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CAP1 (1P4BP)/RASA3 Mediates Galpha (i)-Induced Inhibition of Mitogen-Activated Protein Kinase In Pituitary Cells

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GAP1(IP4BP)/RASA3 Mediates Galpha(i)-Induced Inhibition of Mitogen-Activated Protein Kinase in Pituitary Cells

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This thesis is submitted as a partial fulfillment of the requirements for the degree of Master of Science in Neuroscience.

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Your file Votre référence
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To:

My parents, Mahboobeh and Eissa,

The reasons for my existence,

My sister, Haleh and my wife, Negin,

The meanings of my life.

Abstract

Activation of dopamine D2S receptor (short isoform) inhibits thyrotropin-releasing hormone (TRH)-stimulated p42/p44 mitogen-activated protein kinase (MAPK) phosphorylation in rat pituitary GH4ZR7 cells via Gαi3, but the underlying mechanism is not fully understood.

To identify novel Gαi3 effectors we performed a yeast two-hybrid screen on cDNA library from GH4ZR7 cells using constitutively active Gαi3 as bait and identified RASA3. Gαi3-RASA3 interactions were confirmed using different approaches including yeast mating/β-galactosidase assay, in vitro pull-down assay on bacterially expressed proteins, and co-immunoprecipitation with overexpressed or endogenous proteins in different mammalian cell lines.

To address RASA3 function in dopamine D2S receptor-induced inhibition of MAPK activity endogenous RASA3 was suppressed in GH4ZR7 cells. In these clones D2S-mediated inhibition of TRH-induced phospho-MAPK was reversed by 70-80% compared to parental cells.

Our results provide a novel mechanism for D2S-induced inhibition of MAPK and indicate that RASA3 links Gαi3 to inhibition of the powerful TRH-induced Ras/MAPK activation pathway.

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List of abbreviations

Abbreviation	Full name
aa	Amino acid
AADC	Aromatic L-amino acid decarboxylase
AC	Adenylyl cyclase
BH-4	Tetrahydrobiopterin
Btk	Bruton's tyrosine kinase
cAMP	Cyclic adenosine monophosphate
CAPRI	Ca^{2+}-promoted Ras inactivator
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary cell line
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CTX	Cholera toxin
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32
DAT	Dopamine active transporter
DOPA	Dihydroxy phenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DR	Dopamine receptor
EGF	Epidermal growth factor
ERK	Extracellular-signal regulated kinase
G protein	Guanine nucleotide binding protein
GABA	Gamma-aminobutyric acid
GAP	GTPase activating protein
$GAP1^{IP4BP}$	GAP1 inositol 1,3,4,5 tetrakisphosphate binding
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factor

GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	Glutathione-s-transferase
GTP	Guanosine triphosphate
HEK	Human embryonic kidney cell line
HVA	Homovanillic acid
IP3	Inositol 1,4,5-trisphosphate
IP4	inositol 1,3,4,5-tetrakisphosphate
IPSP	Inhibitory postsynaptic potentials
JNK	C-Jun N-terminal kinase
KDa	Kilodalton
LPA	Lysophosphatidic acid
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
OK	Opossum Kidney cell line
ORF	Open reading frame
OT	Olfactory tubercle
PDE	Phosphodiesterase
PGE1	Prostaglandin E1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PRL	Prolactin

PTX	Pertussis toxin
RASAL	Ras-GTPase-activating-like protein
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinases
SNc	Substantia nigra compacta
TH	Tyrosine hydroxylase
TM	Transmembrane
TRH	Thyrotropin releasing hormone
VIP	Vasoactive intestinal peptide
VTA	Ventral tegmental area

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CHAPTER I

GENERAL INTRODUCTION

Dopaminergic system

Brief history

Dopamine (DA) is an important neurotransmitter that belongs to a group called cathecholamines. Their distinctive structural characteristics are the single amine group, a nucleus of catechol and a side chain of ethylamine or one of its derivatives (Vallone et al., 2000). In the brain, dopamine controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. This neurotransmitter also plays multiple roles in the other organs as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility (Missale et al., 1998b). Dopamine and its consequent functions have been extensively studied since several pathological conditions such as Parkinson's disease, schizophrenia, Tourette's syndrome, and hyperprolactinemia have been linked to a dysregulation of dopaminergic transmission.

Dopamine was first discovered by Arvid Carlsson and Nils-Åke Hillarp at the Laboratory for Chemical Pharmacology of the National Heart Institute of Sweden, in 1952. It was named Dopamine because it was a monoamine, and its synthetic precursor was 3,4-dihydroxyphenylalanine (L-DOPA) (Benes, 2001). In 1957, Balschko suggested a discrete physiological function for DA and few years later, Hornykiewicz considered a role for DA in the CNS (Hornykiewicz, 1966). In the 1960s DA was identified biochemically in the brain and its neuronal localization was histochemically displayed (Lindvall and Bjorklund, 1978). Research in 1970s revealed new interesting evidence of stimulatory or non-stimulatory effects of DA on adenylyl cyclase (AC) activity in

different parts of the brain that led to divide DA receptors (DRs) into two categories: D1 receptors that stimulate AC upon binding to DA and D2 receptors which were not able to activate AC proteins (Spano et al., 1978; Kebabian and Calne, 1979).

Biosynthesis

The precursor for the synthesis of DA is the aromatic amino acid tyrosine. Dopamine is synthesized mainly in region of the central nervous system and the medulla of the adrenal glands from L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine 3-monooxygenase, which is often known by its former name tyrosine hydroxylase (TH). TH is considered the rate-limiting enzyme in this pathway (Feldman et al., 1997). Several endogenous mechanisms have been identified for the regulation of DA synthesis that involve modulation of TH activity. For example, DA and other catecholamines act as end-product inhibitors of TH by competing with the cofactor tetrahydrobiopterin (BH-4) for its binding site on the enzyme (Factor and Weiner, 2002). The second step is the decarboxylation of DOPA, catalyzed by the enzyme aromatic L-amino acid decarboxylase (AADC), which is often referred to as DOPA decarboxylase. The final product of this step is DA.

In neurons, dopamine is packaged after synthesis into vesicles, which are then released in response to the presynaptic action potential and influx of calcium ions (Kelly, 1993). There are three different inactivation mechanisms of neurotransmission: 1) uptake via a specific transporter; 2) enzymatic breakdown; and 3) diffusion. Uptake back to the presynaptic neuron via the dopamine transporter (also known as dopamine active transporter or DAT) has the major role in the inactivation of dopaminergic

neurotransmission (Missale et al., 1998b). DAT is a sodium-dependent membrane spanning protein that binds to DA and moves it from the synapse into a neuron by an electrochemical gradient that forces sodium into the cell and pulls dopamine along. The cytosolic dopamine will face either breakdown by an enzyme or be re-packaged into vesicles and reused. In the case of enzymatic breakdown, DA is subjected to a series of enzymatic metabolic conversion by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). The major metabolites of DA are products of O-methylation and/or oxidative deamination. Major products include 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid, or homovanillic acid (HVA). DOPAC is the major metabolite in the rat brain, while in human brain HVA is the major one (Watson and Wilk, 1975; Wilk and Stanley, 1978).

Dopaminergic brain circuits

DA constitutes about 80% of the catecholamine content in the brain (Vallone et al., 2000). Projections originating from brain areas that synthesize this neurotransmitter are localized in four major systems in the brain: nigro-striatal, mesolimbic, mesocortical and tubero-infundibular systems.

The nigro-striatal pathway projects axons from dopamine-synthesizing neurons of the midbrain nucleus, the substantia nigra compacta (SNc) to the dorsal striatum (caudate nucleus and putamen). This system has an important role in controlling locomotion and its degeneration causes Parkinson's disease, characterized by tremors, rigidity and akinesia (Lang and Lozano, 1998a).

The second system is the mesolimbic pathway, the bundle of dopaminergic fibers associated with the reward circuit. This pathway originates in the ventral tegmental area (VTA) and innervates several structures including ventral striatum (nucleus accumbens), the olfactory tubercle (OT) and parts of the limbic system. The mesolimbic pathway is important for motivating behaviors (Koob and Bloom, 1988; Koob, 1992).

The mesocortical pathway also originates in the ventral tegmental area, but projects to the frontal cortex and surrounding structures. This pathway seems involved in some aspects of learning and memory (Feldman et al., 1997; Lang and Lozano, 1998b).

A fourth dopaminergic pathway worth mentioning is the tubero-infundibular pathway, which connects the hypothalamus to the pituitary gland. Thus, DA is transported to the anterior pituitary where it acts on the pituitary cells including lactotrophs to influence the release and secretion of hormones, such as prolactin. The effect of dopamine on pituitary cells has been explained in details in the following parts, since our project mainly focuses on the inhibitory functions of dopamine neurotransmitter in those cells.

Dopamine receptors

The concept of dopamine receptors was first proposed in 1972 from biochemical studies showing that DA is able to increase adenylyl cyclase activity (Kebabian and Calne, 1979). Further pharmacological and biochemical findings revealed two different populations of dopamine receptors. One group that is positively coupled to AC and the other one, which is independent of the adenosine 3',5'-cyclic monophosphate (cAMP)-generating system. It was shown, in fact, that in the pituitary DA inhibited prolactin

secretion but did not stimulate cAMP formation (Missale et al., 1998b). As we mentioned before, in 1979, Kebabian and Calne (Kebabian and Calne, 1979) summarized these observations and divided dopamine receptors into two main groups: D1 receptors that stimulated AC and D2 group that were negatively coupled to this effector. Although few years later, using gene cloning procedures, three novel DA receptors, D3, D4 and D5 have also been characterized, detailed structural, pharmacological, and biochemical studies demonstrated that all DA receptor subtypes fall into one of the two initially recognized receptor categories (Missale et al., 1998b). Therefore, five distinct dopamine receptors (DRs) have been subdivided into two subfamilies, D1- and D2-like. The D1-like subfamily comprises D1- and D5-R, while the D2-like group includes D2-, D3- and D4-R. Dopaminergic ligands differentiate between the D1- and D2-like receptor subfamilies. However, most of them do not clearly discriminate between members of the same subfamily (Vallone et al., 2000). It is well established that at the molecular level, the subtypes within a class have common signaling properties and similarity of signal transduction pathways is one of the criteria by which subtypes are grouped into classes (Neve et al., 2004).

Dopamine receptors are G protein-coupled receptors (GPCRs), which upon activation by their specific ligands, mediate signals to intracellular effectors mainly by interaction with and activation of heterotrimeric GTP-binding proteins (G proteins). They are members of 7-transmembrane receptors superfamily since they have 7 hydrophobic domains that are predicted to traverse the cell membrane. They all also contain an extracellular N-terminus, an intracellular C-terminus, three intra and three extracellular loops (Please see figure A in Appendices). Analysis of DA receptor structure pointed to

similarities and dissimilarities between D_1-like and D_2-like receptors (Missale et al., 1998b). In the following sections, structural and functional characteristics of each group will be reviewed.

D1-like receptor subfamily

D1-like subfamily was first described as receptors that stimulate adenylyl cyclase in response to a dopamine agonist. Because Gαs is ubiquitously expressed, the ability of D1-like receptors to increase cAMP production in virtually any cell line (Huff, 1997), together with physical and functional coupling of D1-like receptors to Gαs (Sidhu, 1998; Jin et al., 2001), suggests the role of this G protein α subunit to mediate D1-like receptor signaling. But further information revealed that in some tissues like neostriatum, nucleus accumbens and olfactory tubercle, there is a very low Gαs protein expression, while they have significant expression of D1 receptor (Zhuang et al., 2000; Herve et al., 2001). Thus, another Gα subunit, $G\alpha_{olf}$, that was abundantly expressed in those tissues has been suggested as another mediator of D1-like receptor signal to adenylyl cyclase since it is closely related to Gαs and also activates adenylyl cyclase (Jones and Reed, 1989; Neve et al., 2004).

In contrast to the genomic structure of the D2-like receptors, D1-like receptors do not contain introns in their coding regions (Missale et al., 1998b; Vallone et al., 2000) that is a common feature in most G protein-coupled receptors (Dohlman et al., 1987). The D1 receptor has the most widespread and highest level of expression of any of the DA receptors (Dearry et al., 1990; Weiner et al., 1991; Vallone et al., 2000). The D1 receptor

is mainly expressed in striatum (caudate and putamen), nucleus accumbens, olfactory tubercle, cerebral cortex and amygdale (Jackson and Westlind-Danielsson, 1994). The expression of D1R is not limited to central nervous system (CNS) and it is also detected in blood vessels, different regions of kidney and adrenal gland (Missale et al., 1998b). D5 receptor has a more restricted pattern of expression and is mainly found in the hippocampus, the lateral mammillary nucleus, and the parafascicular nucleus of the thalamus (Jackson and Westlind-Danielsson, 1994; Vallone et al., 2000).

Analysis of DA receptor structure showed that D1 and D5 receptors share an 80% identity in their TM domains (Missale et al., 1998b). Both receptors have an extracellular NH2 terminal with the same length, which contains an N-glycosylation site. The prominent structural differences between D1-like and D2-like receptors are long C-terminal tail and short third intracellular loop of D1 and D5 receptors. The latter feature is common in many GPCRs that couple to Gαs protein. The first and second cytoplasmic loops of D1-like receptors, unlike their C-terminal tail and the third intracellular loop, are highly conserved suggesting the important role of the latter structural group in different biological functions of these receptors (Missale et al., 1998b).

Signal transduction

The first biochemical evidence for a dopamine receptor (later known as D1 receptor) was the identification of dopamine-stimulated adenylyl cyclase activity in the retina and rat neostriatum (Brown and Makman, 1972; Kebabian et al., 1972). Its stimulation results in the activation of the protein kinase A (PKA). PKA phosphorylates a number of proteins involved in signal transduction and regulation of gene expression

(Neve et al., 2004). This signaling pathway is mediated by mainly Gαs or $G\alpha_{olf}$, although it seems that βγ subunit also plays an important role. For example, in human embryonic kidney (HEK) 293 cells, depletion of endogenous γ7 subunit reduces D1 receptor stimulation of adenylyl cyclase (Wang et al., 2001).

Activation of D1 receptor leads to several other intracellular signaling effects that we summarize here.

In general, D1-like receptors decrease potassium currents in most cells via stimulation of the protein kinase A-Dopamine and cyclic AMP-Regulated Phosphoprotein 32 kDa (PKA-DARPP-32) signaling cascade that is the opposing effect of D2-like receptors on potassium currents (Neve et al., 2004). D1 receptor stimulation also increases L-type and decreases N, P/Q-type Ca^{2+} channel conductance in most brain areas (Neve et al., 2004).

D1 receptor also regulates cellular activity through manipulation of the phosphorylation state of voltage-gated Na^+ channels. For example, in the neostriatum and hippocampus, D1-PKA-DARPP-32 pathway increases phosphorylation of Ser573 of the Na^+ channel α-subunit. It attenuates transient Na^+ currents and subsequently, excitability of cells (Calabresi et al., 1987; Schiffmann et al., 1995; Cantrell et al., 1997; Schiffmann et al., 1998).

D1-like receptors increase NMDA receptor-mediated responses in neostriatal, hippocampal and cortical neurons (Neve et al., 2004). However, D1R has various effects on $GABA_A$ currents depending on the brain area and cell type being examined. For instance, D1-like receptor stimulation decreases GABA receptor activation in medium spiny neurons from neostriatum and nucleus accumbens, while in large cholinergic

interneurons of the neostriatum, D1-like agonists have been reported to have no effect on GABA receptor inhibitory postsynaptic potentials (IPSPs) (Flores-Hernandez et al., 2000; Pisani et al., 2000; Neve et al., 2004).

There are several reports describing activation of mitogen-activated protein kinases (MAPKs) including ERK, p38 MAP kinase and C-Jun N-terminal kinase (JNK) by D1-like receptors (Zhen et al., 1998; Gerfen et al., 2002). Regulation of the latter two MAP kinase pathways is mediated by PKA, whereas D1-like receptor activation of ERK may be partially independent of PKA but cAMP dependent via activation of Rap GTPase by the cyclic AMP-activated guanine nucleotide-exchange factor Epac (de Rooij et al., 1998; Neve et al., 2004; Weissman et al., 2004).

D2-like receptor subfamily

By 1978, two distinct effects of dopamine agonists on adenylyl cyclase activity in different cells revealed that there are two separate groups of DA receptors. The family that inhibits AC activity is the D2-like receptor group, which comprises D2, D3 and D4 receptors. Among DA receptors, the rat D2-R cDNA was the first to be isolated (Bunzow et al., 1988). Unlike D1-like receptors, the genes encoding the D2, D3 and D4 receptors are interrupted by six, five and three introns respectively (Missale et al., 1998b). In this section we have briefly reviewed some important characteristics of D3 and D4 receptors. D2 receptor has been discussed in details in the following part.

The rat D3 receptor is 466 amino acid (aa) protein and the gene was first cloned from a rat brain cDNA library (Vallone et al., 2000). The D2 and D3 receptors have a

75% protein identity in their TM domains. The human D3 receptor is located on chromosome 3 (Missale et al., 1998b). The distribution of D3 receptor mRNA expression is limited to few areas in the brain such as the islands of Calleja, a few septal nuclei, hypothalamus, and distinct regions of the thalamus and cerebellum (Jackson and Westlind-Danielsson, 1994).

D4 receptor gene is composed of three introns and five coding exons and generates a 387 aa protein. The D2 and D4 receptors share a 53% identity in the TM domains (Missale et al., 1998b). The D4 receptor demonstrates a high expression in the frontal cortex, amygdala, the olfactory bulb, hippocampus, hypothalamus and mesencephalon (Jackson and Westlind-Danielsson, 1994).

D2 receptor

The D2 receptor is a member of D2-like family of DA receptors. The first and original rat D2R cDNA clone contained an open reading frame (ORF) of 1245 nucleotides encoding a 415 aa protein. Later, several groups isolated a spliced variant of this cDNA from different species like rat, mouse, bovine and human and in different tissues including brain, pituitary and retina that encodes a protein that is 29 aa longer. These two variants were named short (D2S) and long (D2L) D2 receptors, respectively (Vallone et al., 2000). The D2-R gene is composed of eight exons, seven of which are coding (Gingrich and Caron, 1993). The alternative splicing of an 87-bp exon between introns 4 and 5, in the sixth exon, generates the D2S and D2L isoforms (Missale et al., 1998b). The two D2 isoforms have identical pharmacology and appear to share

equivalent signaling pathways. The regulatory process of the alternate splicing of the D2 receptor transcript is not completely understood.

The D2 receptor has been found mainly in brain tissues, such as the striatum (caudate and putamen), olfactory tubercle and the core and the shell of nucleus accumbens. This receptor is also expressed in the substantia nigra pars compacta and in the ventral tegmental area (VTA). These are the anatomical regions that give rise to dopaminergic fibers, indicating that, unlike D1-like receptors, the D2 receptors have also a presynaptic location (Vallone et al., 2000). Colocalization with D1 receptors is rare (Missale et al., 1998b), although functional and/or anatomical colocalization of D2R with D1R in striatum has been proposed (Aizman et al., 2000).

D2 receptor localization is also detected outside the brain in different tissues like retina, kidney, vascular system and pituitary gland (Vallone et al., 2000) (for dopamine receptors in pituitary cells, please see the following parts).

By using subtype-specific antibodies against different isoforms of D2 receptor, it is shown that D2 short receptor predominates in the cell bodies and projection axons of the dopaminergic cell groups of the mesencephalon and hypothalamus, whereas the D2 long is more strongly expressed by neurons in the striatum and nucleus accumbens, structures targeted by dopaminergic fibers (Khan et al., 1998a). Moreover, the D2S isoform has much higher expression than D2L in substantia nigra. The localization of D2S receptor in plasma membrane of cell bodies and axons of dopaminergic neurons indicates an autoreceptor role for this protein, whereas presence of D2L isoform in non dopaminergic neurons indicates its main function as a postsynaptic receptor (Khan et al., 1998b; Khan et al., 1998a).

Like other DA receptors, D2R has an extracellular NH2 terminal. There are four potential N-glycosylation sites on it. D2R C-terminal tail is much shorter than D1-like receptors' tail and contains a cysteine residue near its end. The C-terminus in both subfamilies contains phosphorylation and palmitoylation sites which are thought to be involved in agonist-dependent receptor desensitization (Vallone et al., 2000). D2R has a long third intracellular loop that is a common feature of receptors coupling to Gi proteins to inhibit adenylyl cyclase. It also contains, like other GPCRs, two cysteine residues on the second and third extracellular loops, which have been suggested to form a disulfide bridge to stabilize the receptor structure (Civelli et al., 1993; Gingrich and Caron, 1993; Missale et al., 1998b).

