PRELIMINARY CHARACTERIZATION OF GRAVIN, A KINASE SCAFFOLDING PROTEIN

by

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ABSTRACT

Subcellular localization of kinases is considered to be an important method of regulating their activity by limiting access to effectors and key signal dependent substrates. Classes of proteins responsible for such a regulatory localization have been identified for numerous protein kinases including the type II cAMP dependent protein kinase (PKA). This functional class of proteins are collectively referred to as A Kinase Anchoring Proteins (AKAPs). Using a RII overlay procedure to screen a human fetal brain cDNA expression library, we isolated a new AKAP. The original RII binding clone isolated had 99% sequence identity at the nucleotide level to a previously identified cDNA that was designated gravin (Gordon, T., et al, J. Clin. Invest. (1992), 90:992-999). A full length cDNA of 6606 b.p. was obtainedwith a predicted translation product of 1780 amino acids. Analysis of recombinantly-expressed fragments of gravin allowed us to define the primary sequence of its RII binding domain. A synthetic peptide corresponding to this region was able to block the interaction of RII with gravin in a RII overlay. In a collaborative study with Dr. Theresa Klauck we were able to show that gravin binds and inhibits PKC, extending the role of gravin to that of a scaffolding protein. In collaboration with Lorene Langeberg we were able to isolate a gravin/PKA complex from cells using two methods of co-purification. Existing data suggests that gravin modulates membrane cytoskeleton interactions. Immunocytochemistry results confirm that gravin partially localizes with the membrane cytoskeleton, and that six polybasic regions from the amino terminus of gravin are sufficient to determine gravin's subcellular distribution. These polybasic domains have homologous sequence and biochemical similarities with known F-actin and acidic phospholipid binding domains. *In vitro* binding assays were done to determine if the amino terminal polybasic regions could bind F-actin and acidic

phospholipids and contribute to the co-distribution of gravin with the membrane cytoskeleton. The polybasic regions were found to associate with phosphatidylinositol-4,5-bisphosphate. The implications of PIP2 binding and the relationship to gravin's distribution and role as a scaffolding protein in the cell are discussed.

CHAPTER ONE

Introduction

INTRODUCTION TO SIGNAL TRANSDUCTION AND PROTEIN KINASES

In response to changes in the external environment, cells utilize intracellular signaling pathways to initiate the appropriate biochemical and genetic responses (1). The translation and transduction of extracellular information by intracellular signaling pathways is thus essential for maintaining the ordered functioning of the cell. Protein phosphorylation by protein kinases has emerged as an important intracellular mechanism in the transduction of external information into the appropriate biological responses (2).

The number of known protein kinases has escalated into the hundreds. In the budding yeast alone, 120 unique protein kinases have been identified (3). Partial sequencing of the *C. elegans* genome suggests it to have ~270 unique protein kinases (4). Over one hundred mammalian protein kinases have already been identified and it is hypothesized that this total will reach 1000 (3,4). Sequence homology and structural data of known kinases have shown that they all share a conserved catalytic core and comprise a family of related enzymes (5,6). The protein kinase family can be subdivided into two classes, the protein-serine/threonine kinases and the protein-tyrosine kinases (6). Each of these subgroups can be furthur fractionated based on diverse regulatory mechanisms, substrate specificity and activating second messengers (6).

A common mechanism of enhancing the specificity of kinase signaling is their localization to disrete signaling units (7). Tyrosine kinases utilize combinations of src-homology 2 (SH2) and src-homology (SH3) modules to organize specific signaling units in a cell (for a comprehensive review of this subject see (8). On the other hand the serine/threonine protein kinases utilize scaffolding/anchoring molecules to localize and coordinate serial and parallel protein kinase pathways. The serial activation of the MAP kinase pathway in the

yeast mating response is coordinated by Ste5 (9). Ste5 binds Ste11 (a MEKK), Ste7 (a MEK), and FUS3 (a MAPK) thereby coordinating the sequential activation of each member of this pathway and reducing crosstalk between differentially activated MAPK pathways (9). A similar scaffolding protein, JIP-1, has been recently identified for the mammalian MAPK pathway involving JNK (10). Alternatively, the coordination of parallel signaling pathways is represented best by the A-kinase anchoring protein, AKAP79, which binds two distinct broad specificity protein kinases (11,12). AKAP79 originally was identified as an AKAP that tethered the cAMP dependent protein kinase (PKA) to the post-synaptic density (11). AKAP79 was later shown to be a scaffolding protein by binding the calcium/phospholipid dependent protein kinase (PKC) in additon to PKA, thereby coordinating calcium and cAMP second messenger pathways (11,12). The main focus of this thesis is the identification and characterization of an AKAP gravin that, like AKAP79, is a scaffoling protein that binds and localizes PKC and PKA.

cAMP

Rall and Sutherland reported the formation of mononucleotide adenosine-3',5'-phosphoric acid (cAMP) following stimulation of liver, heart, skeletal muscle, and brain particulate tissue extracts incubated with adenosine triphosphate and magnesium ions (13,14). They went on to show that the enzyme responsible for the production of cAMP, adenylyl cyclase (AC), is present in a wide variety of tissue types and throughout many phyla (1). It soon became apparent that the observed stimulation of cAMP production was due to hormonal stimulation of AC in tissue extracts, leading to the idea that cAMP acts as a second messenger, hormones being the first messenger (1). The first AC was cloned in 1989, presently nine mammalian AC isoforms are known (15,16,17). All AC share a 12 transmembrane structure with two intracellular loops that form the catalytic site (15,18,19). AC isoforms exhibit tissue and cell specific expression patterns and differ in their catalytic

response to the regulatory cues and tissue distributions (17). AC isoforms have been identified that are regulated by G-protein subunits, calcium/calmodulin, and phosphorylation (Fig. 1) (17). The cAMP signal is regulated by the presence of an enzyme that degrades cAMP to 5'-AMP. These enzymes are the cyclic nucleotide phosphodiesterases (PDE) and were first purified in 1962 by Butcher and Sutherland (20,21) Seven families of PDEs exist, most families are composed of multiple genes; some of these genes have multiple splice forms (21,22). Like AC, different forms of PDEs have different tissue and cell expression patterns and are subject to a variety of regulatory mechanisms (21,22). Thus, the resultant amplitude and duration of the cAMP signal is controlled by the opposing effects of AC and PDE (1,19). Tissue and cell specific expression patterns of these two enzymes coupled with the differential regulation of AC and PDE isoenzymes enhances specificity of hormonal signaling and downstream biological responses mediated through PKA (1,19).

PKA

PKA was one of the earliest protein kinases purified and over the last 30 years has become one of the most well understood protein kinases (2,23,24). The PKA holoenzyme exists as a tetramer, consisting of two regulatory subunits bound as a dimer and one catalytic subunit bound per regulatory subunit (2,24). Three isoforms (α, β, and γ) of the catalytic subunit of PKA and four isoforms (RI-α, RI-β, RII-α, and RII-β) of the catalytic subunit have been identified (24,25). However, no heterodimerization of regulatory subunits, nor specificity for the binding of catalytic subunit isoforms by regulatory isoforms has been observed. Thus the diversity of holoenzymes formed may only be limited by the requirement for co-expression of the catalytic and regulatory subunits in the same cell. The catalytic subunit binds to an autoinhibitory site on the regulatory subunit, keeping the catalytic subunit in an inactive state (24,25). The cooperative binding of four cAMP

molecules to the regulatory subunits releases active catalytic subunit, which is then free to phosphorylate substrates (2,24,25). PKA is a broad substrate specificity serine/threonine protein kinase in that it phosphorylates a wide variety of substrates (26).

PKA CATALYTIC SUBUNIT

The structure of the catalytic subunit of PKA (C-subunit) was the first eucaryotic protein kinase to be solved by X-ray crystallography (27,28). The high degree of conservation of the catalytic core in the serine/threonine protein kinase family has allowed the PKA catalytic subunit structure to serve as a model for other protein kinases (5). Since then, several other protein kinase structures have been determined (5). These studies have confirmed the conserved nature of the structure and function of protein kinases. The catalytic core of PKA is a bi-lobal structure (5). Simply put, the smaller upper lobe serves to bind ATP while the larger lobe contains the catalytic base and substrate binding pocket (5). Substrate and peptide studies have highlighted Arg-Arg-Xaa-Ser as a minimum consensus sequence of PKA (2).

PKA REGULATORY SUBUNITS

Studies on the fractionation characteristics of the PKA holoenzyme from a variety of tissues on a DEAE-cellulose column indicated that there were two distinct isoforms of PKA. The difference between these two isoforms is due to two forms of regulatory subunit, RI and RII (29,30,31). The regulatory subunits are the major cAMP binding proteins in mammalian cells and are responsible for the cAMP dependency of the catalytic subunit (2). RI and RII subunits have some distinguishing biochemical characteristics. The relative mobility of RI (49 kD) and RII (51 kD) on SDS-PAGE is different. RII can be autophosphorylated by an intramolecular reaction catalyzed by the catalytic subunit,

whereas RI cannot be autophosphorylated (30,31). RI contains a high affinity magnesium binding site, whereas RII does not (31). Two isoforms, an α and β form, of each type (RI and RII) of regulatory subunit have been cloned (32,33,34,35). These studies have served to highlight similarities in the domain structure of the two forms of regulatory subunit (24). The amino terminus of all four regulatory isoforms contains the determinants responsible for homo-dimerization of the regulatory subunits (36,37). The amino terminal half also contains the "hinge" region. The hinge region contains a sequence that resembles a PKA phosphorylation site (24). This site binds and inhibits the catalytic subunit as an autoinhibitor (24). The intramolecular autophosphorylation of the RII subunits mentioned above occurs in this autoinhibitory site. RI is not phosphorylated here because it has an alanine substitution where RII isoforms have a serine (24). The carboxy terminal half of the regulatory subunits contain two cAMP binding sites (25). The cooperative binding of two cAMP molecules to each regulatory subunit (four cAMP per holoenzyme) induces the release of catalytically active C-subunit (25).

Early studies indicated that RI isoforms are cytosolic, while RII isoforms, based on biochemical fractionation and immunocytochemistry, exhibited variable particulate:soluble ratios and discrete sub-cellular distributions in cell fractionation experiments (38,39,40,41). Type II PKA was found to biochemically associate with microtubule-associated protein 2 and bind with high affinity to a CaM binding protein (p75) (42,43,44,45). These observations, coupled with the demonstration of RII dimerization determining the localization of PKA holoenzyme, suggested that type II PKA is localized to discrete regions of the cell through the interaction of the amino terminus of the regulatory subunit binding to targeting proteins, such as MAP2 and p75 (43,46,47). This class of proteins that bind to the amino terminus of RII and localize type II PKA have become known as *A-k*inase *a*nchoring *p*roteins (AKAPs) (48,49).

AKAPS

AKAPs are a class of proteins that bind with high affinity to RII and localize the PKA holoenzyme to a specific subcellular location promoting the preferential phosphorylation of a specific subset of PKA substrates (Fig. 2) (43,48,49,50). Since PKA has a broad substrate specificity the localization of PKA by AKAPs is critical in enhancing the specificity of a PKA signaling pathway. Immunocytological investigations and biochemical analyses have shown that type II PKA is targeted to a variety of subcellular compartments (38,40,42,43,46,51,52). Many of the AKAPs responsible for this subcellular compartmentalization of type II PKA have been cloned and their interaction with RII characterized (Fig. 3). All AKAPs share two functionally related domains. They all possess an amphipathic helix moiety that binds to RII through its hydrophobic face and a targeting domain that determines the subcellular localization of the AKAP (Fig. 2) (49,53). What follows is a brief synopsis of known AKAPs followed by observed functional consequences of PKA anchoring in cells. However, gravin will not be reviewed in this section but will have its own section following the discussion of functional consequences of PKA anchoring.

