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Identification of the Interaction Between Gravin and {mu}-Opioid Receptors

Meifang Liu

IDENTIFICATION OF THE INTERACTION BETWEEN GRAVIN AND μ-OPIOID RECEPTORS

by

Meifang Liu
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This thesis, submitted by Meifang Liu in partial fulfillment of the requirement for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Bryon Grove
(Chairperson)

Jody Rader

This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

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ABSTRACT

The phosphorylation and desensitization of G-protein coupled receptors involve several protein kinases. Two of the major ones are protein kinase A (PKA) and protein kinase C (PKC). It has been reported that gravin, an A-kinase anchoring protein, plays a role in regulating β_2-adrenergic receptor desensitization, resensitization, and internalization by recruiting both PKA and PKC, phosphatase, β-arrestin, and clathrin to the receptor. Similarities between the regulation of μ-opioid receptor and β_2-adrenergic receptor suggest that a similar complex involving gravin may play a role in regulating μ-opioid receptor signal transduction. To investigate this possibility, the current study examined the distribution of gravin, μ-opioid receptor and β_2-adrenergic receptor in SH-SY5Y cells as well as in AN3 CA cells cotransfected with gravin and μ-opioid receptor or β_2-adrenergic receptor vector constructs. This study also examined the distribution of gravin, μ-opioid receptor and β_2-adrenergic receptor transgene in transfected AN3 CA cells after the agonist stimulation. Our data showed that gravin is distributed along the membrane of SH-SY5Y cells and partially colocalized with the μ-opioid receptor and the β_2-adrenergic receptor. The gravin transgene was also concentrated along the membrane of AN3 CA cells. Immunofluorescent microscopy showed extensive colocalization of gravin and either the μ-opioid receptor transgene or the β_2-adrenergic receptor transgene before agonist treatment. However, after agonist treatment, the μ-opioid receptor and β_2-adrenergic receptor transgenes translocated from plasma membrane to a perinuclear location, but there was no redistribution of gravin. The current study provided evidence that the recombinant gravin and the receptor proteins were expressed and distributed in a similar way as the native proteins. This validated their use in studying μ-opioid receptor

and β_2-adrenergic receptor signal transduction and the role of gravin in regulating the function of these receptors. The current study also provided evidence that gravin might not interact with the receptors during the internalization of the receptors.

CHAPTER I

INTRODUCTION

The transduction of signals from the plasma membrane to specific subcellular compartments is a complex and highly regulated series of events. In eukaryotes, protein phosphorylation plays a crucial role in regulating these signaling events by modulating the activity of specific signaling proteins. The family of enzymes catalyzing the phosphorylation of proteins, the protein kinases, represents a diverse group of proteins. To date, more than 300 protein kinases have been found to play key roles in cellular control and Hunter (1994) estimated that there are as many as 2000 conventional protein kinase genes in the human genome. Within this structurely diverse family of intracellular enzymes, the best understood is the cAMP-dependent protein kinase or protein kinase A. Studies of cAMP-dependent protein kinase (PKA) activity provided the first clues about the role of protein phosphorylation in the regulation of signal transduction (Walsh and Van Patten, 1994).

The PKA holoenzyme is a heterotetramer composed of a regulatory (R) subunit dimer that maintains two catalytic (C) subunits in a dormant state. Binding of cAMP to tandom sites in each R subunit releases the active C subunits, which are then free to phosphorylate substrates on serine or threonine residues. As the prototypic model of intracellular kinases, PKA is a multifunctional enzyme with broad substrate specificity (Taylor et al., 1990). Given that PKA is a soluble cytoplasmic enzyme and involved in numerous signaling events, understanding the functional complexities of how the kinase

is activated in the right place and at the right time inside the cell is important. This specificity is achieved, in part, through the compartmentalization of PKA at different subcellular locations through interactions with A-kinase anchoring proteins (AKAPs). Compartmentalization of PKA was first shown in 1974, shortly after PKA was initially purified and characterized (Beavor et al., 1974).

Eukaryotic cells express multiple forms of PKA regulatory and catalytic subunits, which are termed RIα, RIβ, RIIα, RIIβ, Cα, Cβ, and Cγ. These different subunits assemble together as different PKA isoforms. The catalytic subunits show common kinetic features and substrate specificity and therefore the characteristics of the PKA holoenzymes are largely determined by the structure and properties of their regulatory subunits. The R subunits are differentially distributed in mammalian tissues. RIα and RIIα are ubiquitous, whereas RIβ and RIIβ are expressed predominantly in endocrine, brain, fat and reproductive tissues (Taylor et al., 1992). The results of several studies suggested that RI is predominantly in the soluble fraction, where as the majority of RII is associated with the plasma membrane, cytoskeletal, secretory granule, Golgi, and nuclear fractions (Joachim et al., 1990; Pariset et al., 1989; De Camilli et al., 1986). In addition to their distinctive expression and distribution, R subunits differ in their regulation and biochemical properties. The binding affinity to cAMP of RIIβ *in vivo* is lower relative to RIIα and much lower compared to RIα (Taylor et al., 1992). These data imply that holoenzymes containing RI subunits or RII subunits (PKAI and PKAII) decode cAMP signals that differ in duration and intensity: PKAI is activated transiently by weak cAMP signals, whereas PKAII responds to high and persistent cAMP stimulation. The composition and specific biochemical properties of PKA isoenzymes account, in part, for

differential cellular responses to discrete extracellular signals that activate adenylate cyclase.

PKA Anchoring Proteins

It is now generally believed that PKA II is concentrated in particular subcellular compartments through interactions with a family of A-kinase anchoring proteins (AKAPs). AKAPs immobilize PKAII isoforms at specific intracellular locations by binding RII subunits. Although most AKAPs preferentially bind to RII, several AKAPs also bind to RI (Huang et al., 1997). The first RII-binding proteins were identified as contaminating proteins by co-purification with RII after affinity chromatography on cAMP-Sepharose (Sarkar et al., 1984). Currently, the standard method for detection of AKAPs is an overlay assay which is a modification of the Western blot procedure (Carr et al., 1992). This assay is based on the observation that many, if not all, AKAPs retain their ability to bind RII after they have been transferred to nitrocellulose (Lohmann et al., 1984). Using this technique, a family of AKAPs ranging in size from 15 to 300 kDa has been detected in a variety of tissues and cells (Carr et al., 1992). A common feature of AKAPs is that each anchoring protein contains two classes of binding sites: a conserved "anchoring motif" which binds to the RII subunit of PKA and a "target domain" which directs the subcellular localization of the "PKA-AKAP" complex through association with structural proteins, membranes, or cellular organelles (Coghlan et al, 1993). Herberg et al. (2000) showed by using surface plasmon resonance (SPR) that some AKAPs, such as AKAP 79 and AKAP 84, bound both RII alpha and RII beta of PKA; however, the affinity of the AKAPs for different R subunit isoforms differed.

The PKA-AKAP complex model implies that AKAPs must contain a unique targeting site that is responsible for association with subcellular structure. The targeting domain is an essential feature of each AKAP because it determines specificity by targeting the anchored PKA complex to particular organelles. So far, immuno-fluorescence microscopy and subcellular fractionation techniques have identified AKAPs localized to centrosomes (AKAP 350) (Schmidt et al., 1999), the actin cytoskeleton (AKAP 250, and AKAP 75/79/150) (Nauert et al., 1997; Coghlan et al., 1995), the endoplasmic reticulum (AKAP 100) (McCartney et al., 1995), the Golgi (AKAP 85) (Keryer et al., 1993), microtubules (MAP2) (Davare et al., 1999), mitochondria (sAKAP 84) (Lin et al., 1995), the nuclear matrix (AKAP 95) (Coghlan et al., 1994), the plasma membrane (AKAP 15) (Tibbs et al., 1998), and peroxisomes (AKAP 220) (Schillace and Scott, 1999). Although the proposed function of many AKAPs is to target PKA, several AKAPs have been found to serve as scaffolds which simultaneously bind more than one signaling protein and form a signaling complex consisting of enzymes with opposing actions, such as kinases and phosphatases. For example, in neurons, AKAP 79 binds to PKA, protein kinase C (PKC), and the protein phosphatase 2B (PP-2B) (Klauck et al., 1996; Coghlan et al., 1995). The scaffold of AKAP 79 with PKA, PKC, and PP-2B allows AKAP 79 to control the localization of two broad-specificity kinases and a phosphatase. As distinct activation signals are presented at postsynaptic densities of neurons, AKAP 79 might provide a point of convergence for multiple second-messenger signals, such as cAMP, Ca^{2+}, and phospholipids (Faux et al., 1996). Similarly, AKAP 220 has been shown to bind to protein phosphatase 1 (PP1) in addition to PKA (Schillace et al., 1999), suggesting it functions to regulate kinase as well as phosphatase activity.

Gravin

Gravin, also referred to as AKAP 250, is a kinase scaffold protein which binds to both PKA and PKC. The carboxy-terminal fragment of gravin was originally identified as a cytoplasmic antigen recognized by serum from a patient with myasthenia gravis (Gordon et al., 1992). A series of *in vitro* binding experiments showed that the residues 1526-1780 of gravin bind to PKA with high affinity. Structural analysis of this region indicated that it contained an amphipathic helical region similar to that found in other AKAPs. The first 1000 residues of gravin are 69% identical to a murine mitogenic regulatory gene, Src-supresssed C kinase substrate (SSeCKS)/ clone 72, which is a PKC binding protein (Chapline et al., 1996), indicating that gravin may also be a PKC-binding protein. This hypothesis was confirmed by Nauert et al. (1997), who demonstrated that residues 265-556 of gravin bind to PKC in a phosphatidylserine-dependent manner.

Gravin is expressed in many tissue and cell types. It is found in fibroblasts, and smooth muscle cells *in vivo,* as well as in endothelial cells and several adherent tumor cell lines *in vitro* (Grove et al., 1994). In human erythroleukemia cells, phorbol ester can induce gravin expression. Gravin is also widely expressed in the nervous system. It has been reported to be expressed in the cerebral cortex, in the molecular and granular layers of cerebellum, in nerve bundles, in peripheral ganglia, and in sensory organs, such as nerve fibers on the tip of the tongue and taste buds (Grove et al., 1994). Comparable to gravin, SSeCKS is also widely distributed in the nervous system. Immunolabeling of SSeCKS was found in the primary sensory neurons in the dorsal root ganglia, the dorsal horn of the spinal cord, sensory ganglia, medulla, and the molecular layer of cerebellum. This distribution is similar to that described for gravin in the nervous system (Siegel et

al., 2001). Based on the observation that gravin is expressed in the nervous system and evidence that this protein is a multivalent scaffold protein in signal transduction, it is predictable that gravin might play a role in modulating the signaling events in the nervous system.

Immunolocalization studies show that gravin is concentrated at the cell periphery and is enriched in filopodia of erythroleukemia cells (Grove et al., 1994; Nauert et al., 1997; Grove et al., 2001). In cultured human endothelial cells, gravin was not found to be a component of stress fibers, microtubules or intermediate filaments, but associated with the cell cortex, suggesting that gravin may be functionally related to either the plasma membrane or the membrane skeleton (Grove et al., 2001). The wide distribution of gravin further indicates that it is a multifunctional protein and regulates cellular events that involve plasma membrane, possibly by anchoring PKA and PKC to specific sites at the plasma membrane and coordinating phosphorylation and dephosphorylation in signal transduction pathways. A recent study has shown that gravin and PKA forms a dynamic complex with β_2-adrenergic receptors (Shih et al., 1999). The gravin/receptor complex includes PKC, protein phosphatases 2A and 2B, G protein coupled receptor kinase-2, β-arrestin and clathrin (Lin et al., 2000). These complexes may be physiologically important, since gravin deficiency has been reported to inhibit agonist-induced internalization and resensitization of β_2-adrenergic receptors (Shih et al., 1999).

G protein-Coupled Receptors

It is well accepted that neurons communicate by secreting neurotransmitters that bind to specific receptors on postsynaptic cells. A major group of receptors involved in neuronal signaling are the G-protein coupled receptors (GPCRs). More than a thousand

members of this receptor family have been identified. A broad spectrum of extracellular signals, including hormones, neurotransmitters, odorants and light, are detected by G-protein coupled receptor family.

All GPCRs possess seven putative transmembrane domains, and convert extracellular signals into intracellular signals by activating heterotrimeric G-proteins, a protein complex consisting of an α-subunit which carries the guanine-nucleotide binding site, and β- and γ-subunits which form a tightly bound dimer. In response to ligand binding, GPCRs are converted into an active form which functions as a GDP/GTP exchange factor and promotes the exchange of GDP for GTP on the α-subunit. This leads to dissociation of the α-subunit from the Gβγ-dimer, and subsequent activation of G-protein effectors such as ion channels, adenylyl cyclase, or phospholipase C. There are three major types of GPCRs, based on the type of α G-protein they are coupled to and their effect on second messenger signaling. One type of GPCR, (e.g. α-adrenergic receptor) couples to an inhibitory Gα protein and inhibits adenylyl cyclase activity. This results in a decrease in cAMP level and a decrease in PKA activity. Another type of GPCR, (e.g. β_2-adrenergic receptor) couples to a stimulatory Gα protein and activates adenylyl cyclase. This results in increased cAMP levels and enhanced PKA activity. The third group of GPCRs couple to Gα proteins which activate phospholipase C and in turn activate PKC.

Chronic stimulation of GPCRs provokes attenuation of the receptor-mediated signal, or desensitization. Desensitization is an adaptive mechanism in biological systems thought to facilitate responsiveness of the cell to successive multiple extracellular stimuli. For GPCRs, desensitization is a multistep process. The receptor is first uncoupled from

the G-protein and receptor function is attenuated (Lohse et al., 1995). This step involves phosphorylation of the receptor by several kinases, such as PKA, PKC, or G-protein coupled receptor kinases. The phosphorylated receptor is then sequestered from the plasma membrane to an intracellular compartment, where the receptor is dephosphorylated by specific phosphatases and resensitized (Pitcher et al., 1995). When the stimulation is chronically persistent, the receptor is down-regulated through protein degradation and decreased transcription leading to a decrease in receptor number (Handcock et al., 1988).

Two major patterns of rapid desensitization have been characterized. Agonist-specific or homologous desensitization refers to the situation in which receptor activation by the agonist leads to desensitization of the same receptor type. This type of desensitization involves mainly G-protein coupled receptor kinases (Inglese et al., 1993). Heterologous desensitization refers to the situation in which receptor stimulation by the agonist attenuates the response of other receptor types. Heterologous desensitization is believed to result from activation of PKA and PKC, which in turn phosphorylates the heterologous receptors.

Role of Gravin in Regulating G-protein Coupled Receptor Function

The desensitization of GPCRs has been particularly well studied in the β_2-adrenergic receptor/ PKA system. Exposure of β_2-adrenergic receptors to the catecholamines epinephrine or norepinehrine causes rapid desensitization of the receptor-stimulated adenylyl cyclase response. Phosphorylation of the receptor is the first step in desensitization and occurs over a few minutes after agonist exposure (Benovic et al., 1988). Primarily two kinase activities have been implicated in this agonist-induced

phosphorylation: PKA (Clark et al., 1989) and β_2-adrenergic receptor kinase (Lohse et al., 1990). Early studies on phosphorylation of β_2-adrenergic receptor also indicated that β-arrestin is an essential cofactor of β_2-adrenergic receptor kinase (Loshe et al., 1990). Interestingly, in A431 cells, although suppression of PKC has been reported to potentiate rather than to attenuate agonist-induced desensitization (Shih et al., 1994), PKC is required for the association of PKA and β-arrestin with the β_2-adrenergic receptor (Shih et al., 1999; Lin et al., 2000). This suggests that PKC might also play a role in β_2-adrenergic receptor desensitization, but in a manner that differs from that for PKA and β_2-adrenergic receptor kinase, which directly phosphorylate the receptor.

Although the mechanism of desensitization of β_2-adrenergic receptor has been well studied, the desensitization of another important group of GPCRs, the opioid receptor family, is still controversial. Three major types of opioid receptors, μ, δ and κ, have been identified by selective radioactive ligand studies (Chang et al., 1979). Through the use of cloning techniques, the molecular events involved in regulating the activity of these 3 opioid receptor types have been studied in detail. Although each of these receptors has a distinct regional distribution within the brain and unique pharmacological and physiological properties, the primary structures of these receptors show a 65-70% homology with each other (Chen et al., 1993a). All of the three types preferentially activate inhibitory G-proteins (Johnson et al., 1994; Law et al., 1994; Xie et al., 1994), and are capable of regulating the same second messengers. Activation of these receptors also increases phospholipase C activity (Ueda et al., 1995), causes a transient increase in the levels of intracellular Ca^{2+} (Spencer et al., 1997), activates inwardly rectifying K

channels (Henry et al., 1995), and stimulates the mitogen-activated protein kinases Erk-1 and Erk-2 (Fukuda et al., 1996).

Like β_2-adrenergic receptor, the activities of the opioid receptors are attenuated by chronic agonist treatment. Several researchers have reported agonist-induced opioid receptor phosphorylation (Arden et al., 1995; Pei et al., 1995), with some suggesting that phosphorylation correlates with agonist-induced receptor desensitization. This is supported by site-specific mutagenesis studies. Replacement of Thr393 by Ala was shown to blunt DAMGO-induced μ-opioid receptor desensitization (Pak et al., 1997), while El Kouhen and coworkers found phosphorylation on Ser363, Thr370, and Ser375 at the COOH-terminus plays a role in modulating agonist-induced internalization of the μ-opioid receptor (El Kouhen et al., 2001). Phosphorylation by PKA is unlikely the mechanism of agonist-induced desensitization because the opioid receptor mediates inhibition of cAMP formation. On the other hand, it has been reported that phosphorylation by PKC modulates desensitization of the δ-opioid receptor (Ueda et al., 1995) in a *Xenopus* expression system, whereas phosphorylation by PKC Ca^{2+} / calmodulin-dependent protein kinase II (CaM kinase II) (Mestek et al., 1995), and mitogen-activated protein kinase (Polakiewicz et al., 1998) modulates desensitization of the μ-opioid receptor. Studies on dominant negative mutants suggest that desensitization of the δ-opioid receptor may also involve phosphorylation of the receptor by one or more G protein-coupled receptor kinases (Pei et al., 1995). Other studies have been unable to establish a causal relationship between receptor phosphorylation and desensitization. Loh and coworkers compared the rate of μ-opioid receptor phosphorylation and the rate of DAMGO-induced receptor desensitization and they found that agonist-induced receptor

phosphorylation occurred within minutes, whereas the desensitization of the receptor took hours (Kouhen et al., 1999). The absence of correlation between receptor phosphorylation and desensitization was further demonstrated by using μ-opioid receptor mutants in which all the putative phosphorylation sites were removed and receptor phosphorylation was completely abolished. Interestingly, these receptor mutants could still be desensitized by chronic exposure to agonist (Capeyrou et al., 1997). Thus, these experiments suggest that receptor phosphorylation is not a prerequisite for desensitization.