Signal transduction

D2-like receptor signaling is mediated primarily by activation of the heterotrimeric G proteins Gαi/o since most actions of this receptor can be blocked by pertussis toxin (PTX)-catalyzed ADP-ribosylation (Bokoch et al., 1983; Kurose et al., 1983)). D2 receptor has short and long isoforms, which differ by an alternately spliced 29- amino acid sequence located in the third intracellular loop of the D2L receptor (Civelli et al., 1993). Because the i3 loop is implicated in receptor-G protein coupling, this 29 aa insert may lead to different G protein selection by D2S and D2L, although both variants have virtually identical pharmacology and signaling properties (Civelli et al., 1993; Albert, 1994). One of the only major differences is in receptor desensitization, in which the D2S receptor is more sensitive to protein kinase C (PKC)-induced uncoupling and agonist-induced internalization (Liu et al., 1992; Ito et al., 1999). Recently, in GH4

pituitary cells separately transfected with D2L and D2S receptors, it is shown that activation of rat or human D2S inhibits thyrotropin-releasing hormone-induced extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylation, while the D2L receptor failed to inhibit this response (Van et al., 2007). It seems that both receptor isoforms are able to activate multiple Gαi/o subtypes, including Gαi2, Gαi3, and Gαo (Lledo et al., 1992; Liu et al., 1994b), but that interactions with particular G proteins are restricted in a cell-type dependent manner due to compartmentalization or the availability of appropriate effectors and scaffolding proteins (Neve et al., 2004). D2 isoforms are also able to activate Gαz that explains reports of pertussis toxin-insensitive signaling by the D2 receptor (Wong et al., 1992; Senogles, 1994a; Obadiah et al., 1999).

D2 Receptor Regulation of Adenylyl cyclase

The first signaling pathway identified for D2 receptor was inhibition of adenylyl cyclase activity (De Camilli et al., 1979; Stoof and Kebabian, 1981). It is shown that genetic deletion of adenylyl cyclase 5 abolishes D2 receptor-mediated inhibition of adenylyl cyclase in the mouse neostriatum (Neve et al., 2004). D2 receptor inhibits adenylyl cyclase activity in a variety of tissues and cell lines (Huff, 1997). D2 receptor couples negatively to AC in a PTX-sensitive manner that indicates the role of Gαi subunits in this pathway (Taussig et al., 1994). However, new evidence also suggests the role of Gβγ subunits to stimulate types V and VI adenylyl cyclases (Gao et al., 2007). This receptor activation results in depletion of cAMP production via inhibition of stimulated or basal adenylyl cyclase activity. Since the D2 receptor can bind to any subtype of Gαi/o proteins, subtype specificity of signaling has been studied in different cell lines (Albert, 1994; Huff, 1996; Robinson and Caron, 1997). For instance, in rat

pituitary GH4C1, D2 receptor stimulation inhibits basal and Gαs- or forskolin-stimulated AC (Liu et al., 1994b; Senogles, 1994a; Liu et al., 1999a). By using PTX-insensitive Gαi/o mutants, Banihashemi et al showed in GH4ZR7 cells (rat pituitary cells stably transfected by D2S), activation of D2S receptor did not alter very low level basal cAMP production but almost completely suppressed forskolin-stimulated cAMP production through Gαi2 and, to a lesser extent, Gαi3 (Banihashemi and Albert, 2002b). It has also been shown that different Gαi subunits mediate the inhibitory response of dopamine agonists in Balbc/3T3 cells stably transfected with the D2S expression plasmid (BALB-D2S). By using BALB-D2S clones stably expressing PTX-insensitive mutants of Gαi/o subtypes, Ghahremani et al identified an important role for Gαi2, Gαi3 to mediate D2S-induced inhibition of forskolin-stimulated cAMP production, while Gβγ plays a minor role (Ghahremani et al., 2000).

It is worth mentioning that the D2 receptor may use Gβγ subunit to stimulate AC activity. The contribution of this pathway to D2 receptor signaling in neurons is shown by Hopf et al. when they found dopaminergic enhancement of spike firing of nucleus accumbens neurons was prevented by inhibitors of protein kinase A or Gβγ subunit. It seems that cooperation of D1 and D2 receptor stimulation through Gαs or Gαi/o proteins may activate adenylyl cyclase, resulting in enhancement of spike firing (Hopf et al., 2003).

D2 Receptor Regulation of Intracellular calcium mobilization and calcium channels

Activation of D2 receptors induces distinct effects on the intracellular calcium ion level depending on the cell type studied. It has been shown that in cells of mesenchymal origin, D2 receptor activation increases $[Ca^{2+}]_i$. Liu et al. and Vallar et al. demonstrated that in Ltk fibroblast cells, both D2S and D2L receptors induce a rapid stimulation of

inositol(1,4,5)-trisphosphate results in immediate increase of $[Ca^{2+}]_i$ (Vallar et al., 1990; Liu et al., 1992). In D2S transfected BALB/c-3T3 fibroblast cells, likewise, dopamine induced an immediate threefold increase in $[Ca^{2+}]_i$ (Ghahremani et al., 2000). All these effects were blocked significantly by PTX pretreatment, indicating signaling via Gαi/o proteins. It should be mentioned that in BALB-D2S cells, using PTX-insensitive Gαi/o subunits, none of the mutant G proteins rescued the D2S-mediated calcium response after pretreatment with PTX, indicating the possible role of Gβγ subunits in this pathway. The hypothesis was confirmed by using a free Gβγ inactivator, GRK-CT, that considerably reduced dopamine-induced $[Ca^{2+}]_i$ (Ghahremani et al., 2000).

D2 receptor also causes a decrease in intracellular calcium levels by inhibition of inward calcium currents or calcium release from intracellular storage organelles. D2 receptor decreases the activity of L, N, and P/Q-type channels via pertussis toxin-sensitive G proteins (Lledo et al., 1992; Neve et al., 2004). This inhibitory effect seems to be mediated by Gβγ subunit. In GH4C1 cells (Vallar et al., 1990; Seabrook et al., 1994a), NG108-15 cells (Seabrook et al., 1994b), melanotrophs (Williams et al., 1990) and pituitary lactotrophs (Lledo et al., 1992), D2 receptor activation inhibits calcium currents. Although, it is shown D2 receptor has no effect on phosphatidylinositol turnover in many cell lines, this receptor inhibits production of inositol 1,4,5-trisphosphate (IP3) in GH4C1 cells to support inhibitory effect of D2 receptor on calcium channels (Canonico et al., 1983; Enjalbert et al., 1990). In D2S stably transfected rat pituitary cells, GH4ZR7 cells, using PTX-insensitive mutants of Gαi/o subunits or GRK-CT (Gβγ subunit inactivator), revealed that D2S inhibits L-type calcium channels through Gαo and Gβγ subunits (Banihashemi and Albert, 2002b). This is consistent with previous studies using

antisense to Gαo which selectively blocked D2S-induced inhibition of L-type channel activation (Liu et al., 1994b).

D2 Receptor Regulation of Mitogne-Protein Activated Kinase (MAPK)

MAP kinases are Serine/Threonine directed protein kinases that transmit various signals from different stimuli to the cell nucleus and they are involved in many cellular functions, including cell proliferation, differentiation, apoptosis and long term potentiation, etc.

Like many GPCRs, D2 receptor regulates MAPK activity mainly through Gαi/o proteins. The effects of dopamine agonists on MAPK phosphorylation are different depending on cell type and receptor subtype. Since my project is mainly focused on D2R-G protein-MAPK relationship, D2R-induced regulation of MAPK is discussed in detail in a separate section.

D2 Receptor Regulation of Potassium Channels

D2 receptor stimulation exerts a powerful influence on many signaling pathways. It is demonstrated that D2R activation increases outward potassium currents, leading to cell hyperpolarization. This effect has been observed in midbrain dopamine neurons (Lacey et al., 1987), (Liu et al., 1994a), neostriatal D2 receptor-expressing neurons (Greif et al., 1995), and also in rat pituitary lactotrophs (Castelletti et al., 1989; Einhorn et al., 1991) or melanotrophs (Williams et al., 1989). This regulatory system appears to be modulated by G protein mechanisms (Missale et al., 1998b). Like most of the G protein-mediated signaling pathways, different G protein subunits are involved in potassium channel activation in various cell lines. For instance, in anterior pituitary cells, Gαi3

mediates D2-induced increase outward potassium currents (Lledo et al., 1992), while in primary culture of rat mesensephalic neurons Gαo appears to be responsible for this pathway (Liu et al., 1994a).

D2 Receptor Regulation of Arachidonic Acid release

In addition to the signal transduction pathways mentioned before, D2 receptor can also modulate arachidonic acid synthesis. It has been observed that in rat striatal cells and CHO cells, release of arachidonic acid is increased upon activation of D2 receptor that in CHO cells raises intracellular Ca^{2+} concentration (Piomelli et al., 1991; Vallone et al., 2000). Because PTX strongly abolishes D2-induced arachidonic acid release, Gαi/o subunits are likely to be involved in this regulatory pathway (Missale et al., 1998b).

D2 Receptor Regulation of Na^+/H^+ Exchange

D2 receptor also modulates Na^+/H^+ exchangers, which are responsible for regulation of intracellular pH, transcellular Na^+ absorption and cell volume. In D2R-transfected C6 gliomas, Ltk- cells (Neve et al., 1992) and in anterior pituitary cells (Ganz et al., 1990), D2 receptor activation potentiates Na^+/H^+ exchange that in some cases, including primary lactotrophs, is not PTX sensitive (Ganz et al., 1990; Lin et al., 2003). However, in CHO cells, D2 receptor increases extracellular acidification that is blocked by pertussis toxin (Chio et al., 1994b). Therefore, D2 receptor recruits different downstream effectors in different cells to stimulate Na^+/H^+ exchanger.

D2 Receptor Regulation of Phospholipases

D2 receptor also regulates activation of phospholipase in different cells. It is shown that in neostriatal medium spiny neurons, D2R stimulates cytosolic phospholipase Cβ1 (PLCβ1) via Gβγ subunit. Activated PLCβ1 subsequently causes inositol 1,4,5-trisphosphate-induced calcium mobilization (Neve et al., 2004). In Chinese Hamster Ovary (CHO) cells, it has been observed that D2 receptor activates phospholipase A2 that results in potentiation of arachidonic acid release in a PKC, calcium-dependent manner (Piomelli et al., 1991; Vial and Piomelli, 1995), (Kanterman et al., 1991). Finally, D2 receptor stimulates phospholipase D (PLD), which cleaves phosphatidylcholine to form choline and phosphatidic acid (Neve et al., 2004). Senogles et al. showed that short isoform D2 activates PLD that, with low molecular weight G protein RhoA, was involved in anti-proliferative effects of D2S in HEK 293 cells (Senogles, 2003).

D2 Receptor Regulation of mitogenesis and cell proliferation

D2 receptor has also been suggested as an important factor in mitogenesis and cell differentiation. D2 receptors increase [3H]thymidine incorporation in CHO cells (Chio et al., 1994a), (Lajiness et al., 1993), Opossum Kidney (OK) cells (Narkar et al., 2001)) or BALB/c-3T3 cells (Ghahremani et al., 2000) after binding to a dopamine agonist. This regulator pathway can be blocked by PTX indicating the essential role of Gαi/o proteins. Lajiness et al. demonstrated that D2R-induced mitogenesis in CHO cells was cAMP independent but was accompanied by an increase in tyrosine phosphorylation levels that was blocked by tyrosine kinase inhibitor genistein (Lajiness et al., 1993). Furthermore, Narkar et al found that PD 98059 (a selective noncompetitive inhibitor of the MAPK pathway) also suppressed mitogenic effect of D2 receptor in OK cells suggesting the

involvement of p44/42 mitogen-activated protein kinase (MAPK) in this mitogenic response (Narkar et al., 2001). Ghahremani et al. have also shown that persistent activation of D2S receptors in BALB/c-3T3 cells induced cellular transformation that was demonstrated by an increase in focus formation. By using PTX-insensitive Gαi/o mutants, they found that Gαi3 had an important role in mediating D2S-induced cell transformation (Ghahremani et al., 2000).

Contradictory to these results, cell growth inhibition upon D2 receptor activation has been shown in some cell lines. In rat pituitary GH4C1 cells stably transfected with the rat D2 dopamine receptor cDNA, Florio et al. found dopaminergic inhibition of DNA synthesis and concurrent stimulation of phosphotyrosine phosphatase activity. Both of these responses were blocked by PTX or a phosphotyrosine phosphatase inhibitor (vanadate) pretreatment, indicating phosphotyrosine phosphatase as a possible mediator for anti-proliferative action of dopamine (Florio et al., 1992). On the other hand, Senogles et al. observed PTX-insensitive inhibition of [3H]thymidine uptake upon activation of D2S receptor in GH4ZR7 cells that was blocked by down regulation of cellular PKC or treating with PKC inhibitors, such as staurosporine and H7 (Senogles, 1994b). To support Florio's findings, Albert observed suppression of D2-induced inhibition of DNA synthesis after PTX treatment in GH4ZR7 cells, suggesting that the PTX-insensitive activation of protein kinase C epsilon is not the main cellular response to mediate D2 anti-proliferative effect. He demonstrated that depletion of any single $G\alpha_i$ or $G\alpha_o$ subtype abolishes inhibition of DNA synthesis, suggesting that multiple pathways may be involved (Albert, 2002b).

Guanine nucleotide-binding proteins (G proteins)

It is well observed that the maintenance of homeostasis in multicellular organisms is dependent on the continual flow and processing of information through a complex network of cells. Furthermore, to respond to an ever-changing environment, all intercellular signals must be transduced, amplified, and ultimately converted to the appropriate physiological response (Cabrera-Vera et al., 2003). The minimum requirements to send any kind of signal through the cell membrane are three main components: a receptor, an effector and an intermediate signal transducer (Gilman, 1987; Bourne, 1997).

The G-protein-mediated signaling system is one of the most widely used transmembrane signaling mechanisms in mammalian organisms and operates in every cell of these organisms. (Offermanns, 2003). The highly conserved group of molecules known as heterotrimeric guanine nucleotide-binding proteins (G proteins) is the key determinant of this sophisticated molecular machine which is able to receive, to integrate and to process information carried by extracellular signals. Numerous hormones, neurotransmitters, chemokines, autocrine and paracrine factors or sensory stimuli bind to a large superfamily of receptors with seven membrane-spanning regions that activate the G proteins, which consequently, mediate the signals to several distinct intracellular signaling pathways. Changes in the activity of these effectors finally results in changes in cellular functions ranging from short term effects like the control of secretion rates, muscle tone or metabolic processes to long term effects like regulation of growth and differentiation (Offermanns, 2003). These cellular functions in turn regulate

physiological processes such as learning and memory, and organismal homeostasis (Neves et al., 2002).

Brief history

The first receptor-sensitive signal transduction system was reported by Sutherland et al. in 1956 (Milligan and Kostenis, 2006). In the late 1950s and early 1960s, it was known that a broad range of hormones were able to stimulate production of cyclic AMP (cAMP). At that time, Rodbell and Birnbaumer proposed a heterotrimeric complex, consisting AC-independent receptor molecules, for hormone-sensitive adenylyl cyclase (AC) (Birnbaumer and Rodbell, 1969; Rodbell et al., 1970). They found a complex and poorly known model for hormone-mediated signal transduction. At the same time, it was reported that GTP was also important in regulation of the hormone-induced stimulation of AC (Rodbell et al., 1971b; Rodbell et al., 1971a). Meanwhile, it was reasoned that GTP was not a part of the receptor or the effector (in that case PGE1 and AC, respectively), but it could be a transducer or a mediator for that pathway (Krishna and Harwood, 1972). Later, several reports from other scientists revealed that GTP was not restricted to one type of receptor and it would be important in modulation of different receptors (Lefkowitz et al., 1976; Maguire et al., 1976; Ross and Gilman, 1977; Wheeler and Bitensky, 1977). Eventually, Ross and Gilman isolated an approximately 40-kDa protein with the characteristics of a GTP binding protein (Ross and Gilman, 1977).

G protein classification

In the first days of identification of G proteins, it was shown that they act at two AC regulatory pathways; stimulation and inhibition of AC (Birnbaumer, 1990). Those that increased or decreased AC activity were called the Gs or Gi proteins, respectively. After the first four G proteins (Gs, Gt, Gi, and Go) were identified by biochemical purification, a large number of G proteins and their subunits were identified by cDNA cloning (Neves et al., 2002). More recently, heterotrimeric G proteins are typically divided into four main categories based on the primary sequence similarity of the Gα subunits: Gαs, Gαi, Gαq and Gα12,13 (Simon et al., 1991). Members of this family range in size from 39–52 kDa and share between 35% and 95% sequence identity (Oldham and Hamm, 2006).

Structural composition

Heterotrimeric G-proteins consist of an α-subunit which binds and hydrolyses guanosine triphosphate (GTP) as well as of a β- and a γ-subunit (Hepler and Gilman, 1992; Gudermann et al., 1996; Offermanns, 2003). The Gα subunit has structural and functional similarities to other members of the guanine nucleotide binding protein superfamily. Unlike Gα subunit, β- and γ-subunits of heterotrimeric G-proteins form an undissociable complex and represent a functional unit (Offermanns, 2003). In spite of early findings suggesting that only Gα subunits are involved in signal transduction, it is well established now that both Gα and Gβγ subunits participate in a variety of signaling

pathways to provide a highly developed network between G protein mediating systems and other cellular signaling pathways.

Gα subunit

According to our knowledge, more than 20 Gα subunits encoded by 17 different genes are known (Cabrera-Vera et al., 2003), (Venter et al., 2001). They can be divided into four main classes: Gαi/o composed of Gαi1, Gαi2, Gαi3, GαoA, GαoB, Gαz, Gαt1, Gαt2 and Gαg; Gαs including Gαs(S), Gαs(L) and $G\alpha_{olf}$; Gαq consisting Gαq, Gα11, Gα14 and Gα15 or 16; and finally Gα12 having Gα12, Gα13 (Simon et al., 1991; Cabrera-Vera et al., 2003). The range of molecular weights of these proteins is between 39 to 52 kDa and there is 45-80 % identity between family members (Rens-Domiano and Hamm, 1995).

All of the Gα subunits reveal a conserved protein folding composed of two domains, a GTPase domain that is involved in the binding and hydrolysis of GTP and α helical domain to bury the GTP within the core of the protein. The GTPase domain is structurally similar to the family of monomeric G proteins (Oldham and Hamm, 2006). It is composed of a six-stranded β-sheet surrounded by five α-helices. This domain contains highly conserved sequences for binding to inactive Guanine nucleotide diphosphate (GDP), the $Mg2^{+}$-binding domain and the guanine ring-binding motifs. It also hydrolyzes GTP and contains sites for binding to the Gβγ dimer, heptahelical receptors and downstream effector proteins (Oldham and Hamm, 2006). There are three flexible loops near the γ-phosphate binding site on this domain, called Switches I, II and III. These loops undergo significant conformational changes upon GTP-Gα interaction (Coleman et al., 1994; Lambright et al., 1994; Mixon et al., 1995). Mutations in the Switches decrease

the affinity of Gα for Gβγ subunits and leads to dissociation of the trimeric complex (Hamm, 1998). The helical domain consists of six α-helices that form a lid over the nucleotide-binding site, burying bound nucleotides in the core of the protein (Oldham and Hamm, 2006). It has been shown that this domain increases the affinity of Gα for guanine nucleotides (Warner et al., 1998; Remmers et al., 1999) and enhances the GTPase activity of the protein (Markby et al., 1993). Since this domain is the most divergent domain among Gα families, it may play a significant role in directing specificity of receptor- and effector-G protein coupling (Liu et al., 1998).

Gβγ subunit

Gβγ subunit consists of two bound polypeptides that are not dissociable except by denaturation Schmidt, 1992. However, these subunits act functionally and biologically as a monomer. To date, six Gβ subunits, with the average of 36 kDa molecular weight, have been identified. At the amino acid level, they share 50-90% sequence identity, with the long and short splice variants of Gβ5 being the least similar to the other four members of the family (Oldham and Hamm, 2006). The Gß-subunit of heterotrimeric G proteins has a seven-bladed ß-propeller structure containing seven WD-40 repeats (Cabrera-Vera et al., 2003).

In contrast to Gβ subunits, members of the Gγ family are all small proteins, between 7 and 8 kDa, which share 30–80% sequence identity (Downes and Gautam, 1999). Therefore, they have been suggested to determine the functional specificity of the Gβγ subunits. They contain two α-helices connected by a loop. The Gγ-subunits interact with the Gß-subunit through an N-terminal coiled coil and makes extensive contacts

along the base of the Gß-subunit (Cabrera-Vera et al., 2003), which may explain the difficulty in dissociating the two subunits from each other (Clapham and Neer, 1997). The Gßγ dimer binds to a hydrophobic pocket present in Gα-GDP. GTP binding to Gα removes the hydrophobic pocket and reduces the affinity of Gα for Gßγ (Lambright et al., 1994), (Cabrera-Vera et al., 2003). Cloning of different Gβ and Gγ subunits raised the possibility of having various combinations of βγ dimers. Although most β subunits can interact with most γ subunits, there are preferred combinations of isoforms that interact to form a more limited number of distinct dimers. For example, Gγ1 interacts with Gβ1, but not Gβ2 despite its high (87%) sequence identity to Gβ1, and Gβ2 binds to Gγ2 in spite of its similarity to Gγ1 (41% identity) (Pronin and Gautam, 1992; Schmidt et al., 1992; Garritsen and Simonds, 1994; Downes and Gautam, 1999; Oldham and Hamm, 2006).

G protein activation-inactivation cycle

In a typical G protein-mediating signaling pathway, the G protein acts as a controllable molecular switch by coupling/uncoupling the transmembrane heptahelical receptor and intracellular effectors. This role depends on the ability of G-protein α subunits (Gα) to cycle between a resting and an activated state. In the resting state, the Gβγ subunit and the guanosine diphosphate (GDP)-bound α-subunit are associated. This resting conformation is primed for interaction of G protein heterotrimer with an activated transmembrane receptor, which is already stimulated by extracellular stimuli, such as hormones, neurotransmitters, chemokines, light and odorants. This interaction leads to the dissociation of GDP from the α-subunit of the heterotrimeric G-protein. GDP is then replaced by GTP that subsequently induces a conformational change. As a result, GTP-α

subunit complex dissociates from both Gβγ subunits and the receptor. The GTP-bound α-subunit, as well as the βγ-complex, are now able to interact with downstream effectors and regulate their cellular functions (Cabrera-Vera et al., 2003).

