDTA (pH 8.0)), lysed and the cellular proteins denatured by the addition of 10 ml buffer P2 (200mM NaOH, 1% SDS) and the tubes mixed gently and incubated at room temperature for 5 minutes. Next, 10 ml of buffer P3 (3.0M KAc (pH 5.5) was added to precipitate denatured proteins, chromosomal DNA, cellular debris and SDS. Tubes were mixed gently and placed on ice for 20 minutes. The precipitated debris was removed by centrifugation at 16,000 x g for 30 minutes. The clear supernatant was loaded onto a QIAGEN-tip which was pre-equilibrated by applying 10 ml equilibration buffer (750 mM NaCl, 50mM MOPS (3-N-morpholino-propanesulfonic acid, (pKa 7.2), 15% ethanol, 15% (v/v) Triton X-100) to the QIAGEN resin. The salt and pH conditions in the lysate and the selectivity of the resin ensure that only the DNA will bind, while degraded RNA and cellular proteins flow through the column. The QIAGEN-tip was then washed with 60ml of wash buffer (1.0M NaCl, 50 mM MOPS, 15% ethanol) which ensures complete removal of any contaminants and disrupts any non-specific interactions. The DNA was then eluted from the column with elution buffer (1.25 M NaCl, 50 mM Tris-HCl, 1 mM EDTA (pH 8.5)). The eluted plasmid DNA was then precipitated in ice cold 100% ethanol for 20 minutes and then centrifuged at 16,000 x g at 4°C for 20 minutes. The ethanol was poured off and the DNA pellet was air-dried and resuspended in 0.5ml ddH2O. The concentration of the DNA was determined by reading the optical density (OD260) of 2 μl of DNA (1:100) dilution in a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The DNA was then diluted to a concentration of 1.0 μg/μl.

(e) Restriction digests and gel electrophoresis

For restriction digests, restriction endonuclease enzymes (1 to 5 units/μg DNA) were combined with plasmid DNA (approximately 0.5 μg) in a final volume of 20 μl. In addition, 2 μl of 10x buffer was added as recommended by the supplier. Digestions were carried out for one hour at the recommended temperature for the enzyme.
Samples containing restriction digested DNA fragments were combined with 1-2 µl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in ddH2O). The samples were then loaded into wells of an agarose gel submerged in TAE buffer (40 mM Tris, 0.1% (v/v) acetic acid and 1 mM EDTA) and separated by electrophoresis. Electrophoretic mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs. Thus, the fragments are separated by size, with the smallest fragments migrating farthest. By staining the gel with ethidium bromide, these fragments can be made to fluoresce using an UV transilluminator (Bio/Canada Scientific FOTO/UV 26, Mississauga, ON).

(f) DNA sequencing by the Sanger method

The dideoxy chain termination method (also known as the Sanger method), using 7-deaza-GTP and Sequenase version 2.0 was used to determine the base sequence of all constructs. With this technique, 200-250 base pairs can be reliably read from the primer used in the sequencing reaction.

To generate single stranded template for sequencing, 1-2 µg of DNA was denatured by treatment with 2 µl of 2 N NaOH and 2 µl of 2 mM EDTA (pH 8.0) in a total volume of 20 µl. This reaction was incubated for 4 minutes at room temperature, transferred to 37°C for 1 minute and then placed on ice. The DNA was next precipitated by the addition of 10 µl of 1M NaAc (pH 5.2) and 78 µl of ice cold 100% ethanol and allowed to incubate at -20°C for 30 minutes. The DNA was then pelleted by centrifugation at 14,000 rpm for 10 minutes, washed with 70% ethanol and lyophilized.

For each template, a single annealing reaction was used. The dry DNA was dissolved in 5 µl (10 µg/µl) of the appropriate primer (see table 2), immediately followed by 3 µl of ddH2O and 2 µl of the 5x reaction buffer (200 mM Tris-HCl (pH 7.5), 100mM MgCl2 and 250 mM NaCl). The annealing reactions were allowed to proceed for 20 minutes at 37°C.

Four labeling reactions were then performed for each template to be sequenced by adding 1 µl of 0.1% DTT, 2 µl of labeling mix (1.5 µM dCTP, 1.5 µM dGTP, 1.5 µM dTTP) and 1.0 µl of [α-35S]dATP specific activity 1250 Ci/mmol, and 3 U of Sequenase version 2.0 in a total volume of 15 µl. This reaction was mixed thoroughly and allowed to proceed for 5 minutes at room temperature.

The labeling/extension reaction was followed by a termination step in which 3.5 µl of the
labeling reaction was transferred to one of four tubes, each containing 2.5 μl of the 8 μM dideoxy-nucleotides (ddGTP, ddATP, ddCTP and ddTTP). These reactions were incubated for a further 5 minutes at 37°C, when 4 μl of the stop solution was added to each tube (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). Reactions were then stored at -20°C prior to loading on a polyacrylamide gel.

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<th>Primers used for DNA Sequencing</th>
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</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>5'-TAATACGACTCACTATAGGG-3'</td>
</tr>
<tr>
<td>T3</td>
<td>5'-AATTAACCCCTCACTAAAGGG-3'</td>
</tr>
<tr>
<td>PGEXforward</td>
<td>5'-GCATGGCCTTTGCAGGG-3'</td>
</tr>
<tr>
<td>βRK1SEQR</td>
<td>5'-GCAACCCTATAGACTGTAGTC-3'</td>
</tr>
<tr>
<td>D3TM5F</td>
<td>5'-CTACCTGCCCCTGAGTACTGTCTTG-3'</td>
</tr>
<tr>
<td>D3TM6R</td>
<td>5'-CAGCAGACAATGAAGGCCCCCAAGCACAATG-3'</td>
</tr>
<tr>
<td>D3HSEQR</td>
<td>5'-CTGGCTGGACCTGTGGAGTTCTCTG-3'</td>
</tr>
<tr>
<td>PTARGREV</td>
<td>5'-ACTCAAGCTTGGAATTCG-3'</td>
</tr>
</tbody>
</table>

Table 2. Oligonucleotide primers used for DNA sequencing.

(g) High resolution gel electrophoresis

An 8% polyacrylamide gel was made with 8% acrylamide, 0.27% bis-acrylamide, 41% 8M urea, 1x TBE buffer, 0.54 ml ammonium persulfate. Additionally, 60 μl/100ml TEMED (N, N, N', N'-tetramethyl-1,2-diaminoethane) was added prior to pouring the gel to catalyze polymerization.
The gel was then left overnight at room temperature to polymerize. The following day, sequencing samples were denatured at 90°C for 3 minutes and placed on ice (4°C) until loaded on the gel. This method was able to resolve DNA strands differing in length by only a single nucleotide. Samples were loaded in the order G, A, T, C and run for approximately 5 hours at 50W. After this time, the gel was soaked in 10% acetic acid and 12% methanol for one half hour to remove excess urea. The gel was then transferred to a Whatman filter paper and dried in a vacuum drier at 90°C for one hour. The gel was then placed under autoradiographic film (BIOMAX™, Eastman Kodak Co., Rochester, NY) and placed in a cassette overnight.

(h) The Polymerase chain reaction

The polymerase chain reaction (PCR) was used to generate many of the constructs used in this study (see table 3). In brief, the PCR requires several cycles including an initial denaturation phase (D) carried out at 95°C, an annealing phase (A) whose temperature is dependent on the primers being used and an elongation phase (E) carried out at 72°C. The sequence is then repeated and the template is elongated according to the relation 2^n for between 30-40 cycles. Finally, a 4 minute elongation step completes the amplification process. PCR amplifications were carried out in 10 μl reaction volumes, using 1μl (50ng) cDNA template, 1μl buffer (500mM Tris-Cl (pH 8.3), 2.5 mM BSA, 30 mM MgCl₂, and 5% Ficoll 400), 1 μl 2mM dNTP, 0.5 μl each of sense and anti-sense oligonucleotide primers and AmpliTaq DNA polymerase (0.5 U). Prior to thermal cycling, reactions were sealed in capillary tubes (Idaho Technologies, Idaho Falls, ID). All PCR reactions were carried out using an Idaho Technologies Air Thermo-Cycler (Idaho Falls, ID). The samples were then isolated on a 1.0% agarose gel stained with ethidium bromide.

To facilitate subcloning of PCR fragments, the pTarget™ vector (Promega, Madison, WI) was used in select cases. This is a linearized vector with a single thymidine base overhanging at both the 5' and 3' termini. PCR using only AmpliTaq polymerase has the property of leaving a single adenosine base overhang on amplified products. Thus, PCR fragments may be easily subcloned into the pTarget™ vector.

35
### Oligonucleotide Primers and Conditions Used for PCR

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
</table>
| **D3HAT-epitope** | D(95°C); A(48°C); E(72°C); 3 cycles  
|               | D(95°C); A(60°C); E(72°C); 20 cycles          |
| Sense         | 5'-GGGGTACCCCAAGCTTGAGCTGCTGGCCCG-3'           |
| Anti-sense    | 5'-GGGGTACCCGAGATCCACCCGGAACCAGACATGGCATATCAGGCTGTAAGG-3' |
| **D3Loop**    | D(95°C)10s; A(50°C)3s; E(72°C)5s; 40 cycles   |
| Sense         | 5'-GGAGCTCGAGCCATGGGCTATGCTATGGCAGATCTAGTGGTGCTG-3' |
| Anti-sense    | 5'-GTCTCTAGATCGCACACCATTGGTTGTCG-3'           |
| **D3N-Loop**  | D(95°C)10s; A(50°C)3s; E(72°C)5s; 35 cycles   |
| Sense         | 5'-GGAGCTCGAGCCATGGGCTATGCTATGGCAGATCTAGTGGTGCTG-3' |
| Anti-sense    | 5'-GTCTCTAGATCGCACACCATTGGTTGTCG-3'           |
| **D3C-Loop**  | D(95°C)10s; A(50°C)3s; E(72°C)5s; 35 cycles   |
| Sense         | 5'-GGAGCTCGAGCCATGGGCTATGCTATGGCAGATCTAGTGGTGCTG-3' |
| Anti-sense    | 5'-GTCTCTAGATCGCACACCATTGGTTGTCG-3'           |
| **M**         | D(95°C)10s; A(55°C)3s; E(72°C)20s; 40 cycles  |
| Sense         | 5'-GAGGAAATTCCAGACACGGCCCTACCCAC-3'           |
| Anti-sense    | 5'-CTTGGATCTAGGGCATCTGCTGCT-3'                |
| **GRK2**      | D(95°C)10s; A(52°C)3s; E(72°C)8s; 40 cycles   |
| Sense         | 5'-GTCTGGATCTGCGGTGACGACATG-3'                |
| Anti-sense    | 5'-CAGCGAATTCCAGACAGGAGGGAGGACTC-3'          |
| **GRK3**      | D(95°C)10s; A(52°C)3s; E(72°C)8s; 40 cycles   |
| Sense         | 5'-CTCTGGATCTGCGGTGACGACATG-3'                |
| Anti-sense    | 5'-CGTGCGAATTCCAGACAGGAGGGAGGACTC-3'         |

Table 3. Oligonucleotide primers and reaction conditions used for PCR. Engineered restriction sites used for cloning are in boldface and are underlined (see text for explanation).
The D₃ receptor constructs D3Loop, D3N-Loop and D3C-Loop were engineered to contain a unique Xho I restriction site at the 5’ terminus and a unique Xba I site at the 3’ terminus of the DNA product. These sites were subsequently used to subclone the PCR product into the pBluescript vector in an orientation that would render the construct under the control of the T7 promotor to facilitate translation. The initiation sequence of all D₃ constructs was also engineered to match the “Kozak consensus” that is required for efficient translation (see section 3.3 (b)).