MAP2

MAP2, the first AKAP identified, was initially identified when type II PKA was found to co-fractionate with microtubules and that the interaction of PKAII was through the interaction of RII with MAP2 (42). These observations were confirmed by Lohmann and colleagues by showing the co-purification of RII and MAP2 on cAMP-sepharose and by the use of an RII overlay technique (a modified form of a western blot) (43). The further development of the RII-overlay technique and its application to expression cloning of cDNAs has been of the utmost importance in the development of the AKAP field (43,54).

AKAP75/79/150

AKAP75/79/150 was the next AKAP identified and represents bovine, human, and rat homologs of the same AKAP (11). AKAP75 was identified as a calcium/calmodulin (CaM) binding protein of brain and heart that co-purified with RII on a cAMP-sepharose column (44). This led to the molecular cloning of all three homologs (11,45,55). The phosphatase calcineurin (PP-2B) and the calcium/phospholipid protein kinase (PKC) have also been shown to bind to AKAP79, suggesting that AKAP79 is a scaffolding protein that localizes both components of a reversible signal transduction mechanism (12,56). AKAP79 has become the most well characterized AKAP and has served as a model for studies on other AKAPS. Recently, the binding of acidic phospholipids, phosphatidylinositol-4,5-bisphosphate in particular, by three amino terminal basic domains has been shown to target AKAP79 to cell membranes (57).

Ht31

This AKAP is a partial clone from a human thyroid library (49,51,53). Work on this molecule identified the minimal region of an AKAP requisite for RII binding (51). A 23-mer peptide of Ht31 proposed to have an amphipathic helical structure binds to RII and PKA holoenzyme with high affinity (4 and 3.8 nM respectively) and is able to block RII binding to AKAPs in the overlay assay (51). A peptide with a proline mutation, to disrupt the helical structure of the peptide, is unable to block RII-AKAP interactions (53,58,59,60,61,62,63,64). This peptide and the instrumental role it played in early efforts to understand the role of anchored PKA in modulating cell physiology will be discussed later (58,59,60,61,62,63,64).

AKAP95/ mAKAP100

AKAP95 is a nuclear targeted AKAP that contains a DNA binding zinc finger motif in addition to a RII binding motif (65). RII is not found in the nucleus, however, it has been

postulated that AKAP95 interacts with RII during mitosis (66). mAKAP is a muscle specific AKAP that targets PKA to endoplasmic- or sarcoplasmic-reticulum structures (67).

AKAP82

This protein, initially identified as the major fibrous sheath (FS) protein, has been shown to be an AKAP (68). AKAP82 is synthesized as a precursor form and is found as a Triton X-100 soluble protein in the cell body of immature spermatid (69). Following spermiogenesis, AKAP82 is transported to the fibrous sheath of the principal piece of the sperm tail where it is processed and becomes insoluble to Triton X-100 (69).

S-AKAP84/D-AKAP-1

S-AKAP84 tethers RII to the outer mitochondrial membrane of mitochondrion that form the mitochondrial sheath of the sperm midpiece (52,70). Additionally, the expression of S-AKAP84 is developmentally restricted to the nuclear condensed developmental stage of sperm maturation (70). This coincides with the accumulation of type II PKA to the sperm midpiece (70). A region of S-AKAP84 with homology to the mitochondrial targeting domain of NADH-cytochrome b_5 reductase was shown to be sufficient to target S-AKAP84 to the mitochondrial outer membrane (52,70). Several splice variants of this gene have been isolated, which have conserved the mitochondrial targeting and RII binding domains (52).

AKAP220

AKAP220 mRNA is found in heart, brain, lung, kidney, and testis, although it is most highly expressed in testis (71). A carboxy terminal peroxisomal targeting signal and immunocytochemical analyses show this protein to target type II PKA to peroxisomes in testis (71). In addition to binding RII, AKAP220 has recently been shown to bind phosphatase PP-1A (R. Schillace and J. D. Scott unpublished data).

DAKAP550

DAKAP550 is a *D. melanogaster* protein (72). This is not only the first non-mammalian AKAP identified, but is also the first non-vertebrate AKAP identified (72). DAKAP550 binds both mammalian and *Drosophilia* RII isoforms. The binding site is unique among AKAPs in that there are two binding sites (B1 and B2) that are non-contiguous, with B1 having a 20 fold higher affinity than B2 (72). DAKAP550 shows a differential expression pattern during fly development, with highest levels of protein expression in anterior regions (e.g. head), and is expressed throughout development (72).

EZRIN

Ezrin has recently been characterized as a major AKAP in gastric parietal cells (73). Ezrin is a membrane cytoskeleton linker protein that binds F-actin and acidic phospholipids, and is a member of the band 4.1 superfamily (74). It is most homologous with radixin and moesin within this superfamily, and shows more limited homology with the tumor suppressor NF-2 or merlin (74). Radixin and moesin also bind RII in an overlay, however, merlin was not assayed (73). Ezrin is localized to the secretory canaliculus of gastric parietal cells (73). RII is predominantly cytosolic in parietal cells, but translocates to the secretory canaliculus upon gastrin stimulation, suggesting that anchoring of type II PKA by ezrin modulates secretion in parietal cells (73).

AKAP120

AKAP120 was cloned from a gastric parietal cell line cDNA library (75). AKAP220 mRNA is detected in a variety of tissues (brain, pancreas, intestines), but is concentrated in fundic and jejunal mucosa (75).

AKAP KL

AKAP KL was identified by yeast two hybrid and has three splice variants and two possible start sites for six possible isoforms that range in size from 105-133 kDa (76). It is most abundantly expressed in kidney cells where it is found at the apical surface of polarized cells (76).

AKAP15/18

Anchored PKA was shown to be important for the cAMP mediated, voltage dependent modulation of the skeletal muscle L-type calcium channel (59,77,78). An attempt to identify the AKAP responsible identified a 15 kD AKAP that associated with and anchored PKA to the skeletal muscle L-type calcium channel (77). This lab concurrently cloned an 18 kD AKAP that was targeted to cell membranes through three amino terminal acylation modifications (79). AKAP18 is able to promote a cAMP mediated, voltage dependent modulation of the cardiac L-type skeletal channel (79). AKAP18 is also able to augment glucagon like peptide (GLP-1) mediated insulin secretion in a pancreatic beta cell line (79). These data suggest that PKA localized by AKAP18 may be involved in the modulation of various membrane events (79). The 15 kD AKAP associated with the L-type calcium channel was subsequently cloned and found to be identical to AKAP18 (80).

AKAP85 and AKAP350

These AKAPs have been partially purified and their association with a subcellular structure defined, but the molecular clone remains to be isolated. The Golgi complex and the centrosome were identified as areas of intense staining in an immunocytochemical investigation of RII distribution in cells (40). This data was confirmed by subcellular fractionation (40). An 85 kD RII binding protein was found in a Golgi enriched fraction, and its association with RII was confirmed by co-purification on a cAMP agarose column and by co-immunoprecipitation (81). Purification of centrosomes from cells has identified,

by RII overlay, the RII binding component to be a 350 kD protein previously identified as a component of the pericentriolar material recognized by a centosomal specific antibody (82).

FUNCTIONAL CONSEQUENCES OF PKA ANCHORING

Initial studies of AKAPs have focused on their identification, biochemical characterization, sub-cellular distribution, and cell type expression. Nevertheless, the effect of PKA anchoring in cells has been difficult to assess and is currently an area of importance to the advancement of the field. Possibly the most important aspect of the work done so far is the identification of the amphipathic helical nature of the RII binding site of AKAPs (51,53). A peptide synthesized to the RII binding site of Ht31 blocks all RII-AKAP interactions (51). Treatment of cells with this peptide serves to disrupt the anchoring of RII and thus inhibit the modulatory affects of phosphorylation by anchored PKA in the cell (58). A control peptide with a proline mutation to disrupt helical structure is unable to block RII-AKAP interactions and has no modulatory affect on anchored PKA dependent phosphorylations (53,58). These peptides have proved instrumental in early studies aimed at elucidating the role of anchored PKA in cells.

Phosphorylation of AMPA/kainate channels by PKA prevents the rundown of channel current and causes a decrease in spontaneous excitatory postsynaptic currents in hippocampal neurons (83). Perfusion of PKI, a potent peptide inhibitor of PKA, or the anchoring inhibitor peptide, HT31, into primary hippocampal neurons enhanced channel rundown and decreased the amplitude of spontaneous excitatory postsynaptic currents (58). Ht31-proline, a control anchoring inhibitor peptide, had no effect on PKA maintenance of AMPA/kainate channel function (58). This study was the first to demonstrate the relevance of anchored PKA in a physiological setting, and in particular the role of anchored PKA in the regulation of AMPA/kainate channel physiology in hippocampal neurons (58).

Analysis of calcium activated potassium channels in inside-out patches demonstrated a PKA phosphorylation dependent increase in the open probability of the channel that could be blocked by PKI (60). The PKA dependent shift in open probability could also be blocked by incubating the patch with the Ht31 anchoring inhibitor peptide, while the control Ht31-proline peptide had no effect (60). This study highlights a role for anchored PKA in regulating the open probability of calcium activated potassium channels (60).

Endogenous skeletal muscle L-type calcium channels exhibit a time- and voltage-dependent potentiation of channel current and a reduced decay time that requires phosphorylation of the channel by PKA (84). A heterologous expression system was developed to analyze the contribution of anchored PKA to the modulation of skeletal muscle L-type calcium channels (78). Heterologously expressed L-type calcium channels exhibit a similar time- and voltage-dependent potentiation of channel current and decay time that requires PKA phosphorylation (78). Furthermore, application of the Ht31, but not Ht31-proline, inhibited with a similar potency to PKI the time- and voltage-dependent potentiation of skeletal muscle L-type calcium channel currents (78). This culminated in the identification of a 15 kD AKAP that co-purified with the L-type calcium channel that was eventually cloned and found to be identical to AKAP18 (77,79,80).

Glucagon like peptide (GLP-1) potentiates glucose induced insulin secretion in pancreatic beta cells that requires PKA phosphorylation of undetermined substrates (64). The Ht31 peptide was again used to assess the role of anchored PKA in this process. Primary pancreatic beta cells or beta cell lines were transfected with either the Ht31 or Ht31-proline peptides and the affect of GLP-1 treatment on glucose stimulated insulin secretion assayed (64). Ht31 was able to block GLP-1 increases in glucose stimulated insulin secretion, while Ht31-proline had no affect (64). The authors went on to show a decrease in calcium

influx in the Ht31 cells but not the Ht31 proline cells (64). This suggested that one possible site of action for PKA phosphorylation was the L-type calcium channel (64). Application of BAYK8644, an L-type calcium channel agonist, partially prevented Ht31 inhibiton of GLP-1 potentiation of glucose stimulated insulin secretion (64). In a follow up study, heterologous expression of AKAP18 potentiated GLP-1 stimulation of glucose induced insulin secretion (79). These data correlate well with previous studies showing that disruption of PKA anchoring affected calcium influx, that AKAP18 is associated with the L-type calcium channel, and that phosphorylation of the channel by anchored PKA potentiates channel current and prolongs decay time (64,77,79).