Gα protein has a weak intrinsic GTPase activity that leads to hydrolysis of GTP to GDP, reassociation of α and βγ subunits and finally termination of G protein signaling (Please see figure B in Appendices). In the past few years, the application of two well-known bacterial toxins in the field of G protein signaling has given an excellent opportunity to identify the role and function of individual G protein family members. Pertussis toxin produced by Bordetella pertussis catalyzes the adenosine diphosphate (ADP)-ribosylation of the C-terminal cysteine in Gαi family members resulting in uncoupling of receptor and G-protein (West et al., 1985). On the other hand, Cholera toxin from Vibrio cholerae ADP-ribosylates the α-subunit of various G-protein subtypes, including Gαs and Gαt , at an arginine residue at an internal site leading to blockade of its GTPase activity and, consequently, the constitutive activation of the α-subunit (Van Dop et al., 1984; Freissmuth and Gilman, 1989).

G protein coupling

Coupling to upstream receptors

Around 900 genes in human encode G-protein-coupled receptors (Milligan and Kostenis, 2006). These receptors have seven transmembrane spanning domains, with an extracellular N-terminus, intracellular C-terminus and three interhelical loops on each

side of the membrane. They mediate various signals from numerous types of extracellular stimuli to several kinds of intracellular G protein complexes.

Compared to the large number of GPCRs, relatively few types of G proteins mediate signals from many heptahelical receptors. Therefore, each of the G-protein family members must be capable of interacting with many different receptors. It should be mentioned that many receptors are also capable of activating multiple G-protein signaling pathways. These concepts present a very complex network of interacting proteins. The specificity of these interactions is essential for proper signal transduction.

Despite extensive research, the exact mechanism of GPCR-G protein interaction is still not fully understood. As we mentioned, conformational changes in Gα subunit causes release of GDP. It has been shown that the receptor contacts Gα at a site that is more than 20 Å away from the guanine nucleotide binding site (Hamm, 1998; Yang et al., 1999), thus working at a distance to release GDP. One of the theories is that receptor binds the C terminal tail of the Gα-subunit leading to conformational changes that are propagated through Gα to the GDP binding site (Cabrera-Vera et al., 2003). Another issue is the requirement for Gβγ in receptor-G protein interaction that suggests Gβγ may actively participate in GDP release by opening an exit route for the guanine nucleotide to leave the complex (Bourne, 1997). The Gαβγ complex contains a prominent cavity between Gα and Gßγ subunits that is believed to be oriented toward the plasma membrane (Lambright et al., 1996; Bohm et al., 1997). Activated loops of the receptor might use this cavity to tilt Gßγ away from Gα causing the contacts between Gα and Gßγ to be disrupted, including contacts near switch I and the ß3-α2 loop in Gα, the potential

exit route for the nucleotide (Cabrera-Vera et al., 2003). Thus, the Gßγ-dimer actively participates in receptor-mediated G protein activation.

Coupling to the downstream effectors

Until the mid-1990's, researchers believed that the α subunit of G proteins was the primary signaling component. However, it has been shown that following subunit dissociation, both Gα-GTP and Gβγ subunits go on to activate specific downstream effector proteins.

An interesting observation regarding effector activation revealed that the GDP-bound Gα subunit has some ability to interact with effectors, although with 20- to 100-fold reduced potency than the GTP-bound protein (Skiba et al., 1996; Sunahara et al., 1997). This finding suggests that reassociation of Gα with Gßγ is essential for the complete termination of Gα signaling, and inhibition of reassociation could prolong both Gα- and Gßγ-mediated signaling (Cabrera-Vera et al., 2003).

It has been well established that within a family, even closely related Gα subunits stimulates unique complements of effectors. For instance, Gαi2 is required to inhibit forskolin-stimulated AC activity whereas Gαi3 serves to inhibit Gαs-activated AC (Ghahremani et al., 1999). In addition, the α1-adrenergic receptor elevates intracellular Ca^{2+} through two distinct mechanisms by coupling to Gαq and Gα11 (Macrez-Lepretre et al., 1997). Finally, some Gα subunits have only one identified effector, such as cGMP phosphodiesterase for Gαt, whereas others couple to several effector proteins (Oldham and Hamm, 2006).

Gα family

Each Gα subunit interacts with downstream effectors in a highly specific manner. Classical Gα effectors include photoreceptor cGMP phosphodiesterase (PDE) for Gαt, adenylyl cyclase (AC) for Gαs and Gαi, phospholipase Cβ (PLCβ) for Gαq, and p115RhoGEF for Gα13 (Oldham and Hamm, 2006). In the following parts, different Gα family members have been briefly explained.

Gαs subunit

The Gαs pathway is the original cell signaling pathway to be described. Two main members of Gαs family are Gαs and Gαolf subunits. Gαs gene is expressed ubiquitously and gives rise to several splice variants. Long and short Gαs subunits are structurally similar and appear to be functionally indistinguishable (Bray et al., 1986; Kozasa et al., 1988; Mattera et al., 1989; Freissmuth et al., 1991). Another member of this family, $G\alpha_{olf}$, is expressed in olfactory sensory neurons and in the specific parts of brain, digestive and urogenital tracts (Milligan and Kostenis, 2006). All Gαs family members increase AC activity and the main difference between Gαs and $G\alpha_{olf}$ is in their localization. All known adenylyl cyclase isoforms are enhanced by Gαs (Sunahara et al., 1996).

Activation of AC causes increases in cAMP, which acts as a second messenger and enhances multiple cellular machines including ion channels, transcription factors, and metabolic enzymes. The activation of Gαs can also be induced by cholera toxin (CTX) treatment (Neer, 1995). Complete loss of Gαs in mice homozygous for an inactivating Gαs mutation leads to embryonic death (Yu et al., 1998). Heterozygote deletion of Gαs

gene causes opposite effects on energy metabolism depending on whether the maternal or paternal allele is disrupted (Yu et al., 2000).

Gαq subunit

The Gαq pathway is the classical pathway that is activated by calcium-mobilizing hormones and mediates the pertussis toxin insensitive regulation of phospholipase C β - isoforms (PLC-β) to produce the intracellular messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Neves et al., 2002). IP3 triggers the release of calcium from intracellular stores by binding to IP3-sensitive Ca^{2+} channels, whereas DAG activates protein kinase C (PKC) by recruiting this protein to the plasma membrane.

The Gαq family consists of four members: Gαq and Gα11 are expressed ubiquitously and are primary responsible for coupling to PLC-β (Exton, 1996; Rhee, 2001). In contrast, the Gα15/16 is only expressed in a subset of hematopoietic cells, and the expression of Gα14 is limited to several organs like kidney, testis and lung (Amatruda et al., 1991; Wilkie et al., 1991).

It has been shown that Gαq also appears to regulate various isoforms of phospholipase D (Xie et al., 2002). Furthermore, Gαq is reported to activate the transcription factor NF-κB through proline-rich tyrosine kinase2 (PYK2) (Shi and Kehrl, 2001). In mice deficient for Gα14, Gα15/16 and Gα11, no obvious phenotypic changes were observed (Offermanns et al., 1998; Davignon et al., 2000), while the loss of Gαq led to various defects such as ataxia and platelet activation defects in mice (Offermanns, 2003).

Gαi/o subunit

This family consists of several PTX-sensitive Gα subunits with the exception of Gαz (Ho and Wong, 2001). Therefore, PTX treatment provides a power tool to differentiate the role of Gαi/o subunits from other G proteins. The Gαi/o pathway was originally identified by the ability of these Gα subunits to inhibit adenylyl cyclase. Many important hormones and neurotransmitters, such as epinephrine, acetylcholine, dopamine, and serotonin, use this pathway to induce physiological responses (Milligan and Kostenis, 2006). Among the Gαi-family members, Gαi1, Gαi2 and Gαi3 are the most widely expressed proteins. These proteins have been shown to mediate receptor dependent inhibition of various types of adenylyl cyclases (Sunahara et al., 1996). Because the cellular levels of these G-proteins are relatively high, they also represent an important source for Gβγ-complexes, which can regulate a variety of cellular effectors (Offermanns, 2003). Homozygous Gαi1 or Gαi3-deficient mice have no obvious phenotypical changes. Furthermore, inactivation of Gαi2 causes mild and limited phenotypical problems as inflammatory bowel disease and mild platelet activation defect in Gαi2-deficient mice (Offermanns, 2001). Lack of obvious changes in phenotype of deficient mice might be due to the functional similarity and compensation among these Gαi subunits (Offermanns, 2003).

The most abundant G-protein α-subunit in the nervous system is Gαo (Mao et al., 2004). It is also expressed in other tissues like heart, pituitary or pancreas. In spite of its high level of expression in the brain, its function is still poorly understood. In contrast to other G-proteins it appears that most of the effects of Go activation are mediated by its βγ-subunit and little evidence of the effectors for the Gαo subunit is identified

(Offermanns, 2003). Gαo-deficient mice are significantly smaller and weaker than their littermates and have lower postnatal survival rates (Valenzuela et al., 1997; Jiang et al., 1998a; Jiang et al., 1998b). They also suffer from neurological abnormalities such as tremor, seizure and abnormal motor behavior (Offermanns, 2003).

The pertussis toxin insensitive α-subunit Gαz is expressed in various tissues like the brain, platelets or the adrenal gland. It can inhibit adenylyl cyclases types I and V, however, its physiological role is not clearly understood (Offermanns, 2003). It has been observed that mice lacking Gαz are viable and do not show any obvious neurological defects. However, Gαz-deficient mice show altered responses to a variety of psychoactive drugs (Hendry et al., 2000; Yang et al., 2000).

In the Gi/o signaling pathway, both Gα and Gβγ proteins can mediate signals. Gαi and Gαo can regulate various signaling pathways and ion channels. For instance, Gαi proteins can activate inwardly-rectifying K^+ channels (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Wickman and Clapham, 1995). They also regulate Ca^{2+} channels through fast (membrane-delimited) and slow (second messenger-dependent) mechanisms (Hescheler and Schultz, 1994; Hille, 1994).

Historically, Gαi/o subunits were first identified as the inhibitors of AC activity. In most systems, the inhibition of AC is mainly maintained by Gαi proteins, compared to Gαo (Ghahremani et al., 2000). Measuring G protein specificity to AC is difficult because of the existence of different subtypes of AC (Albert et al., 1999; Ghahremani et al., 1999). The specificity of Gαi proteins is dependent on the type of the receptor activated and the type of existing AC, as well as the type of AC activation. For example, it has been shown that Gαi1 inhibits AC V more effectively than AC I (Taussig et al.,

1993). Furthermore, it has been reported that forskolin activates AC I more effectively than AC II, AC V or AC VI; whereas Gαs preferentially stimulates AC II (Sutkowski et al., 1994).

Gαi/o coupled receptors have also been implicated in regulation of growth related processes like MAPK activation, DNA synthesis and cell proliferation. The MAPK regulation by Gαi/o has been discussed in detail in the following sections.

Gβγ subunit

Initially, Gβγ was thought to facilitate the termination of the G-protein signal by passively binding to Gα subunit and accelerating the return of the Gαβγ heterotrimer to the cell membrane (Neer, 1995), and it was only the Gα subunit that could interact with effectors. This belief was challenged when Logothetis et al. showed the first clear evidence that Gβγ could activate a K^+-selective ion channel (I_{KACh}) in cardiac atrial cells (Logothetis et al., 1987). Unlike Gα-subunits, in most of the cases, the conformation of Gβγ-dimers does not significantly change whether Gβγ is in the inactive heterotrimeric complex or in the free active state (Cabrera-Vera et al., 2003).

To date, it has been reported that Gβγ subunit can directly interact and regulate a wide range of effectors. For example, Gβγ directly activates several potassium channels like a K^+-selective ion channel (I_{KACh}) (Logothetis et al., 1987)) or GIRK (Kunkel and Peralta, 1995). This complex also transduces receptor-induced inhibition of calcium channels (De Waard et al., 1997; Zamponi and Snutch, 1998).

Today, it has been shown that Gβγ interact with and activate phospholipase Cβ2 and β3 (Katz et al., 1992; Sternweis, 1994). In some cases, GPCR-induced PI turnover

can be blocked by PTX, indicating that both PTX sensitive (Gαi/o) and PTX-insensitive (Gαq) are involved in this action. Unlike Gαq-induced activation of PLCβ, the Gαi/o-induced increase in PI turnover is not mediated by the Gα subunit but through Gβγ (Clapham and Neer, 1997). Finally, different isoforms of adenylyl cyclase protein such as II, IV, and VII can be stimulated by Gβγ subunit, although AC I, which is mainly expressed in neuronal cells, can be directly inhibited by this complex (Tang and Gilman, 1991).

Mitogen Activated Protein Kinase (MAPK)

Brief history

The initial steps to find the possible candidate mediating the mitogenic effects of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which stimulate receptor tyrosine kinases, resulted in the identification of a tyrosine-phosphorylated protein of 42 kDa (Cooper and Hunter, 1981, 1983). This protein had similar electrophoretic characteristics to those of a phosphotyrosine-containing molecule detected upon activation of PKC with phorbol esters (Kazlauskas and Cooper, 1988). Eventually, this molecule was identified as p42MAPK and was shown to require a dual phosphorylation of both threonine and tyrosine for its full activation (Ray and Sturgill, 1988; Rossomando et al., 1989). Soon after, it was revealed that this protein and its highly related isoform, p44MAPK, belong to family of serine-threonine kinases related to the kinases Fus3 (a yeast MAPK involved in the pheromone response) and Kss1 (kinase suppressor of SST2, a yeast MAPK involved in filamentous growth) from the yeast Saccharomyces cerevisiae, which act as the final step in a kinase cascade that

participates in the pheromone-induced mating response (reviewed in (Gutkind, 2000). It should be mentioned that in mammalian cells, p44MAPK and p42MAPK, also known as ERK1 (extracellular signal-regulated kinase 1) and ERK2, respectively, are involved in many cellular events discussed in the following section.

Classification and function

Signal transduction networks allow cells to receive external stimuli and respond to signals in an appropriate manner. The Mitogen-Activated Protein Kinase (MAPK) signaling pathways have an important role in signal transduction in eukaryotic cells, where they regulate many cellular functions such as mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement, cell survival and apoptosis, as well as cell and neuronal differentiation (Murray, 1998; Schaeffer and Weber, 1999). These protein kinases are generally expressed in all cell types, although they function to regulate specific responses that differ from cell type to cell type (Dhanasekaran and Johnson, 2007).

MAPK proteins constitute evolutionarily well-conserved signaling cascades that are organized in three-kinase modules consisting of a MAP kinase, an activator of MAP kinase (MAP Kinase Kinase or MEK) and a MAP Kinase Kinase Kinase (MEK Kinase, MEKK, MAPK Kinase Kinase, or MAP3K). In a typical signaling pathway, a MAP3K that is activated by extracellular stimuli phosphorylates a MAP2K on its serine and threonine residues, and then this MAP2K stimulates a MAP kinase through

phosphorylation on its serine and tyrosine residues (Minden and Karin, 1997; Dhanasekaran and Premkumar Reddy, 1998; Davis, 2000; Chang and Karin, 2001). Activated MAP kinases translocate from the cytosol to the nucleus where they regulate the activities of various transcription factors through phosphorylation (Khokhlatchev et al., 1998; Brunet et al., 1999).

Until now, four distinct groups of MAPKs have been most intensely studied and characterized in mammalian cells. The first group is the extracellular signal-regulated kinases (ERKs). The ERKs (also known as classical MAP kinases) signaling pathway is preferentially activated in response to growth factors and phorbol ester (a tumor promoter and PKC activator), GPCRs and mitogens, and regulates cell proliferation and cell differentiation.

The second group, c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) with the third group, p38 isoforms, both are responsive to stress stimuli, such as inflammatory cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in inflammation, cell differentiation and apoptosis. And finally, ERK5, which has been found recently, is activated both by growth factors and stress stimuli, and it participates in cell proliferation and differentiation (Dhanasekaran and Johnson, 2007).

Signaling by G Protein-Coupled Receptors to the MAPK Pathway

Although the classical MAPK pathway (ERK1/2) is mainly activated by growth factors and other ligands through receptor tyrosine kinases (RTKs), the activation of MAPKs in response to agonists acting on GPCRs is also well documented (Gutkind,

2000). In the following sections, ERK1/2 regulation by different G proteins has been briefly reviewed.

$G\alpha_s$ regulation of ERK1/2

Gαs is able to stimulate or inhibit ERK1/2 phosphorylation in different cell types. The initial studies focused on defining the oncogenic pathways regulated by the gsp oncogene that encodes the constitutively activated mutants of Gαs revealed that the ectopic expression of Gαs could stimulate ERK1/2 in different cell types (Landis et al., 1989; Lyons et al., 1990; Faure et al., 1994).

To date, it has been known that ERK1/2 can be stimulated by at least three Gαs-dependent mechanisms. Gαs transduces signals from Gs-coupled heptahelical receptors to adenylyl cyclase that converts ATP to cAMP and subsequently to cAMP-mediated activation of protein kinase A (PKA) (Gilman, 1987; Hepler and Gilman, 1992) and EPAC (exchange protein directly activated by cAMP). EPAC is a guanine nucleotide exchange factor (GEF) specific for Rap-1 that enhances GDP–GTP exchange in Rap-1 (de Rooij et al., 1998; Quilliam et al., 2002; Bos, 2006). The GTP-bound Rap-1, then activates B-Raf and the downstream MEK1/2, ERK1/2 proteins (Laroche-Joubert et al., 2002). Another EPAC-independent, cAMP-dependent mechanism to stimulate ERK1/2 is through activation of PKA that is able to phosphorylate Ser-17 of Src (Schmitt and Stork, 2002b; Obara et al., 2004; Weissman et al., 2004; Wang et al., 2006). Subsequently, Src mediates activation of Cbl that recruits Crk-C3G complex (Crk is an oncogene, adaptor protein containing SH2 and SH3 domains and C3G is a guanine nucleotide-exchange factor that activates Rap1). The final step is Crk-SH3 domain-mediated recruitment as

well as activation of C3G, which leads to activation of Rap1 and ERK1/2 module (Ichiba et al., 1999; Schmitt and Stork, 2000, 2002b; Obara et al., 2004; Weissman et al., 2004; Wang et al., 2006). Finally, it has also been reported that Gαs-cAMP-mediated activation of Ras is involved in the stimulation of ERK1/2 modules via PKA-dependent or -independent mechanism in a cell-type-specific manner (reviewed in (Goldsmith and Dhanasekaran, 2007)).

Gαs-mediated cAMP-PKA signaling axis is also able to potently suppress ERK cascade (Cook and McCormick, 1993; Graves et al., 1993; Wu et al., 1993). The primary mechanism by which Gαs inhibits the ERK signaling involves PKA-mediated phosphorylation of a specific isoform of Raf, known as Raf-1 or C-Raf (Wu et al., 1993; Dhillon et al., 2002). It has been shown that Gαs-mediated cAMP-PKA pathway inhibits C-Raf through the phosphorylation of Ser-259 (Dhillon et al., 2002). This C-Raf phosphorylation attenuates the phosphorylation of Ser-338, which is involved in the activation of C-Raf (Dhillon et al., 2002). Therefore it provides a molecular basis for Gαs-mediated inhibition of C-Raf-MEK1/2-ERK1/2 module. Another Gαs inhibitory mechanism is through activation of Rap-1 by PKA or EPAC. The effector domain of Rap1 has close sequence and structural similarities with that of Ras, thus the active Rap1 can bind and sequester C-Raf from being activated by Ras (Bos, 1998; Schmitt and Stork, 2001, 2002a; Goldsmith and Dhanasekaran, 2007) (please see figure C in Appendices).

G_q regulation of ERK1/2

To date, it has been well-established that both Gα and Gβγ subunits of Gq family proteins can stimulate ERK1/2 module. Upon activation by related receptor, the Gαq subunit mediates signals to the downstream pathways via the activation of PLCβ

(Johnson and Dhanasekaran, 1989; Simon et al., 1991). Activated PLCβ hydrolyses PIP2 to DAG and IP3. Both of these effectors can activate protein kinase C (PKC), directly or indirectly via the release of internally stored Ca2+, respectively (Goldsmith and Dhanasekaran, 2007). In the next step, Gαq-activated PKC can stimulate ERK1/2 module by directly phosphorylating and activating C-Raf (Kolch et al., 1993; Ueda et al., 1996; Schonwasser et al., 1998). As an alternative mechanism, Gαq may use Ca^{2+}-calmodulin dependent pathway involving Pyk2, Src and finally Ras proteins that leads to the activation of ERK1/2 module (Lev et al., 1995; Dikic et al., 1996; Della Rocca et al., 1997). In the recent years, another novel Gαq-dependent PKC-independent stimulatory mechanism has been proposed. In this pathway, both DAG and Ca^{2+} activates a calcium- and diacylglycerol-regulated guanine nucleotide exchange factor (CalDAG-GEFI) that can stimulate Rap-1, which in turn, can activate B-Raf and the downstream ERK1/2 module (Guo et al., 2001). It should be mentioned that Gβγ subunit can also stimulate PLCβ2/β3. Therefore, it is possible that they contribute to Gq signaling to ERK1/2 modules in a similar manner by using the DAG/PKC- and/or IP3/Ca^{2+} signaling pathways (Goldsmith and Dhanasekaran, 2007).