The M₄ construct was similarly engineered to contain a unique Eco R1 restriction site at the 5’ end and a unique Bam H1 site at the 3’ end. These sites were again used to subclone the PCR product into the pBluescript vector in an orientation that would render the construct under the control of the T7 promotor to facilitate translation. This was necessary as the original M₄ cDNA obtained from Dr. T. Bonner contained extensive 5’ and 3’ untranslated regions (UTR) that led to lower efficiency of translation.

The GRK2 (303-425) and GRK3 (305-426) constructs were engineered to contain a unique Bam H1 site at the 5’ terminus and a unique Eco R1 site at the 3’ terminus. These sites were subsequently used to subclone the PCR products in frame into the vector pGEX-3X, thereby generating a GST-fusion proteins of the putative SH3 domain of these proteins.
The HAT-epitope Tagged D3 Receptor

Fig. 9 The HA epitope-tagged D3 receptor with hemaglutinin signal sequence and thrombin cleavage site (D3HAT). The signal sequence is removed when the receptor is expressed in mammalian cell lines. Cleavage sites are shown with full arrows. The Kpn I restriction sites used to subclone the PCR fragment are also depicted. For in vitro translation, labeled receptor may be generated using the T7 promoter.

The HA epitope with signal sequence and thrombin cleavage site (HAT-tag) was engineered to contain Kpn I restriction sites at its 5’ and 3’ termini. These sites were used to subclone the epitope tag onto the 5’ end of the D3 cDNA in the pBluescript construct (see Fig. 9). The PCR template used was the hemaglutinin-signal sequence. Directionality of the construct was verified with a restriction digest. Note that upon expression, the signal sequence is cleaved leaving the HA-epitope exposed at the N-terminus of the receptor on the extracellular side of the membrane.

The integrity of all constructs was confirmed by di-deoxy nucleoside sequencing.
3.2 Site-directed Mutagenesis of Dopamine D3 Receptor cDNAs

Site-directed mutagenesis was performed using the Chameleon™ Double-Stranded Site-Directed Mutagenesis Kit available from Stratagene (La Jolla, CA) (see Fig. 10). This protocol was carried out over a four-day period. In a single eppendorf tube, 0.25 pmol of HAT tagged D3 dopamine receptor cDNA was combined with 300 ng (25 pmol) of the Kpn I selection primer, and 300 ng (25 pmol) of the appropriate mutagenic primer. In addition, 2 µl of 10x mutagenesis buffer (100 mM Tris-acetate (pH 7.5), 100 mM MgOAc and 500 mM KOAc (pH 7.5)) and ddH2O was added to a final volume of 20 µl. To anneal the primers to denatured DNA, the eppendorf was then placed on a heat block at 100°C for 5 minutes immediately transferred to ice (4°C) for 5 minutes and then allowed to anneal at room temperature for a further 30 minutes (Fig. 10 (B)).

Primer extension was then initiated by adding 7 µl of the nucleotide mix (2.86 mM of each of dATP, dCTP, dGTP and dTTP, 4.34 mM rATP and 1.43x mutagenesis buffer), and 3 µl of fresh 1:10 enzyme dilution [1 µl of 10x enzyme mix (0.25U/μl native T7 polymerase, 1U/μl of T4 DNA ligase and 0.6 μg/μl single-stranded binding protein) and 9 µl of enzyme dilution buffer (20 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM β-mercaptoethanol, 1mM DTT, 0.1 mM EDTA and 50% (v/v) glycerol)]. This resulted in a total reaction volume of 30 µl for primer extension. The eppendorf tube was then allowed to incubate at 37°C for one hour. Following primer extension, the T4 DNA ligase was inactivated by incubating the mixture at 80°C for 15 minutes and then allowing it to cool to room temperature for a further 15 minutes (Fig. 10 (C)).

Selection for mutagenized DNA was made by adding 1 µl (20U) of the Kpn I restriction enzyme and adding 29 µl of ddH2O (now at a final volume of 60 µl) and allowing the mixture to incubate at 37°C for 3 hours. 6 µl of the DNA was then transformed into XLmutS competent cells (a bacterial strain that is mismatch repair deficient) (see table 1), added to 4 ml LB media and allowed to grow overnight at 37°C with vigorous shaking (Fig. 10 (D)).

The following day, a mini-preparation of the plasmid DNA was performed using the procedure described (see section 3.1 (c)). This DNA was enriched for the mutant plasmid by performing an additional restriction digest with the Kpn I enzyme for a further 7 hours at 37°C (see
section 3.1 (e)). Following the restriction digest (Fig 9 (F)), DNA was transformed into E. Coli XL-1 cells and plated onto LB agar plates with ampicillin selection to prevent contamination.

**Site-Directed Mutagenesis Protocol**

(A) 

(B) 

(D) 

(C) 

(E) 

(F) 

Key: Plasmid D3 cDNA Site-directed mutant

Fig. 10 Site-directed mutagenesis of the dopamine D3 receptor. Mutagenesis of the D3 receptor was performed using the Chameleon\textsuperscript{TM} Double-Stranded Site-Directed Mutagenesis Kit (Stratagene). See text for a detailed explanation.

On the third day, colonies were selected for analysis by restriction digestion and DNA
sequencing by the Sanger method (see section 3.1 (f)). Those colonies that contained mutated D3 cDNA were kept for in vitro experimental analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3mute 1</td>
<td>5’-pGTC AGG CCT GGC TTC CTC CAA CAA ACC CTC-3’</td>
<td>Primer used to mutate generate Pro(236)→Leu(236) point mutation</td>
</tr>
<tr>
<td>D3mute 2</td>
<td>5’-pCT TG AAG CTG GGG CTC CTG CAA CCT CG-3’</td>
<td>Primer used to mutate generate Pro(317)→Leu(317) point mutation</td>
</tr>
<tr>
<td>Kpn I select</td>
<td>5’-pCTA TAG GGC GAA TTG GGT GCC CGG GCC CCC CTC GAG GTC G-3’</td>
<td>Selection primer used to knock out Kpn I cleavage site in the pBluescript MCS</td>
</tr>
</tbody>
</table>

Table 4. Oligonucleotide primers used for site directed mutagenesis (P→L). Targeted residues are in boldface and are underlined.

3.3 *In Vitro* Interaction of GPCR with SH3 containing GST Fusion Proteins

(a) Preparation of Glutathione Sepharose 4B Matrix

The Glutathione Sepharose 4B was purchased from Pharmacia Biotech (Baie d’Urfé, PQ). First, the matrix was resuspended by gentle agitation. In order to prepare a 50% slurry, 1.33 ml of the original Glutathione Sepharose 4B was dispensed per ml of bed volume required. (1 ml of bed volume will bind approximately 5 mg of glutathione-S-transferase (GST). The matrix was then sedimented by centrifugation at 500 x g for 5 minutes and the supernatant discarded. The matrix was then washed with by adding 10 ml of ice cold (4°C) PBS per 1.33 ml of the original Glutathione Sepharose 4B dispensed and mixing gently. Again, the mixture was sedimented by centrifugation at 500 x g for 5 minutes and the supernatant discarded. To create a 50% solution, 1 ml of PBS was added for each original 1.33 ml volume.
(b) *In vitro* translation of GPCR constructs

Using the TNT® Coupled Reticulocyte Lysate System available from Promega (Madison, WI), it was possible to generate labeled GPCR proteins. In a single eppendorf tube, the following reagents were combined: 25 μl TNT® Rabbit Reticulocyte Lysate, 2μl TNT® Reaction Buffer, 1 μl TNT® T7 RNA Polymerase, 1 μl Amino Acid Mixture Minus Cysteine, 4 μl [35S]L-cysteine specific activity 1075 Ci/mmol, 1 μl RNasin® Ribonuclease Inhibitor (40U/μl), 1 μl cDNA in appropriate expression vector (1.0 μg/μl) and 16 μl ddH2O (see table 6). The reaction was then allowed to proceed for 2 hours at 30°C. Quantification of the *in vitro* translated product was then made using the protocol outlined below (see section 3.3 (c)). Also, a small amount of the reaction product was visualized by SDS-PAGE (see Fig. 11) as outlined below (see section 3.3 (f)).

*In Vitro Translation*

![In Vitro Translation Diagram]

**Fig. 11.** *In vitro* translated, [35S]-labeled GPCRs. Signal intensity is a direct function of the number of cysteine residues in the protein. Molecular masses are given in kilodaltons.
In order to obtain maximum efficiency of the translation reaction, the cDNAs used required an initiation codon that matched the "Kozak consensus" (A/GCCAU
gG). Also required was for the gene to be under the control of the T7 promoter. All constructs used were in the pBluescript (Stratagene, La Jolla, CA) vector and met these requirements.

(c) Quantification of in vitro translated product

After the translation reaction had been completed, 2 μl of the reaction mixture was combined with 98 μl of 1M NaOH/2% H2O2, vortexed briefly and incubated at 37°C for 10 minutes. At the end of the incubation, 900 μl of ice-cold 25% TCA/2% casein enzymatic hydrolysate was added to precipitate the translation product. This mixture was incubated at 4°C for a further 30 minutes. Next, 250 μl of the precipitated translation product was bound to Fisherbrand G6 glass fiber filter circles (Pittsburgh, PA) by vacuum filtration followed by a wash step with 1 ml ice-cold TCA and 1 ml acetone. After drying, radioligand bound to the filters was detected by liquid scintillation counting (Packard 4660 scintillation spectrophotometer). Using data on per cent incorporation and number of cysteines present in the translated protein, it was possible to ensure that equimolar amounts of each in vitro translation product were used in each assay.