Receptor internalization is another mechanism for regulating the receptor. Rapid internalization has been demonstrated in both the β_2-adrenergic receptor system and the opioid receptor system. Internalization of the receptor involves clathrin-dependent endocytosis and happens rapidly after agonist stimulation (Gagnon et al., 1998). In the β-adrenergic receptor system, the β_2-adrenergic receptor kinase was found to colocalize with the receptor several minutes after agonist stimulation, indicating its role in agonist-induced receptor internalization (Ruiz-Gomez et al., 1997). Internalization of the receptor has also been reported to involve translocation of phosphoinositide 3-kinase to the plasma membrane, and this process is mediated by β-adrenergic receptor kinase in an agonist-dependent manner (Sathyamangla et al., 2001), suggesting phosphorylation may play a role in the β_2-adrenergic receptor internalization. β-Arrestin, a cytoplasmic cofactor of β_2-adrenergic receptor kinase, acts as an adaptor protein which binds to clathrin (Lin et al., 1997) and plays a role in targeting the receptors to clathrin-coated pits (Krupnick et al., 1997).

Phosphorylation may play a role in agonist-induced opioid receptor internalization. Truncation of a Ser/Thr-rich domain unique to the C-terminus of μ-opioid receptor results in a significant inhibition of internalization and recycling of the receptor (Segredo et al., 1997), suggesting the importance of phosphorylation at the C-terminus in opioid receptor internalization. In addition, the rapid internalization of human δ-opioid receptor could be blocked by a G protein-coupled receptor kinase inhibitor (Hasbi et al., 2000). *In vitro* studies involving the use of either mutant receptors or kinase inhibitors have found that phosphorylation by PKC regulates internalization of both μ-opioid receptor (Ueda et al., 2001) and δ-opioid receptor (Xiang et al., 2001). These observations indicate the importance of G protein-coupled receptor kinase and PKC in modulating internalization of opioid receptors. As in β_2-adrenergic receptor trafficking, internalization of opioid receptor is β-arrestin-dependent and clathrin-dependent (Zhang et al., 1999; Xiang et al., 2000), suggesting the similarities between the mechanism of β_2-adrenergic and opioid receptor internalization.

Recent studies have revealed that members of the AKAP family may play a role in the regulation of GPCR function by recruiting various protein kinases, phosphatases, and other proteins such as β-arrestin or clathrin to the receptor. Fraser et al. (2000) reported that AKAP79 enhances down-stream activity of the MAP kinase pathway by facilitating cAMP-induced phosphorylation of β_2-adrenergic receptor following agonist stimulation. A recent study has also reported that AKAP79 regulates the ability of G-protein coupled receptor kinase 2 to phosphorylate the agonist-occupied receptors (Cong et al., 2001). Besides AKAP79, Malbon and his group have demonstrated that gravin may serve a role as a kinase scaffolding protein that regulates β_2-adrenergic receptor

signaling. Gravin binds to both PKA and PKC, and associates with β_2-adrenergic receptor (Shih et al., 1999), suggesting that gravin is involved in desensitization of the receptor. This complex also includes protein phosphatase 2B, which suggests that gravin is involved in resensitization of the receptor (Shih et al., 1999). Further investigation indicated that gravin was essential to the organization of the signaling complexes composed of the receptor, protein kinases / phosphatases, β-arrestin, and clathrin (Lin et al., 2000). Suppression of the expression of gravin by antisense oligodeoxynucleotides blocked the association of the β_2-adrenergic receptor with these proteins and the sequestration and resensitization of β_2-adrenergic receptor. Fan et al. (2001) have further reported that the cytoplasmic C-terminus of the β_2-adrenergic receptor contains the gravin binding site and the interaction of gravin with the β_2-adrenergic receptor is maintained as the receptor is internalized. These observations suggest that gravin is essential for desensitization of β_2-adrenergic receptor by mediating phosphorylation and internalization of the receptor.

Statement of Problems

The cellular response to hormone or neurotransmitter application via G protein-coupled receptors is a highly regulated event and has been well studied in the β_2-adrenergic receptor system. The formation of a macromolecular complex composed of gravin, β_2-adrenergic receptor, several protein kinases/phosphatases, β-arrestin, and clathrin suggests that gravin plays an important role as a scaffold for these protein-protein interactions in β_2-adrenergic receptor signal transduction. Studies on desensitization and resensitization of the μ-opioid receptor also provide evidence that μ-opioid receptor signaling is regulated by protein kinases and displays arrestin- and clathrin-dependent internalization in response to agonist exposure. This raises the possibility that a complex similar to that associated with β_2-adrenergic receptor may associate with μ-opioid receptor after a signaling molecule binds. Gravin has been found widely distributed in the sensory nervous system, where large amounts of opioid receptor are expressed. Therefore, it is possible that gravin may play a role in regulating μ-opioid receptor signals in the nervous system. Based on this information, my hypothesis is that gravin interacts with μ-opioid receptor during agonist-induced internalization of the receptor. To investigate this hypothesis, the first aim was to determine if gravin codistributed with μ-opioid receptor. This aim was pursued by examining gravin expression and distribution in SH-SY5Y cells, a neuroblastoma cell line that expresses μ-opioid receptor when treated with phorbol esters or retinoic acid (Pahlman et al., 1981 & 1984; Zadina et al., 1993) and in AN3 CA cells cotransfected with gravin and μ-opioid receptor vector constructs. The second aim of this study was to determine if agonist-induced internalization was accompanied by redistribution of gravin. This aim was pursued by 1) generating full-

length gravin and μ-opioid receptor expression vectors and expressing these constructs in AN3 CA cells, a cell line that does not express gravin or μ-opioid receptors and 2) examining the distribution of the gravin and μ-opioid receptor transgenes after the cells were treated with specific agonist.

CHAPTER II

MATERIALS AND METHODS

Materials

Eukaryotic and Bacterial Cell Culture

Eukaryotic Cell Lines

Human neuroblastoma cell line (SH-SY5Y) was a generous gift from Dr. Sandra Roerig; human endometrial carcinoma cell line (AN3 CA) was obtained from ATCC (American Type Culture Collection), Manassas, VA.

Eukaryotic Cell Culture and Transfection

Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, lipofectAMINE (2 μg/ml) reagent and Opti-MEM reduced serum medium from Gibco BRL, Life Technologies, Gaithersburg, MD. Fetal bovine serum (FBS) from Atlanta Biological, Atlanta, GA. Penicillin-streptomycin, trypsin-EDTA (0.05% trypsin), all-trans retinoic acid (RA), isoproterenol, Tyr-D-Ala-Gly-MePhe-Gly(ol)-enkephalin (DAMGO) from Sigma Chemical Company, St. Louis, MO.

Bacterial Cell Line

E. coli strain TOP 10F' used to maintain and propagate plasmid vectors from Invitrogen, Carlsbad, CA. SOC medium was included in the kit.

Bacterial Cell Culture and Transformation

Bacto™ Yeast Extract and Tryptone Peptone from Becton Dickinson, Sparks, MD. Selected agar from Gibco BRL, Life Technologies. Ampicillin (sodium salt) from Sigma Chemical Company, St. Louis, MO.

Oligonucleotides

The primers used to perform RT-PCR reactions were designed by Dr. Bryon Grove and purchased from Genosys, St. Louis, MO. The sequences of these oligonucleotides are shown in Table 1.

Vectors

pcDNA 3.1.V5/His-A and pCR.2.1 from Invitrogen. Expression vector GR81-5534 (gravin expression vector) was designed by Dr. Bryon Grove and constructed by Magdalena Walkiewicz. This expression vector was generated by inserting a full-length gravin sequence (nt 81to 5534) into the multiple cloning site of the pcDNA 3.1.V5/His-A vector in frame with the V5 and polyhistidine tags. Expression vector pcDNA3.1.V5.TGA was designed by Dr. Bryon Grove and constructed by inserting an 18 base pair linker containing a stop codon (TGA) into the Age I site of the pcDNA 3.1.V5/His-A vector.

Enzymes

The restriction endonucleases and other enzymes used in this study include: Age I, BamH I, BstB I, BstE II, Dde I, EcoR I, EcoR V, Hind III, Kpn I, Nhe I, Not I, Sty I, Xba I, T4 DNA ligase from New England Biolabs, Beverly, MA. Advantage-GC cDNA polymerase from Clontech, Palo Alto, CA. *PfuTurbo* DNA polymerase from Stratagene, La Jolla, CA. AMV reverse transcriptase from Promega, Madison, WI.

Table 1. Oligonucleotides Used for Cloning Techniques.

Oligonucleotide	Sequence	Additional Restriction Enzyme Sites for Cloning	Desired Function
MOR4	TAATGGTACCTCCGCCTGACGCTCCTCTC	Kpn I	Forward Primer for MOR clone
MOR2	AAAATCTAGAGGGGCAACGGAGCAGTTTCTG	Xba I	Reverse Primer for MOR clone
MOR3	CAGAGTGGCCAGAGAGGAAAGAGGT		RT Primer for MOR clone
betaadreno1	AAGGGGTACCACACCACAGCCGCTGAA	Kpn I	Forward Primer for β_2-AR clone
betaadreno2	GCCCTCTAGATAGCAGTGAGTCATTTGTACT	Xba I	Reverse Primer1 for β_2-AR clone
betaadreno3	CTGCTCCTCAAATCCCTGCC		RT Primer for β_2-AR clone
Betaadreno5	CTGTTTAGTGTTCTGTTGGGCGGGGGG		Reverse Primer2 for β_2-AR clone

Reverse Transcription and Polymerase Chain Reaction

RNA STAT-60™ kit used to purify mRNA from cultured cells from TEL-TEST, Inc., Friendswood, TX. RNase inhibitor from Promega, Madison, WI. Deoxynucleotide Mix (dNTPs) from Brinkmann Instruments, Inc., Westbury, NY. All polymerase chain reactions were performed on a Thermal Cycler from Perkin-Elmer Corp., Foster City, CA.

DNA purification

DNA fragments were purified from 1% agarose gel using a QIAquick Gel Extraction Kit from Qiagen, Inc., Valencia, CA. Circular DNA from small bacterial cultures was purified using either a Plasmid Mini Kit from Qiagen, or phenol-chloroform purification including phenol, chloroform, isopropanol from Sigma and isoamyl alcohol from Fisher Scientific Company, Fairlawn, NJ. Circular DNA from large bacterial cultures was purified using an EndoFree Maxi Kit from Qiagen, Inc. DNA and RNA concentration was measured on a UV spectrophotometer from Dr. Ann Flower.

Gel Electrophoresis

Agarose and ethidium bromide from Gibco BRL, Life Technologies. Acrylamide, bis-acrylamide and sodium dodecyl sulfate (SDS) from Bio-RAD Laboratory, Hercules, CA. Ammonium persulfate (APS), bromophenol blue, N,N,N',N'-tetramethylethylenediamine (TEMED) from Sigma. Gel documentation included capturing image of the gel using a UVP High Performance Ultraviolet Transilluminator from Ultra Violet Products, Upland CA.

Immunoblotting and Protein Assays

Protease inhibitors used to lyse cells include: aprotinin, calpain inhibitor, ethylenediamine-tetraacetic acid (EDTA) disodium salt, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF) from Sigma.

BCA protein assay kit from Pierce, Rockford, IL.

Nitrocellulose membrane from Bio-RAD. Proteins immobilized on membrane were detected using the CDP-*Star*® detection system from Applied Biosystems, Bedford, MA. This kit includes I-Block™ reagent and 0.25mM Chemiluminescent Substrate.

Immunofluorescence and Confocal Microscopy

Paraformaldehyde was purchased from Polysciences, Inc., Warrington, PA. Normal goat serum and digitonin were purchased from Sigma. FITC-guard mounting medium was purchased from Testog Inc., Chicago, IL. The slides were observed on either a Nikon TE 300 inverted Epi-fluorescence Microscope (Filters: ex580 nm and em620 nm for Cy3, ex480 nm and em535 nm for FITC; Dichroic mirror: 595 nm for Cy3 and 505 nm for FITC) equipped with Hamamatsu "ORCA" digital camera from Dr. Mickael Atkinson or a Fluoview 300 laser scanning confocal system coupled to a IX70 Olympus inverted Epi-fluorescence microscope equipped with Argon laser for FITC and "HeNe" laser for Cy3 from Dept. of Anatomy & Cell Biology.

Antibodies

Monoclonal mouse anti-gravin antibody 2B3-1.1 and polyclonal rabbit anti-gravin antibody Rb7753 were obtained from Dr. Bryon Grove. Monoclonal mouse anti-V5 antibody was obtained from Invitrogen Corporation, Carlsbad, CA. Polyclonal rabbit anti-β_2-adrenergic receptor antibody was obtained from Santa Cruz Company, Santa

Cruz, CA. Polyclonal rabbit anti-μ-opioid receptor antibody was from Neuromics, Minneapolis, MN. Cy3-conjugated Donkey anti-mouse IgG antibody and FITC-conjugated Donkey anti-rabbit IgG antibody were obtained from Jackson ImmunoResearch Laboratories, Inc.. Alkaline phosphatase (AP)-conjugated anti-mouse IgG and AP-conjugated anti-rabbit IgG were obtained from Sigma.

Other Chemicals

Bis [2-hydroxyethyl] imino-tris [hydroxymethyl]methane (Bis-Tris), benzamidine hydrochloride, bovine serum albumin (BSA), calcium chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), glycerol, magnesium chloride ($MgCl_2$), β-mercaptoethanol, potassium phosphate, sodium chloride (NaCl), sodium phosphate (dibasic) (Na_2HPO_4), sodium phosphate (monobasic, anhydrous) (NaH_2PO_4), Tris (hydroxymethyl)-aminomethane (Trizma Base), Triton X-100 from Sigma Chemical Company, St. Louis, MO. Sodium hydroxide (NaOH), hydrochloric acid (HCl) from Fisher Scientific Company, Fairlawn, NJ. Tween-20 from Bio-RAD Laboratory, Hercules, CA. Imidazole from Acros Organics, NJ. Reagent grade water was generated using a Milli-Q water purification system, Millipore Corporation, Bedford, MA.

Methods

Eukaryotic Cell Culture

Human SH-SY5Y cells and human AN3 CA cells were maintained in minimum essential DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37 °C. Fresh medium was added to the culture every 2-3 days and the cells were passaged when confluent.

Retinoic Acid Treatment

SH-SY5Y cells were incubated until the cells were 70-80% confluent. Ten μM

all-trans retinoic acid was added to the culture medium and cells were incubated for 6 days before staining or lysing.

Agonist Treatment

AN3 CA cells seeded on glass coverslips were either co-transfected with GR81-5534/pcDNA3.1.MOR1.V5 or transfected with GR81-5534 or pcDNA3.1.MOR1.V5 alone. The cells were then subject to 5 μM DAMGO for 30 min.

For isoproterenol treatment, AN3 CA cells were either co-transfected with GR81-5534/pcDNA3.1.β_2-AR.V5 (or pcDNA3.1. β_2-AR) or transfected with GR81-5534 or pcDNA3.1.β_2-AR.V5 (or pcDNA3.1. β_2-AR) alone. The cells were exposed to 10 μM isoproterenol for 30 min.

Cloning Techniques

Generating PCR Product

Generating Diethylpyrocarbonate (DEPC) Water. 500μl of DEPC was mixed with 500 ml of Milli-Q water by shaking thoroughly. The DEPC mixed water was stored at RT overnight and then autoclaved for 20 minutes in a liquid cycle.

Purification of mRNA from retinoic acid treated SH-SY5Y cells. Following treatment with retinoic acid (10μM) for 6 days, SH-SY5Y cells grown in monolayer in 25cm^2 flasks were lysed in the culture dish by adding 2 ml of the RNA STAT-60™ and passing the cell lysate several times through a pipette. The homogenate was stored at RT for 5 minutes to permit the complete dissociation of nucleoprotein complexes, then transferred to a microcentrifuge tube and mixed vigorously with 0.4 ml chloroform. Following incubation at RT for 2 min, the mixture was centrifuged at 12,000x g for 15 minutes at 4°C and the upper aqueous phase was collected into a fresh tube. To

precipitate the mRNA, 1 ml of isopropanol was added to the collected phase. The sample was stored at RT for 10 minutes, and then centrifuged at 12,000x g for 10 minutes at 4°C. The RNA pellet was washed with 1 ml of 75% ethanol, air dried and dissolved in 50 µl of DEPC water.

Reverse Transcription. Reverse transcription was performed on 1 µg of mRNA using 10 nM of each of the reverse transcription primer (MOR3, corresponding to the 3' non-coding region of the published human MOR1; or betaadreno3, corresponding to the published human β_2-adrenergic receptor). For each reaction, 1 µg of mRNA and 1 µl (10 nM) of the primers were mixed in 3 µl DEPC H_2O, heated in a water bath at 72 °C for 5 minutes, and then incubated on ice for 5 minutes. The mixture was combined with 0.5 µl (20 U) AMV reverse transcriptase, 0.25 µl (20 U) RNase inhibitor, 1 µl dNTP, and 2 µl 5X AMV reverse transcriptase buffer, and then the desired amount of DEPC H_2O was added to the mixture to bring the volume to 10 µl. The reaction was performed in a water bath at 45 °C for one hour.

Polymerase Chain Reaction. PCR reactions were performed in thin-walled 500 µl micro-reaction tubes in a Perkin-Elmer thermal cycler. The components of each reaction in a 50 µl volume are listed in Table 2. Reaction mixtures were overlaid with one drop of mineral oil to reduce refluxing. The program used for all PCR reaction was as follows: (1) 94 °C for 30 seconds; (2) 35 cycles in which each cycle consisted of a 1 minute 94 °C denaturation step and a 3 minute 68 °C (70 °C for the second β_2-AR clone) annealing and elongation step; (3) 3 minute 68 °C (70 °C for the second β_2-AR clone) extension step. Following amplification, 10 µl of the PCR products was analyzed by submerged agarose gel electrophoresis using a 1% agarose gel.

Table 2. PCR Reactions* Performed in Cloning Technique.

Sequence to be Amplified	Template	Forward Primer	Reverse Primer	Polymerase	Desired Product
MOR1	RT product primed with MOR3	MOR4 (10 nM)	MOR2 (10 nM)	Advantage-GC cDNA polymerase with supplied 5X buffer	A 1381 bp fragment from human MOR sequence
β_2-AR	RT product primed with betaadreno3	betaadreno1 (10 nM)	betaadreno2 (10 nM)	PfuTurbo DNA polymerase** with supplied 10X buffer	A 1358 bp fragment from human β_2-AR sequence
β_2-AR	RT product primed with betaadreno3	betaadreno1 (10 nM)	betaadreno5 (10 nM)	Advantage-GC cDNA polymerase with supplied 5X buffer	A 1422 bp fragment from human β_2-AR sequence

* All reactions were performed in the presence of 1µl of template, 1µl of each primer, 1µl of polymerase, and 1µl of dNTP in a 50µl volume.

** *PfuTurbo* DNA polymerase was supplemented with 1µl of DMSO in this reaction.

Generating Recombinant Protein Expression Vectors

Restriction Endonuclease Digestion. To generate the required overhangs for ligation, the pcDNA3.1.V5.TGA vector and the MOR and the first β_2-AR PCR products were digested by Kpn I and Xba I (See Table 1). The reactions were performed using the PCR product or 1 μg of pcDNA3.1.V5.TGA vector, 1 μl of Kpn I, 1 μl of Xba I, 1 μl of 10X NEBuffer 2, 1 μl of 1 mg/ml BSA in a 37 °C water bath for one hour. The second β_2-AR PCR was ligated into the pCR2.1 vector by TA cloning first, and then digested by EcoR I. The pcDNA3.1.V5/His was also digested by EcoR I to generate the overhangs for ligation.