Regulation of ERK1/2 by Gi

The identification of the activated mutants of Gαi2 as the gip2 oncogene in pituitary adenomas as well as tumors of the ovary and the adrenal cortex (Lyons et al., 1990) indicated the mitogenic activity of Gαi (Goldsmith and Dhanasekaran, 2007). Further studies revealed that expression of the activated Gαi2 was associated with constitutive activation of p44-ERK isoform (Gupta et al., 1992). In addition to activation

of ERK modules, Gα subunits also appear to suppress signals to this cascade. Like Gq signaling pathway, Gβγ subunit of Gi/o proteins can activate MAPK that has been discussed in the next part.

The mechanism of Gαi/o-dependent activation of ERK can be explained by the suppression of two inhibitory pathways. The first mechanism involves its inhibitory effect on adenylyl cyclase (Johnson and Dhanasekaran, 1989; Tang and Gilman, 1992). Consistent with this theory, expression of gip2, the activated mutant of Gαi, led to a decrease in the accumulation of cAMP in cultured cells (Lowndes et al., 1991; Wong et al., 1991). This decrease in cAMP levels and subsequent decrease in PKA activity relieves the inhibitory effect of PKA on C-Raf, therefore, potentiating Ras-c-Raf signaling to ERK module (Radhika and Dhanasekaran, 2001). The observation that the expression of Gαi2 slightly enhances the activation of C-Raf (Pace et al., 1995) supports this theory.

Another possible mechanism by which G protein subunits may also activate MAPK involves inhibitory effects of Gαi/o subunits on Rap1 via Rap1GAP protein. Rap1, initially identified as a biological antagonist of Ras (Kitayama et al., 1989), has received much attention, since it can block MAPK activation by competing with Ras for binding to c-Raf and sequestering c-Raf, or it is able to stimulate MAPK through the activation of B-Raf (Bos et al., 1997; York et al., 1998). Muchizuki et al reported that Gi/o subunits, but not Gαs or Gαq bound specifically an NH2-terminal extended form of Rap1GAP, termed Rap1GAPII. They showed that stimulation of the Gi-coupled m2-muscarinic receptor in 293T cells, translocated rap1GAPII from the cytosol to the membrane and decreased the amount of GTP-bound Rap1. Subsequent release of

sequestered C-Raf can lead to Ras-c-Raf reassociation and the activation of the downstream ERK1/2 module (Mochizuki et al., 1999).

It should be mentioned that the Gi/o regulation of ERK1/2 is cell type-dependent. For example, in nontransformed BALB/c 3T3 fibroblast cells stably transfected with the D2S receptor cDNA, Gαi2 appears to mediate D2S-induced stimulation of p42 and p44 mitogen-activated protein kinase (MAPK) (Ghahremani et al., 2000). On the other hand, it has been reported that in rat pituitary GH4ZR7 cells, activation of D2S receptor leads to strong suppression of ERK1/2 and this signaling pathway is through Gαi3 and Gαo (Banihashemi and Albert, 2002b; Liu et al., 2002b).

How Gαi/o subunits inhibit ERK1/2 module is not completely understood and it seems several different inhibitory mechanisms should be involved. As we mentioned earlier, Gαi is able to decrease adenylyl cyclase activity resulting in blocking PKA and EPAC activation (Johnson and Dhanasekaran, 1989; Tang and Gilman, 1992). Furthermore, Gαi/o subunit can enhance Rap1GAP activity that lead to the consequent decreased levels of Rap1-GTP and B-Raf (Mochizuki et al., 1999). In the case of Gαo, its GDP-bound inactive form binds Rap1GAP, thus preventing its activity and enhancing the accumulation of Rap1-GTP, but upon activation, Gαo releases Rap1GAP that inactivates Rap1. Jordan et al observed when inactivated Gαo was expressed, the amount of activated Rap1 was greatly increased. This effect was not observed with the constitutively active Q205LGαo. Furthermore, they found in inactive Gαo-transfected PC-12 cells, the activity of ERK2 was greatly enhanced, but transfection of constitutively active Q205LGαo strongly attenuates ERK2 phosphorylation (Jordan et al., 1999b).

Recently, we have found a novel mechanism for Gαi3-induced MAPK inhibition that involves RASA3. We have discussed about this pathway in chapter 3.

It has been demonstrated that the βγ subunit of Gi/o family is also involved in activation of ERK by a number of Gi-coupled receptors including m2-muscarcinic, α2-adrenergic, D2 dopamine, A1 adenosine and LPA receptors (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994). Further analyses using a dominant negative mutant of Ras have indicated that the βγ activates ERK1/2 through the Ras protein (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994). Two possible mechanisms for Ras activation by βγ subunit are through phospholipase-C β (PLCβ) and/or phosphoinositide-3-kinase (PI3K), both of which can be activated by the βγ subunit (Camps et al., 1992; Katz et al., 1992; Stephens et al., 1994; Thomason et al., 1994). In the first model, stimulated PLCβ increases intracellular Ca^{2+} and enhances Ca^{2+}-calmodulin-mediated activation of Pyk2 kinase via production of IP3. Eventually, the Pyk2-Src-Shc-dependent Ras-GEF, SOS leads to the activation of Ras (Lev et al., 1995; Dikic et al., 1996; Luttrell et al., 1996; Della Rocca et al., 1997). On the other hand, in Rat1a and COS-7 cells, it has been shown that LPA- or thrombin receptor stimulated release of βγ subunit stimulates ERK1/2 module via a pathway involving PI3K and PI3K-mediated activation of a tyrosine kinase that promotes dynamin II-Grb2 complex formation and finally leads to activation of Ras and the ERK1/2 proteins (Kranenburg et al., 1997), (Kranenburg et al., 1999b, a). It should be mentioned that Gβγ subunit can be involved in Gi-coupled receptor-mediated ERK1/2 phosphorylation by directly activation of Src family, which recruits the Shc and Grb2 adapter proteins to the membrane (Luttrell et al., 1997).

Another interesting mechanism that Gβγ subunit may use to stimulate ERK1/2 is through proteolytic cleavage of latent agonists for the receptor tyrosine kinases. In this pathway, Gβγ subunit may activate metalloproteases, most likely of the ADAM (A disintegrin-like and metalloprotease domain-containing protein) family. These proteinases then cleave inactive 'prohormones' such as transforming growth factor-α (TGF- α), amphiregulin, heparin-binding-epidermal growth factor (HB-EGF) to produce active ligands. Finally, activation of receptor tyrosine kinases by these stimuli may lead to ERK1/2 activation through the classical pathway of Shc/Grb2/SOS/Ras (Massague and Pandiella, 1993; Dong et al., 1999; Gutkind, 2000).

Pituitary gland and lactotrophs

Dopamine receptors in pituitary cells

For the first time, in 1970s, radioligand binding assays demonstrated the presence of D2 receptors in the anterior and intermediate lobes of the pituitary gland (Missale et al., 1998b). D2 receptors are evenly distributed throughout the adenohypophysis with a relatively higher expression level in mature than in young rats (Piano and Pogacnik, 2001). Gene knockout studies of the D2L receptor revealed that it is mainly a post-synaptic receptor, while the D2S receptor is preferentially pre-synaptic and located on dopamine neuron cell bodies (Khan et al., 1998a; Usiello et al., 2000).

In the pituitary gland, dopamine, through D2 receptors expressed on lactotrophs, is a well recognized regulator of cell proliferation, prolactin (PRL) synthesis and secretion. There are different signaling pathways that dopamine utilizes to mediate its

inhibitory signals to the above-mentioned cellular functions. For example, it has been demonstrated that dopamine can inhibit both cAMP-dependent and cAMP-independent hormone secretion (Vallar et al., 1990). Furthermore, activation of D2 receptor initiates opening of K^+ channels and inhibits PI turnover that leads to decrease in $[Ca^{2+}]_i$ (Vallar et al., 1990; Senogles, 1994b; Huff, 1996). D2 stimulation has also been shown to inhibit ERK1/2 phosphorylation (Banihashemi and Albert, 2002b; Liu et al., 2002b). Decreases in $[Ca^{2+}]_i$ level and ERK1/2 activity are known to inhibit PRL secretion.

The inhibitory effects of dopamine on cell proliferation and dopamine production have been best identified in homozygous mice deficient in the gene encoding the D2 receptor, which develop pituitary hyperplasia and adenoma and also chronic hyperprolactinemia (Kelly et al., 1997; Saiardi et al., 1997; Asa et al., 1999; Cristina et al., 2006). Furthermore, knockout mice of the dopamine transporter gene, which results in persistent extracellular hyperdopaminergic tone due to lack of reuptake mechanisms, present with hypoprolactinemia and pituitary hypotrophy (Bosse et al., 1997; Gainetdinov et al., 1999). These results implicate the D2 receptor as the major mediator of the anti-proliferative actions of dopamine in the pituitary.

GH rat pituitary cell lines

GH cells were initially from rat radiation-induced MtT/W5 transplantable pituitary tumor and had been isolated by Tashjian et al. (Tashjian et al., 1968). Since they secreted growth hormone, the cell line was originally named GH cells, although it was demonstrated later that these cells also secrete prolactin and several associated substances. GH cells are different from normal lactotrophs. For example, they are

transformed and if they are injected into animals, they cause tumors (Tashjian et al., 1968). Furthermore, they have smaller hormone storage and higher basal level hormone secretion. They also lack dopamine D2 receptor and its inhibitory signaling pathways. It is worth mentioning that in bromocriptine-resistant prolactinoma, major biochemical defect is decreased density or absence of D2 receptors (Pellegrini et al., 1989; Missale et al., 1993).

In GH4C1 cells, a GH3 subcloned cell line, secretion rate of PRL and GH is enhanced by releasing hormones like thyrotropin releasing hormone (TRH) or vasoactive intestinal peptide (VIP) and is suppressed by inhibitory hormones such as somatostatin (Albert, 1994). Furthermore, several of these hormones are able to regulate the synthesis and gene transcription of prolactin and growth hormone (Tashjian, 1979; Murdoch et al., 1985; Farrow and Gutierrez-Hartmann, 1999). These cells express various types of G protein-coupled receptors such as Gq-coupled receptors (bombesin and TRH) (Westendorf and Schonbrunn, 1983; Hinkle and Phillips, 1984), Gi/o-coupled receptors (Adenosine A1, somatostatin sst1 and sst2 and muscarinic) (Schonbrunn and Tashjian, 1978; Dorflinger and Schonbrunn, 1985; Gu et al., 1995; Gu and Schonbrunn, 1997) and Gs-coupled receptors (VIP, PGE2) (Dorflinger and Schonbrunn, 1983; Albert, 1994). It has been shown that GH cells express several classes of G proteins such like Gαi1-3 and Gαo (Albert, 1994; Liu et al., 1994b; Liu et al., 1999a). They also have different kinds of sodium, potassium and calcium channels and a proportion of cells fire spontaneous action potentials (Koch and Schonbrunn, 1988).

GH4ZR7 cells are GH4C1 cells stably transfected by dopamine D2S receptor, a receptor absent in GH cells but normally present in lactotrophs. It has been reported that

activation of D2S receptor in this cell line suppressed both basal and VIP- or forskolin-stimulated cAMP accumulation (Albert et al., 1990; Banihashemi and Albert, 2002b) and also inhibited TRH- or VIP-stimulated PRL secretion (Albert et al., 1990; Albert, 2002b). D2S receptor stimulation induces membrane hyperpolarization and decreased basal $[Ca^{2+}]_i$ in these cells (Vallar et al., 1990). Finally, it has been demonstrated that activation of this receptor by a dopamine agonist induces a strong suppression of TRH-induced ERK1/2 phosphorylation (Banihashemi and Albert, 2002b). By considering these findings, GH4ZR7 cell line is an excellent model to study the dopamine signal transduction in pituitary cells.

Hypothesis and approach

Hypothesis

We hypothesize RASA3 mediates Gαi3-induced inhibition of mitogen-activated protein kinase in rat pituitary GH4ZR7 cells. Our hypothesis has been supported by two findings. First, Gαi3 plays a crucial role in D2S-induced inhibition of TRH-stimulated MAPK activity in GH4ZR7 cells. Second, RASA3 preferentially interacts with Gαi3.

Approach

To address RASA3 function in dopamine D2S receptor signaling to inhibition of MAPK activity, we initially demonstrated the physical interaction between RASA3 and its upstream interacting protein Gαi3 by different methods including yeast two hybrid/β-galactosidase assay, in vitro pull-down assay and co-immunoprecipitation between overexpressed or endogenous proteins in different cell lines. Most importantly, to address the functional role of RASA3 in D2S-induced inhibition of Ras-ERK1/2 activation, we suppressed endogenous RASA3 protein expression in GH4ZR7 cells and compared the ERK1/2 phosphorylation level upon D2S activation in these cells and their parental cells.

CHAPTER II

RESULTS (MANUSCRIPT)

GAP1(IP4BP)/RASA3 mediates Galpha(i)-induced inhibition of mitogen-activated protein kinase

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Running title: RASA3 mediates D2S-induced ERK1/2 inhibition

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Abstract

Activation of the dopamine D2S receptor (short isoform) inhibits thyrotropin-releasing hormone (TRH)-stimulated p42/p44 mitogen-activated protein kinase (ERK1/2) activation in GH4ZR7 rat pituitary cells, consistent with its actions to inhibit prolactin gene transcription and cell proliferation. The G protein subunit Gαi3 mediates this inhibitory signaling pathway but the underlying mechanism is not fully understood. To identify novel Gαi3 effectors, we performed a yeast two-hybrid screen on a GH4ZR7 cDNA library using constitutively active Gαi3-Q204L as bait and identified multiple clones of the RasGAP cDNA GAP1IP4BP/RASA3. RASA3-Gα interactions were quantified using the yeast mating/β-galactosidase assay, which showed both wild-type and Gαi3-Q204L preferentially interacted with RASA3. Interaction with Gαi3 was verified by in vitro pull-down assay and both inactive and AlF4--activated GST-Gαi3 bound to S-His-RASA3, indicating a direct interaction. Gαi3-RASA3 interaction was further tested by co-expression/co-immunoprecipitation (co-IP) of Flag-tagged RASA3 and wild type or Gαi3-Q204L proteins in HEK-293 cells or between endogenous proteins in GH4ZR7 cells. To address RASA3 function in dopamine D2S receptor-induced inhibition of ERK1/2 activity, endogenous RASA3 protein expression was suppressed (70% knockdown) in GH4ZR7 cells stably transfected with full-length antisense cDNA of RASA3. The selected antisense clones had similar levels of dopamine D2 receptor binding compared to parental GH4ZR7 cells, but in these clones D2S-mediated inhibition of TRH-induced phospho-ERK1/2 was reversed by 70-80% compared to parental GH4ZR7 cells. Our results provide a novel mechanism for dopamine D2S-induced

inhibition of ERK1/2 and indicate that RASA3 links Gαi3 to inhibit Gq-induced activation of the Ras/ERK1/2 pathway.

Introduction

Five dopamine receptor genes are known, and the dopamine-D2 receptor is among the most studied because of its involvement in mental disorders such as schizophrenia, addiction, and Parkinson's disease. In addition, the dopamine-D2 receptor mediates inhibitory regulation of endocrine function, with a primary role in the pituitary to regulate prolactin synthesis, secretion and lactotroph cell proliferation. For example, in homozygous mice deficient in the gene encoding the D2 receptor or lacking dopamine, pituitary adenoma and hyperprolactinemia occur with age (Kelly et al., 1997; Saiardi et al., 1997; Asa et al., 1999; Hnasko et al., 2007). Conversely, mice lacking the gene encoding the dopamine transporter show dopamine hypersecretion that leads to pituitary hypotrophy (Bosse et al., 1997). In addition, dopamine-D2 receptor agonists such as bromocryptine or cabergoline are used clinically to induce regression of pituitary adenomas (Missale et al., 1998a; Trouillas et al., 1999; Colao et al., 2000; Pinzone et al., 2000). The dopamine D2 receptor gene contains an alternately-spliced exon encoding 29 amino acids in the putative third intracellular loop to generate short (D2S) and long (D2L) forms of the receptor (Civelli et al., 1993). Dopamine D2S receptors have been shown to have anti-proliferative actions in pituitary lactrophs, while the D2L receptor did not appear to be anti-proliferative (Iaccarino et al., 2002b). However, the specific signaling mechanisms underlying dopamine D2S actions in pituitary cells remain to be fully elucidated.

GH4ZR7 cells, which are GH4C1 lactotroph cells stably transfected with dopamine D2S receptor, provide a useful model to study the signaling of the dopamine-

D2S receptor in pituitary cells that retains differentiated properties of lactotrophs. These include expression of receptors that stimulate (thyrotropin-releasing hormone (TRH) receptors) or inhibit (somatostatin and muscarinic receptors) the synthesis and secretion of growth hormone (GH) and prolactin (PRL) (Albert et al., 1997). In GH4ZR7 cells, dopamine inhibits cAMP formation, PRL synthesis and secretion, and cell proliferation. Previously, a novel D2S receptor-mediated inhibition of TRH-induced ERK1/2 activation was identified in these cells (Banihashemi and Albert, 2002a; Liu et al., 2002a). Interestingly, D2L receptors did not couple to this pathway (Itzhaki Van-Ham et al., 2007). Activation of ERK1/2 is implicated in TRH induced prolactin transcription (Wang and Maurer, 1999a), and its inhibition is a key pathway for dopamine-D2S-induced inhibition of prolactin transcription (Liu et al., 2005a). This pathway is also observed in primary striatal cultures, suggesting a general role for this D2S receptor-mediated signaling pathway in neuroendocrine tissues (Itzhaki Van-Ham et al., 2007).

The mechanism by which the dopamine-D2S receptor inhibits the Ras-ERK1/2 pathway has been partially characterized. Using a rescue strategy employing transfection of pertussis toxin (PTX)-resistant Gαi/o proteins, we previously found that Gαi3 plays a crucial role in dopamine D2S receptor-induced inhibition of ERK1/2 activation by TRH in GH4ZR7 pituitary cells (Banihashemi and Albert, 2002a). By contrast, blockade of Gβγ signaling did not affect D2S-mediated inhibition of ERK1/2 activation, indicating that the Gαi3, but not Gβγ subunits, are required. We further found that the D2S receptor inhibited TRH-induced activation of MEK and c-Raf, suggesting that Gαi couples to inhibit this pathway upstream of c-Raf, possibly by inactivation of Ras. We hypothesized that a critical downstream effector may directly interact with Gαi3 to mediate dopamine

D2S receptor inhibition of Ras-ERK1/2 signaling. To address this hypothesis we have used two-hybrid screening with a constitutively-active mutant of Gαi3 as bait to probe a cDNA library from GH4 pituitary cells. In this screen we identified RASA3 as a novel Gαi-interacting protein and have validated the Gαi3-RASA3 interaction in pituitary cells. Importantly we also demonstrate the requirement of RASA3 for D2S receptor signaling to inhibit ERK1/2 activation, indicating that RASA3 links Gαi proteins to inhibition of the Ras-ERK1/2 pathway.

Materials and Methods

Materials

Apomorphine, puromycin, TRH, Sepharose G protein beads, anti-ß-actin, anti-Flag antibody and anti-Flag M2 Affinity gel were from Sigma-Aldrich (St. Louis, MO); polyvinylidene difluoride membrane was from PerkinElmer, Inc. (Waltham, MA); enhanced chemiluminescence detection kits were from Roche (Laval, QC); sera and media were obtained from Wisent, Inc. (St-Bruno, Qc). Endonucleases were purchased from New England Biolabs, Inc. (Boston, MA); anti-Gαi3 was from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-phospho-p42/44 ERK1/2 antibody (T202/Y204) and anti-rabbit IgG, HRP-linked antibody were from Cell Signaling Technology, Inc. (Danvers, MA); S-protein HRP conjugate antibody was purchased from Novagen-Merck (Darmstadt, Germany); Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG antibody was obtained from Jackson ImmunoResearch (West Grove,PA). Ni-NTA agarose was from Qiagen (Valencia, CA). Lipofectamine was purchased from Invitrogen, Inc. (Carlsbad, CA). Coomassie (Bradford) Protein Assay Kit was obtained from Pierce (Rockford, IL).

Bacterial expression and protein purification

RASA3 was subcloned into pET30a(+) (Novagen) bacterial vector, which contains an S-tag on N-terminal and His-tag on both N- and C-terminals. E. coli

BL21(DE3) competent cells (Invitrogen, Carlsbad, CA) were transformed by pET30a(+)-RASA3. Bacteria were grown in LB medium containing ampicillin at 37 °C in a shaking incubator at 200 rpm until the optical density at 600 nm or 0.6. Then, they were induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours in LB medium and finally centrifuged at 4,000 x g for 20 minutes at 4 °C. Pellet was resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole) and protease inhibitor and after sonication (6 x 10 seconds) and centrifugation at 30,000 rpm for 30 min, 4 °C, the supernatant was added to Ni-NTA agarose (Qiagen, Valencia, CA). After washing 3x in wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole), the protein was eluted using elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole) according to Qiagen protocol.