(d) In vitro protein-protein interaction assay

GST fusion proteins were prepared on a small scale according to the protocol described by the GST Gene Fusion System (Pharmacia, Baie d’Urfe, PQ). Briefly, 1.5 ml LB-Amp cultures were inoculated with glycerol stocks of the appropriate GST fusion proteins (see table 5), using a sterilized inoculation needle. Cells were grown over night to an OD600 > 0.6. GST fusion protein synthesis was selectively induced using 1mM isopropylthiogalactopyranoside (IPTG) for 2 hours. The cells were then collected by centrifugation at 3,750 rpm and resuspended in 1.5 ml lysis buffer (1% Triton X-100, 1% Tween 20, 10 mM DTT, 10 μg/ml Aprotinin and 10μg/ml Leupeptin). Next, the resuspended cells were sonicated for 10 seconds at power 70 with a Biosonik sonicator (Bronwill Scientific, Rochester, NY). Following disruption, cellular debris was removed by centrifugation at 3,750 rpm for 5 minutes. An aliquot of 50 μl of previously prepared Glutathione Sepharose 4B 50% slurry (see section 3.3 (a)) was then added to 1.5 ml of the lysate and incubated at room temperature
with shaking for 10 minutes. The matrix was then sedimented by centrifugation at 500 x g for 1 minute and washed twice with lysis buffer. This resulted in \( \equiv 10 \) \( \mu \)M purified GST fusion protein as determined by optical density (OD\textsubscript{280}). \textit{In vitro} translated \textsuperscript{35}S-labeled GPCR (2 \( \mu \)l per interaction \( \equiv 0.5 \) pmol) was added to the slurry in a total volume of 100 \( \mu \)l of lysis buffer and incubated for 2 hours at 4\(^\circ\)C with vigorous agitation. Following the interaction, the matrix was sedimented at 500 x g for 1 minute and washed twice with lysis buffer. The matrix was then stored at -20\(^\circ\)C until analyzed by SDS-PAGE.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase only</td>
</tr>
<tr>
<td>Nck</td>
<td>Encodes 3 SH3 domains (1-255) of human Nck</td>
</tr>
<tr>
<td>Grb2(w)</td>
<td>Full length human Grb2 (1-217)</td>
</tr>
<tr>
<td>Grb2(N)</td>
<td>N-terminal SH3 domain (1-58) of human Grb2</td>
</tr>
<tr>
<td>Grb2(C)</td>
<td>C-terminal SH3 domain (159-217) of human Grb2</td>
</tr>
<tr>
<td>Grb2(P49L)</td>
<td>Point Mutation in the N-terminal SH3 domain of Grb2</td>
</tr>
<tr>
<td>c-Abl</td>
<td>SH3 domain of mouse Abl (65-123 of p210 Bcr-Abl)</td>
</tr>
<tr>
<td>Crk(N)</td>
<td>N-terminal SH3 domain of human Crk II (133-184)</td>
</tr>
<tr>
<td>Crk(C)</td>
<td>C-terminal SH3 domain of human Crk II (238-290)</td>
</tr>
<tr>
<td>p85(\alpha)</td>
<td>SH3 domain of bovine p85(\alpha) (1-86)</td>
</tr>
<tr>
<td>PLC(\gamma)</td>
<td>SH3 domain of human PLC(\gamma) (792-851)</td>
</tr>
<tr>
<td>Spectrin</td>
<td>SH3 domain of chicken (\alpha)-chain Spectrin (967-1025)</td>
</tr>
<tr>
<td>c-Src</td>
<td>SH3 domain of chicken c-Src (84-148)</td>
</tr>
<tr>
<td>PGEX-3X GRK2</td>
<td>Putative SH3 domain of bovine GRK2 (303-425)</td>
</tr>
<tr>
<td>PGEX-3X GRK3</td>
<td>Putative SH3 domain of bovine GRK3 (305-426)</td>
</tr>
</tbody>
</table>

Table 5. Description of the GST fusion protein constructs used in the \textit{in vitro} interaction assay (see text for further explanation).

The GST constructs described above (with the exception of GRK2 and 3) were obtained from Drs. M. Anafi and T. Pawson. Their construction was as follows: GST-Nck, GST-Crk(N) and GST-Crk(C) were subcloned into the vector pGEX-2T. GST-Grb2, GST-Grb2(N) and GST-Grb2(C) have been described elsewhere (Lowenstein et al., 1992; Gout et al., 1993). The Grb2(P49L) was created
by site directed mutagenesis of human Grb2. GST-Abl, GST-p85, GST-PLCγ, GST-spectrin and GST-c-Src have been described elsewhere (Gout et al., 1993; Puil et al., 1994; Liu et al., 1996). The constructs GST-GRK2 and GST-GRK3 were subcloned into the vector pGEX-3X. It was not possible to subclone the entire GRK construct as the GST fusion protein proved to be toxic to the bacteria.

(e) Large-scale purification of GST fusion proteins

Cultures of 4 ml LB-Amp were inoculated with glycerol stocks of the GST fusion proteins Grb2(w), Nck and GST in either E. Coli XL-1 or the protease deficient bacterial host strain BL21 using a sterilized inoculation needle. Cultures were grown for 5 hours and then transferred to 500 ml flasks containing LB-Amp and grown overnight with vigorous shaking until an OD600 of 1.0-2.0 was reached. Cultures were then induced for 3 hours by adding 500 µl of 1M isopropyl β-D-thiogalactopyranoside (IPTG) to each 500 ml flask. Bacterial pellets were then purified by centrifugation at 2,600 x g for 10 minutes and the supernatant discarded. Cells were stored at -20°C until needed.

To purify the GST fusion protein of interest, frozen cells were thawed and resuspended in cold (4°C) 16ml lysis buffer/1 ml bacterial pellet (1% Triton X-100, 1% Tween 20, 10 mM DTT, 10 µg/ml Aprotinin and 10µg/ml Leupeptin). This suspension was then sonicated at 4°C for 2 x 10s each at power setting 90 using a Biosonik probe sonicator (Bronwill Scientific, Rochester, NY) as 8 ml volumes in 15ml Nunc tubes. To promote solubilization of the protein, sonicates were combined into 40 ml volumes in 50 ml Nunc tubes and incubated for 1 hour at 4°C with gentle agitation. To remove cellular debris, the tubes were centrifuged at 2,600 x g for 10 minutes and the supernatant collected and retained.

To obtain purification in a single step, 500 µl of the previously prepared Glutathione Sepharose 4B matrix (see section 3.3 (a)) was added for each 40 ml of sonicated GST fusion protein in lysis buffer. This mixture was then incubated for 1 hour at room temperature with gentle agitation. Following incubation, the Glutathione matrix was sedimented by centrifugation at 500 x g for 5 minutes and the supernatant discarded. The matrix was then washed twice with 5 ml of lysis buffer.
and sedimnted after each step by centrifugation at 500 x g for 5 minutes. To create a 50% slurry, the final matrix volume was determined and an equivalent volume of lysis buffer was added.

Quantification of the amount of GST fusion protein isolated was made by removing 20 μl of the 50% slurry, washing twice with 100 μl of PBS and incubating for 15 minutes with glutathione elution buffer (20 mM glutathione, 120 mM NaCl, 0.1% Triton X-100, 100 mM Tris-Cl (pH 8.0)). Determination of the concentration could then be made using a spectrophotometer reading at an OD280. GST fusion proteins were also further visualized by running a small aliquot on an SDS-PAGE gel (see section 3.3 (f)).

(f) In vitro saturation analysis of GPCRs with GST and GST-Grb2(w)

To determine the affinity (Kd) of the interaction between GST fusion proteins and in vitro translated D3 dopamine receptor (or other GPCR construct), saturation analysis was used. The GST fusion protein bound to the Glutathione Sepharose 4B matrix as a 50% slurry was aliquotted into eppendorfs at several concentration points ranging from 0.1 μM to 300 μM. Sufficient unbound 50% slurry was added to each tube to ensure that all held an equivalent volume of matrix. The matrix was then sedimnted at 500 x g for 1 minute to remove any excess liquid in the tubes. 0.5 μl of the In vitro translated GPCR (≈ 0.125 pmol) was then added in a volume of 100 μl lysis buffer, the tubes mixed well and incubated at 4°C for 3 hours with vigorous agitation. Following the incubation, each reaction was washed twice with 100 μl lysis buffer, followed by sedimentation of the matrix at 500 x g at each step. The reaction tubes were then stored at -20°C until analyzed by SDS-PAGE.

(g) SDS-PAGE analysis of in vitro interactions

Samples were thawed on ice (4°C) and 45 μl of 2x sample buffer (0.125 M Tris-HCl (pH6.8), 20%glycerol, 4% (w/v) SDS, 0.005% bromophenol blue, 2.5% β-mercaptoethanol and ddH2O to 10ml) was added to each sample. Samples were then vortexed and denatured on a heat block at 90°C for 3 minutes. To sediment the matrix, each sample was centrifuged for 1 minute at 14,000 rpm. 20 μl of each sample was then loaded onto an 8-16% pre-cast Tris Glycine Novex gel (Helixx Technologies, Scarborough, ON) of the denaturing, reducing type. Approximately 80 ml of running
buffer (0.024 M Tris-Cl, 0.19 M glycine, 0.1% (w/v) SDS) was also required for each gel. Gels were run for approximately 1.5 hours at 125V.

| Constructs Used |
|-----------------|-----------------|
| Construct       | Description                                              |
| pB D3HATWT      | Wild type cDNA of the dopamine D3 receptor. Contains the hemaglutinin signal sequence at its amino terminus, followed by the HA-epitope, followed by a thrombin cleavage site. (*abbreviated as the HAT tag) see Fig. 8 |
| pB D3HATΔ1      | Pro(236)Leu point mutation in the putative amino SH3 binding domain of the 3rd cytoplasmic loop of the D3 dopamine receptor. |
| pB D3HATΔ2      | Pro(317)Leu point mutation in the putative carboxy SH3 binding domain of the 3rd cytoplasmic loop of the D3 dopamine receptor. |
| pB D3HATΔ1/2    | Pro(236/317)Leu double point mutation in the putative amino and carboxy SH3 binding domains of the 3rd cytoplasmic loop of the D3 dopamine receptor. |
| pB D3HATdel     | D3 dopamine receptor containing a deletion of amino acids 224-283 in the 3rd cytoplasmic loop |
| pB β1-AR        | Wild type cDNA of the β1-adrenergic receptor |
| ppA Luciferase  | Luciferase gene (contains a single PXXP motif) |

<table>
<thead>
<tr>
<th>PCR Constructs</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB D3Loop</td>
<td>Amino acids 209-329, corresponding to the 3rd cytoplasmic loop of the D3 dopamine receptor</td>
</tr>
<tr>
<td>pB D3N-Loop</td>
<td>Amino acids 209-287, corresponding to the N-terminal region of the 3rd cytoplasmic loop of the D3 dopamine receptor</td>
</tr>
<tr>
<td>pB D3C-Loop</td>
<td>Amino acids 252-329, corresponding to the C-terminal region of the 3rd cytoplasmic loop of the dopamine D3 receptor</td>
</tr>
<tr>
<td>pB M4PCR</td>
<td>Wild type cDNA of the M4 muscarinic acetylcholine receptor. Construct generated by PCR to eliminate both 5' and 3' untranslated sequences</td>
</tr>
</tbody>
</table>

Table 6. Description of plasmid constructs used for in vitro translations. All constructs were under the control of the T7 promotor.
Following electrophoresis, all gels were washed in 15 ml Fixing solution (12% (w/v) trichloroacetic acid, 3.5% (w/v) 5-sulfosalicylic acid) for 30 minutes, washed twice with ddH₂O and stained (50% Brilliant Blue G-Colloidal Concentrate, 50% methanol) for 1 hour. Following the staining procedure, the gels were destained in the following manner: 1 minute wash with 20 ml destain I (25% methanol, 10% Acetic acid), and 45 minute wash with 40 ml destain II (25% methanol). Again, the gels were washed briefly with ddH₂O and were transferred to a Whatman filter paper. The gels were then dried under a vacuum for 45 minutes at 90°C and then placed under BIOMAX™ autoradiographic film for analysis (Eastman Kodak Co., Rochester, NY). After a 2 day exposure, the dried gels were then placed under a phosphor-imaging screen for quantification using a STORM 860 imager (both from Molecular Dynamics, Sunnyvale, CA). Dissociation constants, $K_d$, were obtained for Grb2(w) by saturation binding analysis. The ligand binding data were analyzed by the nonlinear least-square curve-fitting program Graphpad PRISM™.