Gel Purification. To purify the appropriate DNA fragments, the digestion products were separated electrophoretically on a 1 % agarose gel. The bands at the appropriate sizes were excised using a clean razor blade and the DNA was purified from the gel using a QIAquick Gel Purification Kit. Briefly, 3 volumes of Buffer QG (solubilization buffer) was added to 1 volume of gel and incubated at 50 °C until the gel was dissolved. One volume of isopropanol was then added to the gel mixture and mixed. The mixture was then applied onto a QIA quick spin column and centrifuged at 10,000x g for one minute. After the flow through was discarded, 750 μl of Buffer PE (washing buffer) was placed onto the column and centrifuged at 10,000x g for 1 min. Prior to collecting the DNA, the flow through was discarded and the column was centrifuged for an additional 1 min at 12,000x g. Thirty microliters of Milli-Q water was added onto the column, the column was incubated with the water at RT for 1 min, and then the column was centrifuged at 12,000x g for 1 min. The eluted DNA was collected in a clean 1.5 ml microcentrifuge tube.

Ligation. Following digestion, the MOR1 and the first β_2-AR PCR products were ligated into pcDNA3.1.V5.TGA using standard procedures. The ligation reaction was performed in a clean 1.5 ml microcentrifuge tube containing 1 µl of T_4 DNA Ligase, 200 ng of the digested pcDNA3.1.V5.TGA vector, 40 ng of the digested PCR product, 2 µl of 10X T_4 DNA Ligase buffer. The total volume of the reaction was brought up to 20 µl by Milli-Q water. The mixture was allowed to incubate in a waterbath at 14 °C overnight. The second β_2-AR insert sequence, which was excised from the pCR2.1 vector (see above), was ligated into pcDNA3.1V5/His vector.

Analysis of Clones

Transformation of Competent Bacterial Cells

Frozen competent TOP 10F' cells (50 µl) were thawed on ice, mixed gently with 3 µl of the ligation product and incubated on ice for 30 minutes. Following incubation, the mixture was heated to 42 °C for 30 seconds before 250 µl SOC medium was added, and the mixture was shaken at 225 rpm at 37 °C for one hour. For each transformation, 50 µl and 200 µl of the mixture were spread over the entire surface of 2 LB agar plates containing 50 µg/ml ampicillin using a sterile, bent glass rod. The plates were then inverted and incubated at 37 °C for 12-18 hours or until colonies appeared.

Selection of Colonies

Several 2 ml cultures containing LB broth and 50 µg/ml ampicillin were inoculated with single colonies from the agar plate. The cultures were allowed to grow 8 hours or overnight at 37 °C while shaking at 225 rpm.

Plasmid DNA Purification by Phenol-Chloroform

One and one-half ml of each of the overnight cultures was transferred to a microcentrifuge tube and centrifuged at 1,500x g for 10 min to pellet the cells. Following centrifugation, the supernatant was discarded and each of the pellets was resuspended in 100 µl of solution I (50 mM glucose, 25 nM Tris-Cl, 10 mM EDTA, pH 8.0). The suspensions were then mixed by inversion with 200 µl of solution II (0.2 N NaOH, 1% SDS) and incubated on ice for 5 min. One hundred and fifty µl of solution III (3 M potassium acetate, 11.5 % glacial acetic acid) was then added to each mixture and the solution were mixed by inverting the tube 4-5 times, placed on ice for 5 min, and centrifuged at 12,000x g for 5 min. After centrifugation, the supernatants were transferred to microcentrifuge tubes containing 500 µl phenol/chloroform solution (pH 8.0), mixed by vortexing, and the mixtures were centrifuged for 2 min at 12,000x g. The upper aqueous phases were transferred to microcentrifuge tubes containing 300 µl isopropanol, mixed by vortexing, and centrifuged at 16,000x g for 15 min to pellet the DNA. The supernatants were discarded and the pellets were washed with 1 ml of 75% ethanol by centrifugation at 12,000x g for 5 min. The pellets were then air dried and dissolved in 50 µl Milli-Q water.

Screening for Positive Clones

Positive clones were screened for the presence of specific restriction enzyme sites. For pcDNA3.1.MOR1.V5, the restriction enzymes were EcoR I and Kpn I/Xba I. For pcDNA3.1.β_2-AR.V5, the restriction enzymes used to screen for positive clones were BstE II, EcoRV, and Kpn I/Xba I. For pcDNA3.1.β_2-AR, the restriction enzyme used was EcoR I. Restriction endonuclease digestion was performed in a water bath at the

appropriate temperature for 1 hour in a 10 µl volume containing 2 µl of DNA of the clone to be analyzed, 0.25 µl of each restriction enzyme, and 1 µl of the appropriate10X NEBuffer with or without 1 µl of 10X BSA. The sample was then analyzed by submerged agarose gel electrophoresis using 1% agarose gel. Positive clones were identified by the number of bands and their sizes.

Generating High-quality DNA Used for Diagnostic Analysis

Clones selected for further analysis were cultured overnight at 37 °C in 3 ml of LB broth containing 50 µg/ml ampicillin while shaking at 225 rpm. The plasmid DNA was purified using a QIAgen Plasmid Mini Kit to generate high-quality DNA. The culture was centrifuged at 1,500x g for 10 minutes and the bacterial pellet was resuspended in 0.3 ml of Buffer P1 (50 mM Tris-Cl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0). After resuspension, the culture was mixed gently with 0.3 ml of Buffer P2 (200 mM NaOH, 1 % SDS) and incubated at RT for 5 min. 0.3 ml of Buffer P3 (3.0 M potassium acetate, pH 5.5) was pre-chilled, then mixed immediately with the culture and incubated on ice for 5 min. After incubation, the mixture was centrifuged at 16,000x g for 10 min. The supernatant was removed promptly, applied to a QIAGEN-tip 20, which was equilibrated by 1 ml Buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % isopropanol, 0.15 % Triton X-100), and allowed to enter the resin by gravity flow. The QIAGEN-tip 20 was then washed 4 times with 1 ml of Buffer QC (1.0 M NaCl, 50 mM MOPS, 15 % isopropanol). The plasmid DNA bound to the resin was eluted by 0.8 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-Cl, 15 % isopropanol), and precipitated with 0.56 ml of isopropanol by centrifugation at 16,000x g for 30 min. The DNA pellet was then washed

with 1 ml of 75 % ethanol by centrifugation at 12,000x g for 5 min, air-dried for 5 min and redissolved in 30 µl of Milli-Q water.

Diagnostic restriction analysis

To confirm the presence and the orientation of the insert DNA, diagnostic restriction analysis was performed in a water bath at the appropriate temperature in 10 µl of reaction containing 2 µl of purified plasmid DNA, 1 µl of the appropriate restriction enzymes and 1 µl of 10X NEBuffer with or without 1 µl of 10X BSA for one hour. The restriction enzymes used for analyzing pcDNA3.1.MOR1.V5 were BamH1, BstB I, HindIII/EcoR I, and Kpn I/Xba I. The restriction enzymes used for analyzing pcDNA3.1.β_2-AR.V5 were BstE II, Dde I, Sty I, and Hind III/EcoR V. The restriction enzymes used for analyzing pcDNA3.1.β_2-AR were EcoR V and Not I/BstE II. The reaction products were then subjected to agarose gel electrophoresis using a 1% agarose gel.

DNA Maxi Prep

One hundred ml of LB broth with 100 µg/ml ampicillin was inoculated with 60 µl of a fresh overnight culture of the selected clone and incubated in 37 °C for 12 hours while shaking at 225 rpm. The bacterial cells were then harvested by centrifugation at 2,500x g for 15 min. The pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-Cl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0), followed by gentle mixing with 10 ml of Buffer P2 (200 mM NaOH, 1 % SDS) and incubation at RT for 5 min. Ten ml of pre-chilled Buffer P3 (3.0 M potassium acetate, pH 5.5) was then added to the lysate and mixed by inverting the tube 4-6 times. The lysate was poured into the barrel of a QIAfilter Cartridge and incubated at RT for 10 min. After the cap was removed from the

QIAfilter outlet nozzle, the plunger was gently inserted into the QIAfilter Cartridge and the cell lysate was filtered into a 50 ml tube. The filtered cell lysate was then mixed with 2.5 ml of Buffer ER and incubated at RT for 30 min. The solution was then applied to a QIAGEN-tip 50, which was equilibrated by 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % isopropanol, 0.15 % TritonX-100), and allowed to enter the resin by gravity flow. The QIAGEN-tip 50 was washed twice with 30 ml of Buffer QC (1.0 M NaCl, 50 mM MOPS, 15 % isopropanol) and plasmid DNA was eluted with 15 ml of Buffer QN (1.6 M NaCl, 50 mM MOPS, 15 % isopropanol). After elution, DNA was precipitated by mixing with 10.5 ml isopropanol and centrifuging the mixture immediately at 16,000x g for 30 min in a 50 ml endotoxin-free tube. The DNA pellet was washed with 2.5 ml of endotoxin-free 75 % ethanol by centrifugation at 16,000x g for 10 min. The supernatant was removed and the pellet air dried and redissolved in 30 µl of endotoxin-free Buffer TE (10 mM Tris-Cl, 1 mM EDTA). DNA concentration was determined by UV spectrophotometry.

Transient Transfection

AN3 CA cells were seeded onto glass coverslips in an 8-well plate at 2 x 10^5 cells/well and incubated for 18-24 hours in growth medium containing serum. For each transfection, 1 µg of the vector DNA was mixed with 10 µg of lipofectAMINE™ reagent in 195 µl of Opti-MEM reduced serum medium at RT for 30 minutes. While incubating the mixture, the cells were rinsed 3 times with Opti-MEM medium. The DNA mixture was then mixed with 800 µl Opti-MEM and applied to one well of an 8-well plate. The cells were incubated with the DNA/ LipofectAMINE complex at 37 °C for 5 hours. At the end of the incubation, the DNA/ LipofectAMINE complex in Opti-MEM was

replaced with complete growth medium containing serum and the cells were incubated at 37 °C for 45-48 hours.

Immunofluorescence Microscopy

Cells on glass coverslips were fixed with 3.7 % paraformaldehyde in PBS (58 mM Na_2HPO_4 , 17 mM NaH_2PO_4-H_2O, 68 mM NaCl, pH 7.2) for 10 min, washed twice with PBS 10 min per wash, permiablized with 62 μg/ml digitonin in PBS for 10 minutes, and washed once more with PBS for 10 min. Following this, the cells were incubated in 5%NGS/0.1%BSA/PBS at RT for 30 min and then exposed to primary antibody diluted in 1%NGS/0.1%BSA/PBS for one hour at 37 °C. After exposure to the primary antibody, the cells were washed 3 times in PBS and incubated for one hour at 37 °C in secondary antibody diluted in 0.1%BSA/PBS. The cells were then rinsed with PBS and the coverslips were mounted on glass slides with FITC-guard and observed with either a Nikon TE 300 inverted Epi-fluorescence microscope or a Fluoview 300 laser scanning confocal system. For Nikon TE 300 inverted Epi-fluorescence microscope, the images were collected with Matrox frame capture and analyzed with Metamorph software from Universal Imaging Corp. For Fluoview 300 laser scanning confocal system, the images were collected with photo multiplier tube (PMT) and analyzed with Fluoview software.

Cell Lysis and Immnoblotting Analysis

Preparation of Cell Lysate

Cells grown in 8-well plates or 25 cm^2 flasks were harvested by scraping and washed twice by resuspending in PBS (68 mM NaCl, 58 mM Na_2HPO_4, and 17 mM NaH_2PO_4, pH 7.2) and centrifuging at 1,500 rpm for 10 min. The cell pellet was resuspended in lysis buffer (150 mM NaCl, 20 mM Tris base, 0.01 M EDTA disodium

salt, 10 mM Benzamidine HCL, 1% Triton X-100, and 0.05% Tween-20, pH 7.4) containing 100 μg/ml leupeptin and 1 mM PMSF and incubated on ice for 10 min. The extract was then centrifuged at 16,000x g for 10 min at 4 °C and the supernatant was collected.

BCA Protein Assay

The protein concentration in the cell lysate was determined using a BCA (bicinchoninic acid) assay. The protein standard for the assay was prepared by mixing 200 μl of lysis buffer containing 5 mg/ml of bovine serum albumin (BSA) with 200 μl of lysis buffer containing 10 μg/ml leupeptin to make a 2.5 mg/ml of BSA solution. The 2.5 mg/ml BSA solution was then diluted 8 times by serial dilution into 1.5 ml microcentrifuge tubes containing 200 μl of lysis buffer with 10 μg/ml leupeptin. Samples were prepared by mixing 5 μl of cell lysate with 45 μl of lysis buffer. Ten μl of each mixture was then loaded into a 96-well plate by triplicate, mixed with 200μl of BCA solution from the BCA protein assay kit. The sample and BCA reagent was allowed to react for one hour at 60 °C. The absorption of each sample was measured at λ_{560} on a plate reader and protein concentrations were calculated from the protein standard.

Western Blotting

After the amount of protein in the cell lysate was determined by the BCA protein assay, cells lysates were mixed with appropriate volumes of sample buffer (0.5 M Tris, 10 % SDS, glycerol, β-mercaptoethanol, bromophenol blue) to ensure that the total protein in each sample was the same. Samples were then electrophoresed on 5% SDS-polyacrylamide gels using 20 mA constant current for 1-1.5 hour and transferred electrophoretically to a nitrocellulose paper using150 mA constant current for 2 hours in

transfer buffer (192 mM glycine, 25 mM Tris, 20 % methanol). After 1 hour to overnight incubation in the blocking buffer (0.2 % I-Block™ Reagent, 0.1 % Tween®-20, PBS), the blot was incubated with either an anti-V5 antibody diluted 1:5000 or a polyclonal anti-gravin antibody diluted 1:1000 at RT for 1 hour. The blot was then washed with PBS 4 times for 5 minutes and incubated with an alkaline phosphatase conjugated secondary antibody diluted 1:100,000 at RT for 1 hour, followed by washing with PBS 4 times for 5 minutes. All antibodies were diluted in blocking buffer. For detection, the blot was rinsed in 1X Assay buffer (20 mM Tris, 1 mM $MgCl_2$, pH 9.8) twice for several seconds and exposed to substrate solution (CDP-*Star*® substrate) for 5 min. The blot was then exposed to a standard X-ray film for 1-5 min and the film was developed in a MOHRpro 8 processor.

CHAPTER III

RESULTS

Expression and Distribution of Gravin in SH-SY5Y Cells

Western blot and immunofluorescent microscopy revealed that treatment of SH-SY5Y cells by retinoic acid significantly increased gravin expression. As shown in Figure 1, anti-gravin antiserum strongly detected a ~300 kDa band on immunoblots of extracts from cells treated with 10 mM retinoic acid (RA) for 8 days, but did not detect a band in extracts from untreated cells and only weakly detected a band in extracts from cells treated with ethanol, the solvent used to dissolve the retinoic acid. The ~300 kDa band in the RA treated cells was consistent with the molecular mass of gravin previously found (Grove et al., 1994). The band at ~250 kDa was likely a proteolytic fragment of gravin. Because the protein concentration in the cell extracts was determined by BCA protein assay and an equal amount of protein was loaded into each lane, differences in the density of the bands indicated that the cells treated by RA for 8 days expressed higher levels of gravin than untreated cells and ethanol control cells. Figure 2 illustrates the results of a time course experiment in which SH-SY5Y cells were exposed to RA for various times before they were harvested and equal amounts of total protein were subjected to SDS-PAGE. According to Figure 2, gravin level in the cells started to increase after 4 days of treatment with RA, reached the highest level after 6 and 8 days of treatment and then decreased at 10 and 12 days of treatment.

The effect of RA on gravin expression was confirmed by immunofluorescence microscopy. Cells treated with RA showed a higher level of fluorescent staining than observed in untreated cells (Fig 3). Immunofluorescence microscopy also revealed that gravin was localized primarily at the surface of the cell bodies and the cellular processes (Fig 4). The RA treated cells were also double labeled for gravin and μ-opioid receptor (MOR) and observed using confocal microscopy. Both the anti-MOR antiserum and the anti-gravin antibody detected not only membrane staining, but also partial cytoplasmic staining; however, the anti-gravin antibody detected primarily membrane staining. By image merging, there was partial colocalization of gravin and MOR (Fig 5A). Similarly, in cells double labeled with anti-β_2-adrenergic receptor (β_2-AR) and anti-gravin antibodies, the anti-β_2-AR antibody detected both membrane staining and cytoplasmic staining, and image merging showed partial colocalization of gravin and β_2-AR (Fig 5B).

In order to investigate whether the agonist-induced internalization of the MOR or β_2-AR was accompanied by redistribution of gravin, SH-SY5Y cells were treated with either DAMGO for 60 min or isoproterenol for 30 min. There was no redistribution of either the receptors or gravin in these cells (data not shown).

Characterization of Receptor Expression Vectors

To investigate the interaction of gravin and recombinant MOR and β_2-AR in transfected AN3 CA cells, the expression vectors, pcDNA3.1.MOR1.V5, pcDNA3.1.β_2-AR.V5, and pcDNA3.1.β_2-AR were generated by inserting MOR and β_2-AR sequences into either pcDNA3.1.V5/His or a modified pcDNA3.1.V5.TGA vector.

Characterization of PCR products

Agarose gel electrophoresis of PCR products generated using receptor specific primers revealed the presence of DNA fragments which corresponded in size to the predicted size of 1381, 1358 and 1422 bp for the MOR insert, the first β_2-AR insert and the second β_2-AR insert, respectively (Fig 6). These bands were excised from the gel and ligated into either pcDNA3.1.V5/His or pcDNA3.1.V5.TGA.

Identification of Recombinants

Following ligation and transformation of the ligation products, the appropriate clones were identified by restriction enzyme analysis. Schematic drawings of the pcDNA3.1.MOR1.V5, pcDNA3.1.β_2-AR.V5 and pcDNA3.1.β_2-AR vectors illustrating the restriction enzyme sites used in the analysis are shown in Figure 7.

Clones for pcDNA3.1.MOR1.V5 were identified initially by two criteria: the presence of a unique EcoR I site in the insert DNA and the DNA fragment pattern resulting from double digestion with Kpn I and Xba I. pcDNA3.1.MOR1.V5 linearized by EcoR I digestion was expected to be 6809 bp, 1,381 bp longer than the linearized pcDNA3.1.V5.TGA, due to the presence of the insert DNA. Digestion of pcDNA3.1MOR1.V5 with Kpn I an Xba I was expected to yield two fragments, one 5446 bp in size, the other 1363 bp in size. Of the 6 colonies tested, all were found to meet these criteria and colony 5 was selected for further analysis (Fig 8).

Clones for pcDNA3.1.β_2-AR.V5 were initially identified by the presence of a unique BstE II site, the presence of a unique EcoR V site and the pattern of fragments obtained by double digestion with Kpn I and Xba I. Of the 20 colonies selected, only colony 12 yielded a single band at the predicted size of 6800 bp after BstE II digestion

(Fig 9A). Digestion of plasma DNA from this colony with EcoR V also yielded a single fragment at 6800 bp while double digestion with Kpn I and Xba I yielded a 5446 base pair fragment and a 1358 bp fragment, corresponding in size to the PCR product (Fig 9B).