While the studies mentioned above assess physiological roles of anchored PKA by globally disrupting RII-AKAP interactions, another approach is to add a specific AKAP into a heterologous system that will target PKA to a desired subcellular location. The cardiac isoform of the L-type calcium channel is subject to regulatory phosphorylation by PKA (61). However, studies aimed at the detailed analysis of PKA regulation of the cardiac L-type calcium channel through use of heterologous expression have been unsuccessful, presumably due to a missing component(s) in the heterologous system (61). Gao *et. al* have shown, by transfecting AKAP79, a membrane bound AKAP, into their reconstituted heterologous system, that membrane targeting of PKA is necessary for the appropriate modulation of cardiac L-type calcium channels (61). This result was shown to be due to the localization of PKA, as AKAP79 with a point mutation in the RII binding domain had no effect (61). Additionally, the proper targeting of the AKAP is required for the PKA mediated modulation of the cardiac L-type calcium channel (79). Heterologous expression of AKAP18, another membrane associated AKAP, in the same system as Gao *et. al* also promotes PKA mediated modulation of the cardiac L-type calcium channel (79). However, a mutant AKAP18 that is untargeted and acts like the Ht31 peptide, disrupts cellular targeting of PKA with an anticipated loss of PKA mediated potentiation of cardiac L-type

calcium channels (79). Since two membrane targeted AKAPs were able to mediate similar changes in cardiac L-type calcium channels its brings up the question of specificity of the anchoring in this system. However, it should be noted that these experiments were done by heterologously overexpressing the AKAPs. Thus, it may be that if high enough levels of a membrane targeted AKAP are expressed so that an AKAP that may not be functional in a physiological setting may suffice to redistribute PKA to a location that it may phosphorylate the cardiac L-type calcium channel. Additionally, AKAP18, was initially identified as an AKAP that purified with the skeletal muscle L-type calcium channel suggesting that it may be the endogenous AKAP responsible for the targeting of PKA to the cardiac isoform in endogenous systems.

GRAVIN

Using an RII to screen an expression library we identified gravin, a protein that was originally identified as an autoantigen in myasthenia gravis, as an AKAP. Myasthenia gravis is an autoimmune disease that affects the neuromuscular junction with the nicotinic acetylcholine receptor being the primary autoantigen (85). Autoantibodies in myasthenia gravis also are produced against many other proteins including actin, α-actinin, myosin, filamin, vinculin, and tropomyosin, and this immune response is secondary to the primary immune response (86,87). Screening of a cDNA expression library for additional autoantigens with anti-sera from patients with myasthenia gravis led to the identification of gravin (88). Though gravin autoantigens are specific to myasthenia gravis, there is no correlation between autoantibody levels and severity of pathology, suggesting that the antigravin response arose as a secondary response due to determinant spreading (88,89).

Gravin expression is restricted to adhesive cells (88). For example, human erythroleukemia (HEL) cells normally grow in suspension and do not express gravin, but

treatment with phorbol ester causes the cells to become adhesive and gravin expression to be upregulated (88). An immunohistochemical tissue survey found that gravin is expressed in fibroblasts, neurons, and neural crest derived cells, but suprisingly was not expressed in skeletal muscle (90). Fibroblasts, neurons, and neural crest derived cells all participate in adherent, migratory, or pathfinding behavior and/or are derived developmentally from cells with these characteristics (90). Additionally, gravin expression is higher in non-confluent than confluent cells. Subcellularly, gravin is localized, by immunocytochemistry, to the cortical cytoskeleton (88). Taken together, these observations suggest that gravin may be a component of the cortical cytoskeleton where it may play a role in regulating cell motility and adhesion.

The membrane cytoskeleton consists of the plasma membrane and the underlying cortical cytoskeleton made up of F-actin microfilaments (91). This actin based structure and its interaction with the phospholipid membrane provides organization and mechanical integrity to the cell. Yet, the membrane cytoskeleton must also remain plastic enough to be reorganized in order to accommodate the dynamic processes of endocytosis, exocytosis, motility, cell growth and differentiation (92,93,94,95,96). This reorganization is accomplished through the ordered polymerization/depolymerization of actin filaments coupled with the regulated crosslinking of F-actin to itself and other cytoskeletal structures (e.g. microtubules and intermediate filaments) into higher order structures (96,97). Phosphorylated phosphoinositides, such as PIP2, as well as calcium have emerged as potent effectors for reorganiztion of the membrane cytoskeleton (98,99). For reasons to be discussed in chapter 4, I will be concentrating on the role of PIP2 in actin cytoskeletal reorganization. Thus, in order to better understand possible roles for gravin in the regulation of the membrane cytoskeleton, I will give a brief description of the regulation of the actin cytoskeleton and the contribution of PIP2 binding proteins to this regulation.

ACTIN BINDING PROTEINS

Actin is found as a monomeric form (G-actin), and a filamentous form (F-actin) in the cell. The ratio of G-actin to F-actin in a cell is a highly regulated process that is maintained by the activity of G- and F-actin binding proteins (97). Genetic and biochemical analysis of G- and F-actin binding proteins has elevated our awareness of the dynamic nature of the membrane cytoskeleton and its importance to many physiological processes (92,97,100). Capping and severing proteins, such as gelsolin and severin, bind to the barbed end (fast growing end of an actin filament) and prevent addition of new monomers, resulting in a decrease in the amount of F-actin and an increase in the pools of G-actin (92,95,97). This may lead to a rapid addition of monomers to actin filaments that are uncappped, causing their rapid growth (92,95,97). Cofilin/ADF (actin dissociating factor) also regulate the G- to F-actin ratio of a cell by promoting the dissociation of actin monomers from the pointed ends of actin filaments (92,95,97). A 25-fold enhancement of F-actin depolymerization at the pointed end of actin filaments by ADF/cofilin causes a treadmilling effect of F-actin (92,97). Profilin represents another class of proteins that affect G- to F-actin ratios by sequestering actin monomers (92,95). Like gelsolin, profilin may also contribute to the growth of specific groups of actin filaments by forming concentrated pools of sequestered actin monomers (92,95). However, it should be noted that the above descriptions are simplified and the proteins described above often have additional effects on actin that are less understood and often contrary to their more well known functions described above. The best example of this multi-functionality of an actin binding protein is villin (101,102). Villin contains an amino terminal core that is conserved with proteins of the gelsolin family (severin, fragmin) (101,102,103,104). Villin also contains a unique carboxy terminal fragment called the headpiece (101,102,103,104). The core of villin, like gelsolin, binds G-actin, and severs and caps F-actin in the presence of micromolar calcium concentrations (101,102,103,104). However, at sub-micromolar calcium concentrations the activity of

villin shifts to the bundling of F-actin and this activity is attributed to the headpiece (101,102,103,104).

Actin bundling is a general term used to describe three related activities of F-actin binding proteins. It refers to proteins that can: 1) crosslink actin filaments into networks, 2) bundle proteins into linear arrays, and 3) gelating proteins that alter the property of F-actin solutions to take on the properties of a gel (105,106). These activities are not mutually exclusive and the actin bundling properties of any one protein is determined by its composite structure (95,106,107). This includes the number of actin binding sites per molecule, the oligomeric potential of the actin binding protein, and the spacing of actin binding sites in three dimensional space (95,106,107).

For instance, the MARCKS protein only possesses a single actin binding site and its bundling activity is due to the formation of dimers (108). Talin, on the other hand, contains three actin binding sites and does not need to oligomerize to form higher order F-actin structures (109). The molecular distance between actin binding domains must also be considered as the spacing of the actin binding domains of actinin were shown to effect the nature of the actin bundle formed (110). In addition to the structural organization of actin binding domains their composite activity as a holoenzyme must also be considered. The 34 kD protein of *Dictyostelium* crosslinks and bundles F-actin resulting in the gelation of F-actin (111). Limited proteolysis of this protein releases a 27 kD polypeptide that retains F-actin crosslinking and gelation activities but no longer bundles actin into linear arrays (111). These intrinsic factors that coordinate bundling activity are further regulated by PIP2, calcium, phosphorylation, and pH (101,112,113,114).

PHOSPHORYLATED PHOSPHOINOSITIDES AND THEIR BINDING PROTEINS

The cellular function of phosphorylated phosphoinositides are of interest to researchers for three reasons. PIP2 is the source of PKC activators diacylglycerol and IP3; phosphorylated phosphoinositides have been shown to affect cytoskeletal architecture; and phosphorylated phosphoinositides influence cellular trafficking through the Golgi and associated structures (99). I am going to focus on the effects of PIP2 on the actin cytoskeleton. However these affects are not mutually exclusive with intracellular effects, a topic to which I will return in the final discussion. Early studies on the plasticity of the actin cytoskeleton also highlighted PIP2 as an important mediator of changes to the actin cytoskeleton though unknown mechanisms (98). The past several years have seen a great advancement in the understanding of the role of PIP2 in regulating the plasticity of the actin cytoskeleton (98,115). Phosphoinositides have been shown to regulate both the G-actin/F-actin ratio in a cell and the crosslinking of F-actin to cytoskeletal elements and membrane proteins (115). PIP2 effects these changes to a cell's actin network through direct interactions with a variety of cellular proteins and thus altering either their enzymatic activity or their localization.

Several phosphoinositide binding domains that link proteins to the membrane have been identified. The pleckstrin homology (PH) domain binds with high affinity and specificity to phosphorylated phosphoinositides (116,117,118). Four groups of PH domains can be identified based on their relative affinity (sub-micromolar versus micromolar) for phosphorylated phosphoinositides and their preference for particular phosphorylated species of phosphatidylinositol (119). The specificity of PH domains was perhaps best demonstrated by the analysis of the binding of PKB to phospholipid vesicles through a PH domain (120). The PH domain of PKB exhibited fatty acid preferences (length and saturation) and stereochemical preferences for the inositol head group (120). Crystallographic determination of the structure of the phospho-tyrosine binding (PTB)

domains of Shc and IRS-1 has shown that they adopt a beta sandwich fold that is similar to the PH domain suggesting that these domains may bind acidic phospholipids in addition to phospho-tyrosine residues (121,122,123,124). In fact the PTB domain of Shc was shown to bind to acidic phospholipids (123). Whether this is common feature of all PTB domains is not yet known.

Another well characterized phospholipid binding domain is the CaLB/C2 domain found on a variety of proteins such as synapsin, PKC, and p120-GAP (125,126,127). These domains have calcium dependent, acidic phospholipid binding activity (125,126,127). PH, PTB, and CaLB/C2 domains all have a tertiary structure that contributes to the specificity of the interaction of the protein with the phospholipid.

Another group of membrane binding regions appear to bind non-specifically to acidic phospholipids through electrostatic interactions. This group is typified by AKAP79, and MARCKS (57,108) . Both of these molecules contain polybasic regions that bind to acidic phospholipids and target the respective proteins to membranes (57,108).

The binding of PIP2 by PH, CaLB/C2, and AKAP79/MARCKS like phospholipid binding domains affect the localization and activity of the proteins containing them. For instance, the PH domains of the neuronal Wiscott-Aldrich Syndrome Protein (N-WASP) targets N-WASP to the membrane where N-WASP functions to regulate growth factor dependent F-actin dynamics through a cofilin like domain (128). The binding of 3'-phosphorylated phosphoinositides by the PH domain of the phosphoinositide-dependent protein kinase-1 (PDK1) results in its activation (120,129).