3.4 Mammalian Cell Culture

(a) Cloning of D₃ constructs into the mammalian expression vector pRC/RSV

Cell lines were generated for five D₃ receptor constructs (D3HAT, D3HATA1, D3HATA2, D3HATA½ and D3HATdel). For expression in CHO cells, all five constructs were re-cloned into the pRe/RSV mammalian expression vector (Invitrogen) using the Hind III and Xba I restriction sites.

(b) Transfection of CHO-K1 cells by electroporation

Monolayer CHO-K1 (Chinese hamster ovary) cell cultures grown on 150 mm Nunc plates were dissociated after a 2 minute incubation of cells with trypsin (2.5% w/v trypsin and 0.5 mM EDTA in PBS (2.68 mM KCl, 1.47 mM KH₂PO₄, 137 mM NaCl and 8 mM Na₂HPO₄ (pH 7.4)) followed by aspiration and tapping of the plates. The detached cells were resuspended in PBS (5 x 10⁷ cells /ml) and mixed with sterile plasmid DNA resuspended in PBS (25μg DNA/ 5 x 10⁷ cells). The cells and DNA were chilled on ice for 10 minutes, aliquotted (400μl) into cuvettes and electroporated at 4°C using a BTX 600 Electro Cell Manipulator (San Diego, CA) under the
following conditions: 150V, 2275μF, 72Ω. The electroporated cells were cooled for another 10 minutes on ice and then resuspended with 10 ml media in a 15 ml Falcon tube and plated onto 2 x 100 mm plates. Another 5 ml of media was then added to each plate prior to incubation.

(c) Selection of stable cell lines

Clonal cell lines were selected by growing transfected cells in media containing the selection reagent G418 (400μg/ml). This selected cells, which had incorporated the neomycin resistance gene contained in the mammalian expression vector pRc/RSV. Individual colonies were picked from plates following 2-4 weeks of selection. These picks, or clonal cell lines were expanded. Some cells were stored as freezer stocks, and remaining cell tissue was used for saturation binding analysis to screen clonal cell lines for high expression of dopamine D3 receptors based on [N-methyl-\(^3\)H]YM-09151-2 nemonapride binding. Cell lines expressing receptors at a density of approximately 300-600 fmol/mg protein were used for pharmacological studies.

(d) Ligand binding analysis of stable cell lines

The transfected cell lines were homogenized with a polytron at setting 5 for 20 seconds in binding buffer (50 mM Tris-Cl (pH 7.4), 5 mM EDTA, 1.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 5 mM KCl and 120 mM NaCl). The homogenates were then centrifuged for 15 minutes at 39,000 x g and the supernatant discarded. The pellets were resuspended in binding 15 ml of binding buffer with the polytron for 5 seconds. For saturation binding analysis, 250 μl of the homogenate (\(\approx 20 \mu g\) membrane protein – see section 3.4 (e)) was incubated in duplicate with increasing concentrations (10-1500 pM) of [N-methyl-\(^3\)H]YM-09151-2 nemonapride specific activity 85.5 Ci/mmol. Nonspecific binding was determined by co-incubation with 1 μM haloperidol. The samples were incubated in a final volume of 1 ml for 2 hours and then filtered using a cell harvester (Skatron Instruments, Lier, Norway). Radioligand bound to the filters was detected by liquid scintillation counting (Packard 4660 scintillation spectrophotometer). The values for the density of [\(^3\)H] ligand binding sites (B\(_{max}\)) and dissociation constants of ligands, K\(_d\), were obtained by Scatchard analysis. The ligand binding data were analyzed by the nonlinear least-square curve-fitting program GraphpadPRISM™.

Competition binding analysis was done by co-incubation with 600 pM [\(^3\)H]-YM-09151-2 and
increasing concentrations (10 - 3000 pM) of the ligand either in the presence or absence of 200 µM Gpp(NH)p. Nonspecific binding was determined by co-incubation with 10 µM s-sulpiride. The ligand binding data were analyzed by the nonlinear least-square curve-fitting program Graphpad PRISM™. This program calculates Ki values based on EC50 using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973).

(e) Protein determination of stable cell lines

The BCA Protein Assay (Pierce, Rockford, IL) was used to determine concentrations of solubilized proteins. This assay makes use of the reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium by inclusion of colorimetric detection of Cu⁺ by a unique reagent that contains bicinchoninic acid (BCA). Working reagent was prepared by combining 50 parts of BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.2 N NaOH) with 1 part of BCA reagent B (4% (w/v) CuSO₄). For each sample, 50 µl of cell homogenate was combined with 1.0 ml of the working reagent. A standard curve was also generated using bovine serum albumin (BSA) protein. The samples were vortexed and the reaction tubes incubated at 37°C for 30 minutes. After the reaction, all tubes were cooled to room temperature and the optical densities (OD₅₆₂) of the samples was determined using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). Readings were then converted into concentrations using the standard curve. Approximately 20 µg protein from the membrane homogenate (250 µl) was present for each saturation binding analysis (see section 3.4 (e)).
4.0 RESULTS

4.1 SH3 Binding Domains in the Dopamine D₃ Receptor

Recently, our group was the first to report the presence of SH3 binding domains in the dopamine D₄ receptor. Specifically, it was found that the proline-rich third cytoplasmic loop of the receptor is able to mediate several SH3 interactions in vitro (Oldenhof et al., 1998). To determine whether the dopamine D₃ receptor is capable of interacting with SH3 domain containing proteins, this protein was investigated in an in vitro protein-protein interaction system. Translated (and [³⁵S]-L-cysteine labeled) receptor was precipitated using several proteins that contain SH3 domains. These proteins were expressed as glutathione-S-transferase (GST) fusion proteins with either full-length or SH3 domain alone (for a complete description of all GST fusion proteins used see table 5, section 3.3 (d)). In this experiment, the concentration of GST-SH3 fusion proteins was kept constant (± 10 μM) and the concentration of radio-labeled GPCR or luciferase control protein was equivalent at 0.5 pmol per interaction (± 5 nM -- see section 3.3 (C)).

Of all SH3 domains tested, it was found that under these conditions the full-length HAT-epitope tagged D₃ receptor interacted most strongly with the full-length Grb2 protein. This protein contains two SH3 domains, flanking a central SH2 domain (Lowenstein et al., 1992). In addition, it was found that binding is diminished, but not eliminated, in the Grb2 mutant P49L (fig. 12 (C)). This clone contains a single proline to leucine point mutation at an important, conserved residue in the N-terminal SH3 domain of Grb2. This mutation eliminates binding occurring through the N-terminal SH3 domain (Motto et al., 1994). It was also found that the Nck adapter protein appeared to undergo binding at levels slightly higher than background. In contrast to the work published with the D₄ receptor (Oldenhof et al., 1998), no other SH3 domain that was tested appeared to undergo high affinity interaction with the D₃ receptor under the assay conditions used. Last, there was an additional weak interaction with the G-protein coupled receptor kinases βARK1 (GRK2) and βARK2 (GRK3) (see Fig. 12 (C)). For a more detailed analysis of βARKs, see section 4.2.

To determine which region of the dopamine D₃ receptor is responsible for mediating these protein-protein interactions, a construct was made encoding only the third cytoplasmic loop of the D₃ receptor (amino acids 208-329) (see fig. 12 (D)). At this point, it should be noted that
because of the [\(^{35}\)S]-L-cysteine label used, signal intensity directly correlates to the number of cysteine residues present in the protein. For this reason, loop fragments may not give as strong a signal for an interaction of equivalent (or greater) strength. To improve signal intensity, an additional cysteine residue was engineered by PCR, immediately upstream of the STOP codon. This region corresponded to the TM6 region of the dopamine D\(_3\) receptor (see Fig. 13).

The D3Loop (208-329) fragment displayed a similar binding pattern to the full-length D\(_3\) receptor. Specifically, full-length Grb2 interacted most strongly with the third cytoplasmic loop, followed by the Grb2 mutant P49L. Again, Nck binding appeared to be slightly higher than background. Unlike the full length D\(_3\) receptor, binding to βARKs could not be sustained by the loop alone (see fig. 12 (D)).
**In Vitro Interaction of D3 with SH3 Domains**

Fig 12 *In Vitro* interaction assay of (A) Brilliant blue stained GST-FP (B) Luciferase control (C) D3HAT and (D) D3Loop with different SH3 domains. *In vitro* [35S] labeled proteins were incubated with equivalent amounts of various GST-fusion proteins (≤ 10 μM). Molecular masses are given in kilodaltons.

The luciferase protein contains a PXXP motif, but did not bind to any of the SH3 domains tested in the *in vitro* interaction assay (Fig. 12 (B)). Also, in analogy to other known SH3 binding domains, the dopamine D3 receptor demonstrated specificity by interacting with only a subset of the SH3 domains tested.

In an attempt to further narrow down the region of the third cytoplasmic loop responsible for mediating SH3 interactions in the dopamine D3 receptor, two additional constructs were made. These consisted of the N-terminal region of the third cytoplasmic loop (208-297), named D3N-Loop, and the C-terminal region of the third cytoplasmic loop (252-329), named D3C-Loop. Again, to increase signal intensity, additional cysteine residues were engineered in these
constructs (see Fig 13). Unfortunately neither of these constructs could be translated using the *in vitro* transcription-translation system despite the presence of both an intact Kozak consensus and STOP codons (see section 3.3 (b), Fig. 11). As such, it was not possible to directly determine whether either of these regions of the loop alone could sustain the interaction.