Clones for pcDNA3.1.β_2-AR were initially identified by the presence of a 1438 bp fragment (corresponding to the insert DNA) after EcoR I digestion. All of the 10 colonies selected yielded a 5500 bp fragment and a 1438 bp fragment at predicted sizes (Fig 10).

Diagnostic Restriction Enzyme Analysis

The selected recombinants were further analyzed by restriction enzyme digestions to confirm that the insert DNA was ligated in the forward orientation. Digestion of pcDNA3.1.MOR1.V5 with BamHI, BstB I, Hind III/EcoR I and Kpn I/Xba I yielded fragments which were consistent with the insert DNA having the correct sequence and orientation (Fig 11). Similarly, digestion of pcDNA3.1.β_2-AR.V5 with BstE II, Sty I, and Hind III/EcoR V yielded fragments which were consistent with the insert DNA having the correct sequence and orientation (Fig 12). Digestion of pcDNA3.1.β_2-AR with EcoR V and BstE II/Not I yielded fragments which were consistent with the insert DNA having the correct sequence and orientation (Fig 13).

Expression of Recombinant Proteins in AN3 CA Cells

AN3 CA cells are a human endometrial carcinoma cell line which was originally derived from an undifferentiated metastatic endometrial carcinoma (Fogh et al. 1977). Morphologically, cultured AN3 CA cells display an epithelial-like appearance under the light microscope. Under standard culturing conditions, the efficiency of

transient transfection could reach 30-40%. Preliminary data in our lab has shown that AN3 CA cells do not express endogenous gravin.

To confirm that GR81-5534 was transcribed in AN3 CA cells, cells transfected with GR81-5534 were stained with either an anti-V5 antibody or with monoclonal anti-gravin antibody Mab2B3-1.1 and observed using a confocal microscope. Both antibodies revealed strong signals at the membrane of those cells expressing the transgene (Fig 14). In addition, both the anti-V5 antibody and a polyclonal anti-gravin antibody detected a ~300kDa band on western blots of the transfected cells (Fig 15, lane 1, 2). The molecular weight of the bands was similar to that of the endogenous gravin detected in extracts from SH-SY5Y cells (Fig 15, lane 3).

The epitope-tagged MOR and β_2-AR were initially detected by staining with the anti-V5 antibody. The fluorescent signal was localized at the surface of the transfected cells in both cases (Fig 16). To further confirm that the full-length receptor transgenes were transcribed, AN3 CA cells transfected with either the V5-tagged MOR or the V5-tagged β_2-adrenergic receptor vectors were double labeled using the anti-V5 monoclonal antibody and either a polyclonal anti-MOR antibody or a polyclonal anti-β_2-adrenergic receptor antibody (Fig 17 & 18). The fluorescent labeling by the anti-V5 and anti-receptor antibodies showed extensive colocalization and this colocalization was confirmed by image merging.

Although the anti-V5 antibody displayed primarily plasma membrane staining, the anti-MOR and the anti-β_2-AR antibodies revealed membrane staining and some cytoplasmic staining. To test whether this cytoplasmic staining represented expression of the transgene or endogenous expression of the receptors, non-transfected AN3 CA cells

were stained using either the anti-MOR antibody, the anti-β_2-AR antibody, or the anti-gravin antibody. Both the anti-MOR and the anti-β_2-AR antibodies detected weak cytoplasmic staining, indicating either non-specific staining by the polyclonal antibody or a low level of endogenous expression of these receptors in AN3 CA cells (Fig 19A, B). The non-transfected cells did not label for gravin (Fig 19C).

Coexpression of Gravin and Receptor Transgenes

To determine whether gravin would colocalize with MOR in AN3 CA cells, cells co-transfected with GR81-5534 and pcDNA3.1.MOR1.V5 were double labeled with the anti-gravin antibody and the polyclonal anti-MOR antibody. Both antibodies displayed strong membrane staining and the staining of gravin and MOR showed extensive colocalization (Fig 20, A and B). This colocalization was further confirmed by image merging. To confirm that overexpression of either one of these two proteins did not induce the expression of the other, and to confirm that the antibodies did not cross-react with the recombinant proteins, cells transfected with either GR-81-5534 alone or pcDNA3.1.MOR1.V5 alone were double labeled by both anti-gravin and anti-MOR antibodies. In Figure 20C and D, the cells transfected with only pcDNA3.1.MOR1.V5 showed only anti-MOR reactivity. There was no detectable anti-gravin reactivity in these cells. In Figure 20E and F, the cells transfected with only GR81-5534 reacted with the anti-gravin antibody, but not with the anti-MOR antibody.

In experiments in which AN3 CA cells were co-transfected with GR81-5534 and pcDNA3.1.β_2-AR.V5, and double labeled with anti-gravin and anti-β_2AR antibodies, both antibodies labeled the membrane and displayed extensive colocalization (Fig 21, A and B). In the cells transfected with pcDNA3.1.β_2-AR.V5 only and double labeled with

both the anti-gravin and the anti-β_2-AR antibodies, the anti-β_2-AR antibody labeled the membrane but anti-gravin labeling was negative (Fig 21, C and D). In cells transfected with GR81-5534 and double labeled with both antibodies, only the anti-gravin antibody showed positive staining (Fig 21, E and F).

Effect of Agonist Treatment on Gravin and Receptor Distribution

To determine if agonist mediated internalization of the V5-tagged MOR was accompanied by redistribution of gravin, AN3 CA cells cotransfected with MOR and gravin vector constructs were treated with DAMGO and double labeled with anti-MOR and anti-gravin antibodies. Before agonist treatment, anti-gravin and anti-MOR antibody labeling colocalized at the plasma membrane (Fig 22, A-D). After 30 min DAMGO treatment, anti-MOR antibody labeling displayed a distinct punctate pattern of fluorescence in the perinuclear region of the cell (Fig 22, E and G). This change in anti-MOR labeling occurred in both co-transfected cells and cells transfected only with the MOR vector. On the other hand, the majority of anti-gravin labeling remained at the cell membrane after agonist treatment, and there was no detectable colocalization between the anti-gravin and the anti-MOR labeling (Fig 22, E, F, and H)

To determine whether redistribution of gravin occurs during agonist induced internalization of the V5-tagged β_2-AR, AN3 CA cells cotransfected with both gravin and β_2-adrenergic receptor vector constructs were treated with isoproterenol and double labeled with anti-β_2-AR and anti-gravin antibodies. In cells either co-transfected with GR81-5534 and pcDNA3.1.β_2-AR.V5 vectors or transfected with pcDNA3.1.β_2-AR.V5 alone, anti-β_2-AR labeling showed a distinct punctate pattern of fluorescence at the perinuclear region after 30 min of isoproterenol treatment (Fig 23, E and G). In the

cotransfected cells and the cells transfected with GR81-5534 alone, the anti-gravin antibody detected primarily membrane staining before and after isoproterenol treatment, indicating no change in gravin distribution in response to isoproterenol (Fig 23, F and H). No detectable colocalization of antibody labeling was revealed in cells co-transfected with GR81-5534 and pcDNA3.1.β_2-AR.V5 after isoproterenol treatment (Fig 23, E and F).

To examine the possibility that the V5-epitope fused to the C-terminus of the β_2-AR might affect protein-protein interaction between gravin and the receptors, AN3 CA cells cotransfected with the gravin construct and a β_2-AR construct lacking the V5-epitope were either treated with isoproterenol for 30 min or not treated and double labeled with anti-gravin and anti-β_2-AR antibodies. As can be been in Figure 24, the anti-gravin and anti-β_2-AR labeling colocalized in the absence of isoproterenol (Fig 24, A-D). After isoproterenol treatment, anti-β_2-AR labeling was concentrated in a region adjacent to the nucleus (Fig 24, E), while gravin labeling remained on the cell membrane (Fig 24, F). Control cells transfected with the β_2-adrenergic receptor construct alone displayed redistribution of β_2-adrenergic receptor after isoproterenol treatment (Fig 24, G), while there was no redistribution of gravin in the cells transfected with the gravin construct alone (Fig 24, H).

Figure 1: **Effect of retinoic acid on gravin expression in SH-SY5Y cells** Western blot analysis of gravin expression in SH-SY5Y cells treated with either 10 μM retinoic acid (RA) or ethanol for 6 days, or no treatment. Gravin expression was detected by a polyclonal anti-gravin antibody.

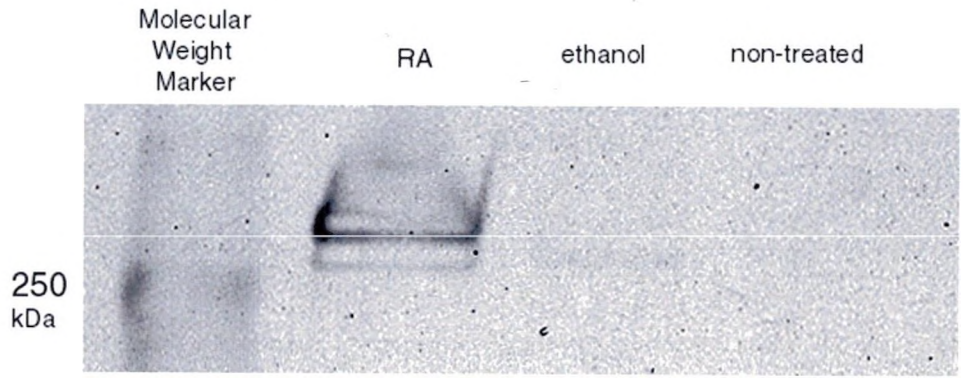

Molecular
Weight
Marker
RA
ethanol
non-treated
250
kDa

Figure 2: **Duration of retinoic acid treatment** Western blot analysis of gravin expression in SH-SY5Y cells treated with 10 μM retinoic acid for 0, 4, 6, 8, 10, and 12 days. Gravin expression was detected by a polyclonal anti-gravin antibody.

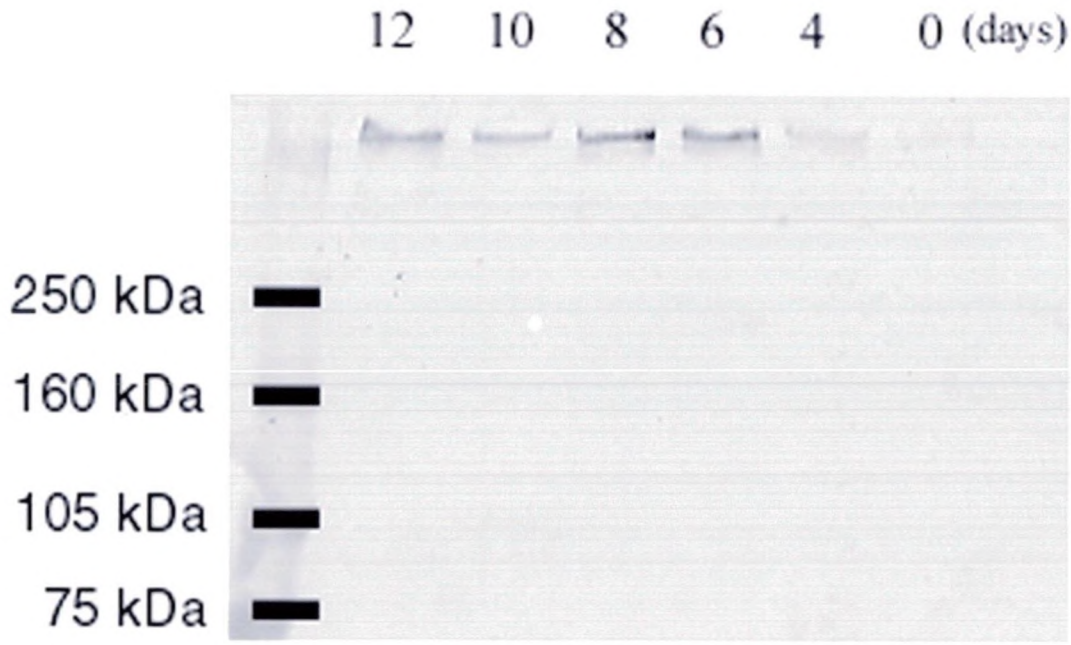

12
10
8
6
4
0 (days)
250 kDa
160 kDa
105 kDa
75 kDa

Figure 3: **Effect of retinoic acid on gravin expression in SH-SY5Y cells**

Immunofluorescent analysis (The magnification of the images is 280X) of gravin (a) without and (b) with 10 μM retinoic acid for 6 days. Gravin expression was detected by a monoclonal anti-gravin antibody and stained by Cy3-conjugated secondary antibody.

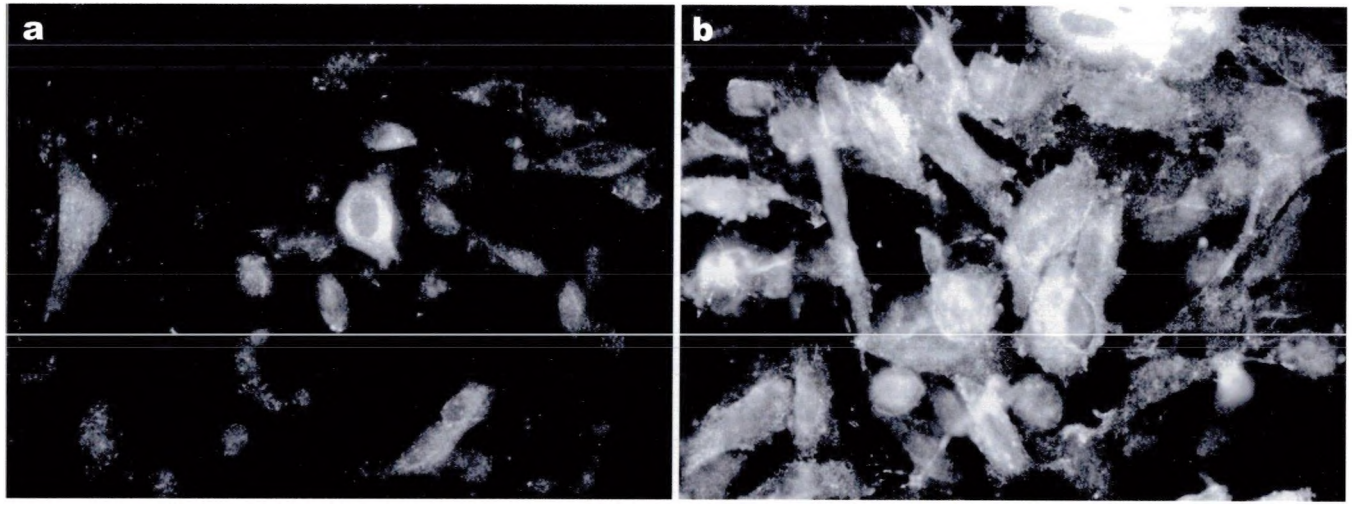

a
b

Figure 4: **Gravin distribution in SH-SY5Y cells treated with retinoic acid for 6 days**

Immunofluorescent image showing gravin distribution in SH-SY5Y cells treated with retinoic acid for 6 days. (a, b): immunofluorescent dye tends to be distributed along margins of cell bodies (arrows) (1200X). (c): immunofluorescent dye reveals puntate staining at cell margins and the edges of neurites (arrows) (950X). (d): immunofluorescent dye reveals puntate staining at the edges of neurites (arrows) (2400X); insert showing more detail at higher magnification (5600X).

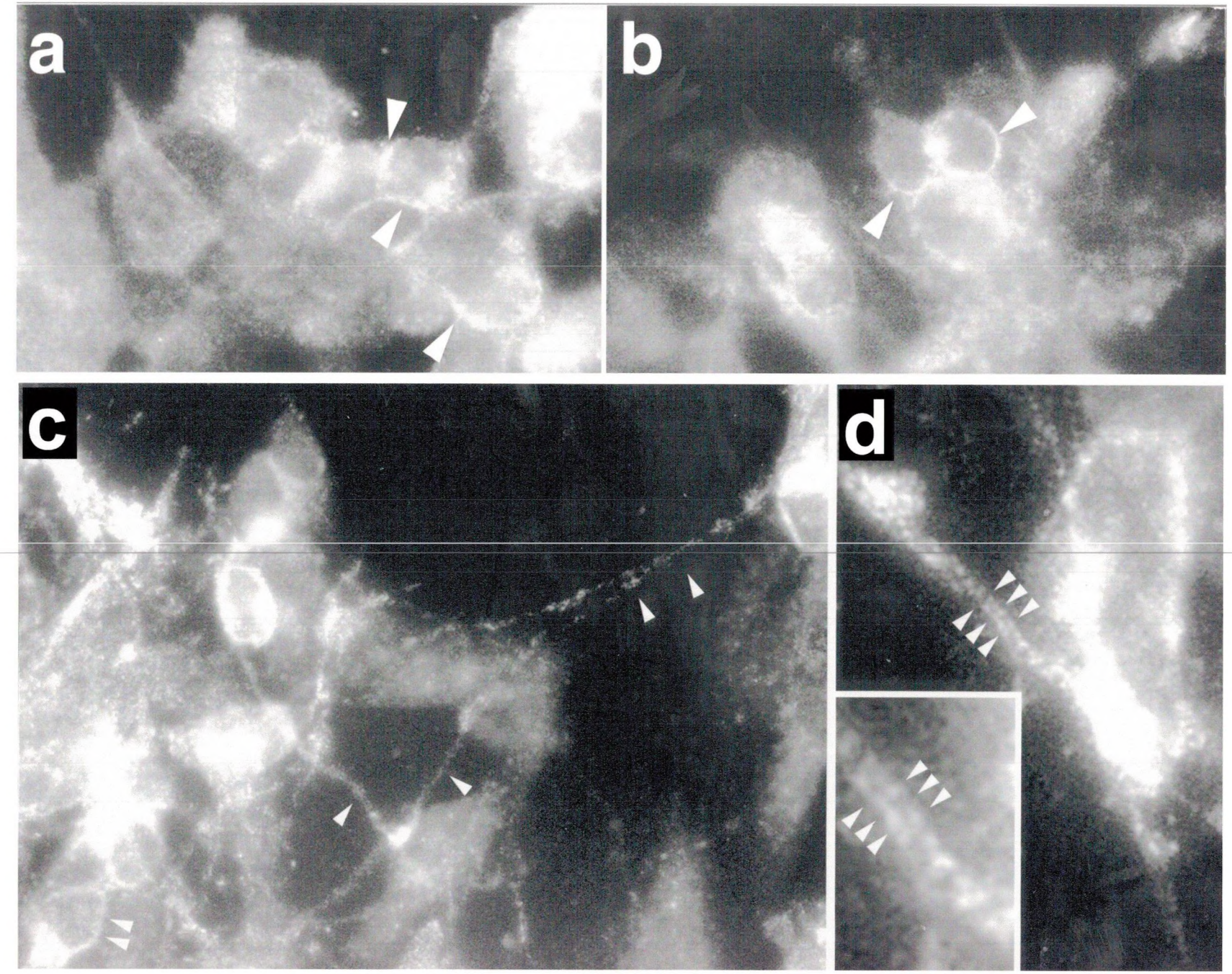
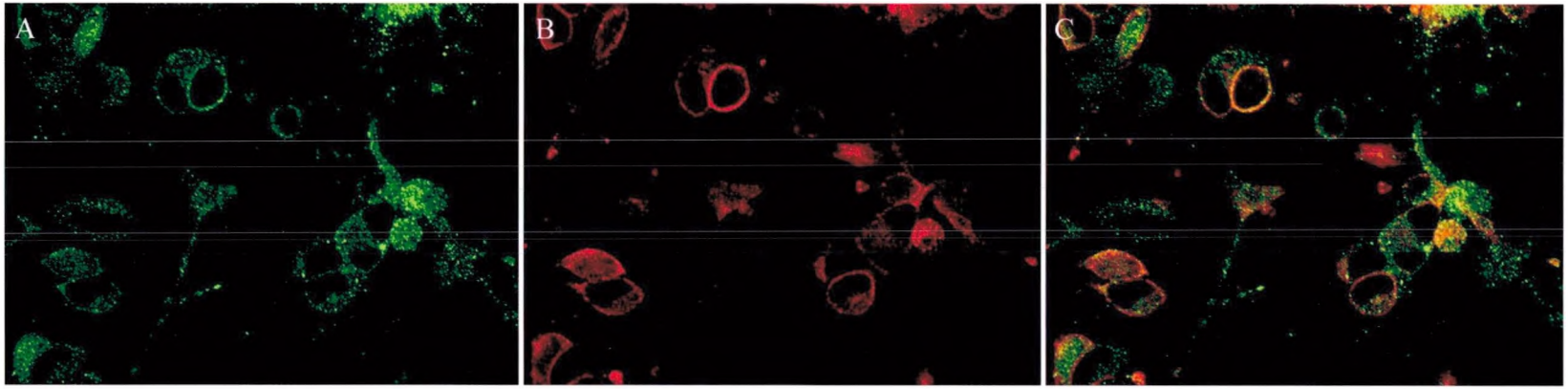

a
b
c
d

Figure 5: **Gravin and MOR or β_2-AR double labeling in SH-SY5Y cells** I. SH-SY5Y cells were double labeled with (A) anti-MOR antibody and (B) anti-gravin antibody, and the images were merged in (C). The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects MOR stained by FITC (A), while the red channel reflects gravin stained by Cy3 (B). Note that there was both membrane staining and cytoplasmic staining for MOR and there was partial colocalization of gravin and MOR at the cell margins. II. SH-SY5Y cells were double labeled with (A) anti-β_2-AR antibody and (B) anti-gravin antibody, and the images were merged in (C). The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects β_2-AR stained by FITC (A), while the red channel reflects gravin stained by Cy3 (B). Note that there was both membrane staining and cytoplasmic staining for β_2-AR and there was partial colocalization of gravin and β_2-AR at the cell margins. *Bar=25μm*