Antibody purification and concentration

Steriflip-GP filters 0.22 μm and PROSEP-G media plugs (Millipore, Billerica, MA) were used to purify serum antibody against full-length RASA3. The antibody was pre-filtered through Steriflip-GP filter device to remove any debris immediately before loading the sample. Next, antibody was added to PROSEP-G media plugs (immobilized recombinant protein G), washed with binding buffer (1.5 M Glycine/NaOH, 3 M NaCl, pH 9.0) and then eluted from the resin by adding elution buffer (0.2 M Glycine/HCl pH 2.5) and brief centrifugation. The antibody was brought to neutral pH by using Neutralization buffer (1 M Tris/HCl pH 9.0). Finally, it was concentrated by Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA).

Yeast Two-hybrid Screening

Oligo-dT-primed cDNA library from GH4ZR7 poly- A+ RNA was constructed in yeast vector pGADT7 (Clontech) and over 80% of clones contained inserts of average size 900-bp. The library was screened with constitutively-active human Gαi3Q204L from UMR cDNA Resource Center (Rolla, MO) as bait. The yeast cells were grown at 30 °C for 5–7 days. Transformants (106) were selected on SD-Leu- Trp- His- Ade- plates and screened by X-gal (5-bromo-4-chloro-3-indoyl-β-D-galctopyranoside) (Wisent, QC) overlay assay. DNA from positive clones was extracted and sequenced.

Yeast mating/β-galactosidase assay

Gαi subunits and RASA3 constructs were subcloned in pAS2-1 and pACT2 vectors (Clontech, Palo Alto, CA), respectively. Y187 and AH109 yeast strains were transformed with individual Gα subunits and full-length RASA3, and selected for positive transformants on SD-Trp- and SD-Leu- plates, respectively. Resultant colonies were mated and selected on SD-Leu- Trp- His- for 3-5 days at 30 °C. A quantitative β-galactosidase assay using ONPG (2-nitophenyl β-D-galactopyranoside) (Sigma) as substrate was performed.

In Vitro Pull-down assay and Western Blotting

Bacterially-expressed S-6XHis-RASA3 (pET, Novagen) and GST- tagged Gαi3 (pGEX, Amersham) proteins were used as bait and prey, respectively. BL21 (DE3) competent cells were transformed by either pET or pGEX vectors containing RASA3 or Gαi3, respectively. They were induced with IPTG (Isopropyl-β-D-thiogalactoside) for

three hours, harvested and resuspended in lysis buffer(50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole), sonicated (6 X 10 seconds) and finally centrifuged 20,000 x g for 30 minutes at 4 °C. The RASA3 supernatant was incubated with Ni-NTA agarose (Qiagen) on shaker for 1 h at 4 °C and then washed 3 times with washing buffer containing 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole. Then, Gαi3 supernatant was added and incubated with the resin on shaker for 1 hour at 4 °C. Washing steps were repeated. The resin was incubated with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole) for 15 minutes on shaker at 4 °C and finally eluted. To activate Gαi3, separate lysis, washing and elution buffer containing 30 μM AlCl3, 5 mM MgCl2 and 10 mM NaF were used. The proteins were resolved on an SDS-10% polyacrylamide gel that was transferred onto a polyvinylidene difluoride membrane and blocked at 22°C for 1 h in 5% skimmed milk in TBS buffer. The membrane was blotted with anti-S antigen (Novagen) or anti-Gαi3 (Upstate) antibodies overnight at 4 °C, and then horseradish peroxidase-linked anti rabbit secondary antibody was added to detect anti Gαi3 antibody. The membrane was incubated with chemiluminescence substrate (Roche) and exposed to Kodak BioMax MR film.

Co-immunoprecipitation assay

The pcDNA3-FLAG-RASA3 construct was transiently co-transfected with either pcDNA3-Gαi3 or pcDNA3-Gαi3-QL into HEK-293 cells by the calcium phosphate co-precipitation method. Forty-eight hours after transfection, the cells were scraped in RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1%(w/v) SDS, 5 mM EDTA, 1% (w/v) NP-40, 0.5% (w/v) DSS, 10 mM NaF, 10 mM Na2PPi) and left at 4 °C on shaker for 3 h,

and lysed further by passing through 25G needle. The cell lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatants were incubated with ~40 µl of Protein G Sepharose, Fast Flow (Sigma) at 4 °C for an hour in a pre-clear phase. They were centrifuged again and the supernatants were incubated with ~40 µl of anti-FLAG M2-agarose affinity gel (Sigma) on shaker at 4°C overnight. The gel was washed three times with RIPA lysis buffer, resuspended in 2x SDS-loading buffer and boiled for 5 min to elute the proteins. The eluted supernatants were resolved on an SDS-10% polyacrylamide gel and analyzed by Western blotting. For co-immunoprecipitation of endogenous RASA3 and Gαi3, GH4ZR7 cells were treated with either apomorphine or ddH2O (vehicle) for 15 minutes. The cells were scraped in RIPA lysis buffer and lysed as mentioned above. After pre-clear phase, the supernatants were incubated with 4 µl anti-Gαi3 antibody at 4 °C overnight. The next day, ~40 µl of Protein G Sepharose, Fast Flow (Sigma) was added to each tube and incubated at 4 °C for an hour. Washing and elution were as described above.

Stable Transfection

GH4ZR7 cells were co-transfected with plasmids for antisense RASA3 (5 µg) and pGK-puro (0.5 µg) per 10 mm dish using Lipofectamine (Invitrogen)(1.5x, w/v). The transfected cells were cultured in Ham's F-10 + 8% fetal bovine serum containing puromycin (20 µg/ml) for 3–4 weeks. Antibiotic-resistant clones were picked (34 clones) and tested for expression of the corresponding RASA3 protein by Western blot analysis.

Ligand binding

Dopamine-D2 receptor density was measured by specific binding of the antagonist [3H]spiperone. Cell membranes were prepared from 15-cm dishes by replacing the medium with hypotonic buffer (15 mmol/L Tris–HCl, pH 7.4, 2.5 mmol/L MgCl2, 0.2 mmol/L EDTA). The cells were scraped from the plate and centrifuged at 500 g for 15 min at 4 °C and the pellet was resuspended in cold TME buffer (75 mmol/L Tris, pH 7.4, 12.5 mmol/L MgCl2, 1 mmol/L EDTA). The cells were lysed further by passing gently through 25G needle and centrifuged at 12 000 g for 30 min at 4°C and resuspended in TME. For binding assay, aliquots of 100 μg/tube membrane preparation were added to triplicate tubes containing 0.2 ml of TME + 0.1% ascorbic acid with 9000 cpm of [3H]spiperone ± 10–6 M apomorphine. After 30 min incubation at room temperature, the reactions were terminated by addition of 1 ml ice-cold 50 mM Tris, pH 7.4. The samples were filtered through GF/C glass microfiber filters (Whatman, Clifton, NJ) and washed 3x with 3 ml of ice-cold 50 mM Tris, pH 7.4. The filters were then combined with 3 ml of scintillation fluid (InterSciences Inc., Markham, ON), and radioactivity was detected using the Packard TRI-CARB 2100TR scintillation counter (PerkinElmer Life Sciences). The receptor density was normalized to protein concentration determined by Bradford assay.

Measurement of Phospho-ERK1/2 in GH4ZR7 cells

Equal numbers of cells (3x105 cells per well) were plated in six-well plates. At 80% confluence, the cells were placed in serum-free Ham's F-10 medium (1 h, 37 °C).

Cells were treated with the indicated drugs at 37 °C, and after the indicated time the plates were transferred on ice and washed two times with cold PBS. Cells were lysed in 100 µl of lysis buffer containing equal volumes of 2.5x SDS loading and RIPA buffers, stored on ice, sonicated for 6 sec, and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant (30 µl) was heated (100 °C, 5 min) and rapidly cooled on ice. Samples were centrifuged 30 sec and were separated by SDS-PAGE, and subjected to Western blot analysis. Phosphorylation was detected using (1:1000) anti-phospho-p42/44 ERK1/2 (ERK1/2). The corresponding bands for ERK1 and ERK2 were digitally quantified using Adobe Photoshop. The results were normalized to the β-actin control.

Results

RASA3, a novel Gαi3-interacting protein

To identify novel Gαi3 effectors involved in Gαi3-induced inhibition of ERK1/2 activation in pituitary cells, we constructed an oligo-dT-primed cDNA library from GH4ZR7 pituitary cell poly-A+ RNA for yeast two hybrid screening with constitutively-active Gαi3Q204L as bait. In two sets of screening we identified 6/6 positive/106 and 4/4 positive/106 clones that encoded 834 amino acid rat RASA3 (AB020479), also known as Ras p21 protein activator 3, GTPase activating protein III or GAPIII, and inositol 1,3,4,5-tetrakisphosphate-binding protein or GAP1(IP4BP) (Fig. 1A). Sequence analysis revealed that this protein is highly conserved in human, rat and mouse species (Fig. 1B). RASA3 is a member of the Ras GTPase-activating proteins (RasGAPs), which inactivate Ras or related small G-proteins by enhancing their weak intrinsic GTPase activity. RASA3 is a member of GAP1 subfamily, which includes GAP1m, CAPRI (Ca2+-promoted Ras inactivator) and RASAL (Ras-GTPase-activating-like protein). RASA3 has a modular structure (Fig. 1C) with two C2 domains, a conserved 330-aa RasGAP domain, pleckstrin homology (PH) and Bruton's kinase (BTK) domains (Kupzig et al., 2005). RASA3 shows GAP activity against both Rap, Ras and R-Ras (Ohba et al., 2000; Kupzig et al., 2006). RASA3 is associated with the plasma membrane via PH domain-PIP2 interaction, and activated by inositol 1,3,4,5-tetrakisphosphate (IP4) binding to the BTK domain (Cozier et al., 2000; Cozier et al., 2003). As a RasGAP protein, RASA3 could directly couple Gαi proteins to inhibition of ras activation, and hence its role was examined.

RASA3 expression in GH4ZR7 cells

Since commercially available anti-peptide antibodies against RASA3 lacked the sensitivity or specificity to detect endogenous RASA3 (data not shown), a rabbit polyclonal antibody was raised against full-length recombinant rat RASA3, named D40. By Western blot analysis, the D40 antibody detected purified recombinant RASA3 (data not shown) and endogenous RASA3 (96 Kd) in GH4ZR7 cell extracts, while preimmune serum failed to detect RASA3, although some non-specific products were present (Fig. 2A). Furthermore, in GH4ZR7 cells depleted of RASA3 using antisense construct (RASA3-AS30 clone, see below) a reduced level of RASA3 was detected by the purified D40 antibody, while in Balb-D2S fibroblast cells (BDY11) stably transfected with sense RASA3 (BDY11-21) the antibody detected an increase in RASA3 protein (Fig. 2B). These experiments validated the specificity of the D40 antibody for detection of endogenous rat RASA3 in GH4ZR7 cells.

Gαi3/RASA3 interactions

RASA3-Gαi interactions were quantified using the yeast mating/β-galactosidase assay (Fig. 3). Both wild-type and constitutively-active Gαi3 interacted with RASA3, while only constitutively-active but not wild-type Gαi1, Gαi2 and Gαz, interacted strongly with RASA3, while Gαo showed a weak interaction. By contrast, Gαs and Gαcone failed to interact with RASA3 (i.e., no colonies were obtained), suggesting that RASA3 preferentially interacts with Gαi family proteins. The preferential interaction of

RASA3 with both wild-type and active Gαi3 is consistent with the preferential role of Gαi3 in D2S receptor-induced inhibition of ERK1/2 signaling.

To verify the RASA3-Gαi3 interaction, in vitro pull-down assays using bacterially expressed S-6XHis-RASA3 and GST-tagged Gαi3 proteins were done. Ni-NTA agarose bound to S-6XHis-RASA3 with little or no contaminating GST-tagged Gαi3. On the other hand, in the presence of S-6XHis-RASA3, both non-activated and AlF4--activated Gαi3 bound to RASA3 (Fig. 4), consistent with interactions observed in yeast mating assays.

The RASA3-Gαi3 interaction was further tested by co-transfection and co-immunoprecipitation of Flag-tagged RASA3 and wild type or constitutively active Gαi3 proteins in HEK-293 cells (Fig. 5). Transfected Flag-RASA3 was successfully immunoprecipitated by anti-Flag M2 Affinity gel, with no Flag-RASA3 detected in non-transfected cells. Immunoprecipitation of Flag-RASA3 co-immunoprecipitated both wild type or constitutively active forms of Gαi3, with no Gαi3 present in immunoprecipitate from non-transfected cells. Interestingly, much more of the constitutively active form of Gαi3 (Gαi3QL) was bound to RASA3, consistent with an increased association of active Gαi3 with RASA3.

The interaction between endogenous Gαi3 and RasGAP3 in GH4ZR7 cells was assessed. GH4ZR7 cells were untreated or treated with apomorphine (1 μM) to activate dopamine D2S receptor signaling and cell lysates were prepared. The Gαi3 proteins were immunoprecipitated using anti-Gαi3 antibody and endogenous proteins detected using anti-Gαi3 and the D40 anti-RASA3 antibody to detect endogenous proteins (Fig. 6). When cell extracts were run on columns without anti-Gαi3 antibody, no Gαi3 or RASA3

was detected. By contrast when Gαi3 was immunoprecipitated, both endogenous Gαi3 and RASA3 were present in immunoprecipitates from untreated and treated GH4ZR7 cells. These results show a specific interaction is present between Gαi3 and RASA3 in unstimulated, as well as D2S-stimulated cells. Since only a small proportion of Gαi3 protein is activated upon D2S receptor activation, the enhanced RASA3 interaction that we observed with constitutively active Gαi3 may not be detectable upon receptor stimulation.

Functional role of RASA3 in ERK1/2 pathway

To address RASA3 function in D2S-induced signaling to inhibit ERK1/2 activation, GH4ZR7 cells were stably transfected with a full-length antisense RASA3 construct. As shown in Fig. 7, several GH4ZR7 clones stably transfected with AS-RASA3 show significant knockdown of RASA3 determined using D40 antibody in Western blot analysis. Clones RASA3-AS30 and RASA3-AS20 displayed the greatest knockdown of RASA3 protein levels (to about 30%) and were examined further for signaling to ERK1/2 compared to parental GH4ZR7 cells.

Inhibition of TRH-induced ERK1/2 phosphorylation by dopamine-D2S receptor signaling was examined in these clones (Fig. 8). Basal phosphorylation of ERK1/2 was undetectable and was not altered by the dopamine agonist apomorphine. TRH greatly increased ERK1/2 phosphorylation, and this effect was completely blocked by co-activation of dopamine-D2S receptors using apomorphine. Apomorphine-induced inhibition of TRH action was greatly suppressed in RASA3-AS30 and AS20 clones (Fig. 8A, B, respectively), by 70-80% compared to maximal D2S-induced inhibition in

GH4ZR7 cells (Fig. 8C). Importantly, the AS20 and AS30 clones displayed similar levels of dopamine D2 receptor density compared to parental GH4ZR7 cells (83±8, 97±4, and 115±8 fmol/mg, respectively), indicating that the reduced D2S receptor signaling was not due to fewer receptors. The pronounced reduction of D2S-mediated inhibition of ERK1/2 phosphorylation in GH4ZR7 clones depleted of RASA3 indicates a critical role for RASA3 in mediating D2S receptor signaling to inhibit ERK1/2 activation.

Discussion

RASA3, a linker between Gαi3 and Ras proteins

Our results delineate the obligatory role of a novel Gαi3 target, RASA3, in signaling of the dopamine-D2S receptor to inhibit ERK1/2 activation in GH4ZR7 pituitary cells. RASA3 was the predominant cDNA clone identified in the two-hybrid screen with constitutively active Gαi3-QL mutant, and its interaction with Gαi proteins was validated by three different approaches. First, yeast mating assay verified that RASA3 can interact with all constitutively-active Gαi, Gαo and Gαz mutants, but only with wild-type Gαi3 (Fig. 3). Second, purified recombinant RASA3 interacted with wild-type Gαi3 in the pull down assay (Fig. 4). Third, transfected Flag-RASA3 immunoprecipitated both wild-type Gαi3 and Gαi3-QL, with greater interaction with the latter (Fig. 5). Finally we found that endogenous RASA3 and Gαi3 interact, since anti-Gαi3 immunoprecipitated RASA3 (Fig. 6). Although both wild-type and constitutively active Gαi3 interacted with RASA3, there appeared to be a greater interaction with active Gαi3. This suggests that upon activation of dopamine D2S receptors, active Gαi3 may recruit RASA3 to inactivate membrane-bound Ras, leading to inhibition of Ras-ERK1/2 activation. However, activation of wild-type Gαi3 by apomorphine or AlF4- (Fig. 4, 6) did not enhance its interaction with RASA3 as clearly as for constitutively active Gαi3Q204L, perhaps because mutational activation is more pronounced and persistent. To address its role in D2 receptor signaling, depletion of RASA3 was done using antisense cDNA constructs (Fig. 7). We have found that stable transfection of full-length antisense constructs in GH4 cells provides excellent specificity for knockdown individual

Gαi/o subunits, which are highly (90% aa-identity) conserved (Albert and Morris, 1994; Liu et al., 1994c; Liu et al., 1999b). Depletion of RASA3 in two independent clones strongly blocked dopamine D2S-induced inhibition of TRH-induced ERK1/2 activation (Fig. 8), which clearly displays the critical role of RASA3 in this pathway. Furthermore, suppression of RASA3 had no effect on basal or TRH-induced ERK1/2 activity in antisense clones (Fig. 8). Together these data support a critical role for RASA3 in mediating D2S receptor signaling via Gαi3 to inhibit TRH-induced ERK1/2 activation

The preferential coupling of the dopamine D2S receptor via Gαi3 to inhibit ERK1/2 activation was suggested by previous studies of G-protein specificity in GH4ZR7 cells using a rescue strategy with stable transfection of PTX-insensitive Gα subunits cDNA's (Banihashemi and Albert, 2002a). Using this approach, we showed that the dopamine-D2S receptor signals through different subsets of Gi/Go proteins to inhibit cAMP formation (Gαi2 and Gαi3), decrease calcium influx (Gαo/Gβγ), and block TRH-induced ERK1/2 activation (Gαi3 and Gαo) (Banihashemi and Albert, 2002a). Thus, although Gαi3 may be preferentially involved in D2S receptor-mediated inhibition of TRH-induced c-Raf-ERK1/2 signaling, Gαo could also play a role since expression of Gαo partially rescued D2S signaling to this pathway. However, the preferential interaction of RASA3 with both non-activated and active forms of Gαi3 but not other non-activated Gαi/Gαo subunits suggests a preference for wild-type Gαi3 (Fig. 3). This is consistent with a "pre-coupled" state of Gαi3 and RASA3, which may explain the preference of D2S receptor for Gαi3 in signaling to this pathway.

Liu and colleagues also found in GH4ZR7 cells that dopamine-D2S-induced inhibition of basal ERK1/2 activity is mediated through Gαo, but not Gαi2 (Liu et al.,

2002a). In our studies, the dopamine-D2S receptor inhibited both TRH-stimulated c-Raf and basal B-raf activity (Banihashemi and Albert, 2002a). B-raf inhibition is likely mediated by Gαo, which upon activation mobilizes Rap1GAP to inhibit Rap1-mediated B-raf-ERK1/2 signaling (Jordan et al., 1999a). However, since RASA3 has dual activity against both Ras and Rap proteins (Cullen et al., 1995; Kupzig et al., 2006), it is possible that RASA3 also mediates D2S receptor/Gαo-mediated inhibition of basal Rap-B-Raf activity.

We have recently shown that somatostatin and muscarinic receptors can mediate inhibition of TRH-induced ERK1/2 activation (Itzhaki Van-Ham et al., 2007), consistent with previous findings that these receptors are able to couple via Gαi3 in these cells (Liu et al., 1999b; Albert, 2002a). However, not all Gαi-coupled receptors coupled to this pathway, as D2L receptors failed to inhibit TRH-induced ERK1/2 activation (Itzhaki Van-Ham et al., 2007). The lack of coupling of the D2L receptor may reflect inefficient coupling of this receptor to Gαi3 in these cells, but this has not been tested. Thus, receptors that couple to Gαi3 would be predicted to mediate this signaling to inhibit ERK1/2 in cells that express RASA3.

RASA3: structure, function and regulation

RASA3 is a member of the GAP1 family of RasGAP proteins, based on their sequence homology, particularly in the RasGAP domain. The GAP1 proteins all have similar primary structure containing C2, RasGAP, PH and BTK domains (Suppl. Fig. 1), although based on the tertiary structure of p120GAP, it is believed that protein is folded so that both N-terminal C2 domains and the C-terminal PH/Btk domains are in close

proximity (Yarwood et al., 2006). GAP1m is the most closely related variant of RASA3, with similar C2 domains that lack key residues required for Ca2+-binding, thus these two proteins are not regulated by [Ca2+]i, unlike CAPRI and RASAL (Lockyer et al., 1997; Lockyer et al., 1999a; Cozier et al., 2000; Kupzig et al., 2005; Liu et al., 2005c; Yarwood et al., 2006). Interestingly, although not regulated by calcium, Gα12 has been shown to bind directly to GAP1m via its PH/BTK domain, leading to its activation (Jiang et al., 1998b). Our identification of the Gαi3-RASA3 interaction is the first evidence that RasGAPs are connected to Gi/Go protein signaling. In yeast mating assays, the C-terminal portion of RASGAP3 containing PH/BTK domains did not interact with Gαi3 (data not shown), suggesting that the interaction domain may be larger or different than that of GAP1m and Gα12. Thus both GAP1m and RASA3 link to G-proteins to inhibit Ras-ERK1/2 signaling, although via different G-protein pathways.