### D3 Constructs

![D3 Constructs Diagram]

**Fig. 13** The dopamine D₃ receptor constructs used in this study. Boxes depict the position of PXXP motifs within the third cytoplasmic loop. Arrows depict the conserved prolines that were targeted for site-directed mutagenesis. Termination signals are shown with an asterisk.
4.2 G protein Coupled Receptor Kinases (GRKs) as SH3 Domains

At present, it is not known which SH3 domains function as binding partners for the dopamine D₃ receptor in vivo. In an attempt to pinpoint potential in vivo binding partners for GPCRs, primary amino acid comparison of several SH3 containing proteins with the βARK1 (GRK2) and βARK2 (GRK3) kinases revealed that several of the important residues were conserved. Both βARKs possess a high degree of homology within their putative SH3 domains (see fig.14). To test for interaction with GPCRs, GST fusion proteins were made of regions 303 - 425 of βARK1 and 305 - 426 of βARK2. Both clones contained substantial flanking regions N- and C-terminal to their putative SH3 domains (see section 3.1 (h) and 3.3 (d) for a detailed explanation of cloning).

In this experiment, the concentration of GST-βARK was ≈ 1 μM. Due to the poor solubility of the GST fusion proteins, it was not possible to obtain appreciably higher levels of expression. In contrast to other GPCRs tested (data not shown), it was found that there is a weak interaction between the wild-type dopamine D₃ receptor and both the G-protein coupled receptor kinases βARK1 and 2 (see Fig. 13 (C)). This region of interaction could be maintained by only a small (approx. 120 amino acid) region highly conserved by both βARK 1 and 2 (see fig. 14).

**SH3 Domain Alignments of Proteins Used**

![Consensus (Manouchehri, 1994)](Fig. 14. Primary amino acid alignment of some SH3 domains. The βARK1 (GRK2) and βARK2 (GRK3) kinases possess many of the residues conserved in SH3 domains.)
When GST-βARK fusion proteins were tested against the D3 Loop (208-329), the interaction was not sustained (see Fig. 12 (D)). Thus, it is possible that the GST-βARK1 and 2 fusion proteins interact with the D3 receptor; however, this interaction occurs by a mechanism distinct from that of Grb2. Also, as the interaction appeared to be fairly strong at relatively low levels of GST fusion proteins, it is likely that the affinity is in a range higher than those obtained by SH3 domain interactions.

4.3 D3 Binding to Grb2 does not Involve SH2 Domains

The full-length GST-Grb2 protein contains an SH2 domain flanked by two SH3 domains (Lowenstein et al., 1992). SH2 domains are known to undergo binding to phosphotyrosine residues with an affinity in the 10 – 100 nM range (Pawson, 1995). Because the third cytoplasmic loop of the dopamine D3 receptor contains three distinct tyrosine (Y) residues, it is possible that residual tyrosine kinases in the in vitro translation reaction could target these residues. Under this scenario, phosphorylated tyrosines could serve as binding partners for the SH2 domain in Grb2.

To address the question of tyrosine phosphorylation in the third cytoplasmic loop of D3, the tyrosine kinase inhibitor genistein was added to the in vitro translation reaction. Genistein is a highly specific inhibitor of tyrosine kinases and has little effect on serine/threonine kinases or other ATP analogue-driven enzymes (Akiyama et al., 1987; Linassier et al., 1990). Genistein has an IC50 of 22 µM. To eliminate tyrosine kinase activity, the inhibitor was added to the translation reaction at a concentration of 250 µM. The in vitro interactions were then carried out using identical conditions as before (see section 4.1). It was found that genistein did not inhibit binding to any of the GST-FPs tested including Grb2 (w) and βARKs (see fig. 15).
**In Vitro Interaction +/- Genistein**

<table>
<thead>
<tr>
<th>GST</th>
<th>Nick</th>
<th>Grb2(w)</th>
<th>Grb2(P49L)</th>
<th>ARK1</th>
<th>ARK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3HAT</td>
<td>55</td>
<td>37</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3HAT + genistein</td>
<td>55</td>
<td>37</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(250 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luciferase</td>
<td>55</td>
<td>37</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luciferase + genistein</td>
<td>55</td>
<td>37</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(250 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 15. In vitro** interaction of D3HAT in the presence an absence of 250 μM of the tyrosine kinase inhibitor genistein. Molecular masses are given in kilodaltons.

4.4 Saturation Analysis of D₃ Binding to Grb2

To quantify the strength of the interaction between the full length Grb2 adapter and the dopamine D₃ receptor, saturation binding analysis was employed. To do this, the in vitro interaction assay was performed at several concentrations of GST-fusion protein ranging from 100 nM to 300 μM using a constant amount (± 0.125 pmol or 0.125 nM) of [³⁵S]-labeled receptor (see section 4.1 (b)) for each interaction. This experiment was performed in triplicate for both full-length D3HAT and D3Loop using the full-length Grb2 adapter. To establish baseline, the experiment was simultaneously performed using GST alone at identical concentration points. The amount of labeled receptor added was one fourth that used for the visual interaction assays. It was determined that D3HAT had an affinity \( (K_d) \) of \( 7.76 \pm 3.23 \) μM (N=3) for the full-length D3HAT receptor.
Grb2 protein. At the same time, the affinity of the D3 Loop by itself was $2.22 \pm 0.52 \mu M$ (N=3) for full-length Grb2(w). Thus, the interaction with the third cytoplasmic loop was significantly higher ($P < 0.05$) than for the full-length receptor as determined by using the unpaired t-test (see Figs. 16 and 17).
Fig. 16. Saturation analysis of D3HAT with Grb2(w). A constant amount of \textit{in vitro} [\textsuperscript{35}S]-L-cysteine labeled receptor protein was incubated with increasing concentrations of GST-Grb2. The data was quantified using a STORM 860 imager. (A) The brilliant blue stain of precipitated GST-Grb2 and the eluted [\textsuperscript{35}S]-D3HAT receptor. (B) A typical saturation curve plotted from phospho-imager data. The affinity ($K_d$) was determined from 3 independent experiments ± standard deviation.
Fig. 17. Saturation analysis of D3Loop with Grb2(w). A constant amount of *in vitro* \[^{35}\text{S}\]-L-cysteine labeled receptor protein was incubated with increasing concentrations of GST-Grb2. The data was quantified using a STORM 860 imager. (A) The brilliant blue stain of precipitated GST-Grb2 and the eluted \[^{35}\text{S}\]-D3Loop. (B) A typical saturation curve plotted from phospho-imager data. The affinity (K_d) was determined from 3 independent experiments ± standard deviation.
4.5 Role of PXXP Motifs in SH3 Binding in the D₃ receptor

Until very recently, all known SH3 ligands were proline-rich and contained PXXP motifs. The dopamine D₃ receptor contains two such motifs in its third cytoplasmic loop, one at both its N- and C-terminal regions. To determine whether either of these motifs were important for interactions with SH3 containing proteins, three site-directed mutant receptor constructs were generated. The first, D3HATmute1, changed residue 236 from proline to leucine (the first proline in the N-terminal PXXP motif). The second construct, D3HATmute2, targeted residue 317 (the second proline in the C-terminal PXXP motif), and similarly substituted proline for leucine. The third construct, D3HATmute1/2, contained both point mutations (see Fig. 13).

*In vitro* interactions were then performed using those GST-fusion proteins that had previously been shown to bind to the D₃ receptor. Assay conditions were the same as before. *In vitro* labeled GPCR was added at a concentration of ≅ 5 nM and the GST-FP was present at a concentration of ≅ 10 μM. It was found that binding in the proline-mutated dopamine receptors was not significantly altered from the full-length wild-type D₃ (see Fig. 18). However, when a truncated form of the D₃ receptor, missing the N-terminal region of the third cytoplasmic loop (D3HATdel, missing amino acids 224-283) was tested, it was found that binding to Grb2 had been abolished (see fig. 18).
SH3 Interactions with D3 Mutants

Fig. 18. Role of the third cytoplasmic loop and PXXP motifs in SH3 domain binding in the dopamine D3 receptor. Molecular masses are given in kilodaltons.
To quantitatively determine whether Grb2 binding to mutant and truncated forms of the receptor had been significantly altered, binding was monitored at defined points along the saturation curve. As binding of both D3HAT and D3Loop was saturated over a narrow concentration range, concentrations for GST-Grb2 were selected a distance of two log units apart, near the calculated $K_d$ (see Fig. 19). Specifically, it was found that at 55 µM GST-Grb2, binding for all clones had reached saturation. Higher concentrations of GST-Grb2 did not increase binding (data not shown). There were no significant differences in maximal binding between mutant and wild-type D3 receptors. Binding of D3Loop was not determined at 55 µM GST-Grb2. However, saturation binding analysis (Fig. 17) revealed that binding to the D3Loop had saturated at this point. At 5.0 µM Grb2, there was no significant change between any of the P→L point mutations from the full-length D3 receptor. However, the D3Loop construct bound with significantly higher affinity ($P < 0.001$). In addition, binding of the truncated form of the receptor, D3HATdel, was significantly reduced ($P < 0.001$) from wild-type. Similarly, at 0.5 µM Grb2, it was found that D3Loop had significantly higher affinity than full-length D3HAT ($P < 0.01$) and D3HATdel again showed decreased affinity ($P < 0.05$). The double point mutant had a slightly increased affinity ($P < 0.05$) for Grb2 (see fig. 18). All levels of significance were determined through use of the unpaired t-test.
**Effect of Point Mutations and Loop Fragmentation on D3-SH3 Binding**

**(A)**

![Diagram](image)

**(B)**

![Graph](image)

Fig. 19. The effect of point mutations and Loop fragmentation on Grb2 binding. The *in vitro* interaction was performed at 55 μM, 5 μM and 0.5 μM GST-Grb2. (A) Brilliant blue precipitated GST fusion protein and *in vitro* labeled receptor. D3HAT is shown as an example. (B) Differences in affinity for GST-Grb2. Statistically significant data are indicated as follows: (*) P < 0.05, (**) P < 0.01 and (***) P < 0.001.
4.6 *In vitro* interaction of GPCRs with SH3 Domains

Numerous GPCRs also contain the minimal PXXP motif for SH3 binding within either their third cytoplasmic loop or C-terminal tails, or both. To determine whether SH3 binding is limited to the dopamine receptors, or a trait of GPCRs in general, two other receptors were tested for *in vitro* interactions with SH3 domains. Both the β₁ adrenergic and the M₄ muscarinic acetylcholine receptors are extremely proline-rich, and contain several of the minimal PXXP motifs within their intracellular domains (see section 1.7, Fig.8).