I. Double label gravin/μ-opioid receptor



II. Double label gravin/β_2-adrenergic receptor

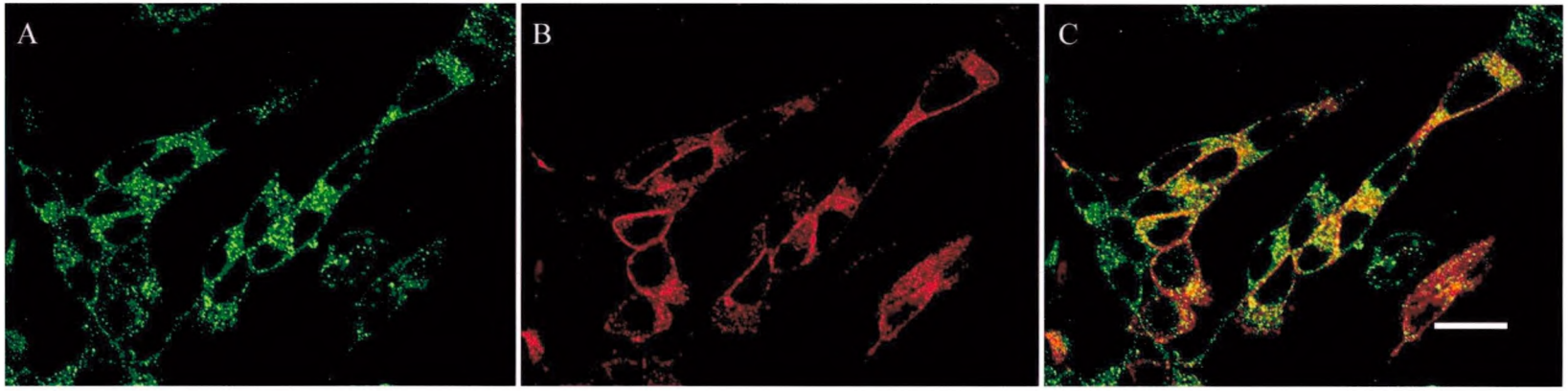


Figure 6: **DNA sequences amplified by PCR** DNA sequence encoding human μ-opioid receptor and β_2-adrenergic receptor was PCR amplified from messenger RNA isolated from SH-SY5Y cells that had been treated with retinoic acid for 6 days. The PCR product was separated on a 1% agarose gel. The presence of the band at 1381 base pairs, 1358 base pairs and 1422 base pairs indicated the presence of the amplified μ-opioid receptor and two β_2-adrenergic receptor sequences, respectively.

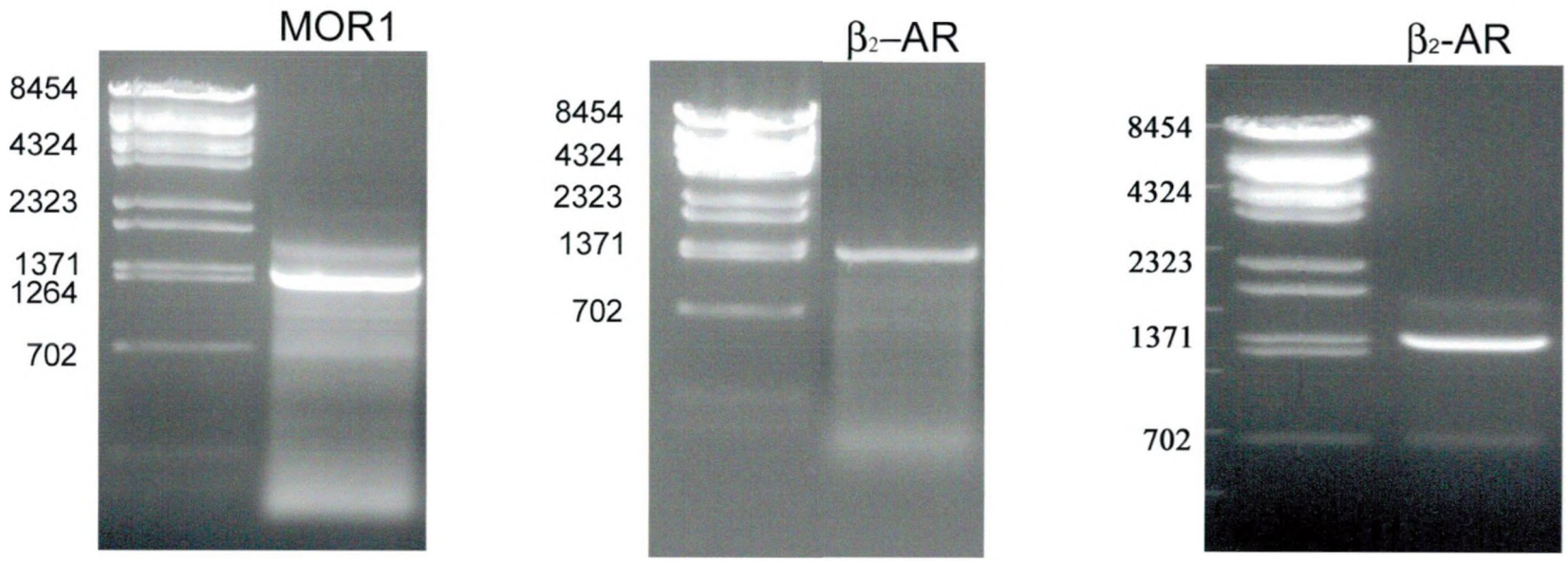

MOR1
8454
4324
2323
1371
1264
702
β2–AR
8454
4324
2323
1371
702
β2-AR
8454
4324
2323
1371
702

Figure 7: **Schematic drawing of (A) pcDNA3.1.MOR1.V5, (B) pcDNA3.1.β_2-AR.V5 and (C) pcDNA3.1.β_2-AR including restriction enzyme sites.**

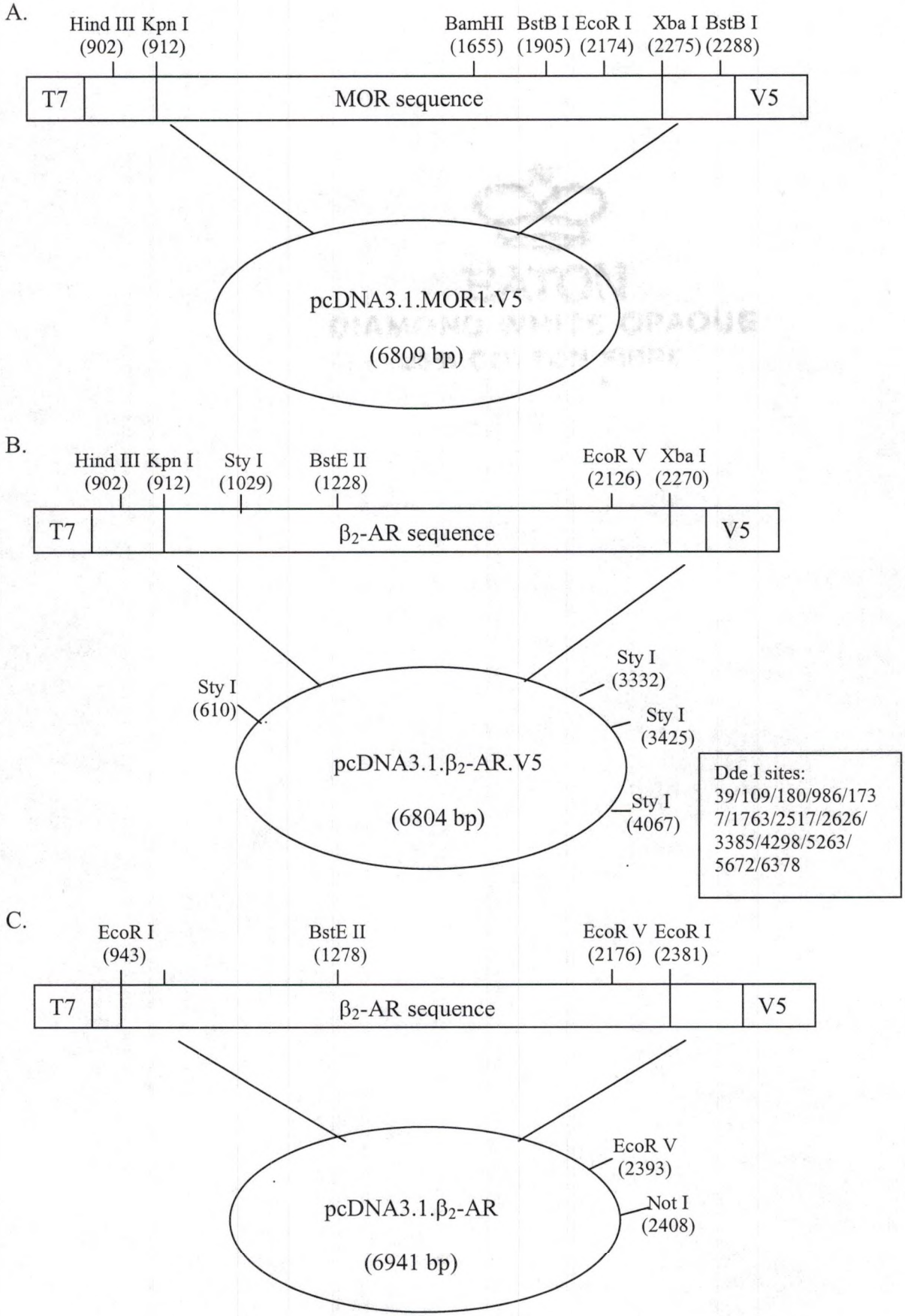
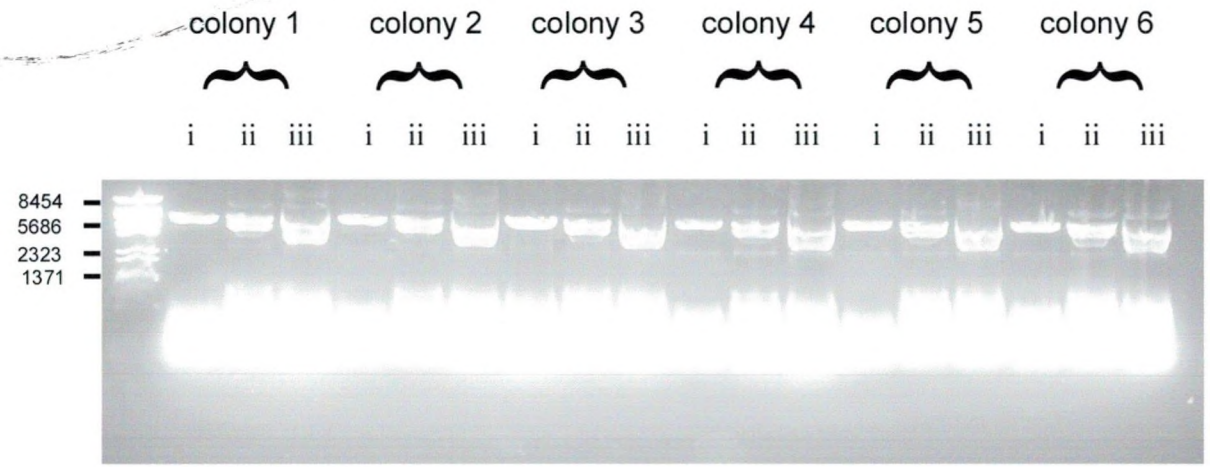

A.
Hind III (902)
Kpn I (912)
BamHI (1655)
BstB I (1905)
EcoR I (2174)
Xba I (2275)
BstB I (2288)
T7
MOR sequence
V5
pcDNA3.1.MOR1.V5
(6809 bp)
B.
Hind III (902)
Kpn I (912)
Sty I (1029)
BstE II (1228)
EcoR V (2126)
Xba I (2270)
T7
β2-AR sequence
V5
Sty I (610)
Sty I (3332)
Sty I (3425)
Sty I (4067)
pcDNA3.1.β2-AR.V5
(6804 bp)
Dde I sites:
39/109/180/986/1737/1763/2517/2626/3385/4298/5263/5672/6378
C.
EcoR I (943)
BstE II (1278)
EcoR V (2176)
EcoR I (2381)
T7
β2-AR sequence
V5
EcoR V (2393)
Not I (2408)
pcDNA3.1.β2-AR
(6941 bp)

Figure 8: **Screening for positive clones for pcDNA3.1.MOR1.V5** DNA samples from 6 colonies were digested by either EcoR I or Kpn I/Xba I, and separated on 1% agarose gel to screen the positive clones. The presence of one band at 6809 base pairs by EcoR I digestion and two bands at 1363 base pairs and 5446 base pairs by Kpn I/Xba I digestion indicated a positive clone.



i: EcoR I
ii: Kpn I/Xba I
iii: uncut DNA

Figure 9: **Screening for positive clones for pcDNA3.1.β_2-AR.V5**

(A): DNA samples from 20 colonies (1-20) were digested by BstE II and separated on a 1% agarose gel. The presence of one band at ~6800 base pairs suggested a positive clone.

(B): Colony 12 was further selected and digested by BstE II, EcoR V and Kpn I/Xba I. The presence of one band at 6804 base pairs by EcoR V digestion, 1358 base pair larger than the fragment of pcDNA3.1V5.TGA digested by EcoR V, and two bands at 1358 base pairs and 5446 base pairs by Kpn I/Xba I digestion confirmed the presence of the insert DNA.

Lane 1: Molecular weight marker

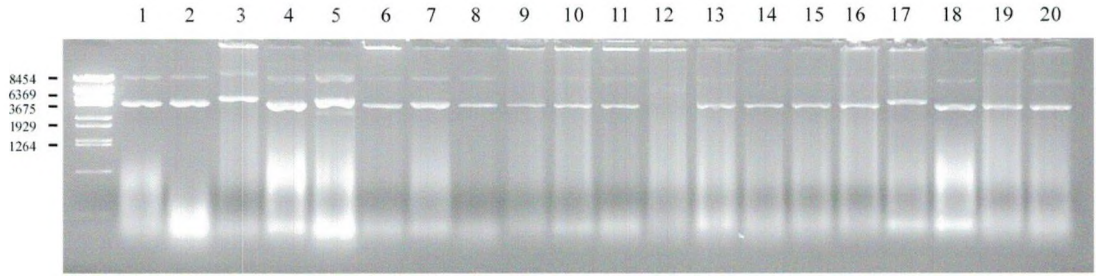
Lane 2: BstE II digestion

Lane 3: EcoR V digestion

Lane 4: Kpn I/ Xba I digestion

Lane 5: pcDNA3.1V5.TGA digested by EcoR V

A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
8454
6369
3675
1929
1264

B.

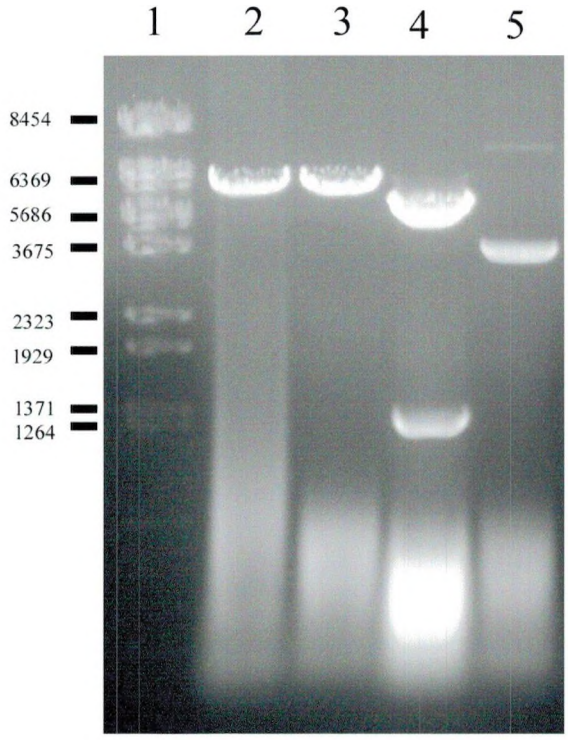

1 2 3 4 5
8454
6369
5686
3675
2323
1929
1371
1264

Figure 10: **Screening for positive clones for pcDNA3.1.β_2-AR** DNA samples from 10 colonies (1-10) were digested by EcoR I and separated on a 1% agarose gel. The presence of the band at 1438 base pairs suggested positive clones.