Additionally, a variety of actin modulating proteins contain PIP2 binding domains. This has been observed for proteins that modulate the G-actin/F-actin ratio and for proteins that

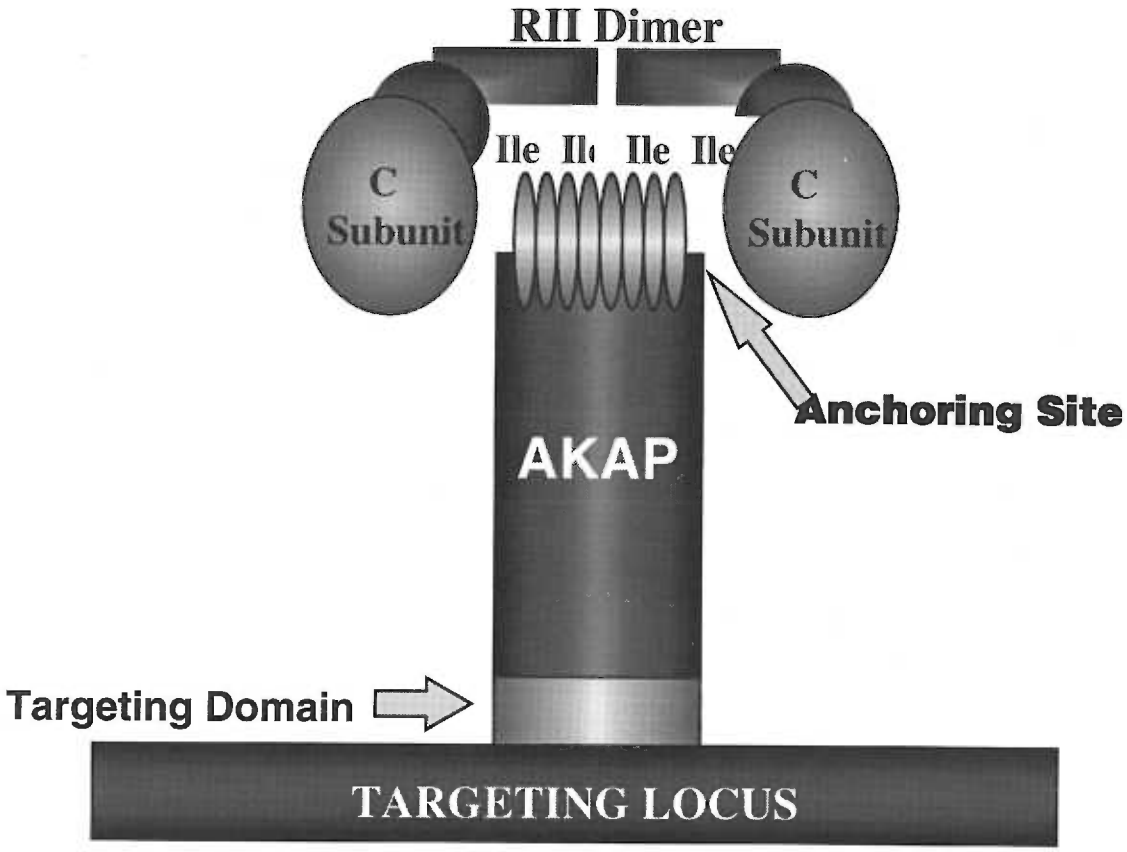
regulate the higher order structure of actin networks. For instance, the F-actin severing activity of gelsolin is increased in the presence of PIP2, and the actin bundling activity of filamin is inhibited in the presence of PIP2, whereas PIP2 promotes the binding of talin to F-actin (98,99).

CHAPTER ONE FIGURE LEGENDS

CHAPTER 1, FIGURE. 1 Activation OF PKA. Schematic showing the activation of adenylyl cyclase (AC) by G-protein coupled receptors, calcium/calmodulin, and phosphorylation by PKC. Activated AC produces cAMP from ATP. Four cAMP molecules bind cooperatively to the PKA regulatory subunits (2 cAMP/regulatory subunit) promoting the dissociation of active catalytic subunits.

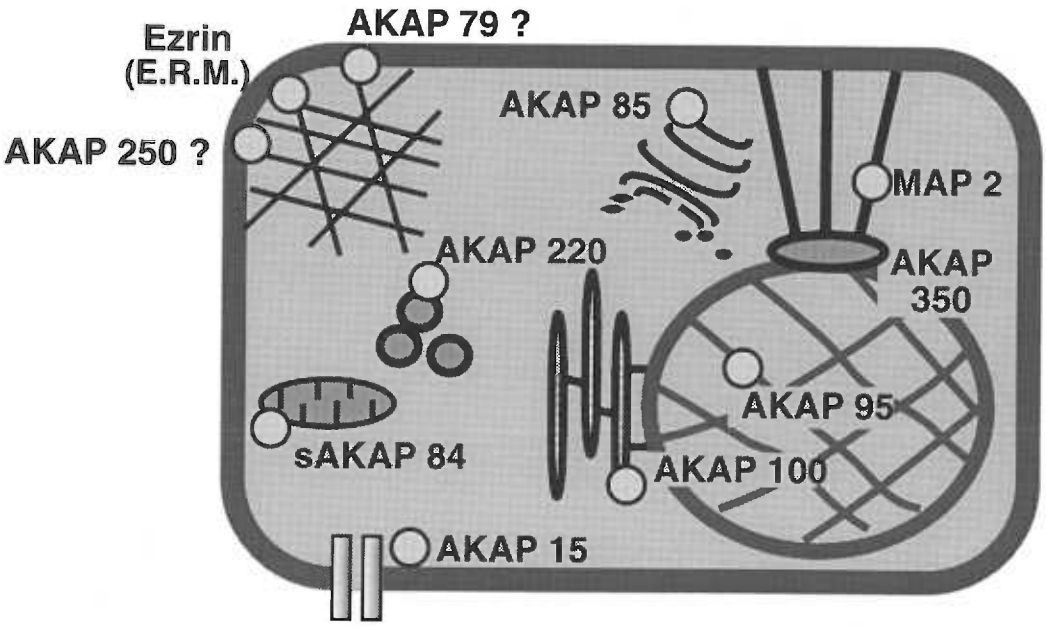
CHAPTER 1, FIGURE. 2 Targeting OF PKA through AKAPs. Schematic showing the binding of type II PKA to an AKAP. The RII dimer binds to an amphipathic helical structure on the AKAP. In turn the AKAP is targeted to a discrete subcellular region through a targeting domain.

CHAPTER 1, FIGURE. 3 Subcellular Targeting Of A Variety Of AKAPs. A selection of AKAPs and their subcellular targets are represented. Refer to text for more detailed descriptions of AKAPs and their targets.



CHAPTER 1, FIGURE 2

Subcellular distribution of AKAPs



CHAPTER 1, FIGURE 3

CHAPTER TWO

Cloning and characterization of the RII binding site of AKAP220

Lester, L. B., Coghlan, V. M., Nauert, B., Scott, J. D. (1996) J. Biol. Chem, 272:9460-9465.

INTRODUCTION

Cellular responses to many hormones are mediated by intracellular signals which ultimately change the phosphorylation state of key proteins (130). The transfer of phosphate groups to and from proteins is the function of specific kinases and phosphatases, and therefore, it is the activity and location of these enzymes which ultimately determines the response to a hormonal signal (131). PKA, cyclic AMP dependent kinase, responds to activation of Gs and the subsequent increases in intracellular cAMP by changing the phosphorylation state of specific proteins (130). It is critical that only certain proteins are activated by a particular hormone, therefore, PKA and the intracellular messenger cAMP are localized in specific cellular compartments (47). Activation of specific pools of compartmentalized PKA would increase the selectivity and intensity of a hormonal response. To facilitate this, the distribution and location of PKA must be regulated (49).

The cellular location of type II PKA is dictated by the regulatory (RII) subunit (132). A significant proportion of RII is found in the particulate fraction (29,34,38,41). RII is associated with the plasma membrane, cytoskeleton, endoplasmic reticulum, secretory granules and nucleus (40,42,44,65,133,134,135). The sub-cellular location of RII is maintained through a high affinity interaction with a family of anchoring proteins called, A-kinase anchoring protein (AKAPs) (136). Previous reports have characterized the interaction of PKA with AKAPs, the most generalized being the need for RII dimerization prior to the interaction (132). The interaction between PKA and AKAPs occurs at the first 5 amino acids of RII and appears to require a conserved amphipathic helix in the AKAPs (53). Synthetic peptides patterned after the amphipathic helix region of the AKAPs can bind RII with nanomolar affinity and block the interaction of RII with other AKAPs. These anchoring inhibitory peptides have been used to disrupt the RII-AKAP interaction in several tissues and uncouple the regulatory effect of PKA phosphorylation. Previously

studied AKAPs have localized RII to specific subcellular areas in various cell types (49). Therefore, each AKAP appears to contain a unique binding motif that allows for the compartmentalization of PKA at multiple subcellular sites. These findings support the targeting subunit hypothesis.

In this report, we describe the cloning and characterization of a novel AKAP, called AKAP 220. Analysis of this protein indicates that it is most abundantly expressed in testis and pancreas where it is co-localized with PKA.

METHODS AND MATERIALS

Cloning of AKAP220 cDNA The initial AKAP220 clone was obtained by screening a rat pituitary (GH_4C_1) cDNA λ Zap library by a direct overlay method with anti-RIIα as a probe as previously described (54). Plaques were blotted in duplicate onto isopropyl- 1-thio-β-D-galactopyranoside-soaked nitrocellulose filters as described (11). Secondary and tertiary screening was performed on all positive clones by a modified overlay procedure with a ^{32}P-labeled RIIα as a probe. Sequencing of the cDNA was performed by either a dideoxy chain termination method of Sanger (137) or an automated TAQ dideoxy terminator cycle method (ABI). DNA screening of a random-primed rat olfactory bulb cDNA library was done using 500-1,000 bp fragments from the 5' end of the previous clone. The cDNA was random primed with ^{32}P labeled as described (11). The labeled 1,000 bp-cDNA from the C-terminal clone was used to probe a human and rat mRNA Northern blot (multi tissue, Clontech) at 42°C in 50% formamide. The blot was probe with ^{32}P-Radiolabeled β-actin cDNA under similar conditions.

Expression of AKAP220--A 1203 bp fragment of cDNA was amplified by polymerase chain reaction. Primers were designed to create a Nde I site at the 5' end and a Bam H1

site at the 3' end of the polymerase chain reaction product. After digestion with both restriction enzymes, the insert was ligated into the bacterial expression vector, pET 16d (Novagen). Protein expression occurred after IPTG stimulation for 2 hours. Crude cell lysates were fractionated by centrifugation at 10,000 x g and the recombinant protein was recovered from the particulate fraction by solubilization in 6 M urea, 5 mM imidazole, 0.5 mM NaCl and 20 mM tris-HCL, ph 7.9. The protein was refolded by gradual dialysis at 4°C against 40 mM MOPS, 0.1 mM EDTA and 0.5 mM dithiothreitol.