Again, in the *in vitro* protein-protein interaction assay, both the β₁-adrenergic receptor and the M₄ muscarinic acetylcholine receptor were done as described before. GST fusion proteins were present in the assay at approximately equivalent concentrations (≈ 10 μM). It was found that the β₁-adrenergic receptor interacted most strongly with the full-length Grb2 protein and much less strongly with the Nck adapter. The M₄ receptor similarly displayed strongest binding with full-length Grb2 and much weaker binding with individual SH3 domains within the Grb2 protein. There was also a weak interaction with the Nck adapter protein (see Fig. 20). In contrast to the dopamine D₃ receptor, no interaction was detected for either the β₁ or the M₄ receptors with the Grb2 P49L mutant.
**In Vitro Interaction of GPCRs with SH3 Domains**

(A) GST-FP

(B) β1

(C) M4

(D) Luciferase

**Fig. 20** *In vitro* Interaction of GPCRs with different SH3 domains. *In vitro* [35S]-L-cysteine β1-adrenergic receptor, M4 muscarinic receptor and control (luciferase) proteins were incubated with equivalent amounts of various GST fusion proteins (± 10 μM). The top panel shows the relative amounts of GST fusion protein (Brilliant blue stain) used to precipitate the *in vitro* labeled GPCRs. Molecular masses are given in kilodaltons.
4.7 Pharmacological Analysis of Wild-Type and Mutant HAT-tagged D₃ Receptors

For expression in mammalian cells, the cDNAs encoding the wild-type and mutant D₃ receptors were subcloned into the eukaryotic expression vector pRc/RSV and transfected into CHO-K1 cells (see section 3.4). Colonies that expressed dopamine D₃ receptors were selected by G418-resistance. The level of D₃ receptor expression was then determined by saturation analysis of [³H]YM-09151-2 nemonapride binding. Individually, 10 - 15 cell lines were generated for each D₃ receptor clone. Clonal cell lines with representative affinities (K_d) for nemonapride were selected for further analysis based on two criteria. First, the reported affinity of the D₃ receptor for nemonapride is 0.17 ± 0.03 nM (Vile et al., 1995). Thus, it was not known what effect, if any, point mutations would have on binding affinity; however, those with representative K_d values were taken for additional analysis. Second, it was desirable to utilize cell lines with the highest levels of receptor density (B_max). As such, cell lines with levels of expression between 358 - 700 fmol/mg protein were used in subsequent pharmacological experiments. Once selected, additional independent experiments were performed to obtain statistically relevant data.

In this manner, cell lines were created for all full-length HAT epitope tagged D₃ receptor constructs. This included the wild-type D₃ receptor, all point mutants and the third cytoplasmic deletion mutant. For all cell lines, [³H] YM-09151-2 nemonapride binding was concentration-dependent, saturable and displaceable with cold haloperidol (a dopamine receptor antagonist).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg)</th>
<th>$K_i$ Quinpirole (nM)</th>
<th>$K_i$ Raclopride (nM)</th>
<th>$K_i$ Spiperone (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3HAT</td>
<td>0.136 ± 0.074 (n=4)</td>
<td>0.626 ± 0.056 (n=4)</td>
<td>259 (n=2)</td>
<td>29.1 (n=2)</td>
<td>1.61 (n=2)</td>
</tr>
<tr>
<td>D3HAT Δ1</td>
<td>0.147 ± 0.025 (n=4)</td>
<td>0.518 ± 0.118 (n=4)</td>
<td>256 (n=2)</td>
<td>13.7 (n=2)</td>
<td>5.48 (n=2)</td>
</tr>
<tr>
<td>D3HAT Δ2</td>
<td>0.440 ± 0.121 (n=4)</td>
<td>0.358 ± 0.169 (n=4)</td>
<td>434 (n=1)</td>
<td>18.5 (n=1)</td>
<td>9.74 (n=1)</td>
</tr>
<tr>
<td>D3HAT Δ1/2</td>
<td>0.127 ± 0.061 (n=4)</td>
<td>0.695 ± 0.287 (n=4)</td>
<td>184 (n=2)</td>
<td>13.3 (n=2)</td>
<td>3.23 (n=2)</td>
</tr>
<tr>
<td>D3HAT Del</td>
<td>0.147 ± 0.035 (n=3)</td>
<td>0.607 ± 0.224 (n=3)</td>
<td>299 (n=2)</td>
<td>9.74 (n=2)</td>
<td>1.59 (n=2)</td>
</tr>
</tbody>
</table>

Table 7 $[^3]H$-nemonapride binding of CHO-D3 cell lines. $K_d$ represents the dissociation constant (affinity) of the ligand for the receptor. $K_i$ represents affinity in competition of $[^3]H$-nemonapride. $B_{max}$ is a measure of receptor density. (n) represents the number of independent, duplicate experiments performed.

Competition analysis of $[^3]H$-nemonapride binding with dopamine was performed in the presence and absence of 200 µM of the non-hydrolyzable GTP analog Gpp[NH]p. G protein coupling as observed by Gpp[NH]p, was performed in duplicate and analyzed using both the one and two-site competition binding models with the program Graphpad PRISM. The best-fit line was plotted (see Fig. 21). It was found that the D3HAT wild-type displayed high affinity dopamine binding that was not sensitive to the inclusion of Gpp[NH]p (lowered from 5.02 nM to 5.98 nM in the presence of Gpp[NH]p). Similarly, there was little G-shifting observed in any of the P→L point mutant cell lines. Specifically, the first proline mutant, D3HATmute1 (Δ236), displayed high affinity for dopamine (15.4 nM) with little change to low affinity (19.7 nM) upon inclusion of Gpp[NH]p. The $K_i$ value of dopamine for D3HATmute2 demonstrated that binding was much reduced compared to wild-type -- lowered to approximately 10 µM. This reduction in affinity for dopamine appeared to be reversed to a slightly higher affinity by the inclusion of Gpp[NH]p (to 6.73 nM). Upon introduction of a both point mutations into the same receptor, binding affinity for dopamine was restored to high affinity (7.7 nM). The double-point-mutant did not appear to undergo significant loss of affinity for dopamine when Gpp[NH]p was included.
(there was a G-shift to 4.5 nM). In the D₃ receptor lacking the N-terminal (Δ224-283) amino acids of its third cytoplasmic loop, the high affinity for dopamine was reversed to a low affinity state (from 4.60 nM to 19.2nM). This suggests that the D₃ deletion mutant may indeed be coupling to G proteins in the CHO-K1 cell line.

Competition binding analysis of [³H]-nemonapride binding was also performed to determine rank order of potency. This was done using the ligands quinpirole (a D₂-like agonist), spiperone (an antagonist) and raclopride (an antagonist). These results were plotted in Fig. 22 and Ki values summarized in table 7. In all cases spiperone had the highest affinity for D₃ sites followed by raclopride and quinpirole.
Fig. 21 Competition binding analysis of $[^3H]$-nemonapride binding with increasing concentrations of dopamine in the presence (■) or absence (▲) of 200 µM Gpp[NH]p. Data was analyzed using both the one and two-site model. The best-fit curve is plotted. This data is representative of competition curves (2 independent experiments). $K_i$ represents affinity for dopamine using the one-site model (average value of experiments).
**Competition Binding Analysis of CHO-D3HAT Cell Lines – Rank Order**

Fig. 22 Competition binding analysis of $[^3]$H-nemonapride binding with increasing concentrations of drug: (■) quinpiroie (▲) raclopride and (▼) spiperone. This data is representative of typical competition curves. $K_i$ values were determined and summarized in table 7.
5.0 DISCUSSION

The main objective of this project was to characterize SH3 binding domains in the dopamine D3 receptor in vitro. The D3 receptor contains two SH3 binding motifs (sequences that contain the PXXP consensus) within its third cytoplasmic loop. Earlier work with the dopamine D4 receptor has implicated analogous motifs in the N- and C-terminal regions of the third loop as SH3 binding sites (Oldenhof et al., 1998). It was not possible to directly test the role of these proline-rich regions in the interaction as the third cytoplasmic loop of the D4 receptor is difficult to target with site directed mutagenesis. Thus, the data presented indicate that the D3 receptor can similarly bind the Grb2 adapter protein with high affinity and that this binding appears to be located within the third cytoplasmic loop. However, traditional PXXP motifs do not appear to be involved in SH3 binding in the dopamine D3 receptor.

5.1 SH3 Binding Domains in the Dopamine D3 Receptor

To date, the possibility of SH3 binding domains in GPCRs, including the dopamine receptors, remains largely uninvestigated. This is despite the presence of numerous “core” SH3 binding motifs (PXXP) in regions of these receptors that would be necessary for functional coupling. Such regions include the third cytoplasmic loop and C-terminal tail. As noted in section 1.1, these regions in particular are the source of much of the variability between GPCR subgroups and could be sites important for coupling to previously unappreciated effector pathways. To determine whether SH3 binding to the dopamine D3 receptor is possible, we have employed an in vitro protein-protein interaction assay based on glutathione-S-transferase (GST) to test individual SH3 domains for an interaction. Several different SH3 domains were selected for analysis including the adapter proteins Nck, Crk and Grb2, the kinases c-Abl and c-Src, the cytoskeletal protein spectrin, PLCγ, and the p85α subunit of phosphatidylinositol-3 kinase.

The specificity of the protein-protein interactions was revealed by the luciferase control protein. Luciferase contains a single PXXP motif, but did not interact with any of the SH3 domains tested. In addition, the dopamine D3 receptor could interact with only a subset of the SH3 domains tested. This observation of specificity is consistent with all reports of SH3 domain interactions to date.

In this study, we have shown that of the SH3 domains tested, the dopamine D3 receptor undergoes a high affinity interaction in vitro with the full length Grb2 adapter protein. In
addition, there was a much weaker SH3 interaction with the Nck adapter. It was also found that binding was diminished, but not eliminated in the Grb2 mutant P49L. This clone contains a single proline to leucine point mutation at an important conserved residue in the N-terminal SH3 domain of Grb2. This mutation eliminates binding occurring through the N-terminal SH3 domain (Motto et al., 1994). Reduction of the protein-protein interaction with the Grb2 P49L mutant suggests that both the N- and C-terminal SH3 domains of Grb2 contribute to binding in the D3 receptor. However, neither of the SH3 domains of Grb2 could interact with the D3 receptor in isolation. The fact that individual SH3 domains within Grb2 failed to interact with the D3 receptor means that binding should be interpreted with caution. Grb2 binding has been shown to undergo cooperative binding in some cases, requiring both of its SH3 domains in tandem for high affinity interactions (Anafi et al., 1997; Oldenhof et al., 1998).