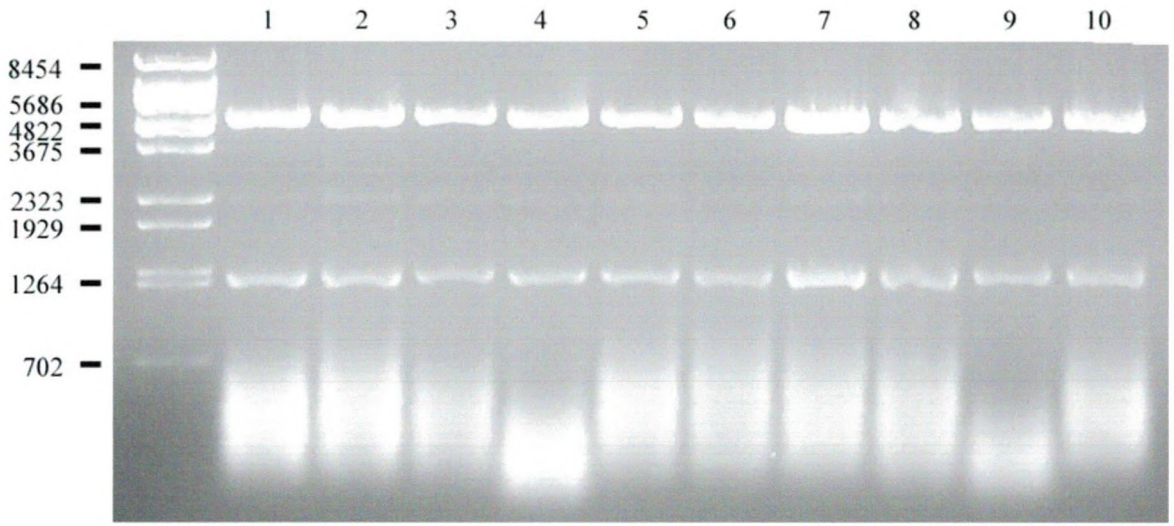

1
2
3
4
5
6
7
8
9
10
8454
5686
4822
3675
2323
1929
1264
702

Figure 11: **Identification and orientation of insert DNA for pcDNA3.1.MOR1.V5**

DNA sample was digested by BamHI, BstBI, Hind III/EcoR I, and Kpn I/Xba I to confirm the presence of the insert DNA and the orientation of the insert. The existance of the band at 1363 base pairs by Kpn I/Xba I digestion, which is the same size as the band obtained from the PCR product digested by Kpn I/Xba I, confirmed the presence of the insert. The 6809 base pair fragment by BamHI digestion, 1363 base pairs larger than the fragment of pcDNA3.1V5.TGA digested by BamHI, also confirmed the presence of the insert. The smaller band at 383 base pair by BstBI digestion and the smaller band at 1272 base pairs by Hind III/EcoR I digestion indicated the right orientation of the insert DNA.

Lane 1: Molecular weight marker

Lane 2: BamHI digestion

Lane 3: BstBI digestion

Lane 4: HindIII/EcoR I digestion

Lane 5: Kpn I/Xba I digestion

Lane 6: undigested supercoiled pcDNA3.1.MOR1.V5

Lane 7: pcDNA3.1V5.TGA digested by BamH1

Lane 8: PCR amplified MOR1 sequence digested by Kpn I/Xba I

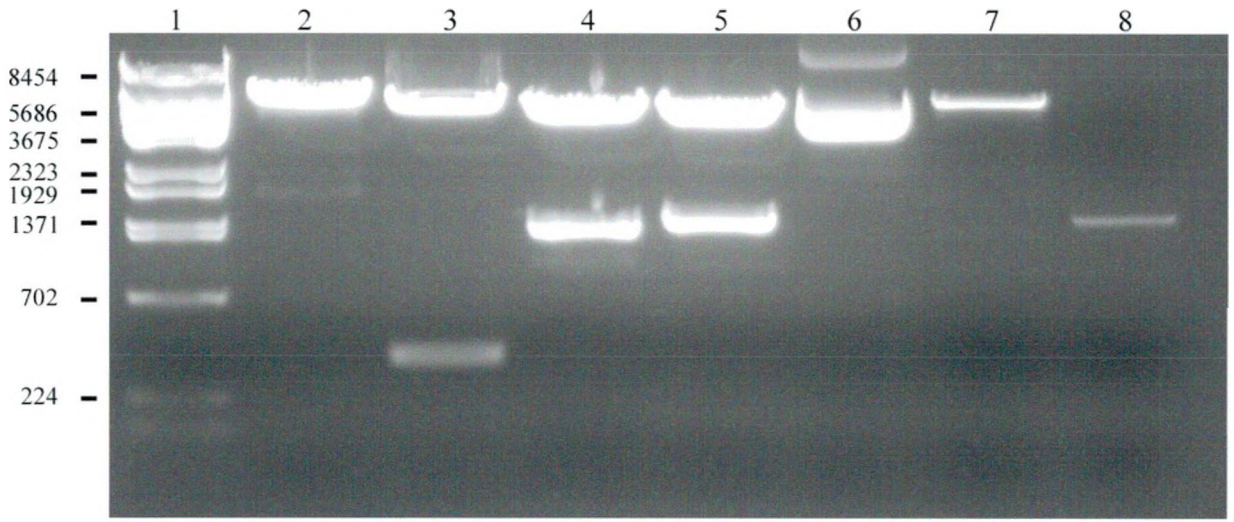

1
2
3
4
5
6
7
8
8454
5686
3675
2323
1929
1371
702
224

Figure 12: **Identification and orientation of insert DNA for pcDNA3.1. β_2-AR.V5** The presence of the insert DNA was already confirmed by EcoR V and Kpn I/Xba I digestion. The full-length recombinant DNA is 6804 base pairs, according to BstE II digestion. The smaller band at 1224 base pairs by Hind III/EcorR V digestion and the migration pattern of the Dde I and Sty I digestion fragments indicated the right orientation of the insert DNA.

Lane 1: Molecular weight marker

Lane 2: Bst EII digestion

Lane 3: Dde I digestion

Lane 4: Sty I digestion

Lane 5: Hind III/EcoR V digestion

Lane 6: undigested supercoiled pcDNA3.1. β_2-AR.V5

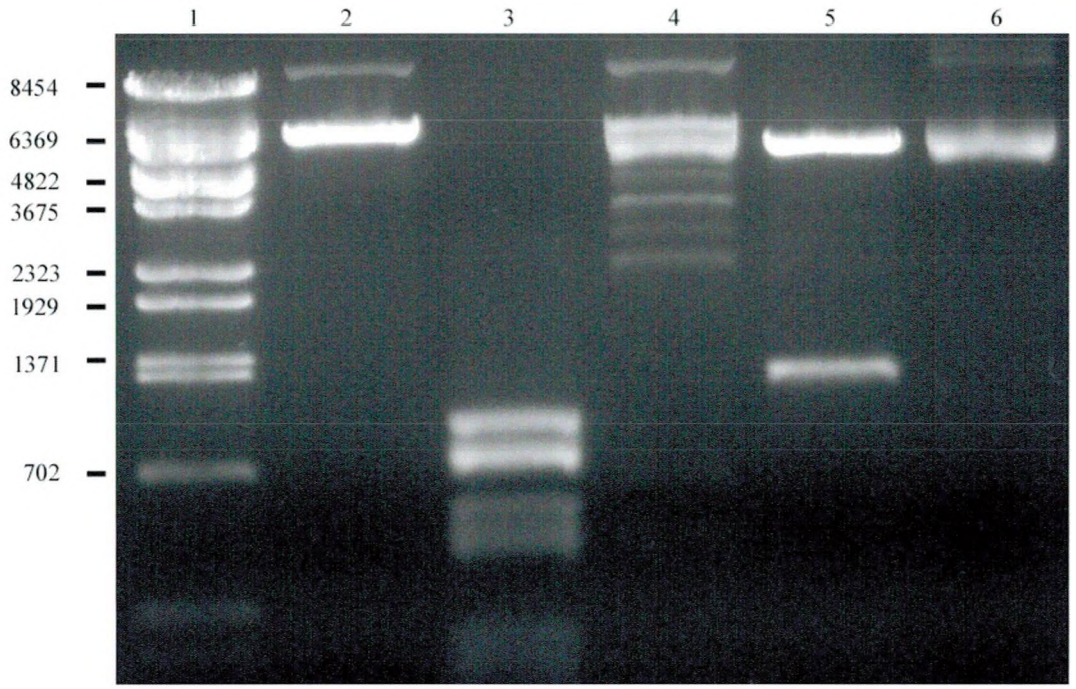

1
2
3
4
5
6
8454
6369
4822
3675
2323
1929
1371
702

Figure 13 **Identification and orientation of insert DNA for pcDNA3.1. β_2-AR** DNA sample was digested by EcoR V and Not I/ BstE II to confirm the presence of the insert DNA and the orientation of the insert. The presence of the band at 217 base pairs by EcoR V digestion and the presence of the band at 1130 base pairs by Not I/ BstE II digestion confirmed the presence and the right orientation of the insert DNA.

HMWM: High molecular weight marker

LMWM: Low molecular weight marker

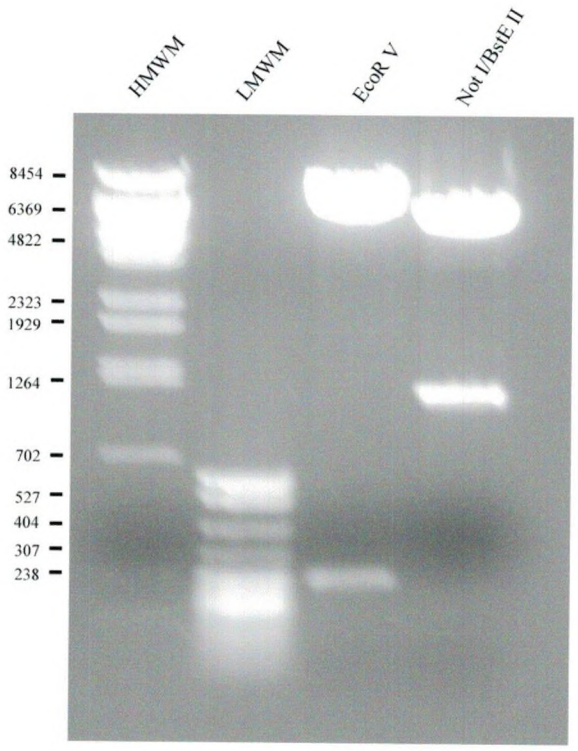

HMWM
LMWM
EcoR V
Not I/BstE II
8454
6369
4822
2323
1929
1264
702
527
404
307
238

Figure 14: **Immunofluorescence microscopy showing expression of the gravin fusion protein in AN3 CA cells** AN3 CA cells grown on glass coverslips were transiently transfected with GR81-5534. The expression of gravin was identified by (A) anti-V5 antibody and (B) anti-gravin antibody. The distribution of gravin was analyzed by confocal microscopy. Both anti-V5 and anti-gravin antibodies revealed strong fluorescence at the cell margins. *Bar=7.5μm*

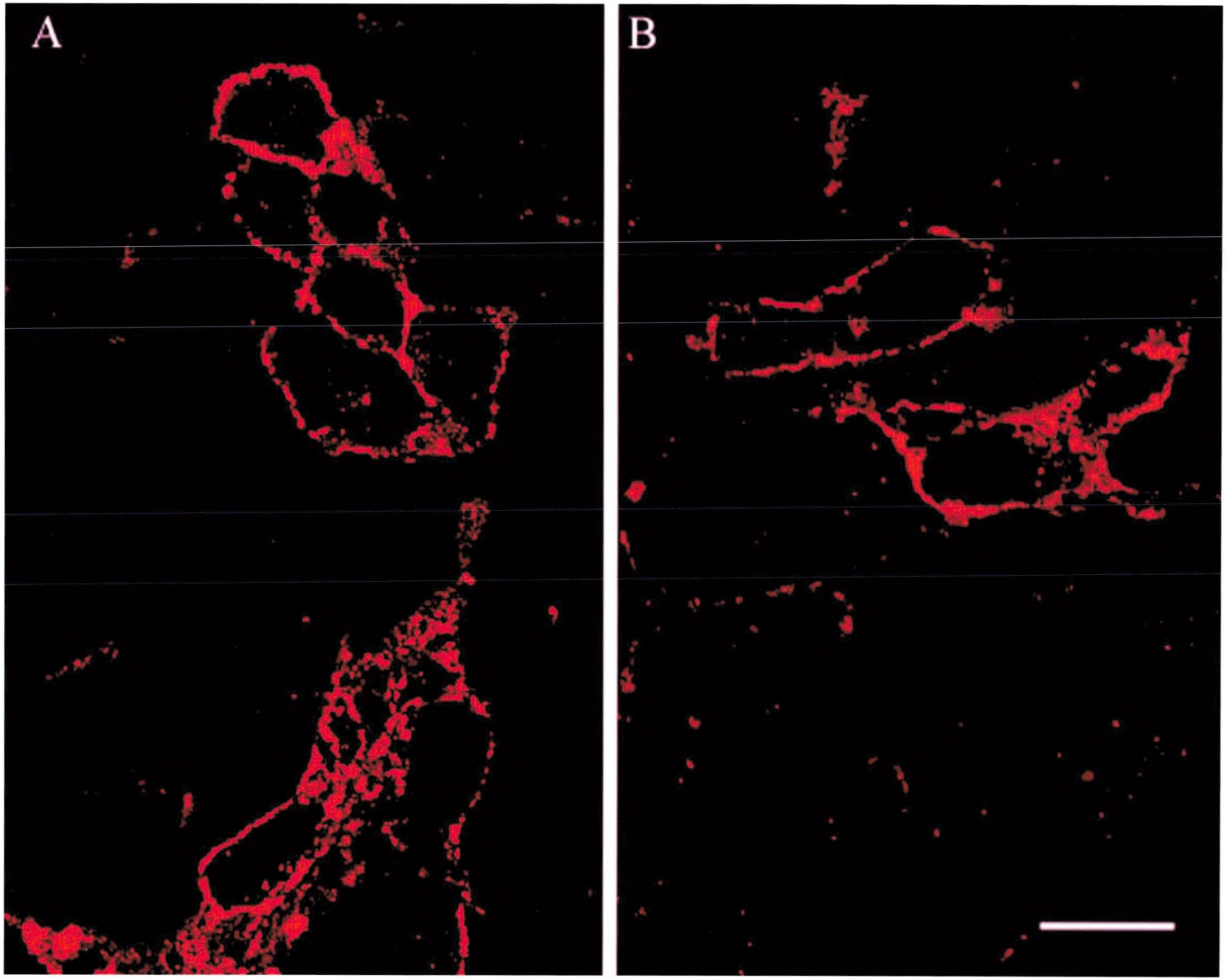

A
B

Figure 15: **Western blot analysis showing expression of gravin fusion protein in AN3 CA cells** AN3 CA cells were transiently transfected with GR81-5534. The cells were lysed and the expression of the gravin fusion protein was detected by either an anti-V5 antibody (lane 1) or a polyclonal anti-gravin antibody (lane 2) using enhanced chemiluminnescence. The control experiment included cell lysate from SH-SY5Y cells treated with retinoic acid for 6 days, and immunoblotted with the anti-gravin antibody (lane 3).

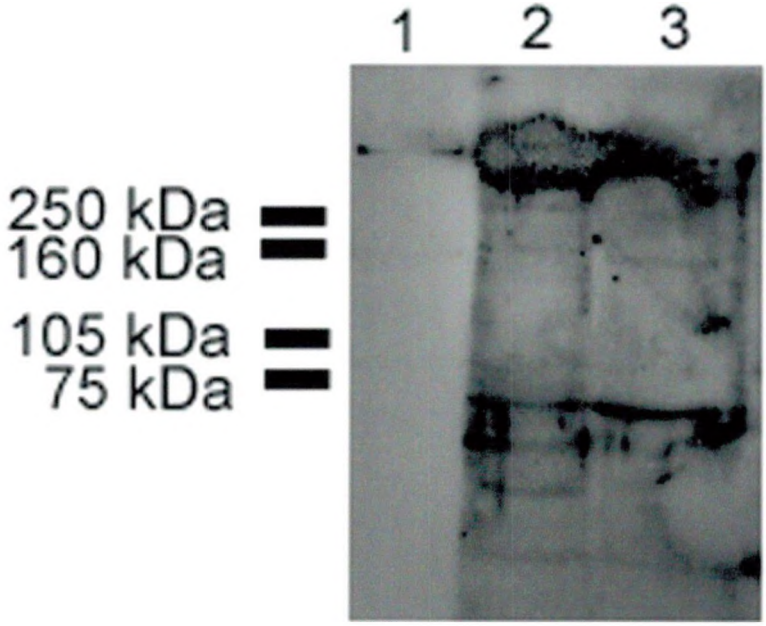

1
2
3
250 kDa
160 kDa
105 kDa
75 kDa

Figure 16: **Immunofluorescence microscopy showing expression of MOR1 and β_2-adrenergic receptor fusion protein in AN3 CA cells** AN3 CA cells grown on glass coverslips were transiently transfected with (A) pcDNA3.1.MOR1.V5 or (B) pcDNA3.1.β_2-AR.V5. The expression and distribution of MOR or β_2-AR were detected by anti-V5 antibody and analyzed by confocal microscopy. The anti-V5 antibody detected both fusion proteins at the membrane of the transfected cells. *Bar=25 μm*

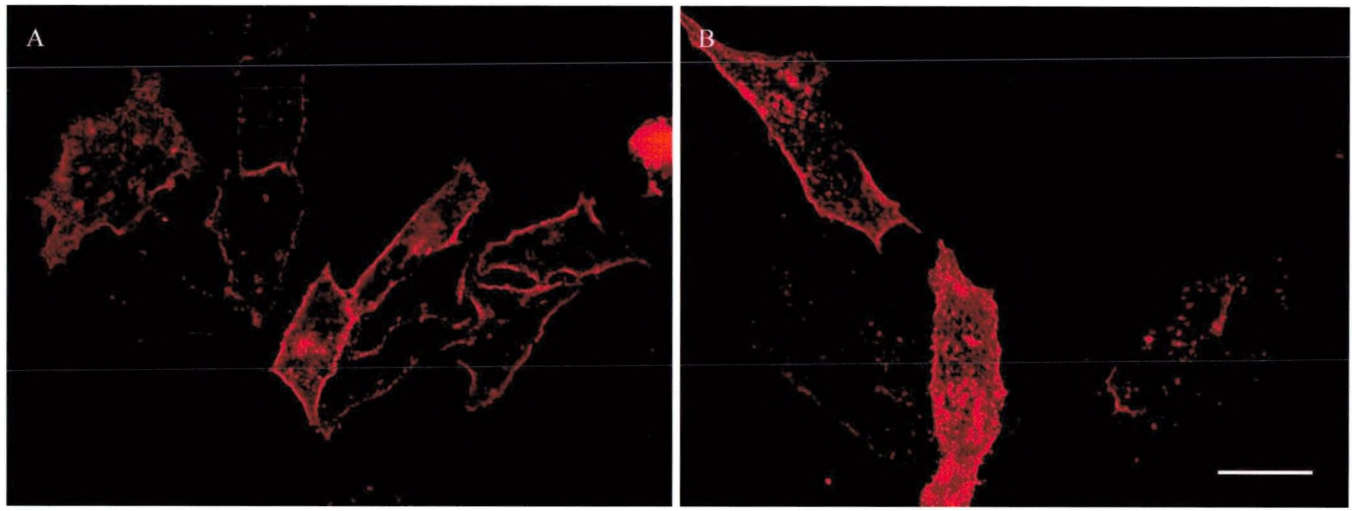

A
B

Figure 17: **Immunofluoresence microscopy showing expression of the pcDNA3.1.MOR1.V5 transgene in AN3 CA cells** AN3 CA cells grown on glass coverslips were transiently transfected with pcDNA3.1.MOR1.V5 and double labeled with (A) an anti-MOR antibody and (B) an anti-V5 antibody, and the images were merged in (C). The anti-MOR was detected by an FITC-conjugated secondary antibody and revealed as green fluorescence under confocal microscopy (A). The anti-V5 antibody was detected by a Cy3-conjugated secondary antibody and revealed as red fluorescence under confocal microscopy (B). Both anti-V5 and anti-MOR antibodies revealed membrane staining and image merging showed overlap of MOR1 and the V5-epitope (C). *Bar=25μm*


A
B
C

Figure 18: **Immunofluoresence microscopy showing expression of the pcDNA3.1.β_2-AR.V5 transgene in AN3 CA cells** AN3 CA cells grown on glass coverslips were transiently transfected with pcDNA3.1. β_2-AR.V5 and double labeled with (A) an anti-β_2-AR antibody and (B) an anti-V5 antibody, and the images were merged in (C). The anti-β_2-AR antibody was detected by an FITC-conjugated secondary antibody and revealed as green fluorescence under confocal microscopy (A). The anti-V5 antibody was detected by a Cy3-conjugated secondary antibody and revealed as red fluorescence under confocal microscopy (B). Both anti-V5 and anti-β_2-AR antibodies revealed membrane staining and image merging showed overlap of β_2-AR and the V5-epitope (C).