In addition to its regulation by Gαi protein, the PH domain of RASA3 binds to both PIP3 (phosphatidylinositol 3,4,5-trisphosphate) and PIP2 (phosphatidylinositol 4,5-bisphosphate), resulting in its constitutive association with the plasma membrane (Cozier et al., 2000; Yarwood et al., 2006). Among GAP1 family members, RASA3 is uniquely regulated by IP4 produced from inositol 1,4,5-trisphosphate upon phospholipase C activation. IP4 binds the PH domain and is thought to compete with PIP2, resulting in activation of RasGAP activity (Cozier et al., 2000; Yarwood et al., 2006). The TRH receptor signals via Gαq/11, which activates phospholipase C. Stimulation of phospholipase C leads to the formation of IP3 and diacylglycerol, release of calcium stores and activation of PKC (Jones et al., 2007), which is thought to mediate Ras-ERK1/2 activation (Wang and Maurer, 1999a; Gutkind, 2000; Smith et al., 2001).

Oppositely, the generation of IP4 and activation of RASA3 may inhibit ERK1/2 activation. It is possible that IP4- and Gαi3-mediated activation of RASA3 are synergistic, and that activation of both messengers is required for RASA3 activation in vivo. TRH induced a robust stimulation of ERK1/2 phosphorylation (Banihashemi and Albert, 2002a; Itzhaki Van-Ham et al., 2007) (Fig. 8), suggesting that IP4-activated RASA3 was not able to inhibit ERK1/2 phosphorylation. But, upon activation of D2S receptor-Gαi3, TRH-induced ERK1/2 activation was almost completely suppressed, suggesting a synergistic effect of both IP4 and Gαi3 on activation of RASA3 that overcomes stimulatory effect of TRH on ERK1/2 phosphorylation. Future studies in vitro and in vivo are necessary to address whether such synergy occurs.

Roles of D2S receptor-Gαi-RASA3 signaling

Several lines of evidence indicate that dopamine-D2 receptors negatively regulate the proliferation and differentiation of lactotrophs in vivo, as well as inhibiting PRL synthesis and secretion (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001). However, the specific signaling pathways involved in D2 receptor actions on cell proliferation and PRL synthesis have not been clarified.

In GH4 cells, TRH-induced ERK1/2 activation mediates increase in PRL transcription (Wang and Maurer, 1999a; Smith et al., 2001), and inhibition of ERK1/2 is required for dopamine-D2S receptor mediated inhibition of PRL gene transcription (Liu et al., 2002a; Liu et al., 2005a). Since inhibition of RASA3 largely prevented apomorphine-induced inhibition of TRH-mediated ERK1/2 activation (Fig. 8), it is likely that this pathway is critical to inhibit TRH-induced PRL transcription. However, it

remains unclear whether this pathway also inhibits basal PRL synthesis, as we were unable to detect basal ERK1/2 phosphorylation under our conditions. As discussed above, since RASA3 inactivates both Ras and Rap, it is possible that it mediates inhibition of basal ERK1/2 activity. In addition, while dopamine D2 receptor activation inhibits cell proliferation, the role of inhibition of ERK1/2 activation remains unclear, as multiple G proteins (and presumably alternate signaling pathways) are involved in inhibition of cell proliferation (Albert, 2002a).

Since we found in GH4ZR7 cells that D2S signaling to ERK1/2 is inhibited upon depletion of RASA3, it is possible that other cell types that express RASA3 can mediate inhibitory Gαi signaling to ERK1/2. RASA3 RNA is expressed in different human tissues including brain, skeletal muscles, spleen, peripheral blood leukocytes and platelets suggesting roles in all of these tissues (Lockyer et al., 1999b; McNulty et al., 2001). In the brain, immunoreactivity for RASA3 is highest in the CA1 of the hippocampus, amygdala, cerebellum and pyriform cortex (Signore et al., 1999). As discussed above, other Gαi-coupled receptors have the potential to couple to RASA3 to inhibit ERK1/2 activation, and the role of RASA3 may depend on the receptors present. More recently we have shown in primary rat striatal cultures that the D2-selective agonist quinpirole inhibited potassium-stimulated ERK1/2 activation (Itzhaki Van-Ham et al., 2007). However, the role of RASA3 in inhibitory regulation of ERK1/2 activation in striatal cells remains to be elucidated. The widespread distribution of RASA3 and Gαi proteins suggests an important role for this coupling in a variety of physiological processes involving regulation of ERK1/2 signaling. For example, if Gαi-RASA3 signaling is

important in vivo, the development of pharmacological compounds could provide novel anti-proliferative agents by activating RASA3 to inhibit cell proliferation.

Conclusion

In this study we identified a novel Gαi-interacting protein, RASA3, and determined the importance of Gi-RASA3 coupling in the negative regulation of ERK1/2 activation by the D2S receptor. Our results indicate that the D2S receptor couples via Gαi-RASA3 to inhibit TRH-induced ERK1/2 activation in pituitary cells, suggesting that RASA3 may be a general mediator of Gαi-induced inhibition of ERK1/2 activation.

Footnotes

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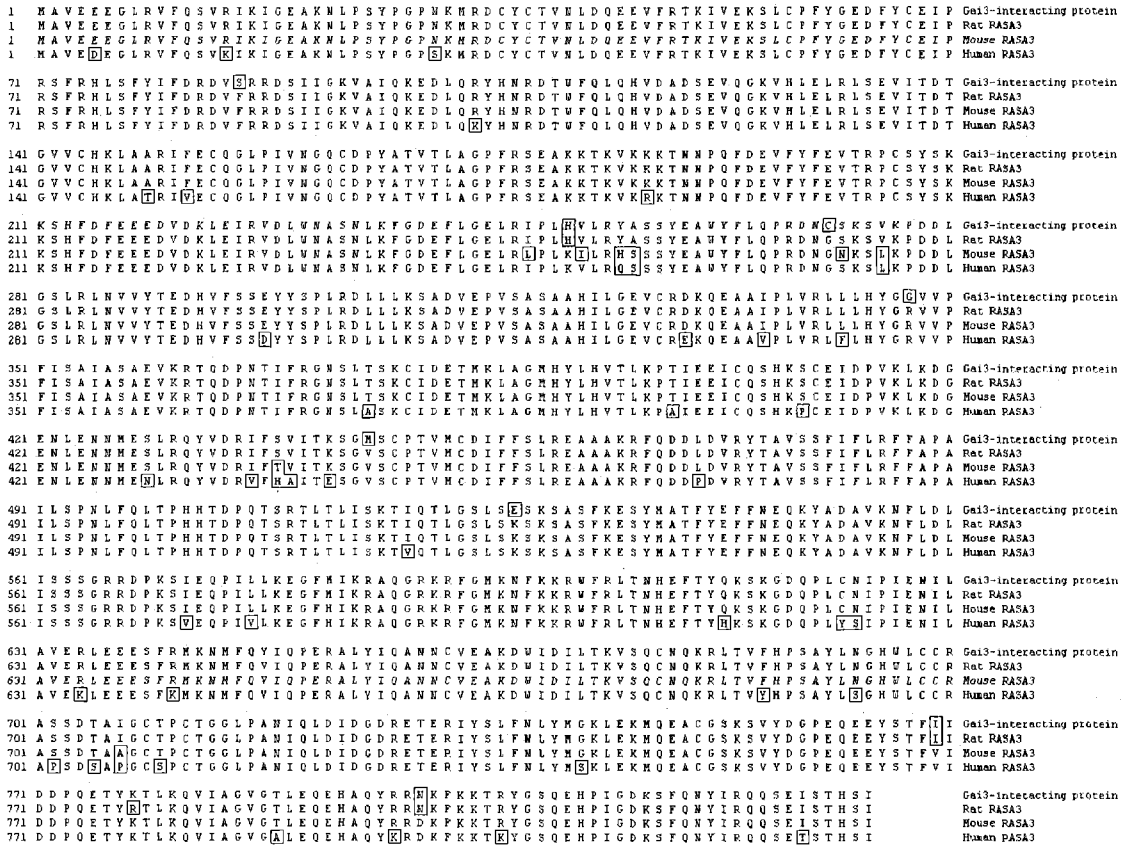
The abbreviations used are D2S, short isoform of dopamine D2 receptor; TRH, thyrotropin-releasing hormone; GH, growth hormone; PRL, prolactin; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; GAP, GTPase activating protein; IP4, inositol 1,3,4,5-tetrakisphosphate; CAPRI, Ca2+-promoted Ras inactivator; RASAL, Ras-GTPase-activating-like protein; PH, pleckstrin homology; BTK, Bruton's kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.

Figure 1

A


Gai3-interacting protein
Rat RASA3
Mouse RASA3
Human RASA3

B

Percent identity

	1	2	3	4		
1		99.3	98.1	94	1	Gαi3-interacted protein
2	0.7		98.6	94.5	2	Rat RASA3
3	1.9	1.4		94.9	3	Mouse RASA3
4	6.2	5.7	5.3		4	Human RASA3
	1	2	3	4		

Divergence

C

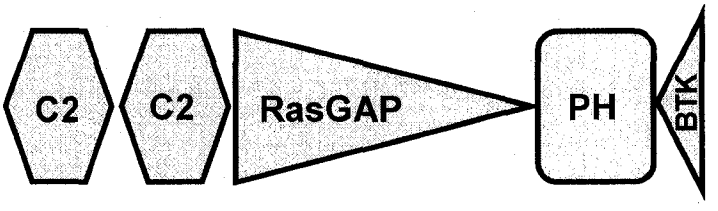


Fig. 1. RASA3, a Gαi3-QL-interacting clone from yeast two hybrid screen of GH4ZR7 cDNA library. **A)** Amino acid sequences of rat, mouse and human RASA3 aligned with Gai3-interacting clone using DNAStar MegAlign program. **B)** Percent amino acid identity between the new Gαi3-interacter and rat, mouse and human RASA3. **C)** Schematic representation of functional domains of RASA3.

Figure 2

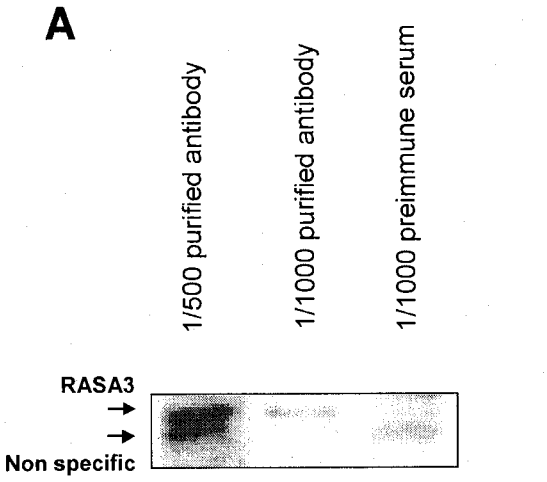

A
1/500 purified antibody
1/1000 purified antibody
1/1000 preimmune serum
RASA3
Non specific
B
RASA3
GH4ZR7
RASA3-AS30
BDYII
BDYII-21

Fig. 2. Presence of RASA3 in GH4ZR7 cells detected using a polyclonal anti-RASA3 antibody. **A)** PROSEP-G-purified D40 (full-length RASA3 antibody) detected the endogenous RASA3 protein in GH4ZR7 cell lysate (50 μg/lane) with 1/500 and 1/1000 dilution, while 1/1000 dilution of preimmune serum (Pre-imm) failed to recognize the protein. **B)** PROSEP-G-purified D40 was used to detect endogenous level of RASA3 (arrow) in GH4ZR7 or BDYII cells and two derivative stable cell lines of overexpressed or depleted RASA3. GH4ZR7: GH4C1 cells stably transfected by D2S (short isoform of dopamine D2 receptor); RASA3-AS30: GH4ZR7 stably transfected by antisense RASA3; BDYII: Balb-c/3T3 cells stably transfected by D2S; BDYII-21: BDYII cells stably transfected by sense RASA3. The same total protein concentration (50 μg/lane) was loaded in each well.

Figure 3

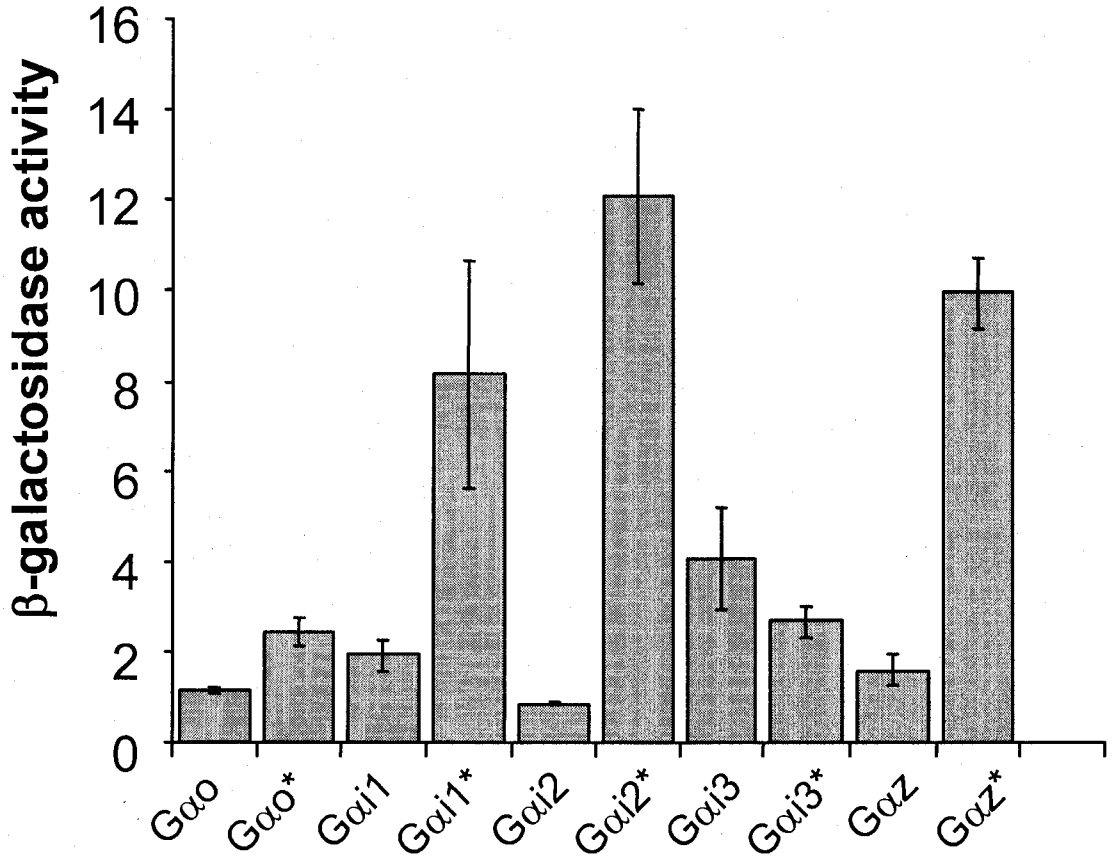

β-galactosidase activity
16
14
12
10
8
6
4
2
0
Gαo
Gαo*
Gαi1
Gαi1*
Gαi2
Gαi2*
Gαi3
Gαi3*
Gαz
Gαz*

Fig. 3. Specificity of RASA3-Ga interaction. Interactions between RASA3 and the indicated wild-type or constitutively active mutant (*) Gα proteins were examined by yeast mating assay. Quantitative β-galactosidase assay was performed on cell lysates, and data were normalized to the positive control pCL1 from the same experiment presented as mean ± S.E. (n = 4-5).

Figure 4

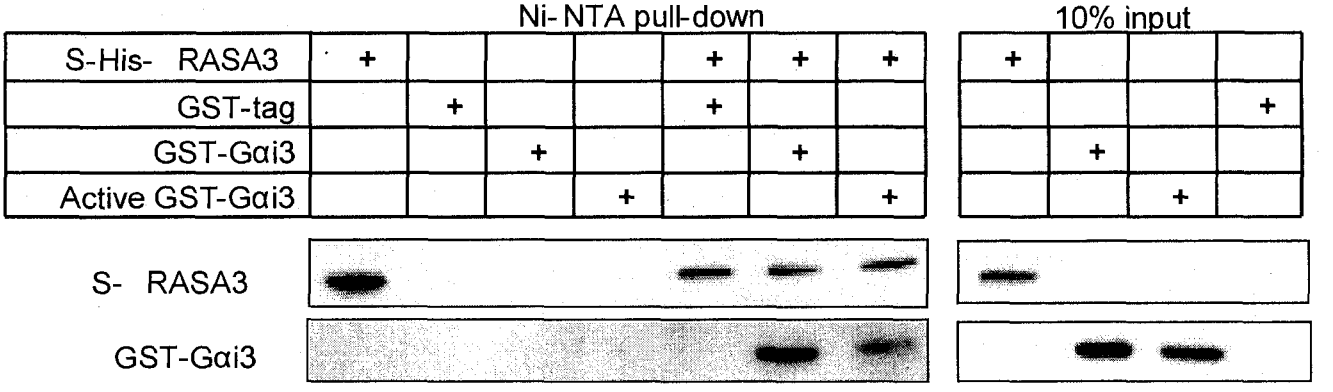

Ni-NTA pull-down
10% input
S-His- RASA3
GST-tag
GST-Gαi3
Active GST-Gαi3
S- RASA3
GST-Gαi3

Fig. 4. Direct interaction between RASA3 and Gai3 in vitro. For in vitro pull-down assay, bacterially expressed GST or GST-Gαi3 fusion protein was incubated with S-6xHis-RASA3 protein and the His-tag was pulled down using Ni-NTA beads. The proteins were resolved by SDS-PAGE and immunoblotted using anti-S (1/5000) or anti-Gαi3 (1/1000) antibodies. $AlF4^-$ was used to activate GST-Gαi3.

Figure 5

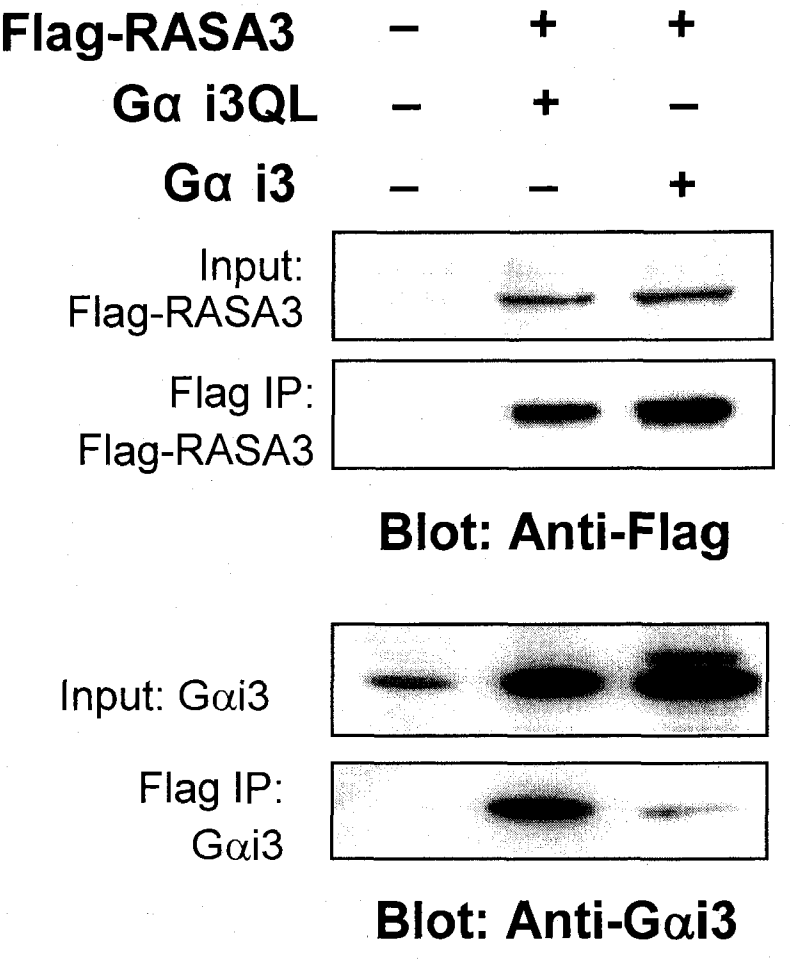

Flag-RASA3 – + +
Gα i3QL – + –
Gα i3 – – +
Input: Flag-RASA3
Flag IP: Flag-RASA3
Blot: Anti-Flag
Input: Gαi3
Flag IP: Gαi3
Blot: Anti-Gαi3

Fig. 5. Preferential interaction between RASA3 and constitutively active Gai3 in cells. HEK293 cells were transiently cotransfected with pcDNA3FLAG-RASA3 with pcDNA3-Gαi3 (wild-type or constitutively active mutant). For coimmunoprecipitation, cell lysate was incubated with anti-FLAG M2-agarose affinity beads, eluted, and resolved by SDS-PAGE. Membranes were blotted with either anti-Flag antibody (1/1000) or specific anti-Gαi3 antibody (1/1000). Note that RASA3 interacted with both Gαi3 wild-type and constitutively active forms, but the latter showed the stronger interaction.