Binding of the Grb2 adapter protein to the dopamine D3 receptor was specific and saturable. It was determined that the full-length D3 receptor had a Kd of approximately 8 μM for Grb2. This affinity is approximately 20-fold higher than reported for D4.2 and D4.4 and approximately 6-fold higher than for D4.7, under identical assay conditions (Oldenhof et al., 1998). Other characterized SH3 interactions such as those of the p85α subunit of PI3K and c-Src are also typically in the 5-100 micromolar range (Yu et al., 1994; Pawson, 1995). When the third cytoplasmic loop (residues 208-329) of the D3 receptor was tested in isolation, there was a significant increase in affinity for the Grb2 adapter protein to Kd ≈ 2 μM. This observation is consistent with that observed in the D4 loop which had a Kd of ≈ 10 μM under identical assay conditions (Oldenhof et al., 1998). The reason for this Kd shift is unknown at present. The minimal structural conformation that is required for binding to SH3 domains is the left-handed poly-proline type II (PPII) helix. Large flanking sequences are unnecessary for SH3 binding domains to form. Indeed, the vast majority of studies into SH3 binding domains have utilized peptides of approximately 10-12 amino acids in length (Cheadle et al., 1994; Rickles et al., 1994; Sparks et al., 1994; Yu et al., 1994; Alexandropoulos et al., 1995; Feng et al., 1995; Rickles et al., 1995; Grabs et al., 1997). Also, GPCRs contain seven transmembrane helices and as such, are extremely hydrophobic in nature. It is likely, that when the D3 receptor is not constrained by its TM domains, its third cytoplasmic loop is capable of adopting a conformation similar to that of the native membrane-bound receptor. This soluble form is much more favourable for interactions with SH3 domains.
There is a considerable amount of evidence supporting ligand induced conformational changes in the GPCRs. It is thought that one specific change is enhanced accessibility of the third cytoplasmic loop to the cell interior. It has been suggested that such enhanced accessibility would facilitate binding to G proteins or other effectors. Similarly, enhanced accessibility to the third loop of the dopamine D₂-like receptors could be important for a novel effector system, mediated by SH3 domains.

Last, a positive *in vitro* interaction with the Grb2 adapter does not indicate a functional contact *in vivo*. Similarly, the lack of an interaction with some of the SH3 domains tested does not mean that they will not interact in the cell. GPCRs are integral membrane proteins and as such, are subject to conformational and entropic constraints when expressed in the cell. Local concentrations and orientation of binding partners may prove to be equally important contributors. In addition, the buffer conditions used in the *in vitro* interaction assay contain high concentrations of solubilizing detergents (1% Triton X-100, and 1% Tween 20) that may be less favourable for detecting specific protein-protein interactions.

### 5.2 SH3 Binding to the Dopamine D₃ Receptor Does Not Involve PXXP Motifs

At present, it is well accepted that the ligands with highest affinity for SH3 domains contain a core PXXP motif. Notable exceptions include intramolecular SH3 binding in c-Src (Xu et al., 1997) and its family member Hck (Sicheri et al., 1997), as well as Bruton’s tyrosine kinase (Btk) (Matsushita et al., 1998) and the tumour suppressor, p53 (Gorina and Pavletich, 1996). The dopamine D₃ receptor contains two distinct PXXP motifs within the N- and C-terminal regions of the third cytoplasmic loop. To determine the role of these PXXP motifs in SH3 binding to the D₃ receptor, site directed mutagenesis was employed. A single proline residue was mutated to leucine in each of the PXXP motifs, either alone or in combination. Similar site-directed mutants have previously been shown to effectively abolish SH3 binding to proline-rich peptides *in vitro* (Feng et al., 1994).

Despite the intrinsic solubilizing nature of the lysis buffer used to carry out the protein-protein interactions, direct comparisons may be made between the dopamine D₃ receptor and its mutants. This analysis is indicative of the specific role of proline residues in SH3 mediated binding to Grb2.
Thus, quantitative analysis of Grb2 binding affinity to the dopamine D₃ receptor was performed. Comparisons were made to the wild-type D₃ receptor. Measurement of SH3 binding was performed over a concentration range of two log units, surrounding the calculated $K_d$ of D₃ for Grb2. It was found that for both the mutant and wild-type D₃ receptors, binding was specific and saturable. At 55 µM GST-Grb2, all receptor constructs had reached maximal binding. This was an expected result as the calculated $K_d$ for D3HAT was $\approx 8$ µM. At 5 µM GST-Grb2, both the wild-type and point mutants had reached 25% of maximum binding. Again, this result is consistent with the calculated $K_d$. At 0.5 µM GST-Grb2, a small but significant increase ($P < 0.05$) in binding was observed with the double-point-mutant (D3HATmute1/2 (Δ236/317)). This was an unexpected result as disruption of PXXP motifs would be expected to alter PPII helix formation and consequently binding to SH3 domains. Thus, it was concluded that site-directed mutagenesis of proline residues in the N- and C-terminal PXXP motifs had no detrimental effects on SH3 binding to the dopamine D₃ receptor. No differences in affinity were observed for binding to the Grb2 adapter. This result is in direct opposition to the earlier findings of Feng et al. that mutation of conserved proline residues to alanine decreased binding from $K_d \approx 75$ µM to $> 500$ µM (Feng et al., 1994). Thus, it does not appear likely that SH3 binding in the dopamine D₃ receptor occurs through traditional PXXP motifs.

To further narrow down the region responsible for SH3 binding in the D₃ receptor third cytoplasmic loop, a series of deletion constructs were also made. One construct, D3HATdel, lacked residues 224-283 in the N-terminal region of the loop. This region also included the N-terminal PXXP motif. Initially, when this mutant was tested for interactions with SH3 domains, no binding was observed. However, when visualized using a STORM 860 phospho-imager (which is up to 1000x more sensitive than film), it was noted that the interaction was preserved, but had been reduced significantly. As such, in this mutant receptor, it was again found that saturation binding was achieved at 55 µM GST-Grb2. However, at 5 µM and 0.5 µM GST-Grb2, binding had been significantly diminished ($P < 0.05$) from wild-type by almost 67% and 50% respectively. It was concluded that the SH3 binding region is contained within the C-terminal region of the D3Loop. Furthermore, the N-terminal region is a modulator of this SH3 interaction. This modulatory activity could occur by inducing structural constraints that are favourable for SH3 binding in vitro. At the present time, it is not known what phenotypic changes, if any, deletion of the N-terminal region of the loop will induce in the cell.
In an attempt to further narrow down the region of the loop responsible for binding to SH3 domains, two additional clones were generated encoding fragments of the third cytoplasmic loop. One clone, (D3N-Loop) encoded the N-terminal 78 amino acids, while the other (D3C-Loop) encoded the C-terminal 77 amino acids of the loop. The region of overlap was 35 amino acids. It was expected that all binding would be contained within the C-terminal region, but with reduced affinity as predicted by deletion analysis. Unfortunately, it was not possible to generate \[^{35}S\]-labeled fragments of these constructs. It was not clear why the transcription-translation failed as both constructs contained both an intact Kozak consensus and STOP codon.

One limitation of the majority of studies into SH3-ligand interactions, is that they deal primarily with combinatorial peptide libraries. To date, when predicting SH3 binding sites, researchers have tended to screen with biased peptide libraries (containing PXXP motifs) and then conduct homology searches for proteins that contain these motifs. A distinct disadvantage of this technique is its failure to predict novel SH3 binding domains such as the exceptions outlined above. With the recent discovery of novel SH3 ligands, it is becoming clear that proline residues may not be an absolute requirement for binding. One very recent report has suggested that N-substituted amino acids may form the principal restriction. Under this model, proline residues are favoured not because of their particular side chain shape or inherent rigidity, but because they constitute the only naturally occurring amino acid with amide N-substitution (Nguyen et al. 1998). Furthermore, other reports have suggested that PPII helix formation may be all that is required to form SH3 binding domains. This secondary structure is relatively common in proteins and despite its namesake, does not necessarily require proline residues for its formation (Adzhubei and Sternberg, 1993). Beyond, PXXP motifs, other structural features may prove to be equally important for binding. Steric limitations, arrangement at the plasma membrane and physical proximity of SH3 domains and their binding partners are examples of other factors that could be important. Ultimately, as more research is focussed toward \textit{in vivo} SH3-ligand interactions, the list of novel effector systems will rise.
Summary of D3-SH3 Interactions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Strength of Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3HAT</td>
<td>++</td>
</tr>
<tr>
<td>D3HATmute1</td>
<td>++</td>
</tr>
<tr>
<td>D3HATmute2</td>
<td>++</td>
</tr>
<tr>
<td>D3HATmute1/2</td>
<td>++</td>
</tr>
<tr>
<td>D3HATdel (Δ224-283)</td>
<td>+/-</td>
</tr>
<tr>
<td>D3Loop (209-329)</td>
<td>+++</td>
</tr>
<tr>
<td>D3N-Loop (209-287)</td>
<td>Will not translate</td>
</tr>
<tr>
<td>D3C-Loop (252-329)</td>
<td>Will not translate</td>
</tr>
</tbody>
</table>

Fig. 23 Summary of in vitro protein-protein interactions between the dopamine D3 receptor and the Grb2 adapter protein. Strength of the interaction is indicated: (+++) very strong, (+++) strong, (+/-) weak.

5.3 SH3 Binding Domains in GPCRs

Beyond the dopamine receptors, several other GPCRs contain potential SH3 binding motifs within either their third cytoplasmic loop, C-terminal tail or both. These are regions where functional contacts could be made with effector systems. In particular, PXXP motifs appear in several subgroups of GPCRs including the α- and β-adrenergic, the muscarinic and dopamine receptors. The incidence of PXXP motifs in these receptors is 10-fold higher than would be expected from random amino acid distribution. The idea that at least select GPCRs could contain
SH3 binding motifs has been proposed at least once before, but the idea has never been fully pursued experimentally (Ren et al., 1993).

In this study, we have shown that beyond the dopamine D₃ receptor, and the recently published D₄, at least two additional GPCRs appear to be capable of mediating SH3 interactions in vitro. Specifically, the β₁-adrenergic receptor and the M₄ muscarinic acetylcholine receptor were tested using the same GST-SH3 fusion proteins and assay conditions that demonstrated SH3 binding in the D₂-like receptors. It was found that both receptors demonstrated highest affinity for the Grb2-like adapter protein, and little affinity for the other SH3 domains tested. Thus, SH3 binding may not be a phenomenon restricted to the dopamine receptors. Instead, it may be a phenomenon of GPCRs in general.

5.4 βARK1 and 2 as SH3 Binding Domains

At present, it is not known what SH3 interactions may occur with the dopamine receptors in vivo. A scan of the primary sequences of potential binding partners revealed that the βARK1 (GRK2) and βARK2 (GRK3) kinases, which have been implicated in homologous desensitization of GPCRs, contain several of the conserved residues found in characterized SH3 domains. To address the question of whether βARK1 or 2 can act as SH3 binding partners for the dopamine D₃ receptor, GST fusion proteins were generated for these kinases. It was not possible to create fusions encoding the full-length protein for either βARK. Invariably, clones recovered subsequent to bacterial transformation contained frame-shift or nonsense mutations. It was concluded that GST-βARK is incompatible with expression in E.Coli. For this reason, fusion proteins were generated using only those regions of βARK corresponding to the putative SH3 domain. Also included was substantial flanking sequence to the putative SH3 binding region (the βARK1 construct encoded residues 303 - 425, while the βARK2 construct encoded residues 305 - 426).