Bar=25μm


C
B
A

Figure 19: **Immunofluorescence microscopy of anti-MOR, anti-β_2-AR, and anti-gravin labeling in non-transfected AN3 CA cells** AN3 CA cells were labeled with (A) anti-MOR, (B) anti-β_2-AR, or (C) anti-gravin antibodies and analyzed under confocal microscopy. *Bar=25μm*

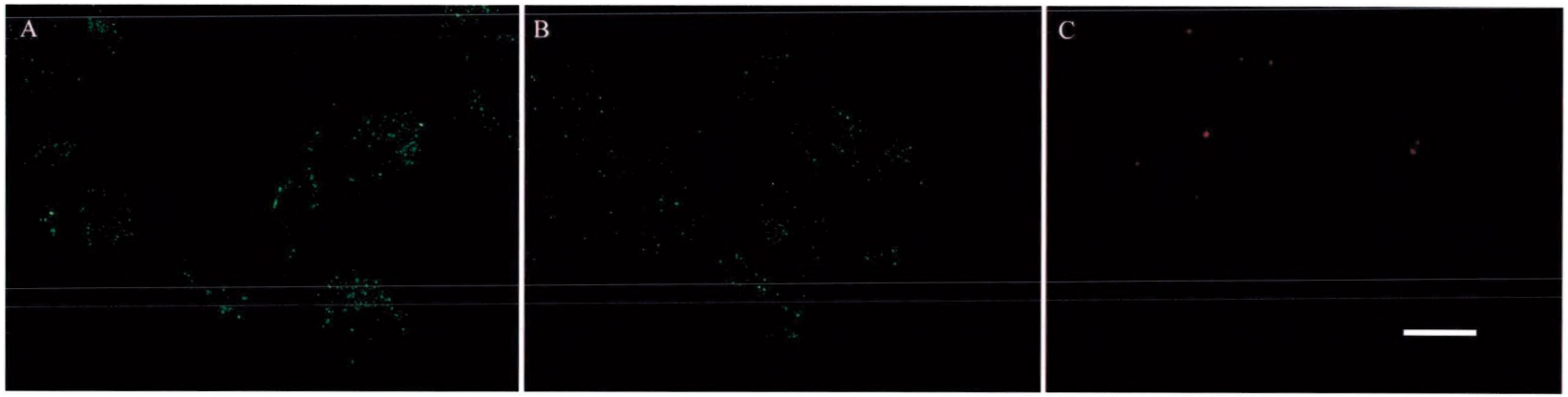

A
B
C

Figure 20: **Immunofluorescence microscopy showing colocalization of gravin and MOR in AN3 CA cells** AN3 CA cells were co-transfected with GR81-5534/ pcDNA3.1.MOR1.V5 (A, B), transfected with pcDNA3.1.MOR1.V5 alone (C, D), or transfected with GR81-5534 alone (E, F). All the transfectants were double labeled with anti-gravin and anti-MOR antibodies. The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects MOR1 stained by FITC (A, C, E), while the red channel reflects gravin stained by Cy3 (B, D, F). Note the colocalization of gravin and MOR1 at the cell margins. *Bar=25μm*

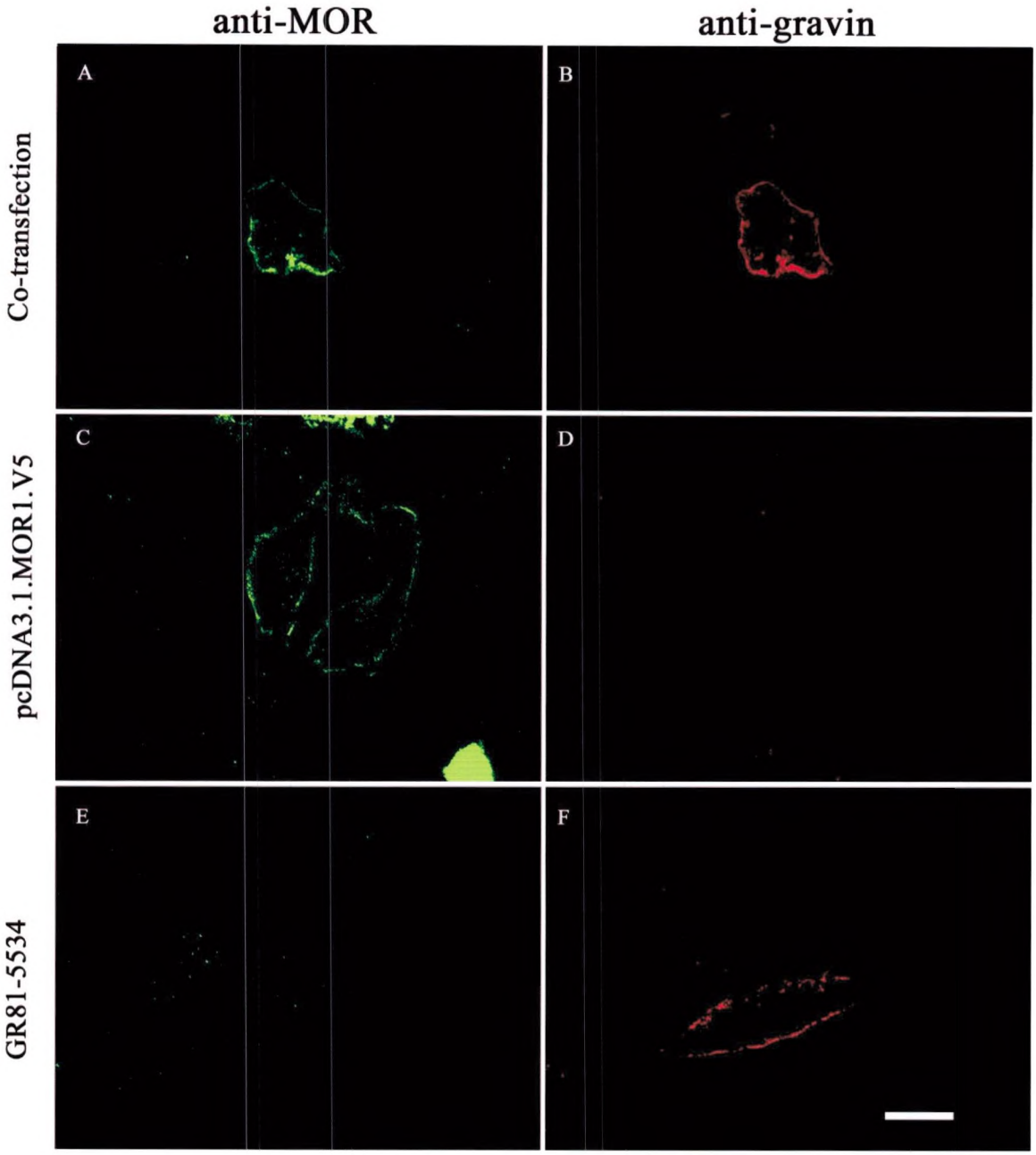

anti-MOR
anti-gravin
A
B
C
D
E
F
Co-transfection
pcDNA3.1.MOR1.V5
GR81-5534

Figure 21: **Immunofluorescence microscopy showing colocalization of gravin and β_2-adrenergic receptor in AN3 CA cells** AN3 CA cells were co-transfected with GR81-5534/ pcDNA3.1.β_2-AR.V5 (A, B), transfected with pcDNA3.1.β_2-AR.V5 alone (C, D), or transfected with GR81-5534 alone (E, F). All the transfectants were double labeled with anti-gravin and anti-β_2-AR antibodies. The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects β_2-AR stained by FITC (A, C, E), while the red channel reflects gravin stained by Cy3 (B, D, F). Note the colocalization of gravin and β_2-adrenergic receptor at the cell margins. *Bar=25 μm*

Co-transfection
pcDNA3.1.β2-AR.V5
GR81-5534
anti-β2-AR
anti-gravin
A
B
C
D
E
F

Figure 22: **Immunofluorescence microscopy showing the effect of DAMGO treatment on MOR and gravin distribution in AN3 CA cells** AN3 CA cells were co-transfected with GR81-5534/ pcDNA3.1.MOR1.V5 (A, B, E, F), transfected with pcDNA3.1.MOR1.V5 alone (C, G), or transfected with GR81-5534 alone (D, H). The cells were double labeled with anti-gravin and anti-MOR antibody before (A-D) and after (E-H) 10 μM DAMGO stimulation for 30 min. The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects MOR stained by FITC (A, C, E, G), while the red channel reflects gravin stained by Cy3 (B, D, F, H). Note the translocation of MOR, but not gravin, after DAMGO stimulation. *Bar=25μm*

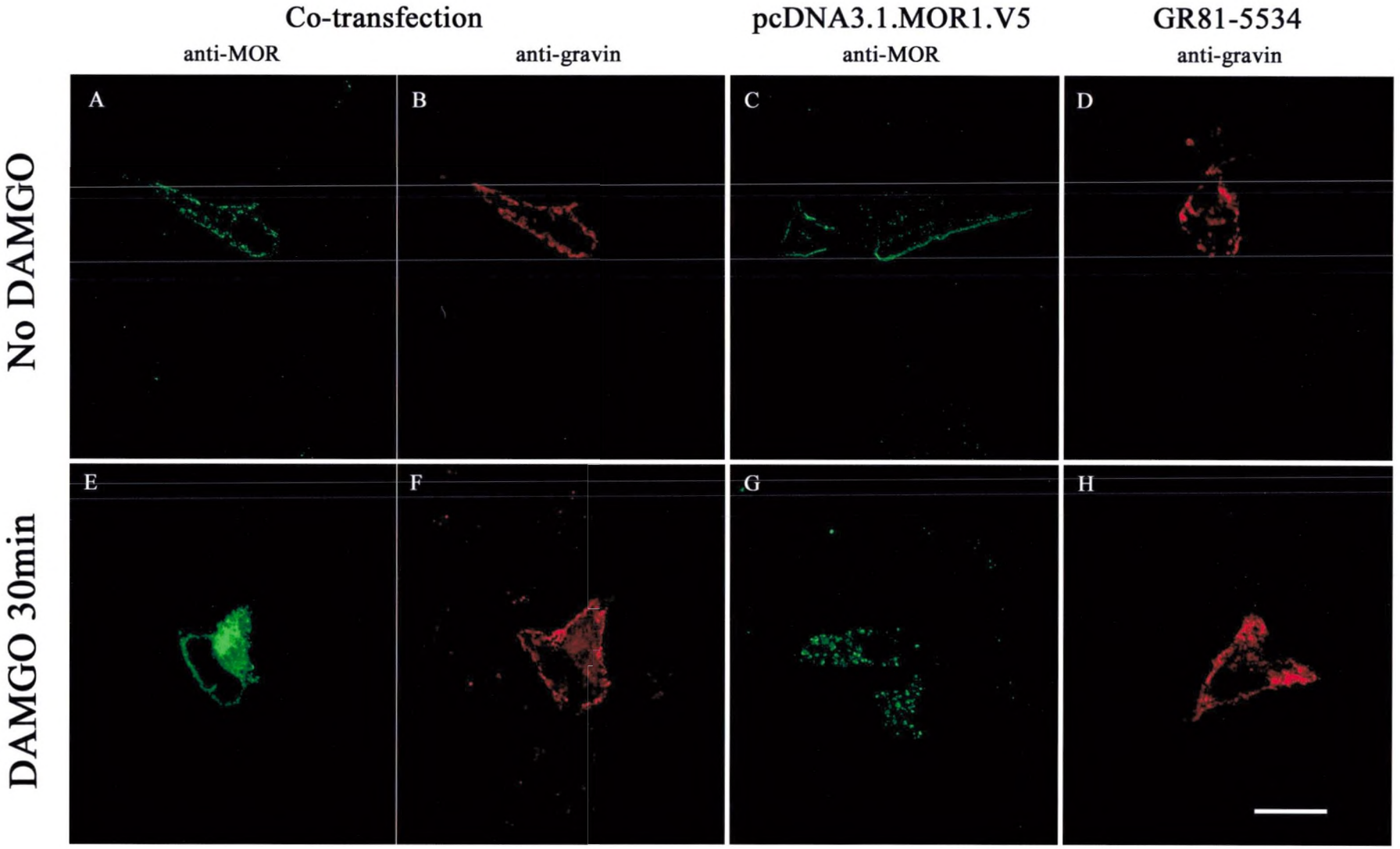

Co-transfection
anti-MOR
anti-gravin
pcDNA3.1.MOR1.V5
anti-MOR
GR81-5534
anti-gravin
No DAMGO
DAMGO 30min
A
B
C
D
E
F
G
H

Figure 23: **Immunofluorescence microscopy showing the effect of isoproterenol on β_2-AR and gravin distribution in AN3 CA cells** AN3 CA cells were co-transfected with GR81-5534/ pcDNA3.1.β_2-AR.V5 (A, B, E, F), transfected with pcDNA3.1.β_2-AR.V5 alone (C, G), or transfected with GR81-5534 alone (D, H). The cells were double labeled with anti-gravin and anti-β_2-AR antibody before (A-D) and after (E-H) 10 μM isoproterenol stimulation for 30 min. The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects β_2-AR stained by FITC (A, C, E, G), while the red channel reflects gravin stained by Cy3 (B, D, F, H). Note the translocation of β_2-adrenergic receptor, but not gravin, after isoproterenol stimulation. *Bar=25μm*

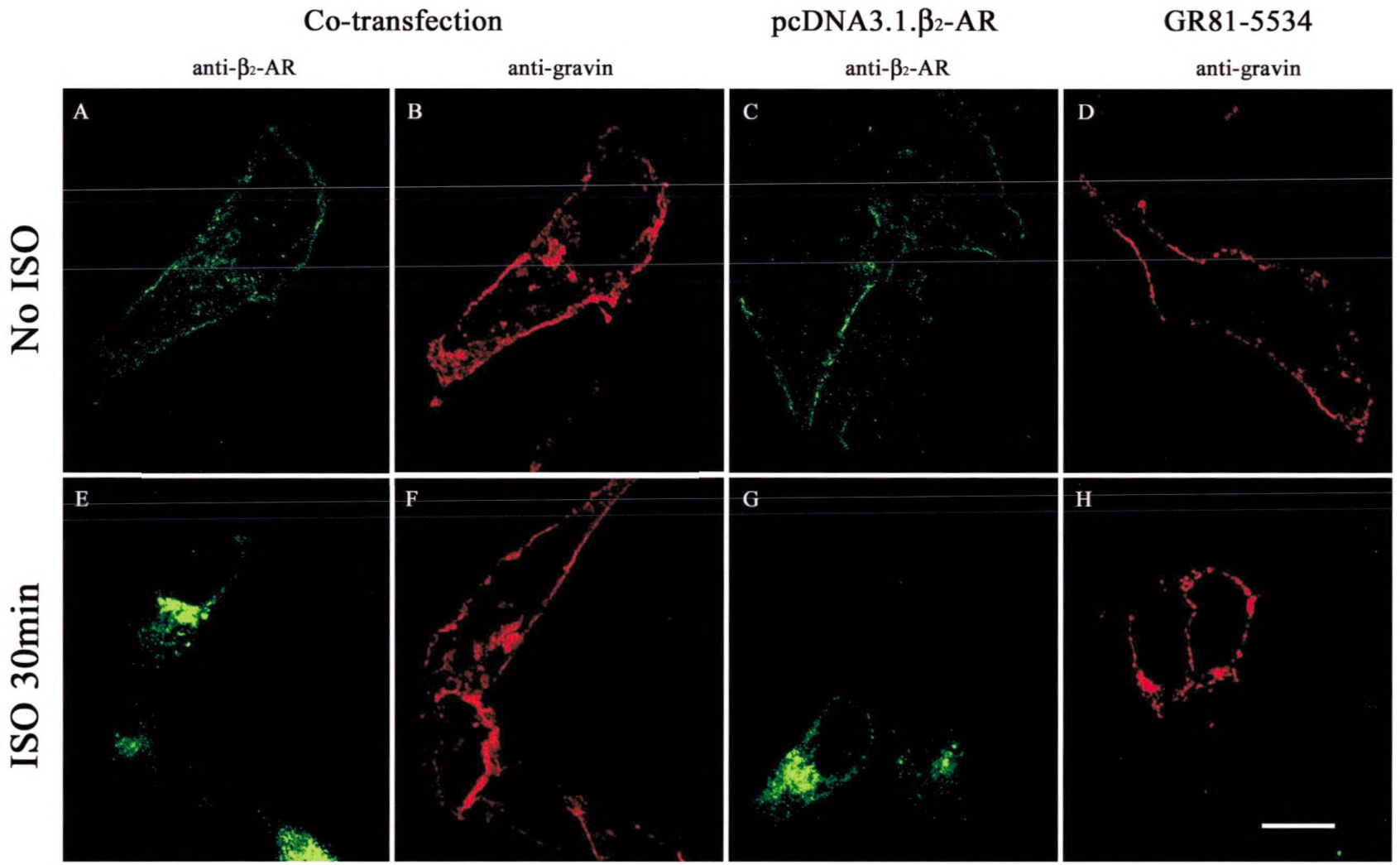
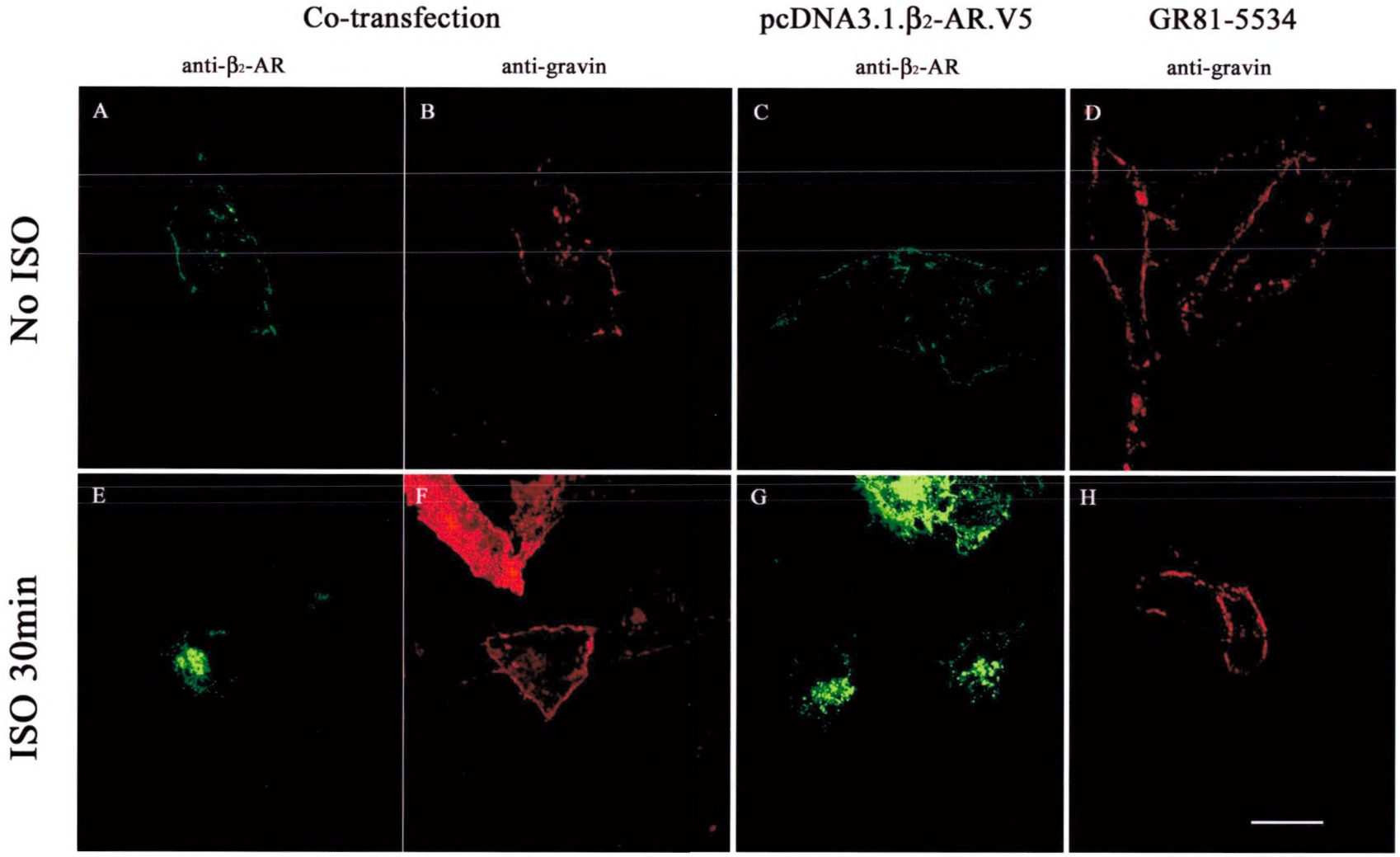