RII Overlay Proteins were separated by SDS-PAGE and electrotransfered to PVDF membranes (Immobilon, Millipore Corp.) as described (11). Murine recombinant RII α was phosphorylated using the C subunit of PKA and ^{32}P ATP. The blots were blocked in 1% blotto and incubated with 1 X 10^5 cpm of ^{32}P labeled RIIα per 10 milliliters as previously described (11). Control experiments were performed by pre-incubating blots with 0.5 μM anchoring inhibitor peptide, Ht-31 (53). Quantitative overlays were performed using slotblot placement of a concentration range of expressed AKAP220 and Ht -31 as described (132). Quantitation was based on protein immunoblots. Films were digitally scanned and analyzed by densitometry with the National Institutes of Health Image (version 1.55).

Western Blot- Equal concentration of proteins (75μg-150μg) were separated on 7.5% SDS-PAGE and electrotransfered to PVDF membranes (Immobilon, Millipore Corp.). Rabbit poly-clonal anti-bodies to the His-Tag purified AKAP 220 (produced by bethyl Laboratories, Inc., Montgomery, TX) were affinity purified using a homogenous preparation of recombinant AKAP220 protein coupled to Affi-Gel 15 (Bio-Rad) and used at 1:200 dilution. The PKA catalytic sub-unit antibody was used at 1:250 dilution.

RESULTS

Cloning of AKAP220--Clones encoding RII-binding proteins were isolated from a GH_4C_1 cDNA expression library as previously described (65) using radiolabeled RIIα as a probe. One clone (GH_4-12) was 2100 bp in length and contained a continuous open-reading frame of 336 amino acids (Fig 1A). The size of the transcript was determined by probing a northern blot of various rat tissues with radiolabeled DNA from the original clone. The mRNA for GH_4-12 showed two predominant message sizes of 9.7 and 7.3 kB in rat heart, liver, lung, kidney and testis whereas a third message of 5.5 kb was detected in rat brain (Fig. 2). These mRNA sizes suggested that the GH4-12 clone encompassed a partial fragment of the full length message. Therefore, a 1000 bp Pst1 fragment excised from the 5' end of the GH4-12 clone was used to screen rat cDNA libraries for more complete transcripts of the message. Three overlapping clones were obtained from the rat olfactory bulb cDNA library that extended the GH4-12 sequence to 9730 bp. The contiguous sequence of GH4-12, presented in figure 1A, contains the open-reading frame of 3,386 bp that encodes for a 1,129 amino acid protein. The full length sequence has been placed in the GenBank. Although the nucleotide sequence predicts a protein of molecular M_r 124,472, RII overlay and western blot analysis of rat tissues indicates the protein migrates on SDS-polyacrylamide gels at approximately 220 kD (see below). Therefore, we have named this protein AKAP220.

Comparison of the full-length AKAP220 sequence to DNA and protein data bases did not identify any over-all homology to known protein sequences. However, a consensus nucleotide binding motif was identified between residues 1058-1065, and the last three amino acids Cys-Arg-Leu fulfill the criteria for a C-terminal peroxisomal (microbody) targeting signal (Fig 1A). In addition, residues 905-918 are likely to represent an RII-

binding site as this region exhibits high probability for forming an amphipathic α-helical wheel (Fig. 1B) and is similar to the RII-binding regions of other AKAPs (Fig. 1C).

Expression and Characterization of AKAP220: 761-1129 fragment-- Since the original cDNA was isolated by interaction cloning using RII we knew the RII binding region was contained within this region that represented the carboxy terminal 336 residues of the full length AKAP220. This fragment of AKAP220, encoding the C-terminal 366 amino acids was expressed in *E.Coli* using the pET16D His tag expression vector. A 58 kDa His tag fusion protein was detected in bacterial extracts of cells induced with IPTG and purified by affinity chromatography on His-binding resin (Fig. 3A). This fragment was specifically recognized by a polyclonal AKAP220 antibody raised against the protein (Fig. 3B). The recombinant protein fragment bound ^{32}P -labeled RIIα as assessed by a direct overlay method (Fig. 3C). Control experiments demonstrated that RII binding was inhibited by pre-incubating the blot with 0.5μM anchoring inhibitor peptide Ht 31 (Fig.3 D). These findings confirm that the RII-binding region of AKAP220 is located in the C-terminal third of the molecule.

The binding affinity of AKAP 220 fragment for RII was measured by a quantitative overlay procedure. Various concentrations of AKAP220 (761-1129) and the human thyroid AKAP, Ht 31, (from 2.0 ng/ 100μl to 125 ng/100μl) were immobilized on nitrocellulose and probed with ^{32}P-RIIα at a specific activity of 1.0 cpm/pmol. The RII binding of immobilized AKAP 220 and Ht31 was detected by autoradiography and measured by densitometry. The half-maximal binding values were calculated at 20 ng and 25 ng for AKAP220 and Ht31 respectively (Fig. 4). This data indicates that AKAP 220 encodes for a protein with high affinity for RII.

In this chapter, I describe the cloning and characterization of a novel A-kinase anchoring protein, called AKAP 220. AKAP220 shares many properties with other previously characterized AKAPs including a high affinity for RII (53).

DISCUSSION

Previous studies suggest that AKAPs possess a conserved RII-binding site that is composed of an amphipathic helix (53). The RII binding site of AKAP 220 is in the C-terminal third of the protein since this was the cDNA originally selected by an RII interaction cloning strategy. The binding site probably resides at residues 905-918 as this sequence exhibits a high probability of amphipathic helix formation and shares sequence homology with the RII binding regions of AKAP95, Ht 31 and MAP 2 (Fig 1B) (53). In addition, a recombinant fragment encompassing this region (residues 711-1129) exhibits a similar affinity for RII, binding RII with nanomolar affinity (Fig 3). Moreover, the anchoring inhibitor peptide, Ht31 (393-415), blocks the interaction of the AKAP 220 fragment with RII. Collectively, these findings suggest that AKAP220 contains all the hallmarks of a prototypic RII binding protein or AKAP.

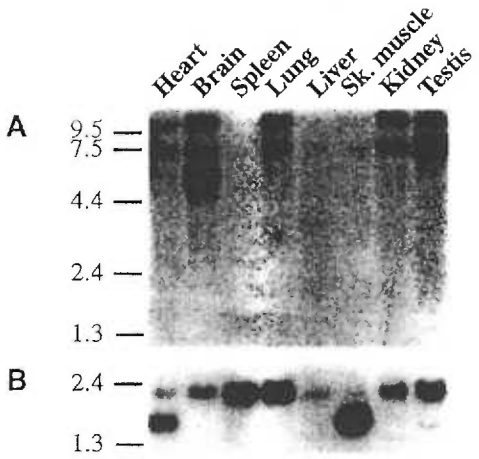
CHAPTER TWO FIGURE LEGENDS

CHAPTER 2, FIGURE 1 Sequence Of AKAP220. A) The nucleotide sequence and the deduced amino acid sequence of the cDNA encoding the A kinase anchoring protein, AKAP220. The boxed area indicates the putative RII binding region, while the peroxisome-targeting sequence is underlined and in *italics*. **B)** Helical wheel representation of AKAP220 (residues 905-918) drawn as an α-helix of 3.6 amino acids/turn. The shaded area indicates the hydrophilic residues, and the black area indicates the hydrophobic residues. **C)** Sequence homology between AKAP220 (residues 905-918) and the RII binding regions of two other AKAPs, AKAP15O and HT31. The shaded area indicates amino acid identity and conserved amino acids are indicated (*).

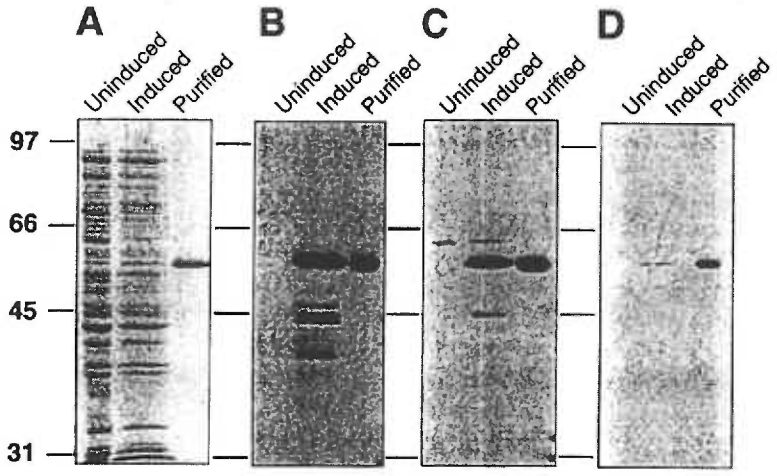
CHAPTER 2, FIGURE 2 The Tissue Distribution Of The AKAP220 mRNA. A) 2 micrograms of poly(A) mRNA from rat tissues (rat MTN, Clontech) were probed with a ^{32}P-radiolabeled 936-bp. fragment excised from the 3' end of GH4-12 as described under "Experimental Procedures". **B)** The same filter was probed with ^{32}P-radiolabeled beta-actin. Hybridizing mRNA species were detected by autoradiography. The tissue source of each RNA is indicated above each lane. Kilobase markers are indicated beside each panel.

CHAPTER 2, FIGURE 3 Recombinant AKAP220 fragment specifically binds RIIα. A fragment of the AKAP220 cDNA (encoding residues 761-1129 of the protein) was expressed using the pET16b bacterial expression vector. Bacterial extracts, induced or uninduced (100 μg), or purified protein (10μg) were separated by electrophoresis on 10% (w/v) SDS-PAGE and electrotransferred to PVDF membranes. **A)** Gels were stained with Coomassie blue dye. **B)** The recombinant AKAP220 fragment was detected by Western blot with affinity purified antibodies. RII binding proteins were detected by a solid-phase binding assay using ^{32}P-radiolabeled RII as a probe in the absence **(C)** or in the presence **(D)** of 1μM anchoring inhibitor peptide, HT31 (493-515). Sample sources are indicated above each lane, and the molecular weight markers are indicated beside each panel.

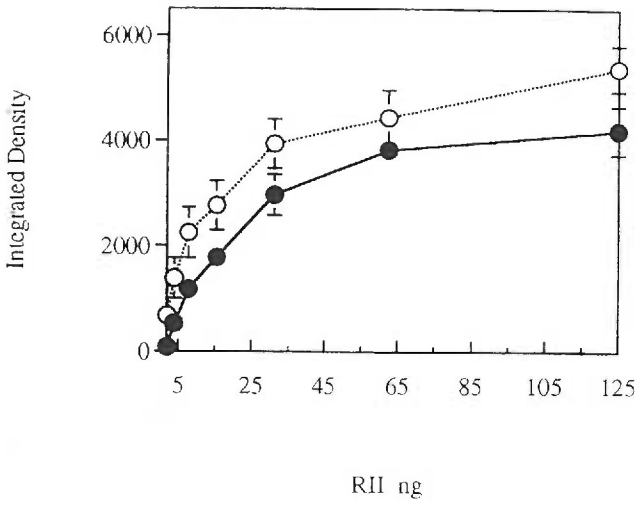
CHAPTER 2, FIGURE 4 Estimation of the AKAP220 binding affinity for RIIα. Binding of ^{32}P-radiolabled RII to the COOH-terminal fragment of AKAP220 (residues 761-1129) and a corresponding fragment of the human thyroid anchoring protein HT31 was measured by a semiquantitative overlay procedure. Aliquots of the purified protein ranging from 5 to 125 ng was immobilized onto nitrocellulose filters using a slot-blot apparatus. Individual filters were probed with excess ^{32}P-radiolabeled RII (specific activity: 1.5-2.1 X 10^5 cpm/nmol). Detection of immobilized RII was by autoradiography. Quantitation of binding over the range of protein concentrations was determined by densitometry of the radiographs. Signals were normalized for the specific activities of each RII probe. Binding curves for AKAP220 (open cirlces) and HT31 (filled circles) are presented from three experiments; the standard deviation of the integrated density is indicated.