Figure 6

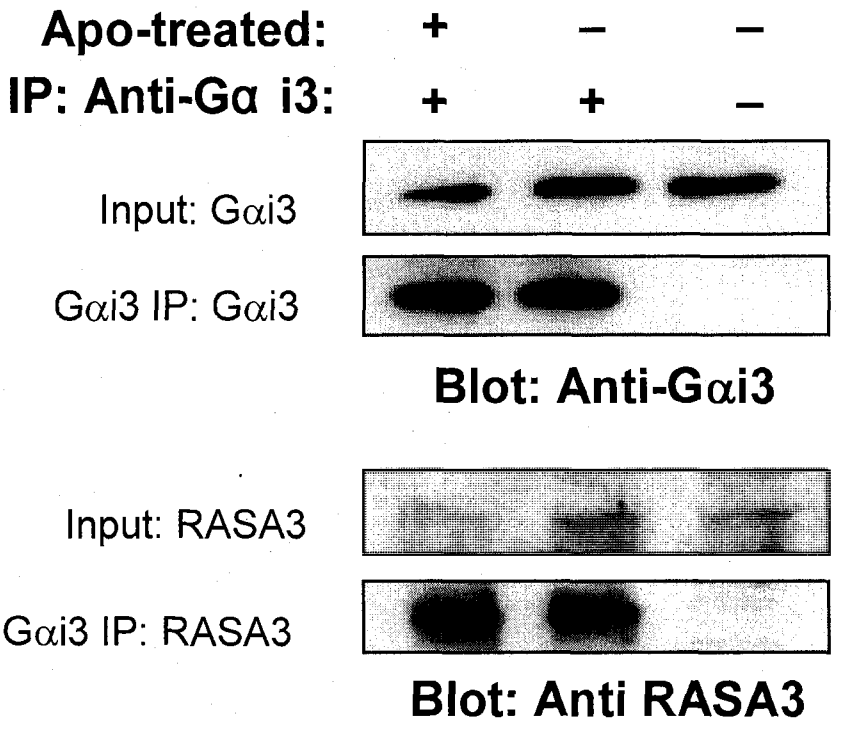

Apo-treated: + – –
IP: Anti-Gα i3: + + –
Input: Gαi3
Gαi3 IP: Gαi3
Blot: Anti-Gαi3
Input: RASA3
Gαi3 IP: RASA3
Blot: Anti RASA3

Fig. 6. Interaction between endogenous RASA3 and Gαi3 in GH4ZR7 cells. For assay, the cells were incubated or not with apomorphine (1μM) for 15 minutes followed by cell lysis. The cell lysate was incubated with anti-Gαi3 antibody and protein G Sepharose, eluted, and resolved by SDS-PAGE. Membranes were blotted with either D40 anti-RASA3 antibody (1/1000) or anti-Gαi3 antibody (1/1000). Note that in both unstimulated, as well as apomorphine-stimulated cells RASA3 interact with Gαi3, but not in the absence of anti-Gαi3 antibody (negative control).

Figure 7

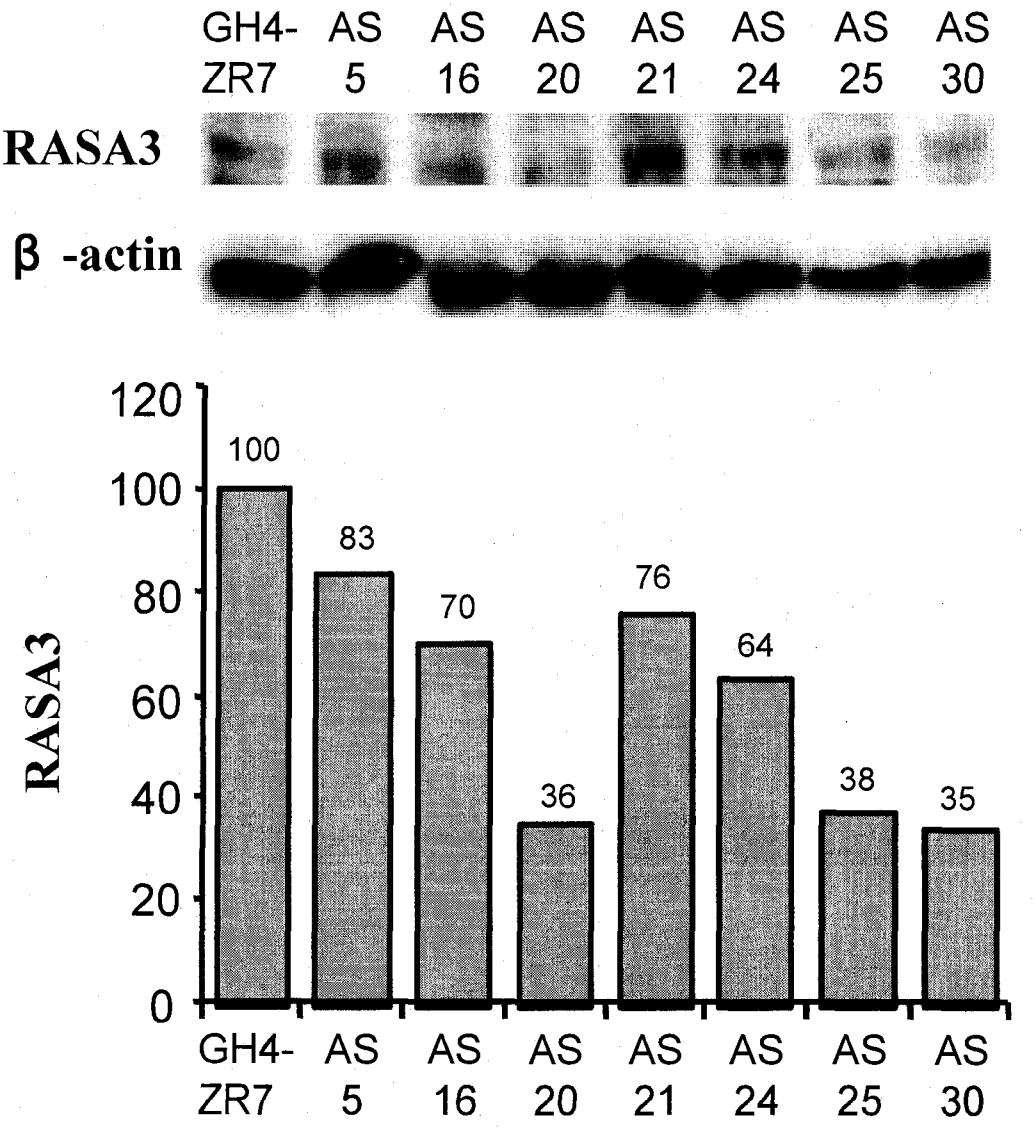

GH4-ZR7
AS 5
AS 16
AS 20
AS 21
AS 24
AS 25
AS 30
RASA3
β -actin
RASA3
120
100
80
60
40
20
0
100
83
70
36
76
64
38
35

Fig. 7. Depletion of RASA3 in GH4ZR7 clones stably transfected with antisense RASA3. GH4ZR7 cells were stably transfected with pcDNA-antisense RASA3, and stable clones selected and screened for endogenous RASA3, as detected by D40 antibody (1/1000). Above is a representative Western blot, while below is quantification of RASA3 protein normalized to β-actin expressed as % of GH4ZR7 content (100%). Clones RASA3-AS30 and RASA3-AS20 were selected for further experiments as RASA3-depleted clones.

Figure 8

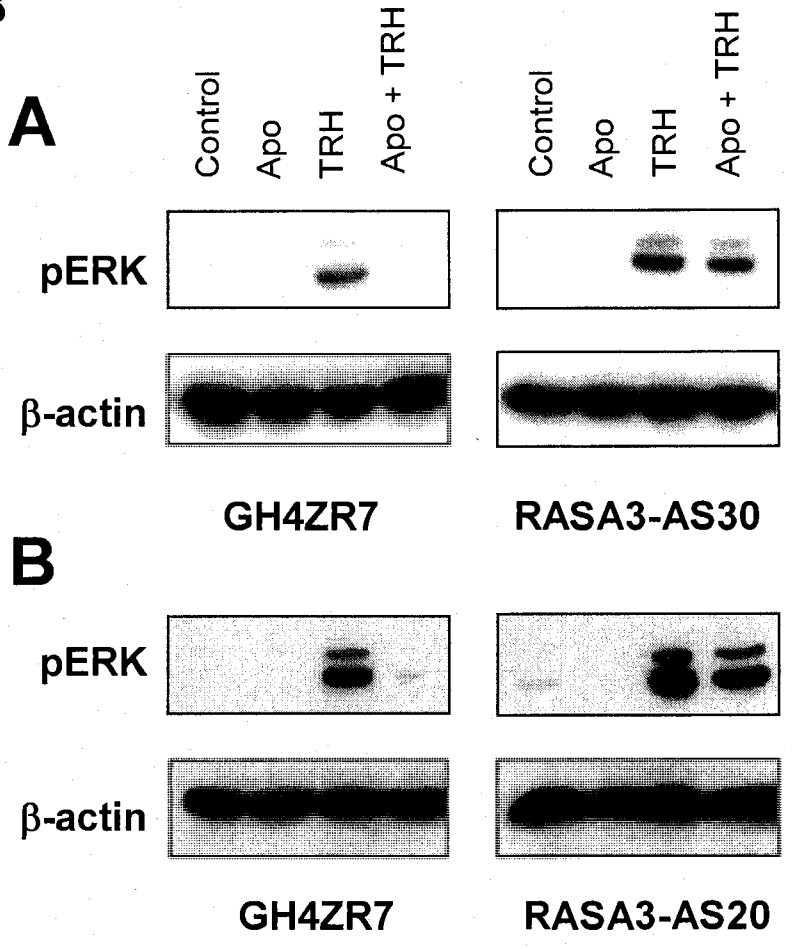
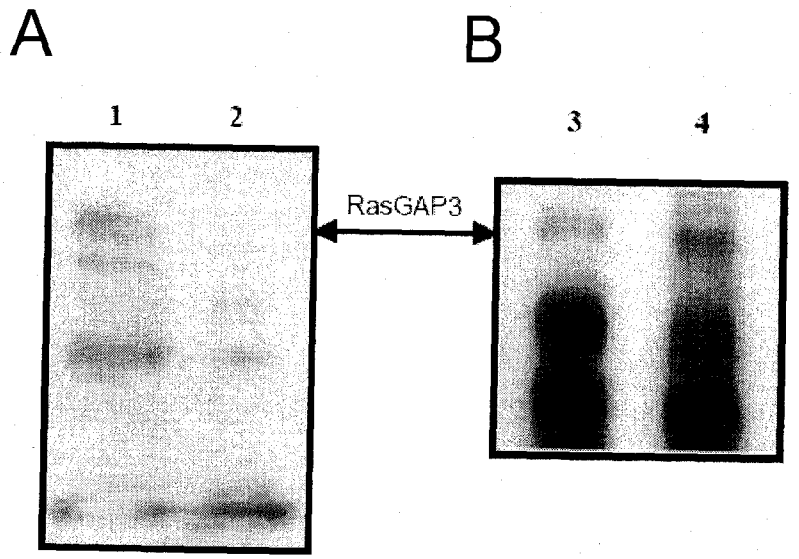

A
Control
Apo
TRH
Apo + TRH
Control
Apo
TRH
Apo + TRH
pERK
β-actin
GH4ZR7
RASA3-AS30
B
pERK
β-actin
GH4ZR7
RASA3-AS20
C
GH4ZR7
RASA3-AS30
RASA3-AS20
D2S-induced ERK Inhibition (%control)
120
100
80
60
40
20
0
100
100
**
**
24
22
**
**
30
23
ERK1
ERK2

Fig. 8. Suppression of dopamine D2S-induced ERK1/2 inhibition in GH4ZR7 cells transfected with antisense RASA3. Cells were pretreated or not with 1 μM apomorphine (Apo) for 15 min followed by addition of 1 μM TRH or control for 7 minutes, as indicated. Western blot analysis of lysates was done using specific antibody against phospho-ERK1/2 (1/1000). Membranes were reprobed with β-actin antibody (1/2000 as a loading control. Representative blots from GH4ZR7 compared to in RASA3-AS30 (A) and RASA3-AS20 clones (B) are shown. (C) Data from three independent experiments are presented as mean±S.E. of % apomorphine-induced inhibition of TRH-induced pERK1/2 in clones compared to parental GH4ZR7 cells (100%). **$P < 0.05$ by paired two-tailed t-test.

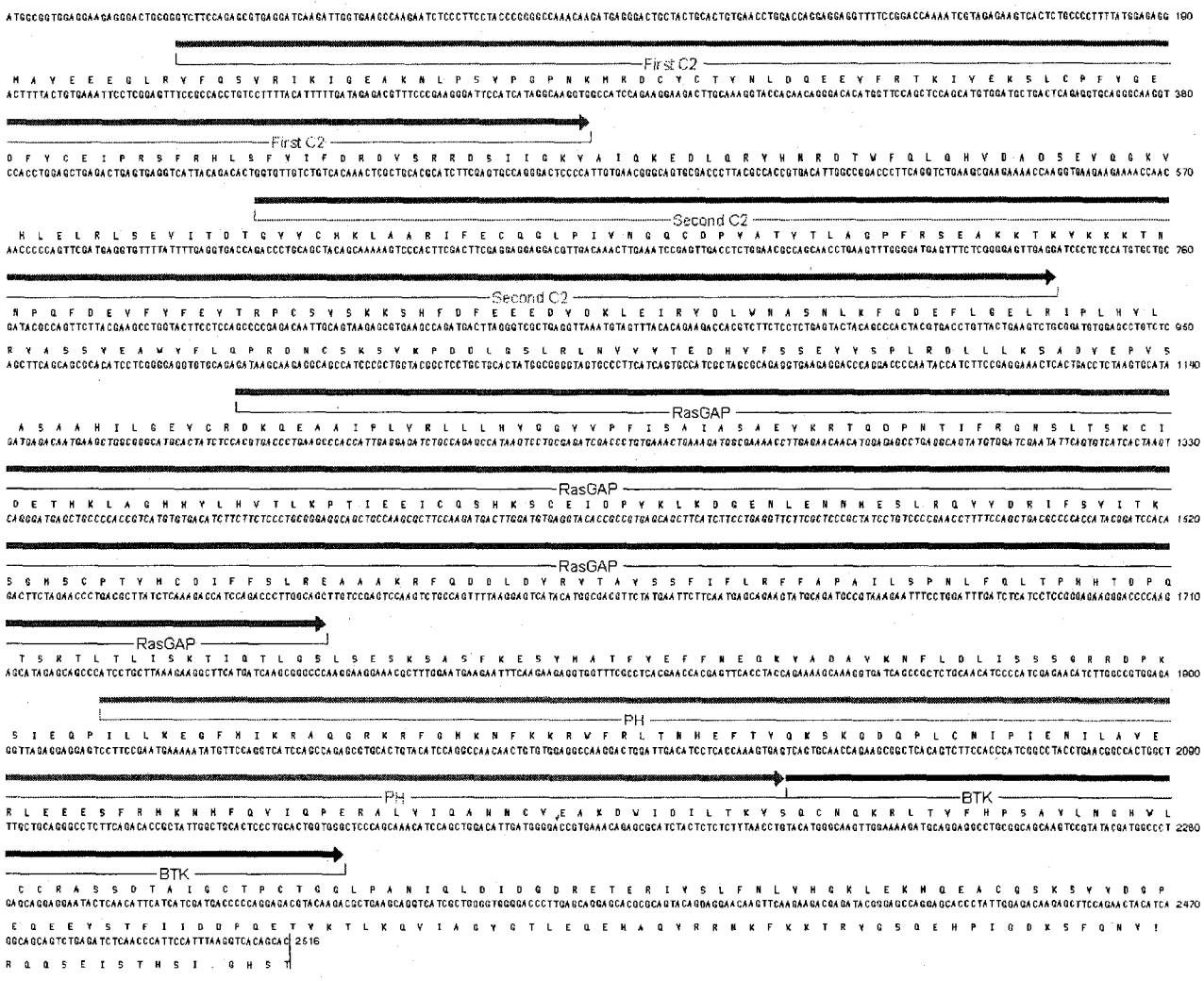
Suppl. figure 1



Supp. Fig. 1A,B 1,2- Purified RASA3 and pET-30a(+) vector alone blotted by D40, specific RASA3 antibody, respectively.

3,4- Purified RASA3 blotted by anti S-tag and D40, specific RASA3 antibody, respectively.

Suppl. figure 2



Supp. Fig. 2 Functional domains of RASA3. Surrounding the RasGAP domain at the N-terminal end are tandem C2 domain repeats. C-terminal to the RasGAP domain is a PH domain that contains a Btk motif.

CHAPTER III

GENERAL DISCUSSION

Dopamine, in the central nervous system, governs a broad range of physiological functions, from the control of locomotor activity and behavior to the synthesis of pituitary hormones (Picetti et al., 1997). The dopamine D2 receptor is a member of seven transmembrane domain GPCRs and is highly expressed in the pituitary gland (Missale et al., 1998b). This receptor regulates many signaling pathways. It is well known that dopamine D2 receptor agonists, such as bromocriptine, pergolide, quinagolide, cabergoline, and lisuride, can inhibit PRL synthesis and secretion by binding to receptors located on normal as well as tumorous pituitary cells (Ivan et al., 2005) and bromocriptine has been used as an effective drug for pituitary adenoma for many years (Colao et al., 1995; Kanasaki et al., 2000).

Recently, gene knockout studies in mice have demonstrated the role of D2 receptor in the formation of pituitary gland as well as prolactin secretion. In D2R-deficient mice, there was a hyperproliferation of lactotrophs resulting in pituitary prolactinoma and also chronic hyperprolactinemia (Kelly et al., 1997; Saiardi et al., 1997; Cristina et al., 2006). Recently, Hnasko et al. used a viral-mediated gene transfer to selectively restore dopamine to the dorsal striatum of dopamine-deficient mice which permits the mice to survive without necessary daily treatment with 3,4-L-dihydroxyphenylalanine (L-dopa). They demonstrated that mice chronically lacking tuberoinfundibular dopamine secrete large amounts of prolactin because of the development of severely enlarged pituitary glands mainly consisting of hyperplastic hypertrophic lactotrophs and multifocal prolactinomas (Hnasko et al., 2007).

Conversely, knockout mice lacking the dopamine transporter gene, display an augmentation of dopaminergic tone due to lack of the dopamine reuptake mechanism that

leads to an opposite pituitary phenotype, namely hypoprolactinoma and pituitary hypotrophy (Bosse et al., 1997; Gainetdinov et al., 1999). Although, many studies have demonstrated the crucial role of the dopamine D2 receptor in prolactin synthesis, secretion and cell proliferation in pituitary cells, the underlying mechanisms are still not well understood. Among multiple regulatory pathways involved in cell proliferation and PRL synthesis, MAPK cascades are known to have an important role (Liu et al., 2002b). Furthermore, although activation of MAPK pathways are known to be crucial in mediating stimulatory responses of the prolactin gene to growth factors (Schweppe et al., 1997; Castillo et al., 1998), thyrotropin-releasing hormone (TRH) (Wang and Maurer, 1999b), and estrogen (Watters et al., 2000), the specific pathways that mediate inhibition of MAPK regulation in the dopaminergic suppression of prolactin need to be clarified.

My thesis is mainly focused on the mechanism of the inhibitory action of the dopamine D2S receptor on the Ras-ERK1/2 cascade in pituitary cells.

D2 modulation of MAPK activation

The mitogen-activated protein kinases are a group of serine/threonine kinases, which are phosphorylated/stimulated by a cascade of protein kinases to induce responses such as proliferation, differentiation, apoptosis, and long-term potentiation. This signaling cascade is an important cellular pathway and is used by many growth factors, hormones, and neurotransmitters to modulate diverse physiological functions (Kim et al., 2004). It has also been shown that various GPCRs are able to activate MAPK and that this may allow plasma membrane receptor systems to influence different cellular processes, from cell proliferation to hormone secretion.

Activation of MAPKs by D2 receptor has been well-documented in a wide range of cultured mesenchymal cells, including COS (Faure et al., 1994), Balb-c/3T3 (Ghahremani et al., 2000), Chinese hamster ovary (Oak et al., 2001) and C6 glioma (Luo et al., 1998). It also activates MAPKs in some tissues like brain slices (Yan et al., 1999; Calabresi et al., 2001) or lung epithelium (Guerrero et al., 2001). This activation is generally blocked by the ADP-ribosylating agent pertussis toxin (Faure et al., 1994; Ghahremani et al., 2000; Oak et al., 2001), indicating a requirement for the class of Gi/o proteins (Liu et al., 2002b). As mentioned in chapter I, both α and $\beta\gamma$ subunits can mediate this stimulatory signal (please see "Regulation of ERK1/2 by Gi" in the introduction).

Unlike mesenchymal cells, MAPK regulation by D2 receptor in pituitary gland is unique because there are several independent findings demonstrating that dopamine or its agonists lower the levels of activated (phosphorylated) MAPKs, ERK1 and ERK2. For instance, Ohmichi et al showed that in both GH3 and primary cultures of rat anterior pituitary cells, TRH activated ERK1/2, while dopamine was able to reduce that MAPK phosphorylation (Ohmichi et al., 1994b). They also have shown that in GH3 rat pituitary cells, TRH activates MAPK through a PKC-dependent pathway as well as a second pathway possibly involving tyrosine phosphorylation (Ohmichi et al., 1994a). Inhibition of MAPK by dopamine-D2 signaling has also been observed in GH4ZR7 cells (Banihashemi and Albert, 2002b; Liu et al., 2002b; Liu et al., 2005b) and in primary cultures of rat pituitary cells (Liu et al., 2002b). Inhibition of MAPK by D2 receptors is required for inhibition of PRL transcription (Liu et al., 2002b; Liu et al., 2005b). Taken

together, these studies indicate a key role for inhibition of MAPK in dopamine-D2 receptor actions in pituitary cells, especially for inhibition of PRL transcription.