Co-transfection
pcDNA3.1.β2-AR
GR81-5534
anti-β2-AR
anti-gravin
anti-β2-AR
anti-gravin
No ISO
ISO 30min
A
B
C
D
E
F
G
H

Figure 24: **Immunofluorescence microscopy showing the effect of isoproterenol on untagged β_2-AR and gravin distribution in AN3 CA cells** AN3 CA cells were co-transfected with GR81-5534/ pcDNA3.1.β_2-AR (A, B, E, F), transfected with pcDNA3.1.β_2-AR alone (C, G), or transfected with GR81-5534 alone (D, H). The cells were double labeled with anti-gravin and anti-β_2-AR antibody before (A-D) and after (E-H) 10 μM isoproterenol stimulation for 30 min. The distribution of the antigens was analyzed by confocal microscopy. The green channel reflects β_2-AR stained by FITC (A, C, E, G), while the red channel reflects gravin stained by Cy3 (B, D, F, H). Note the translocation of β_2-adrenergic receptor, but not gravin, after isoproterenol stimulation. *Bar=25μm*


Co-transfection
pcDNA3.1.β2-AR.V5
GR81-5534
anti-β2-AR
anti-gravin
anti-β2-AR
anti-gravin
No ISO
ISO 30min
A
B
C
D
E
F
G
H

CHAPTER IV

DISCUSSION

The present study investigated the hypothesis that gravin interacts with μ-opioid receptors and β_2-adrenergic receptors during agonist-induced receptor internalization. Immunofluorescent microscopy and immunoblotting data showed that gravin was upregulated by retinoic acid in SH-SY5Y cells and that gravin was localized at the cell membrane. The present study, the first to describe the distribution of μ-opioid receptor in SH-SY5Y cells using immunofluorescent staining and the first to describe the expression and distribution of β_2-adrenergic receptor in SH-SY5Y cells, also revealed that gravin partially colocalized with μ-opioid receptor and β_2-adrenergic receptor in these cells. Recombinant gravin, μ-opioid receptor, and β_2-adrenergic receptor expressed in AN3 CA cells also localized to the membrane of the transfected cells. Extensive colocalization of gravin and μ-opioid receptor or β_2-adrenergic receptor was observed in each of the cotransfected cells. Although the recombinant receptors underwent internalization after agonist treatment, this was not accompanied by gravin redistribution.

The pattern of gravin expression in SH-SY5Y cells corresponded with that of μ-opioid receptor. Western blot analysis and immunofluorescence microscopy revealed that gravin expression was upregulated by retinoic acid treatment. Increased gravin expression was observed after 4 days of retinoic acid treatment and reached the highest levels at 6 to 8 days of treatment. This pattern of expression in response to retinoic acid

was similar to that reported for μ-opioid receptor in SH-SY5Y cells. It has been reported that either retinoic acid or phorbol ester treatment resulted in a significant increase in the number of μ-opioid receptor without affecting the affinity of agonist binding in SH-SY5Y cells (Zadina et al., 1993). Six days of retinoic acid treatment resulted in a ~175% increase in μ-opioid receptor expression (Zadina et al., 1994). Because of the large amount of expression of μ-opioid receptor in this cell line, SH-SY5Y cells have proven useful as a model system for studying μ-opioid receptor in terms of the acute action of opiates, or desensitization, resensitization, or down-regulation of the receptor. The similarity of the expression pattern between gravin and μ-opioid receptor in response to retinoic acid is consistent with the hypothesis that gravin interacts with μ-opioid receptors and provided the possibility to study gravin and μ-opioid receptor interaction in SH-SY5Y cells.

The distribution of gravin in retinoic acid treated SH-SY5Y cells is also consistent with the hypothesis that gravin may regulate μ-opioid receptor activity. Immunofluorescence microscopy revealed that in addition to being present in the cytoplasmic compartment, gravin was localized at the membrane of the cell bodies and the neurites, consistent with the localization of gravin at the cell membrane in HEL and human umbilical vein endothelial cells (Nauert et al., 1997; Grove et al., 2001). Given that gravin is known to bind PKA and PKC, the membrane localization of this protein suggests that it may be involved in membrane associated PKA and PKC signaling events. At present, the specific PKA and PKC signaling events in which gravin might participate are unknown. However, several studies indicate that μ-opioid receptor activity is regulated by PKA and PKC. For instance, Smart and Lambert (1995) reported that

desensitization of μ-opioid receptor in SH-SY5Y cells involved both PKA and PKC. In addition, Kramer et al. (1999) showed that exposure of SH-SY5Y cells to DAMGO elicited a pronounced translocation of PKC from cytosol to plasma membrane. Studies on other cell lines have also indicated that phosphorylation by PKC is required for desensitization and internalization of μ-opioid receptor (Mestek, et al., 1995; Ueda et al., 2001). The involvement of PKA and PKC in regulating μ-opioid receptor, together with the membrane localization of gravin in SH-SY5Y cells supports the hypothesis that gravin might play a role in regulating μ-opioid receptor function by recruiting both PKA and PKC to the receptor.

Data arising from the colabeling of SH-SY5Y cells for gravin and μ-opioid receptors is also consistent with the idea that gravin may interact with μ-opioid receptors. Immunofluorescence microscopy of cells double labeled with anti-gravin and anti-MOR antibodies revealed partial colocalization of gravin and μ-opioid receptors at the cell surface and in the cytoplasm. Immunofluorescence microscopy of colabeled cells revealed two populations of μ-opioid receptor labeling, one at the cell surface, and the other within the cytoplasm. Several studies have demonstrated that μ-opioid receptors are localized at the cell membrane in different cell lines (Ueda et al., 2001; Sternini et al., 1996; Ruiz-Gomez et al., 1997; Gagnon et al., 1998). However, immunofluorescence microscopy data has not yet been published for SH-SY5Y cells. While the cytoplasmic μ-opioid receptor labeling may represent non-specific antibody labeling, it may also represent receptors undergoing intracellular trafficking.

Gravin also colocalized partially with β_2-adrenergic receptors in retinoic acid treated SH-SY5Y cells. Immunofluorescence microscopy showed anti-β_2-AR antibody

labeling both at the membrane and in the cytoplasm. In addition, anti-β_2-AR labeling partially codistributed with the anti-gravin labeling. SH-SY5Y cells were colabeled for gravin and β_2-adrenergic receptor because recent reports indicating that gravin may interact with β_2-adrenergic receptor (Shih et al., 1999) raised the possibility that gravin expression in these cells may be linked to β_2-adrenergic receptor expression as well. However, β_2-adrenergic receptor had not been reported to be expressed in SH-SY5Y cells. The presence of positive anti-β_2-AR labeling in the cells and the fact that β_2-adrenergic receptor cDNAs were amplified by RT-PCR from SH-SY5Y mRNA in the present study confirmed that β_2-adrenergic receptor is expressed in SH-SY5Y cells. Partial colocalization of the anti-β_2-AR and anti-gravin label is consistent with previous reports of gravin-β_2-AR interactions (Shih et al., 1999) and suggests that any interactions between gravin and GPCRs expressed in SH-SY5Y cells may involve β_2-adrenergic receptor as well as μ-opioid receptor.

The interaction between gravin and μ-opioid receptor or β_2-adrenergic receptor was also investigated in AN3 CA cells transfected with gravin and the receptor constructs. Initial characterization of the transfected cells confirmed that the transgenes were expressed. Not only did the protein specific antibodies react specifically with the transgene, but the gravin, μ-opioid receptor and β_2-adrenergic receptor transgenes expressed in the AN3 CA cells displayed membrane localization, consistent with the distribution pattern in SH-SY5Y cells or other cell lines (Grove and Bruchey, 2001; Ueda et al., 2001; Ruiz-Gomez et al., 1997). In the case of the gravin transgene, both the anti-gravin and the anti-V5 antibody labeling revealed concentration of the transgene at the cell membrane. In addition, western blotting with both the anti-gravin antibody and the

anti-V5 antibody detected bands in transfected AN3 CA cell extracts which were approximately the same molecular weight as the endogenous gravin from SH-SY5Y cells. In case of the μ-opioid receptor and β_2-adrenergic receptor transgenes, immunofluorescence microscopy of transfected cells double labeled with the anti-V5 antibody and the receptor-specific antibodies showed colocalization of the epitope tag and receptor labeling. These validate the use of these fusion proteins in the current study.

Extensive colocalization of gravin and μ-opioid receptor or β_2-adrenergic receptor at the cell membrane was revealed in the cells cotransfected with both gravin and the receptor protein vectors. This colocalization was not due to cross-reactivity of the antibodies, because cells transfected with either one of the vectors were not recognized by the antibody against the other protein. Studies of β_2-adrenergic receptor regulation demonstrated the involvement of gravin in the phosphorylation and internalization of the β_2-adrenergic receptor and suggested that gravin may act as a scaffold which recruits the receptor, protein kinases, β-arrestin, and clathrin (Lin et al., 2000). The finding in the current study that gravin and β_2-adrenergic receptor colocalized is consistent with these observations. It has been also reported that internalization of μ-opioid receptor is β-arrestin-dependent and clathrin-dependent (Xiang et al., 2000), and involves phosphorylation of the receptor by PKC (Ueda et al., 2001). This suggests that gravin may be involved in this event, a hypothesis which is supported by the colocalization of gravin and the μ-opioid receptor.

Although gravin appeared to colocalized with the μ-opioid receptor and β_2-adrenergic receptor transgenes in untreated cotransfected cells, agonist induced receptor internalization was not accompanied by gravin internalization. After agonist treatment,

the μ-opioid receptor and β_2-adrenergic receptor transgenes displayed significant translocation from the cell membrane to the perinuclear location, indicating that the receptors were responsive to the agonist and that the epitope did not affect receptor trafficking. However, the majority of gravin remained on the cell membrane and no detectable colocalization between gravin and the internalized receptors was observed. The rapid internalization of μ-opioid receptor and β_2-adrenergic receptor has been reported in other cell lines (Ueda et al., 2001; Sternini et al., 1996; Ruiz-Gomez et al., 1997; Gagnon et al., 1998). The consistency between the current data and the data from these other studies indicated that the receptors were functioning appropriately in the cotransfected AN3 CA cells. The use of a β_2-adrenergic receptor transgene lacking the epitope tag ruled out the possibility that the epitope tag at the C-terminus of the receptor, where the gravin binding site is located (Fan et al., 2001), might have disrupted protein-protein interaction. Although the possibility still exists that the epitope tag at the C-terminus of gravin may have interfered with gravin binding to the receptor and that use of an untagged gravin transgene might lead to an alternative result, the current data suggests that the internalization of the receptors is not accompanied by gravin redistribution. In addition, the finding that the receptors in the cells transfected with only the receptor protein vectors still underwent significant internalization upon agonist stimulation suggests that gravin might not be crucial for internalization of the receptors.

Although gravin did not colocalize with the μ-opioid receptor or β_2-adrenergic receptor after agonist treatment, the possibility still exists that gravin may be functionally related to the receptors during the internalization procedure. It has been reported that the internalization of the receptor is a rapid event that happens within minutes after the

agonist stimulation (Gagnon et al., 1998). If gravin interacts with the receptors either prior to or immediately after agonist stimulation, but the interaction is not retained during the internalization event, approaches other than immunofluorescent staining would be necessary to investigate gravin-receptor interaction. For example, one approach would be to use fluorescence resonance energy transfer (FRET) microscopy, a technique to study the protein-protein interaction within the living cells (Periasamy A, 2001). Another approach to study gravin-receptor interaction might include immunoprecipitation, which has been used in other studies to successfully identify a gravin-β_2-adrenergic receptor interaction (Shih et al., 1999). A third approach might include two-hybrid system, which has also proved to be a successful way to identify protein-protein interaction in other studies (Li et al., 2001).

On the other hand, the lack of colocalization between gravin and μ-opioid receptor after agonist treatment could indicate that gravin may not interact with μ-opioid receptor. Such a conclusion would be consistent with the view of some investigators that PKA and PKC may not play a primary role in regulating μ-opioid receptor activity. For instance, the initial effect of μ-opioid receptor activation is to decrease cAMP level and decrease PKA activity. Therefore, it is understandable that PKA may not be the initial kinase that phosphorylates the receptor. Consistent with this is the finding by one group of researchers that forskolin did not stimulate δ-opioid receptor phosphorylation in 293 cells (Pei et al., 1995). This group also found that down-regulation of PKC expression failed to affect agonist-induced receptor phosphorylation, indicating PKC did not play a primary role in opioid receptor phosphorylation after agonist treatment (Pei et al., 1995). In addition, although PKC has been shown to translocate from the cytosol to the cell

membrane after DAMGO stimulation in SH-SY5Y cells, this translocation occured 2-6 hours after DAMGO treatment and PKC was postulated to contribute to the down-regulation of the μ-opioid receptor expression (Kramer et al., 1999). Another study on μ-opioid receptor also indicated that the effect of PKC in regulating μ-opioid receptor was a long-term effect, which affected the mRNA level of the μ-opioid receptor, rather than a short-term effect, which affected the process of desensitization or internalization by phosphorylation (Gies et al., 1997). Given that receptor phosphorylation is the initial step after the agonist stimulation and leads to the internalization of the receptor, a lack of PKA and PKC involvement in this event could account for the finding in the current study that gravin was not required for the internalization of the receptor. However, the role of PKA and PKC in regulating opioid receptor desensitization and internalization remains controversial and further studies will be required to resolve the role of these kinases in receptor regulation.

The finding in the current study that gravin did not colocalize with the β_2-adrenergic receptor after the agonist treatment differs from what has been reported by others. Through a series of studies, Malbon and colleagues have reported that gravin is associate with β_2-adrenergic receptor and plays a role in phosphorylation and internalization of β_2-adrenergic receptor after agonist treatment in A431 cells (Shih et al., 1999; Lin et al., 2000). In part, the results in the current study may differ from those reported by others because of differences between A431 cells and transfected AN3 CA cells. It is well known that phosphorylation of β_2-adrenergic receptors requires several different kinase activities, including PKA and PKC activity. It has been reported that there are 2 different isoforms of PKA and 10 different isoforms of PKC. To date, it is not

clear which isoform(s) of PKA or PKC are responsible for β_2-adrenergic receptor phosphorylation in A431 cells. It is possible that different sets of protein kinase isoforms may be expressed in different cell lines and those responsible for β_2-adrenergic receptor phosphorylation in A431 cells may not be expressed in AN3 CA cells to an appropriate level. It is not clear whether the binding between gravin and PKA or PKC is isoform-specific and therefore the lack of specific isoform(s) of PKA or PKC may interfere with the formation of a gravin-mediated β_2-adrenergic receptor signal complex after agonist stimulation. This possibility may be confirmed by identifying the PKA or PKC isoforms expressed in A431 cells and comparing them to those expressed in AN3 CA cells.

Although it has been postulated that gravin may be a component of the internalization mechanism for β_2-adrenergic receptor (Lin et al., 2000), gravin was not required for β_2-adrenergic receptor internalization in the current study. This difference in results may in part be due to diversity in the internalization mechanism among different cell lines. For example, catalytically inactive G-protein coupled receptor kinase 2 dominant-negative mutants blocked m2 muscarinic acetylcholine receptor (m2 mAChR) internalization in COS7 cells, but had no effect on m2 mAChR internalization in BHK-21 and HEK 293 cells (Tsuga et al., 1994; Pals-Rylaarsdam et al., 1995). Experiments testing the effects of β-arrestin and dynamin dominant-negative mutants on the internalization of the β_2-adrenergic receptor indicated that β-arrestins specifically target β_2-adrenergic receptor for endocytosis via clathrin-coated vesicles (Zhang et al., 1996). However, whereas the β_2-adrenergic receptors underwent rapid internalization in HEK 293 cells, they internalized poorly in COS7 cells even after overexpression of β-arrestin (Zhang et al., 1996). Menard et al. (1997) also found that the maximal extent of β_2-

adrenergic receptor internalization in response to agonist activation was markedly lower in COS7 cells than in HEK 293. These observations indicate the internalization of the β_2-adrenergic receptor is regulated by the cellular environment in which the receptor is expressed. Given these observations, it is possible that the internalization mechanisms in A431 cells differ from those in AN3 CA cells so that gravin is required in one situation but not in the other.

Although the current study did not identify the functional interaction of gravin and μ-opioid receptor and β_2-adrenergic receptor, the recombinant protein system used in AN3 CA cells provided a good model to further pursue this hypothesis due to the appropriate recombinant protein distribution, expression, and receptor trafficking observed in the transfected cells. Future studies may include the use of different techniques, or possibly different cell lines to further identify gravin-receptor interaction or other aspects of the receptor functions.

CHAPTER V

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