CHAPTER TWO, FIGURE 2



CHAPTER TWO, FIGURE 3



CHAPTER TWO, FIGURE 4

CHAPTER THREE

Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein.

Nauert, B., Klauck, T. K., Langeberg, L. K., and Scott, J. D. (1997) Curr. Biol. 7:52-62.

INTRODUCTION

Changes in cell physiology are modulated by the input of external signals and the subsequent activation of intracellular signal transduction pathways (1). A major mechanism of intracellular signal transduction is the regulation of the phosphorylation state of cellular proteins by kinases and phosphatases (2,138). The changes in the phosphorylation state of proteins has important effects on cellular physiology (1). These physiological effects may take form in the modulation of the activity of an enzyme or modifying the role of a structural protein in the maintenance of cellular architecture (23,139,140). Cells possess many diverse kinase and phosphatase signaling pathways, they are, however, activated by a relatively limited set of second messengers. Different extracellular signals using the same second messenger pathways produce distinct changes in the phosphorylation state of particular substrate proteins (141). The control of stimulus specific signaling pathways is one of the major questions facing the signal transduction field today. One hypothesis put forth to explain this phenomenon is the *targeting hypothesis* (131). The targeting hypothesis proposes that localized intracellular compartments of signaling complexes are maintained to enhance the specificity of a signaling pathway in response to an external stimulus. The consequence of this mechanism is the compartmentalization of second messenger, kinase/phosphatase, and substrate proteins, thereby strengthening the specificity of a signaling pathway while decreasing indiscriminate background phosphorylation (7,19,49,131,136,142).

Maintenance of a kinase or phosphatase to a specific signaling compartment is proposed to occur through the association of an enzyme with a *targeting protein* or *subunit* (143,144). Tyrosine kinase (PTK) and tyrosine phosphatase (PTPase) activity is coupled to downstream cytoplasmic enzymes through adapter proteins that contain SH2 and SH3 domains (8). Modular proteins like Grb2, p85, IRS-1, Crk, and Nck consist of a single SH2 domain that recognizes certain phosphotyrosyl residues on signaling enzymes, and

two SH3 domains that bind to a PXXP motif on a separate set of target proteins (8). In addition the pheromone mating response in yeast is initiated through a G-protein linked receptor that activates a yeast MAP kinase (145). This process proceeds efficiently because each enzyme in the cascade is associated with a scaffold protein called sterile 5 (STE 5) (9,146,147). Clustering of successive members in the MAP kinase cascade is optimal for the tight regulation of the pathway and prevents cross-talk between the six functionally distinct MAP kinase modules in yeast (145).

Work on broad specificity serine/threonine protein kinases and phosphatases has demonstrated that these signaling molecules also possess mechanisms for targeting. Numerous phosphatase targeting subunits have been identified which are specific for the three major classes of phosphatase catalytic subunits PP-1, PP-2A and PP-2B (131,148,149,56,150,151). Three distinct classes of protein kinase C (PKC) targeting proteins have been identified (152,153,154). In addition both Cdk5 and protein kinase N (PKN) have been shown to be targeted to neurofilaments (155,156). Cdk5 is bound to neurofilaments through its activator, p35, whereas PKN binds directly through its regulatory domain (156). PKN is also targeted to actinin (157).

Immunocytological investigations and biochemical analyses have shown that type II PKA is targeted to a variety of subcellular compartments (38,42,43,40,11,65,70,71,52). This compartmentalization of the cAMP-dependent protein kinase (PKA) occurs through the interaction of the regulatory subunits (R) with a functionally related family of 30 or so A-Kinase Anchoring Proteins, (AKAPs) (47,50). The molecular cloning and subsequent biochemical and cellular analysis of many of these AKAPs has confirmed the capacity of these molecules to bind and localize type II PKA. While many of the identified AKAPs have discrete subcellular distributions, some AKAPs have overlapping subcellular distributions yet use distinct mechanisms of targeting. This is best represented by

AKAP79 and AKAP18; both AKAPs are targeted to the cell membrane (57,79). AKAP79 is bound through an interaction with acidic phospholipids (57), while AKAP18 is associated with the membrane via covalent myristate and palmitate modifications (79).

The most characterized AKAP is AKAP79 which serves to maintain a signaling scaffold by targeting PKA, PKC, and protein phosphatase 2B (PP-2B) to the cell membrane in primary hippocampal neurons, and HEK cells (11,12,56,57,158). AKAP79 has several functional domains and resembles STE 5 in that deletion analysis, peptide studies, and co-precipitation techniques have demonstrated that each enzyme binds to a distinct domain of the anchoring protein (12,53,56). The binding of kinases and a phosphatase by AKAP79 suggests a model for reversible phosphorylation in which the opposing effects of kinase and phosphatase action are compartmentalized by a common anchoring protein (159).

In this chapter I will discuss the cloning and characterization of a second multivalent kinase-scaffold protein called gravin. A fragment of gravin was originally identified as a cytoplasmic antigen recognized by sera from patients with myasthenia gravis (88). However, we now show that distinct regions of the protein function as an AKAP and a PKC substrate/binding protein. *In vitro* binding studies have mapped the binding sites for both kinases, and co-purification experiments have allowed us to propose that gravin may function as a kinase scaffold protein that coordinates the intracellular locations of PKA and PKC.

MATERIALS AND METHODS

Cloning of the gravin cDNAs. A 3023 bp partial cDNA encoding an RII binding protein was isolated by expression cloning from a human fetal brain lambda-ZAP cDNA library using a modified form of the RII overlay procedure as previously described (71). Clones encoding the full-length gravin message were obtained by screening a human heart lambda-

ZAP cDNA library using an α-^{32}P dCTP random primed 1676 bp Eco RI-Spe I fragment from the original clone as a probe. Nucleotide sequencing was performed by the Sanger dideoxy method (137) or by automated sequencing using an Applied Biosystems Incorporated sequencer (Foster City, CA).

Bacterial expression of recombinant proteins. A 1953 bp insert encompassing the entire open reading frame of the original gravin cDNA was subcloned into the bacterial expression vector pET16b. This was accomplished by amplifying this fragment using the polymerase chain reaction (PCR) with the original HF 9 clone as the template. Primers were synthesized that generated an Nde I site (CCGCCATGGTGCATATGTCCGAGTCCAGTGAGC) at the 5' end and a Bam HI site (GGAGGATCCAGAGATTCTGTAGTTCTG) at the 3' end to facilitate subcloning into the vector. A similar strategy was used to generate the RII-binding site constructs encompassing residues 1130-1780, 1130-1525 and 1526-1780 of gravin. The first two constructs shared a common 5' primer (CCGCCATGGTGCATATGTCCGAGTCCAGTG AGC) but had distinct 3' primers: (GCGCGGATCCGCACTCACTTTGACCTCCTG) for residues 1130-1780 and (GCGCGGATCCGCTATCACGTGAGCTTGTGT) for residues 1130-1525. The 1526-1780 construct was amplified using a unique 5' primer (CCGCCATGGTGCATATGGTAGCAATTGAGGATTTAG) in conjunction with the 3' primer (GGAGGATCCAGAGATTCTGTAGTTCTG) used to subclone the full length clone. Likewise, the gravin 265-556 fragment was generated by a PCR based strategy to map the PKC-binding site. This insert was amplified from the orginal gravin cDNA using a 5' primer (CCGCCATGGTGCATATGAAAGAGGAAGGAGAAGAG) which encoded an Nde 1 site and a 3' primer (GGAGGATCCAGAGCTGTCCGGAGAATCGGC) which encompassed a Bam H1 site.

Each gravin construct was transfected into E. coli and the recombinant Histag fusion proteins were induced by IPTG. Each recombinant protein was purified according to previously published methods (56). The 1130-1780 gravin fragment was used for production of a polyclonal antibody (Bethyl Labs, Montgomery, TX), hereafter referred to as R3698.

Surface Plasmon Resonance Measurements. A recombinant fragment encompassing residues 1526-1780 of gravin was coupled to a carboxymethyldextran IAsys cuvette using standard EDC/NHS coupling chemistry (160). The cuvette was activated by treating with 0.4 M EDC/0.1 M NHS for 8 min and washed extensively with PBST (PBS + 0.05% Tween-20). Coupling of the gravin 1526-1780 fragment (25 μg/ml) was accomplished in 10 mM formate buffer, pH 3.6 for 10 min at room temperature. Uncoupled protein was washed out with PBST and free amines were blocked with 1M ethanolamine, pH 8.5 for 2 min at room temperature. After washing with PBST, a stable baseline was established for 10 min before data collection. All binding experiments were performed with a recombinant fragment of RIIα (RII 1-45) which binds AKAPs with a similar affinity as the full-length protein. Previous experiments have indicated that release of RIIα 1-45 from the binding surface can be performed under conditions that are less harmful to the immobilized anchoring protein than studies using full length RII. Binding experiments were performed over a range concentrations from 25 to 150 nM in volumes of 200 μl. The binding surface was regenerated between binding measurements using 60% ethanol with no decrease in extent measurements over the duration of an experiment. Data collection was done over 3 second intervals and was analyzed using the Fastfit™ software which was provided with the IAsys instrument.

Solid-phase overlays and Western blots. RII overlays, western blots and PKC-overlays were performed essentially as previously described (12,132). Immunochemical detection

of RII was obtained with an affinity purified rabbit antibody to murine RIIα. The PKC overlay was probed with a monoclonal antibody that recognizes both α and β PKC (Transduction Labs, Lexington, KY). The catalytic subunit of PKA was detected in using affinity purified antibodies to an N-terminal peptide of the human PKA C subunit (gift from Dr. Steven Pelech, Kinetek Biotechnology Corp, Vancouver, B.C., Canada).

PKC Inhibition Assay. PKC was assayed as described (161) in a reaction containing 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM DTT, 100 μM [γ^{32}P] adenosine triphosphate (ATP) (500 cpm/pmol), phosphatidylserine (20 μg/ml), and epidermal growth factor receptor peptide (VRKRTLPRL) as substrate at 30°C for 10 min. PKC βII (20 ng/μl) was diluted 1:10 in 20 mM Tris (pH 7.9), 1 mg/ml bovine serum albumin (BSA) and 1 mM DTT. Inhibition constants ($I.C._{50}$) were determined over an inhibition concentration range of 0.1 to 10 μM gravin 265-556 fragment.