Although the inhibitory action of D2R agonists on MAPKs in pituitary cells appears consistent with their inhibitory actions on prolactin synthesis and cell proliferation, there are controversial observations in the literature. In 2002, Iaccarino et al. generated transgenic mice overexpressing either D2L or D2S in lactotrophs. They demonstrated that increased expression of D2S, but not of D2L, resulted in pituitary hypoplasia as already expected. But, surprisingly they found that overexpession of D2S also led to MAPK induction. They also evaluated the levels of phospho-ERKs in pituitary tumor extracts from anterior lobes of D2R-null mice and observed a significant decrease in MAPK activation compared with wild type female mice (Iaccarino et al., 2002a). It is worth mentioning here that in a normal situation, two isoforms of D2 receptor are in a well-defined ratio *in vivo* and changing this ratio by overexpressing D2L or D2S could lead to unexpected physiological responses. Furthermore, the whole pituitary anterior lobe, consisting of several different cell types, was dissected and homogenized. Thus, the overall increased level of phospho-MAPKs could be from other cells not only lactotrophs. This is particularly likely in the D2S-overexpressing mice, which lack lactrotrophs.

GH4ZR7 cells are derived from rat pituitary tumors and stably transfected by D2S cDNA, a receptor absent in GH cells but present in normal lactotrophs. It has been shown that upon activation of D2S receptor in this cell line, TRH-induced ERK1/2 activation was strongly inhibited, a finding contradicting that in D2S overexpressing transgenic mice but consistent with the inhibitory effect of dopamine on prolactin synthesis and cell

proliferation (Banihashemi and Albert, 2002b; Liu et al., 2002b). To further examine the cell context for dopaminergic suppression of ERK1/2, Liu et al. measured the dopamine response in primary rat pituitary cells and found that dopamine or bromocriptine reduced phospho-ERK levels, showing that D2R-dependent regulation of MAPK in normal pituitary cells parallels the response in the GH4ZR7 model (Liu et al., 2002b). Moreover, in striatal cultures, the D2-selective agonist quinpirole inhibited potassium-stimulated ERK1/2 phosphorylation, indicating the presence of D2-induced MAPK inhibition pathway in neurons (Van et al., 2007).

By generating GH4ZR7 stable cell lines transfected by different PTX-insensitive Gαi/o subunits or GRK-ct (a selective Gßγ scavenger), Banihashemi et al. showed that D2S inhibitory effect on TRH-induced MAPK phosphorylation was through Gαi3 and Gαo but not Gαi2 or Gβγ. This concept that Gαi2 and Gβγ are not involved in inhibition of MAPK is consistent with our current knowledge that Gαi2 and Gβγ subunits are the main mediators of Gi/o-induced MAPK stimulation (please refer to "Regulation of ERK1/2 by Gi" in the introduction). Indeed, in both GH3D2L and GH3D2S cell lines (GH3 cells stably transfected by D2L and D2S, respectively) dopamine treatment slightly increases ERK phosphorylation compared to parental GH3 cells (An et al., 2003) that could be through the Gαi2 or Gβγ subunits. However, in GH4ZR7 cells, no basal or D2S-induced phosphorylation of MAPK was detected by using antibody specific for dual phosphorylated MAPK (Banihashemi and Albert, 2002b). In another case, dopamine decreased the level of basal phospho-MAPK that was quantified by its ability to phosphorylate the substrate ETS protein, ELK1 (Liu et al., 2002b).

To further show the complexity and specificity of D2 regulation of MAPK, I should mention that although activation of rat or human dopamine-D2S receptors inhibits thyrotropin-releasing hormone-induced ERK1/2 phosphorylation, long isoform of D2 receptor was unable to mediate this response (Van et al., 2007). Furthermore, in D2S and D2L overexpressing transgenic mice, whereas D2S overexpression leads to a significant reduction of prolactin synthesis, D2L greatly elevates prolactin secretion (Iaccarino et al., 2002a).

Overall, it seems that D2 regulation of ERKs in pituitary cells utilizes different receptor isoforms and G proteins and there is a cross talk between this pathway and other cellular regulatory pathways.

Role of RASA3 in D2S-induced MAPK inhibition

The demonstration of inhibitory action of D2S receptor on basal and TRH-induced MAPK phosphorylation in pituitary cells raised an intriguing question: which pathways and what proteins mediate the signal from the cell membrane to ERK1/2 module? Banihashemi et al. showed that Gαo and Gαi3 were required mediators and while Gαo was mainly involved in inhibition of B-Raf and basal MAPK activation, the other one played a role in suppression of TRH-induced MAPK phosphorylation. It has been shown that TRH recruits Gαq subunit to signal to Ras and C-Raf proteins (Please see "Gq regulation of ERK1/2" in the introduction). To date, Gαi3 has been connected to neither Ras nor C-Raf and my thesis is the first evidence demonstrating a specific link between Gαi3 and Ras/C-Raf/MEK/ERK module.

To identify the possible interacting proteins to a known protein, such as Gαi3 in our case, yeast two hybrid screening of a cDNA library of the relevant cell type provides

one of the best strategies. By yeast two-hybrid screening of cDNA library from GH4ZR7 cells with constitutive active Gαi3, we identified GAP1(IP4BP) or RASA3 and the interaction was further confirmed by β-galactosidase assay.

It has been shown that RASA3 inactivates Ras and Rap proteins by enhancing their weak intrinsic GTPase activity (Cullen et al., 1995; Kupzig et al., 2006). RASA3 is a member of the GAP1 family due to its consensus 330-aa RasGAP domain and is unique because it is stimulated by inositol 1,3,4,5-tetrakisphosphate (IP4) (Kupzig et al., 2005). IP4 is generated by a Ca^{2+}-regulated IP3 3-kinase following receptor-mediated PLC activation (Cullen and Lockyer, 2002). This protein has also high affinity to bind to PIP2 and PIP3 (Yarwood et al., 2006) and is constitutively associated with the plasma membrane via PH domain-PIP2 interaction (Cozier et al., 2000). Since both PIP2 and IP4 have the same binding site on PH domain of RASA3 (Cullen et al., 1995; Cozier et al., 2000), it has been suggested that the binding of IP4 to this site serves to remove the inhibitory influence of PIP2 and consequently allowing activation of the Ras-GAP activity (Yarwood et al., 2006).

As mentioned in chapter I, stimulation of Gαo can lead to inhibition of Rap1/B-Raf/MEK/ERK pathway by activating Rap1GAP (Jordan et al., 1999b). Therefore, in a similar manner, RASA3 may be the best candidate to link Ras/C-Raf/MEK/ERK to another inhibitory Gα subunit, Gαi3. Figures 3-6 illustrate different approaches used to demonstrate the interaction between RASA3 and Gαi3 proteins. In vitro pull-down assay (fig. 4) depicts the direct interaction between these proteins; moreover yeast mating and co-immunoprecipitation assays (fig. 3, 5, 6) further support the interaction in yeast or mammalian cells. Furthermore, co-immunoprecipitation assay using overexpressed

proteins suggests that stimulated-Gαi3 has greater affinity for binding to RASA3 than inactive GDP-bound Gαi3 (fig. 5). Conversely, in the case of Gαo, its GDP-bound inactive form strongly binds Rap1GAP, but upon activation it inhibits Rap1 by releasing Rap1GAP protein (Gutkind, 2000).

The functional importance of RASA3 in the inhibitory effect of D2S receptor/Gαi signaling on MAPK has been further supported by showing that depletion of endogenous level of RASA3 in pituitary cells leads to suppression of D2S/Gαi3-induced MAPK inhibition (Fig 8). As shown in figure 8, the same pattern of suppression was observed in two independent clones suggesting a reproducible effect among antisense clones. It is worth mentioning that suppression level of D2S signal was around 70-80 %, and could be due to the partial suppression of endogenous RASA3 in stable antisense clones, which was about the same magnitude.

To further delineate the role of RASA3 in GH4ZR7 cells, clones depleted of RASA3 by antisense can be examined for other dopamine D2S receptor signaling pathways including inhibition of cAMP and $[Ca^{2+}]_i$. It is expected that, unlike MAPK cascade, these pathways will not be altered since they do not involve the MAPK cascade but are mediated by direct coupling of G-protein subunits to other effectors (adenylyl cyclase or calcium channels). Using the IP-kinase assay (Banihashemi and Albert, 2002b), the effect of RASA3 depletion on D2S induced inhibition of c-Raf vs. B-Raf could be tested. Since RASA3 can affect Ras and Rap activation, both D2S-induced inhibition of Rap-B-Raf and Ras-C-Raf pathways should be attenuated. The RASA3-depleted GH4ZR7 cells should be further analyzed for D2S induced inhibition of cell proliferation, PRL secretion and PRL gene transcription or synthesis (Albert, 2002b). It

is expected that blockade of MAPK inhibition will prevent D2S actions to inhibit PRL synthesis but may not abolish D2S-induced cell proliferation, as it has been shown that additional G proteins (and presumably alternate signaling pathways) are involved in inhibition of cell proliferation (Albert, 2002b).

To further verify the importance of this pathway, primary cultures of pituitary or striatal cells, in which dopamine D2-induced inhibition of MAPK has been observed (Liu et al., 2002b; Van et al., 2007), will be examined. To suppress the endogenous RASA3, transfection of full-length antisense or siRNA may be used, although siRNA transfection has not been published for pituitary cultures, and transfection efficiency of striatal neurons is low. To solve this possible problem, a bicistronic vector (Rees et al., 1996) with siRNA/GFP can be used to identify transfected cells, and immunostaining for phosphoMAPK may be used to examine MAPK activation in these cells following quinpirole treatment. The D2-selective agonist quinpirole will be used since in primary cultures D1 receptors respond to apomorphine complicating the interpretation (Van et al., 2007).

By these studies we will determine the importance of Gi-interacting protein RASA3 in regulation of cell proliferation by the D2S receptor that may ultimately reveal a novel pharmacological target to provide anti-cancer agents by activating RasGAP3 to inhibit cell proliferation. However, to illustrate the actual physiological importance of these findings caution should be used when interpreting the data. Before reaching any firm conclusion, these observations have to be validated by appropriate studies in native environments, where lactotrophs are in close contact with adjacent other lactotrophs and non-prolactin producing cells.

Conclusion

In this thesis, I have investigated the role of a novel Gai-interacting protein, RASA3, in the inhibitory response of dopamine D2S receptor on TRH-induced MAPK phosphorylation in rat pituitary GH4ZR7 cells. I have shown with different approaches that RASA3 preferentially interacts with Gai3 subunit. I have also demonstrated that depletion of this protein in the cellular context leads to suppression of D2S inhibitory pathway on TRH-induced ERK1/2 activation.

CHAPTER IV

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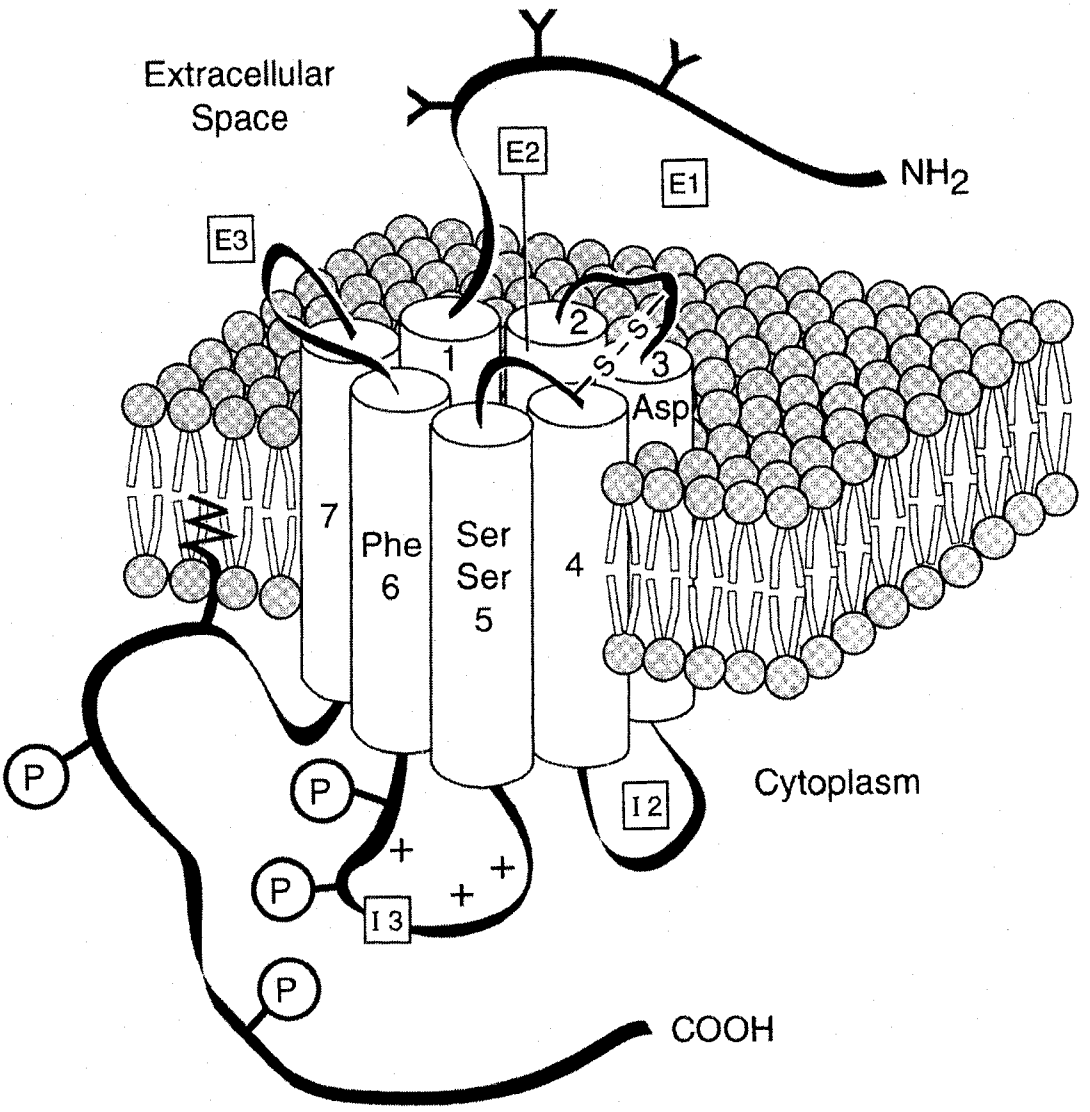
APPENDICES

Figure A



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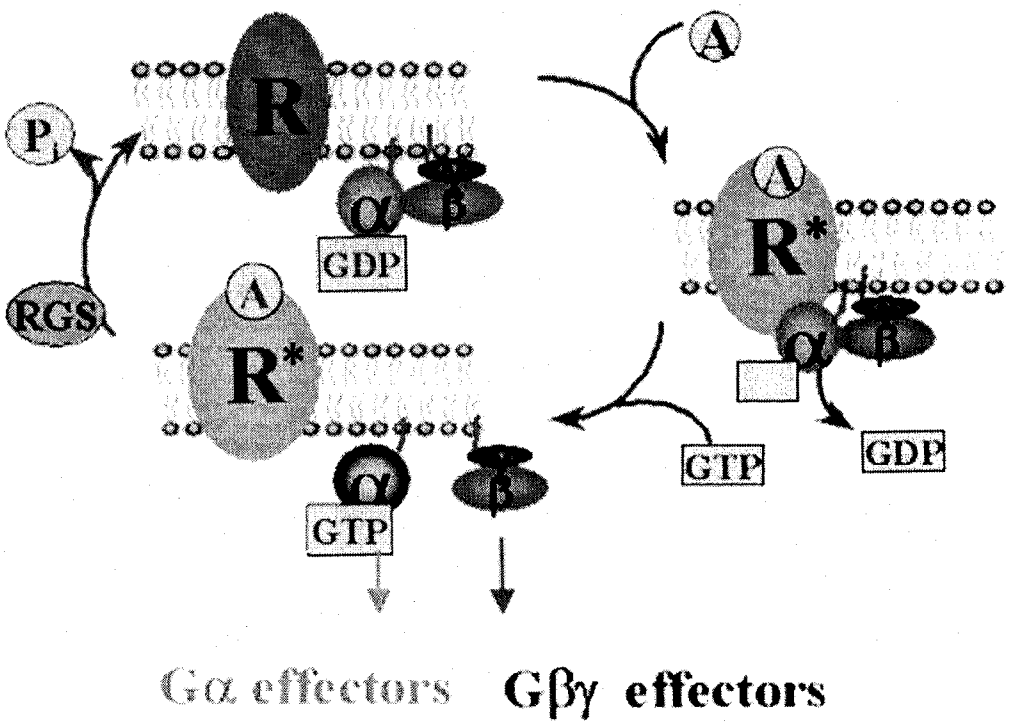
Dopamine receptor structure. Structural features of D1-like receptors are represented.

Figure B



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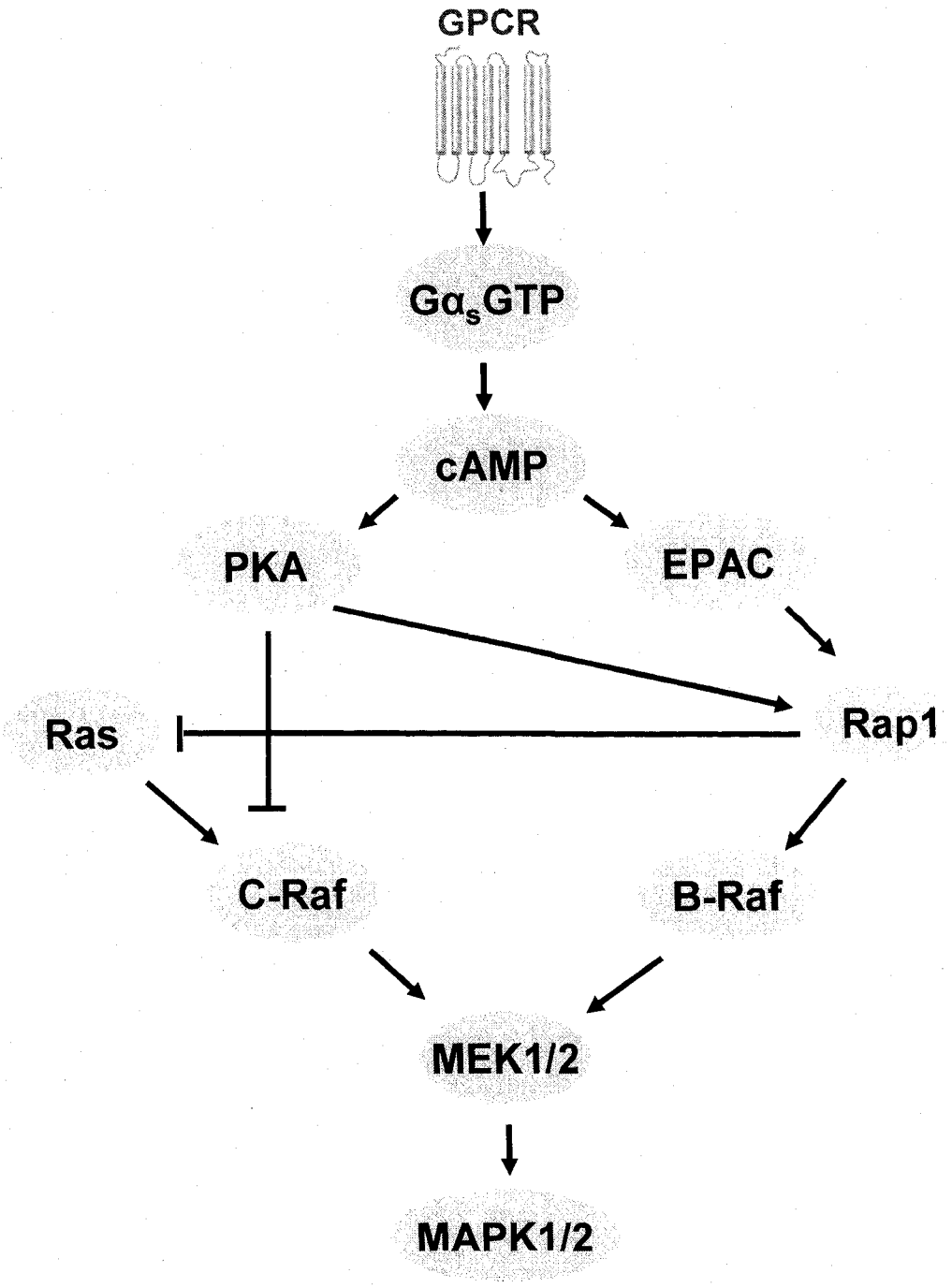
Receptor-mediated G protein activation. 'A' is the ligand and R* shows the active receptor.

Figure C



Signaling pathways for Gαs-induced MAPK1/2 inhibition. For more details, please see "Gαs regulation of ERK1/2" in chapter I.