Structurally, all GRKs possess a central, catalytic kinase domain 263-266 amino acids in length that is flanked by large N- and C-terminal regulatory domains. As the amino terminal domain appears to share a degree of homology between kinases, it has been suggested that this region may share a common function such as receptor recognition (Ingleson et al. 1993). In contrast, the C-terminal domains tend to be variable and in βARK1 and 2 contains sites for
interaction with the βγ subunit of heterotrimeric G proteins (Pitcher et al. 1992). The domains of βARK1 and 2 chosen for the in vitro interaction assay corresponded to the C-terminal end of the catalytic domain, just upstream of a calmodulin binding site (Pronin et al. 1997; Levay et al. 1998). Additional proteins that interact with βARK1 in vitro have been isolated although these have yet to be identified (Carman et al. 1998). Three of the candidate genes had molecular weights in the range of the dopamine D3 receptor (38 – 44 kDa).

It was found that both of the GST-βARK fusions were capable of interacting with the full-length dopamine D3 receptor. Again, this interaction may or may not be indicative of an actual protein-protein interaction in vitro. Calmodulin binding to βARK1 has been reported to be in the 50 - 100 nM range (Levay et al. 1998). This level of affinity would be consistent with a sensitivity detectable by our in vitro interaction assay. Furthermore, the interaction of βARKs with the D3 receptor likely occurred by a mechanism distinct from that of the Grb2 SH3 domains as the third cytoplasmic loop did not appear to be capable of interacting when expressed by itself. Furthermore, lack of tyrosine phosphorylation in the D3 receptor did not appear to inhibit binding to either βARK kinase. Interestingly, the D3 construct containing a deletion of the amino acids in the N-terminal region of the third cytoplasmic loop also did not appear to interact with either of the GST-βARK fusions. It is possible that the protein-protein interaction was not visible due to low affinity. Thus, this region of the loop appeared to be important not only for modulating the interaction with SH3 domains, but the interaction with βARKs as well.

5.5 Pharmacological Characterization of HAT-epitope Tagged D3 Receptors and Mutants

To determine the pharmacological profile of wild-type and mutant HAT-epitope tagged D3 receptors, stable cell lines were generated for several of the D3 constructs used in this study. Receptors were expressed as stable cell lines in Chinese Hamster Ovary (CHO-K1) cells using the mammalian expression vector pRSV. Several distinct stable cell lines were generated for each clone. Those cell lines expressing dopamine D3 receptors with Kd values closest to the published value were likely to be representative and were retained for additional pharmacological analysis. Also, to facilitate comparison between clones, cell lines with similar levels of expression of D3 receptors, as measured by [3H]-YM 09151-2 (nemonapride) binding,
were chosen for analysis. As such, expression levels ranging from 0.358 fmol/mg protein to 0.695 fmol/mg protein were subsequently used.

Saturation analysis with [³¹H]-nemonapride revealed both Kₐ and Bₘₐₓ for all cell lines generated. It was found that in almost all lines, the calculated affinity for nemonapride correlated very closely with the published value of Kₐ = 0.17 ± 0.03 nM. The notable exception to this was the D3HATmute2 clone. All cell lines generated for this receptor had an affinity for nemonapride that was reduced by at least 2 to 3-fold from the wild-type.

(a) Dopamine and Guanine-nucleotide Binding Effects

Guanine nucleotide effects on dopamine binding were evaluated by measuring [²³H]-nemonapride binding in the presence and absence of the non-hydrolyzable GTP analog Gpp[NH]p. This GTP analog converts the high affinity binding of dopamine to a single, low affinity state. Such GTP sensitivity toward agonist binding is a characteristic of GPCRs and indicates coupling to endogenous G proteins present in CHO cells.

Dopamine competition analysis revealed that the wild-type dopamine D₃ receptors were in a high affinity state, with a Kᵢ value of approximately 5 nM. This is consistent with other studies of dopamine competition, which reported affinities in the 10 - 25 nM range for the wild-type receptor (Sokoloff et al., 1990; Sokoloff et al., 1992; Freedman et al., 1994; MacKenzie et al., 1994). When the proline point mutants were investigated, it was found that mutation of P²³⁶ to leucine lowered the affinity of the receptor for dopamine to ≈ 15nM, a change of 3-fold. The mutation of P³¹⁷ to leucine led to a striking difference in that there was a drastic reduction in affinity of the mutant receptor for dopamine. Dopamine binding in this cell line was reduced by approximately 1000 fold compared to wild-type (Kᵢ ≈ 10 μM). It was concluded that mutation of P³¹⁷ led to conversion of the receptor to the low-affinity state. Moreover, mutation of both proline residues in the D₃ third cytoplasmic loop apparently restores affinity for dopamine (Kᵢ ≈ 8 nM). In effect, mutation of P²³⁶ is compensatory for mutation of P³¹⁷ as high-affinity dopamine binding could be fully restored by targeting this residue.

Little to no change in affinity for dopamine in the presence of the non-hydrolyzable GTP analog Gpp[NH]p was observed in the wild-type HAT-epitope tagged dopamine D₃ receptor or any of the D₃ receptors containing point mutations. This result was consistent with other observations for D₃ receptors, which also failed to observe a G-shift in CHO cells (Sokoloff et
al., 1990; Sokoloff et al., 1992; MacKenzie et al., 1994). This observation does not imply that the dopamine D3 receptor is incapable of coupling to G proteins. The dopamine D3 receptor is known to couple to G proteins as it has been shown to weakly inhibit adenylyl cyclase in some cell lines (Chio et al., 1994; Potenza et al., 1994; Robinson et al., 1994; McAllister et al., 1995) as well as mediate effects on mitogenesis and Ca2+ and K+ currents (Chio et al., 1994; Pilon et al., 1994; Potenza et al., 1994; Seabrook et al., 1994; Liu et al., 1996; Werner et al., 1996). It is probable that the CHO-K1 cell line does not endogenously express G proteins that have a high affinity for the D3 receptor. Interestingly, the deletion mutant exhibited a G-shift, significantly favouring the two-site model of binding \( (P < 0.05) \). In the case of the third cytoplasmic loop deletion, the change in \( K_i \) in the presence of Gpp[NH]p was more than 4-fold. The reasons for this change in G-shifting are unknown at present. However, the data is suggestive of functional coupling to G proteins in CHO cells for these D3 mutants. If G protein coupling has occurred, the G-shift was uniphasic and therefore likely complete. It is possible that the N-terminal region of the third cytoplasmic loop blocks coupling to G proteins endogenously expressed in CHO cells.

(b) Rank Order of Potency

To further investigate effects of point mutations and third cytoplasmic loop fragmentation on the dopamine D3 receptor, competition binding analysis was employed to provide rank order of potency for these receptors. In all cases, the indicated rank order of potency was Spiperone > Raclopride > Quinpirole (i.e. \( K_i \) (Spiperone) < \( K_i \) (Raclopride) < \( K_i \) (Quinpirole)). This rank order was identical to other published data on the dopamine D3 receptor (Sokoloff et al., 1990; Assie et al., 1993; Freedman et al., 1994; MacKenzie et al., 1994; Vile et al., 1995). However, in all cases, \( K_i \) values were lowered by 5 - 20 fold from published values. The D3 receptor containing a mutation in P317 again differed slightly from the remaining clones. This receptor demonstrated much less selectivity between spiperone and raclopride; however, more replicates need to be completed to determine the significance of this result. It is possible that this mutation induces structural changes in the receptor that alter ligand binding.

As there was general agreement of \( K_i \) values between wild-type and mutant receptors, it is possible that the HAT-epitope had a negative effect on ligand binding. This epitope has been used with success on other dopamine receptors, and did not significantly alter agonist binding.
(Guan et al., 1992; von Zastrow and Kobilka, 1992; von Zastrow et al., 1993). To determine whether the HAT epitope is responsible for differences in binding, comparisons will have to be made with non-epitope tagged D3 receptor.

5.6 Conclusions

Our finding that there are SH3 binding sites in the dopamine D3 receptor could indicate that there are novel SH3-mediated effector systems yet to be discovered in GPCRs. Recently, evidence has uncovered other protein-protein interactions for GPCRs that are mediated by SH2 domains including Jak2 kinase (Ali et al., 1997), phospholipase Cγ (Venema et al., 1998), as well as calmodulin and protein kinase C (Minakami et al., 1997). In addition, the SH2 mediated association of Grb2 with the β2-adrenergic receptor tail has also been reported (Minakami et al., 1997; Malbon and Karoor, 1998; Shih and Malbon, 1998; Karoor et al. 1998). Protein-protein interactions of GPCRs with Grb2 may be of particular interest as these may have implications for receptor internalization and signaling via MAP kinase.

In the dopamine D3 receptor, SH3 binding does not appear to be mediated through traditional PXXP motifs. Instead, binding is localized in the C-terminal region of the third cytoplasmic loop. The N-terminal region of the loop seems to be important for regulating the strength of not just SH3 interactions, but coupling to βARK kinases as well.

5.7 Future Directions

SH3 binding to the dopamine D3 receptor can now be localized to the C-terminal of the third cytoplasmic loop, a region 61 amino acids long. Thus, it will be necessary to systematically narrow down this region even further to determine what the minimum residues required for SH3 binding in the D3 receptor are. This will be done using a combination of PCR-based approaches and loop deletion constructs similar to those already employed in this study.

Once the precise SH3 binding domains in the dopamine D3 receptor have been located, it will be necessary to determine what role, if any, SH3 binding plays in the cell. In the dopamine D4 receptor, it has been shown that the regions implicated in SH3 binding also lie in regions that are involved in the control of receptor internalization (Oldenhof et al., 1998). However, this study was unable to definitively link SH3 interactions with these events. Once it has been
determined which regions are necessary for SH3 binding, it will be possible to generate point mutations through site-directed mutagenesis that effectively uncouple the D3 receptor from SH3 (Feng et al., 1994). Both the point mutations and other SH3 binding domain-deleted constructs will prove valuable for study in whole-cell systems.

In the future, it will be interesting to determine which SH3 containing proteins (including the adapter, Grb2) functionally couple to the D3 receptor in the cell. This aspect of the interaction could be examined by co-immunoprecipitation techniques, searching for interactions with some common SH3 domains using available antibodies. An alternate approach is to use the yeast-two-hybrid system. In theory, this technique would allow screening for novel partners.

The fact that the third cytoplasmic loop has been found to be the source of the interaction is a potential source of interest. It will be interesting to investigate the relationship between SH3 interactions and G protein coupling for the receptor. For example, does SH3 act as a regulator of G protein coupling? Also, the third cytoplasmic loop is thought to become more accessible to intracellular signaling cascades upon agonist exposure. Thus, it will also be necessary to determine whether D3-SH3 binding is inducible by exposure to ligands.

Last, the Grb2 adapter protein has been linked to the MAP kinase pathway (see (Lopez-Ilasaca, 1998) for review). It will be necessary to test whether D3 mutants that have been uncoupled from the Grb2 adapter in vitro are similarly uncoupled from MAPK through this pathway.
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"If your experiment needs statistics, you ought to have done a better experiment"

-Ernest Rutherford