Cell culture and preparation of cell lysates. Human erythroleukemia (HEL 92.1.7, ATCC TIB 180) cells were grown in RPMI 1640 containing 12% fetal calf serum and 4 mM glutamine. Gravin expression was induced by culturing with 40 nM phorbol myristate acetate for 18 hr. Cell lysates were prepared from either adherent cells grown in the presence of PMA, rinsed with PBS and scraped from the interior of 150 cm^2 flasks or from suspension cultures of HEL cells grown in the absence of PMA. Cell pellets were washed twice with PBS prior to resuspension in 20 mM TrisHCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.25% Triton X-100, 0.05% Tween 20, 0.02% NaN_3, 10 mM benzamidine, 2μg/ml pepstatin, 2μg/ml leupeptin, 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Lysis Buffer) and incubation on ice for 10 min. The extract was then centrifuged for 10 min at 16,000 x g at 4°C and the cell lysate supernatant was collected. Protein concentrations were measured using the Bio-Rad *DC* Protein Assay kit. HEK 293

and Cos 7 cells were plated at 30 % density on a 10 cm dish. 24 hours later they were transfected using the calcium phosphate method. The cells were washed with PBS the following day and fed with fresh media. The cells were grown an additional 48 hours before harvesting. Cell pellets were washed twice with PBS prior to resuspension in 20 mM TrisHCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1.0% Triton X-100, 0.05% Tween 20, 0.02% NaN_3, 10 mM benzamidine, 2μg/ml pepstatin, 2μg/ml leupeptin, 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Lysis Buffer) and incubation on ice for 10 min. The extract was then centrifuged for 10 min at 16,000 x g at 4^0C and the cell lysate supernatant was collected. . Protein concentrations were measured using the Bio-Rad *DC* Protein Assay kit.

Immunocytochemistry and In situ RII overlays. HEL cells were grown on glass coverslips in the presence of 40 nM PMA for 18 hr, rinsed with PBS, fixed in 3.7% formaldehyde and extracted in -20° C absolute acetone. Cells were rehydrated for 1hr in PBS and 0.2% BSA and then incubated with either affinity purified anti-gravin antibody, R3698, at 0.5 μg/ml or pre-immune IgG at 0.5 μg/ml. After 1 hr the coverslips were carefully washed in PBS and 0.2% BSA and incubated with either a mixture of FITC conjugated donkey anti-rabbit secondary antibody (1:100 dilution, Jackson ImmunoReasearch Laboratories Inc, West Grove, PA) and rhodamine conjugated phalloidin (1 unit/coverslip, Molecular Probes, Inc, Eugene, OR) or secondary antibody alone. *In situ* RII-overlays were performed essentially as described (65). Prior to incubation with primary antibody, cells were incubated with 80 nM recombinant murine RIIα for 2 hr and unbound RII removed by washing three times in PBS and 0.2% BSA. The immobilized RIIα was detected immunochemically with affinity purified goat anti-murine RII (1 μg/ml) and Texas red conjugated donkey anti-goat IgG secondary (1:100 dilution, Jackson ImmunoReasearch Laboratories Inc, West Grove, PA). Control coverslips were treated with the antibody to RII in the absence of exogenous murine RII.

Cells were examined using a Leica confocal laser scanning system equipped with a Leitz Fluovert-FU inverted microscope and an argon/krypton laser.

Immunoprecipitation. HEL cell lysates (200µl of 15 mg/ml) prepared as described above, were incubated with either 15 µg of affinity purified anti-gravin, or 15 µg of pre-immune IgG at 4° C for 18 hr. Immune complexes were isolated by the addition of 200 µl of 10% (v/v) Protein A-Sepharose CL-4B (Sigma, St Louis, MO) which had been pre-equilibrated in Lysis Buffer. Following incubation at 4°C for 90 min the beads were washed once in 0.5 M NaCl Lysis Buffer and four times in excess 20 mM TrisHCl, pH 7.4, 150 mM NaCl. The PKA catalytic subunit was released from the immune complex by incubating the Protein-A beads in 200 µl 1 mM cAMP, 20 mM TrisHCl, pH 7.4, 150 mM NaCl for 15 min. The eluate was TCA precipitated prior to analysis on a 4-15% SDS-PAGE gel, electroblotted onto nitrocellulose and the C subunit was detected, as previously described. For the immunoprecipitation and detection of gravin, elution was accomplished by boiling the washed beads in SDS-PAGE sample buffer, separation of proteins on a 4-15% denaturing PAGE gel (5µg/lane), transfer to nitrocellulose and analysis by gravin western, PKC overlay and RII overlay western as described previously (12).

Co-purification of gravin and RII by cAMP sepharose. HEL cell lysates (400 µl of 15 mg/ml, prepared as described above with the addition of 10 mM IBMX to the buffer), were incubated with 200 µl packed cAMP-agarose (Sigma, St Louis, MO) which had been equilibrated in Lysis Buffer with 10 mM IBMX. The slurry was gently mixed for 18 hr at 4^0C and then washed with 1.5 ml Lysis Buffer with 1 M NaCl followed by four 1.5 ml washes with 20 mM TrisHCl, pH 7.4, 150 mM NaCl. Elution was accomplished by incubating the beads in 0.5ml 75 mM cAMP, 20 mM TrisHCl, pH 7.4, 150 mM NaCl for 30 min at room termperature. The final wash and the eluate were TCA precipitated and the entire sample loaded into a single lane on a 4-15% SDS-PAGE gel. The separated proteins

were blotted to nitrocellulose and gravin was identified by western analysis as described above.

RESULTS

Isolation of Gravin clones - To isolate cDNAs encoding potential RII binding proteins, a human fetal brain λ-ZAP cDNA library was screened by a modified overlay procedure using radiolabeled RIIα as a probe (43). Eight RII binding clones were identified, plaque purified and the ends of each insert were sequenced. Two of the clones represented known sequences. One matched MAP2, a previously identified AKAP (42) and the 3' end of another clone, called HF 9, was identical to a partial clone encoding a protein called gravin, which was originally isolated by screening a Human Umbilical Vein Endothelial Cell (HUVEC) cDNA library with serum from a myasthenia gravis patient (88). The other six clones had no homology to any known protein in the Genbank.

Further sequencing of HF 9 showed that the cDNA was 3023 base pairs in length and encoded a continuous open reading frame of 651 amino acids. Northern blot analysis using a ^{32}P random primed 1676 base pair Eco RI-Spe I fragment of HF 9 as a probe indicated that gravin mRNA was selectively expressed in certain human tissues. Two predominant mRNA species of 8.4 kb and 6.7 kb were detected in all tissues but predominated in liver, brain and lung, whereas an additional 5.5 kb message was detected in brain (data not shown). Due to the larger sizes of all the gravin messages, we concluded that HF 9 clone represented a partial transcript of the mRNA. Therefore, the 1676 base pair fragment was used to further screen the human fetal brain cDNA library for more complete transcripts and five additional clones were obtained that yielded an additional 600 base pairs of coding region. As an alternative strategy a human heart cDNA library was

expressed in *E. coli* using the pET16b Histag bacterial expression/affinity purification system (Figure 3B). A 452 residue fragment encompassing residues 1130-1582 bound ^{32}P radiolabeled RIIα in the overlay, whereas a smaller fragment, residues 1130-1525, which lacked the proposed RII-binding region was unable to bind RIIα (Figure 3C). Two additional experiments provided evidence that the putative amphipathic helix region was sufficient for RII-binding. A third fragment encompassing residues 1526-1780 of gravin bound RII in the overlay (Figure 3C) and a synthetic peptide covering residues 1537-1563 blocked all RII-binding in the overlay (Figure 3D). In addition, the anchoring inhibitor peptide Ht31(493-515) which is a competitive inhibitor of RII/AKAP interactions (53,58) also blocked RII binding to gravin as assessed by the overlay assay. The Ht31-Pro control peptide did not (data not shown). When combined, these results suggest that gravin is an AKAP and the principal RII-binding site involves residues 1537-1563.

This finding was further substantiated when the binding affinity of the gravin 1526-1780 fragment for recombinant fragment of RIIα was measured by surface plasmon resonance (SPR). The binding properties of the immobilized gravin fragment were measured over a range of RIIα 1-45 concentrations from 25 to 150 nM (Figure 4A). Uniform pseudo-first order binding was recorded with a k_{ass} of 160006 ± 9700 sec^{-1} (n=3) and with a k_{dis} of 0.016 ± 0.001 sec^{-1} (n=3) (Figure 4B). These values were used to calculate a dissociation constant (KD) of 100 nM (n=3) for the RII/gravin fragment interaction (Figure 4B). The nanomolar binding constant for RII/gravin is consistent with the notion that both proteins may associate *in situ*.

Gravin is a PKC-binding protein. - On the basis of sequence homology to clone 72 (Figure 2A) we postulated that the amino terminal regions of gravin may bind PKC. Therefore, a 290 amino acid fragment corresponding to residues 265-556 of gravin was expressed in E. coli (Figure 5A). The affinity purified gravin 265-556 fragment bound PKC in a

phosphatidylserine (PS) dependent manner as assessed by an overlay assay (Figure 5B&C), whereas a C-terminal fragment, residues 1130-1582, did not (Figure 5B&C). Binding studies were performed with a mixture of PKC α, β and γ isoforms. Neither of the gravin fragments bound to PKC in the absence of PS (data not shown) which confirms other reports that phospholipid is a co-factor in the PKC/binding protein complex (154,164). Unfortunately, the excessive refractive properties of the PS moiety prevented affinity measurements of PKC/gravin interaction by surface plasmon resonance.

It has been suggested that polybasic regions participate in formation of a phospholipid bridge between the PKC and its binding protein (154,164). We have shown that a polybasic region on another scaffold protein, AKAP79, forms the PKC binding site (12). Since the AKAP79 32-51 peptide also blocked PKC binding to gravin (Figure 5D) it seems that both anchoring proteins bind to a similar site on PKC. Further similarity to AKAP79 is demonstrated when the gravin 265-556 fragment was able to inhibit PKC activity toward a peptide substrate with an $I.C._{50}$ of 0.50 ± 0.12 μM (n=4) (Figure 5E), whereas the RII binding peptide did not inhibit the kinase (data not shown). There are two polybasic regions in the gravin 265-556 fragment located between residues 295-316 and 514-536 (Figure 2A). Interestingly, peptides to either polybasic region blocked PKC/gravin interactions as assessed by the overlay (data not shown). Collectively, these experiments show that protein kinase C binds gravin *in vitro* at one or more polybasic sites located between residues 265-556 of the protein. Furthermore, interaction with gravin appears to inhibit PKC activity.

Induction of Gravin in Human Erythroleukemia Cells - Previous studies have suggested that gravin is expressed in a variety of cell types, including neurons, fibroblasts and endothelial cells (88,90). An immunochemical survey of cell-lines indicated that gravin was expressed in MG-63 cells, HEK-293 cells (data not shown) and human

erythroleukemia (HEL) cells. Phorbol ester treatment of HEL cells induces morphological, functional and biochemical changes that are characteristic of macrophage-like cells (165). One hallmark of this process is the robust induction of gravin (88). Therefore, we decided to examine the PKA and PKC binding protein profile of HEL cells after prolonged exposure to phorbol esters. Human erythroleukemia cells were grown in the presence of 40 nM phorbol myristyl acetate (PMA) for 18 hours and extracts from control and treated cells were subjected to western blot with an affinity purified antibody against residues 1130 to 1780 of gravin. PMA treatment caused an induction of a 250 kDa protein that specifically reacted with anti-gravin antibodies (Figure 6A). Subsequent overlay assays demonstrated that PMA treatment induced the expression of a 250 kDa PKC-binding protein (Figure 6B) and an RII-binding protein of the same size (Figure 6C). These results confirm that phorbol ester treatment induces gravin expression in HEL cells and suggest that the native protein binds PKA and PKC *in vitro*.

Concomitant with the macrophage-like shift, the HEL cells become more adherent and display a considerable cytoplasmic spread (165). This sometimes results in the formation of actin stress fibers and causes a general flattening of the cell. In order to establish whether gravin aligned with the actin cytoskeleton, phorbol ester treated HEL cells were stained with rhodamine phalloidin as a marker for actin. All of the cells displayed a concentration of actin to the periphery (Figure 7A). In contrast, gravin staining was predominantly cytoplasmic and only a subset of the cells (approximately 25%) expressed large quantities of the protein (Figure 7B). Variable levels of gravin expression were not unexpected as HEL cells represent a heterogeneous population at different stages of differentiation (165). Superimposition of both images show that gravin and actin exhibit distinct but overlapping subcellular locations (Figure 7C). Control experiments were negative when cells were stained with preimmune serum (Figure 7D). More detailed confocal analysis of HEL cells detected gravin staining toward the periphery of the cell and

enriched in filopodia at the adherent surface (Figure 8 A-D). These findings are consistent with the notion that gravin is a component of the membrane cytoskeleton that may function in some capacity to enhance cell adhesion.

Dissection of the Gravin Signaling complex - In vitro binding studies suggested that gravin is a kinase scaffold protein. Therefore, co-localization experiments were initiated to determine whether a gravin signaling complex could be detected in HEL cells. Fixed and permeabilized cells pretreated with PMA were overlayed with recombinant RIIα (Figure 9 A-D). RII-binding *in situ* was detected with antibodies that specifically recognize murine RII (Figure 9B) and mimicked the staining pattern for gravin (Figure 9 A&C). Since control experiments confirmed that the anti-murine RII antibodies did not detect the endogenous human RII (Figure 9D), we have concluded that the increased RII staining was due to direct association with gravin. This conclusion was supported by additional control experiments showing that *in situ* RII-binding was blocked by incubation with the Ht 31 anchoring inhibitor peptide (data not shown). Parallel experiments which attempted to detect PKC binding by *in situ* overlay were unsuccessful.

Finally, the gravin signaling complex was isolated by two complementary biochemical methods: immunoprecipitation and affinity chromatography on cAMP-agarose. Immunoprecipitation with gravin antibodies specifically isolated a 250 kDa protein that could be faintly detected when SDS gels were stained with Coomassie Blue (data not shown). This 250 kDa protein was only present in immunoprecipitates using the affinity purified gravin antibodies and was not detected in control experiments performed with preimmune serum. Western blot and overlay assays confirmed that the 250 kDa protein was gravin (Figure 10A), a PKC-binding protein (Figure 10B) and an AKAP (Figure 10C). Moreover, co-precipitation of the PKA holoenzyme was demonstrated by detection of the C subunit in fractions eluted from the immunoprecipitate with cAMP but not in

experimental fractions treated with preimmune serum (Figure 10D). We were unable to detect R subunit in the immunoprecipitates because the 54 kDa protein migrates with the same mobility as the IgG heavy chain (Figure 10C). However, the R subunit/gravin complex was purified from PMA induced HEL cell extracts by affinity chromatography on cAMP-agarose (Figure 11A). After extensive washing in high salt buffers gravin was eluted from the affinity resin with 75mM cAMP (Figure 11B). Since free gravin is refractive to the affinity resin we concluded that the protein detected in the eluate was associated with the regulatory subunit. Both co-purification techniques strongly suggest that the PKA holoenzyme is associated with gravin *in vivo*. However, preliminary attempts to co-purify PKC were unsuccessful, and technical manipulations of the purification techniques are being utilized to better understand the interaction between PKC and gravin.

DISCUSSION

We have cloned a novel human AKAP called gravin using a modified form of the RII overlay. A carboxy terminal fragment of gravin was originally identified as a cytoplasmic antigen recognized by sera from patients with the disease myasthenia gravis (MG) (88). MG is an autoimmune disease that affects the neuromuscular junction. The primary autoantigen is the nicotinic acetylcholine receptor while only 31 % of MG patients have autoantibodies against gravin (85,88). Gravin auto-antibodies are, however, specific to MG, as no anti-gravin antibodies were detected in other autoimmune diseases (systemic lupus erythematous, progressive systemic sclerosis) (88). Suprisingly, even though the pathology of MG occurs at the neuromuscular junction, an immunohistochemical survey of baboon tissues showed that gravin was not expressed in either muscle or neurons at the neuromuscular junction, and thus does not appear to be a source of pathology in MG (90). Gravin was, however, expressed in fibroblast and muscle progenitor cells between muscle fibers (90).

We have assembled a full length cDNA of 6606 base pairs, with an open reading frame (ORF) encoding a protein of 1780 amino acids. Heterologous expression of the ORF in HEK and COS cells produces a protein that migrates with the same mobility of 250 kDa as the endogenously expressed protein (Fig. 12). A second cDNA isoform of gravin has been cloned that encodes for a protein of 1684 residues (Kokame, K., 1997, unpublished, accession #2081607). Residues 1-109 of our clone are not present in this new clone. In addition, this alternative form contains a unique 10 residue. amino terminus before continuing with residue 110 of our clone.

A rat gene with 70% homology, which is possibly the rat homolog of gravin, has been independently cloned by two groups (162,164). Both groups have isolated this clone as a possible anti-oncogene. One group, referring to their clone as *s*rc *s*uppr*e*ssed *C k*inase *s*ubstrate (SSeCKS), identified their gene while screening for mRNA transcripts whose expression decreased upon transformation of fibroblasts with a constituitively active form of src (166). The second group, referring to their clone as clone72, identified rat gravin as a PKC-substrate/binding protein (164) whose expression decreased in rat embryonic fibroblasts through a series of increasingly transformed cellular states. In comparison to human gravin, SSeCKS/clone72 migrates at 207 kDa consistent with the predicted smaller ORF of 1596 amino acids (163,167). Unlike the report of an alternative form of gravin, the amino termini of both rat clones are identical and no subsequent rat clones containing an alternate amino terminus have been reported (163,167). We propose that gravin and SSeCKS/clone 72 are both members of an emerging class of mammalian scaffold proteins which contribute to the organization of signal transduction events by coordinating multiple kinase signaling pathways (143).

Our studies support residues 1537-1563 of gravin as the RII-binding site. A peptide made to this sequence blocks RII-binding in the overlay assay Additionally analysis of a protein fragment containing the RII binding domain by surface plasmon resonance (SPR) indicated the RII-gravin interaction to be 100 nM. This sequence is identical in SSeCKS/clone 72, so it is likely that SSeCKS/clone 72 will also bind RII. Gravin's RII-binding sequence has 10 out of 14 residues which are conserved in the RII-binding region of another mammalian scaffold protein, AKAP79, which binds PKA, PKC and PP-2B (11,12,56). The identification of a conserved RII-binding sequence in gravin, SSeCKS/clone 72, and AKAP79 was rather surprising as it has been proposed that a lack of sequence identity between the AKAPs is due to a conservation of secondary structure in the RII-binding motif (53). Therefore, gravin, SSeCKS/clone 72, and AKAP 79 may be members of a subfamily of AKAPs which bind more than one kinase or phosphatase.

Although we have demonstrated that gravin anchors PKA *in vivo*, the question of whether it is also an intracellular PKC-binding protein is not so clear. Three functionally distinct classes of PKC-binding proteins have been identified by gel overlay and yeast two-hybrid (142,143). PKC substrate/binding proteins and *r*eceptors for *a*ctivated *C-k*inase (RACKs) have been detected by the gel-overlay procedure, and bind to activated forms of PKC (153,154). On the other hand *p*roteins that *i*nteract with *C-k*inase (Picks), have been isolated in two-hybrid screens (152), and contain a PDZ domain that binds to a PDZ binding domain on the carboxy terminus of PKC- α (168). On the basis of the homology to clone72, we would propose that gravin is a PKC substrate/binding protein. Consequently, we have located a region of 290 amino acids that supports PKC-binding in the presence of calcium and phosphatidylserine, and blocks kinase activity *in vitro*. However, there is some ambiguity as to where the principal PKC-binding site(s) are located within this region. It has been suggested that phosphatidylserine (PS) supports a ternary complex of PKC and polybasic regions on the substrate/binding protein (169).

Accordingly, there are two polybasic regions in the gravin 265-556 fragment, both of which block PKC-binding in the overlay (T.M. Klauck and J.D. Scott unpublished observation). Furthermore, both polybasic regions (residues 295-316 and residues 514-536) resemble the PKC-binding site on AKAP79 which also blocks binding in the overlay and inhibits the kinase (12). One difference between the binding site on AKAP79 and gravin is that both potential PKC-binding domains of gravin are substrates, while the polybasic PKC binding domain on AKAP79 is not a substrate for PKC. At this time it is unclear how both domains of gravin contribute to PKC binding.

Despite evidence of PKC-binding *in vitro* we have been unable to detect the gravin/PKC complex inside cells. There are, however, several plausible explanations for this. First of all, phosphorylation regulates the association of substrate/binding proteins with PKC; consequently, the substrate/binding protein may interact with the kinase transiently (169). Substrate/binding proteins may therefore represent PKC substrates that have a slow rate of dissociation following the phosphotransfer reaction (170). The net effect of such a kinetic mechanism would be that substrate/binding proteins could buffer the amount of active PKC available to conventional substrates, rather than perform a scaffolding function. This may explain why sub-micromolar concentrations of the gravin 265-556 fragment inhibit kinase activity in the presence of excess EGFR peptide substrate. Secondly, cell extraction buffers and techniques used to isolate the gravin signaling complex from cells may remove co-factors required for the PKC/gravin interaction, or induce the release of PKC from gravin. Lastly, the prolonged exposure to phorbol esters, which is necessary to induce high level gravin expression in HEL cells, is known to cause the proteolytic degradation of some PKC isoforms and this will undoubtedly decrease the amount of enzyme available to associate with gravin (171). Nevertheless, the temporal dynamics of a PKC-gravin targeting interaction in HEL must remain open until there is definitive evidence that the complex is formed *in vivo.*

Several lines of evidence suggest gravin is in a class of molecules involved in two distinct but overlapping cellular processes: 1) cell migration/adherence, and 2) the cell cycle. A tissue survey has shown that gravin exhibits a restricted cellular distribution and is predominantly expressed in fibroblasts, neurons, and cells derived from the neural crest (90). Each of these cell-types participates in adherent, migratory or path-seeking behavior (90). In addition, phorbol ester induced adhesion of HEL cells (165) is concomitant with an increase in gravin expression (Figure 7) that is localized to ruffles, lamellapodia and filopodia, areas of the cell involved in cell adhesion and motility. In addition, loss of an adherent phenotype upon transformation of REF52 fibroblasts with an SV40 derivative, or Rat-6 fibroblasts transformed with a constituitively active *ras* or *src* oncogene, is coincident with the down-regulation of clone72 (164), or SSeCKS (162) respectively. The loss of SSeCKS/clone72 expression upon fibroblast transformation also suggests that SSeCKS/clone72 may be a type II tumor suppressor molecule (162). This concept agrees with the suppression of mitosis by cell-cell contact in confluent fibroblasts and the associated increase in gravin expression (172). This is contrary to an oncogenic state where inhibitory mitotic cues from cell-cell or cell-matrix interactions are over-ridden with an associated loss of adherence (100).

The mechanism of regulation of cell adhesion/motility or tumor suppression by type II tumor suppressors is not well understood. However, this class of tumor suppressors are cytoskeletal components that link membrane and cytoskeletal events, and this suggests that changes in cellular architecture may affect the mitotic competency of a cell (94,100). The effect on membrane linked cytoskeletal organization of the cell by type II tumor suppressors could modulate a cell's repertoire of potential responses to extracellular initiated controls on the cell cycle. These effects may be due to; 1) effects on cytoskeletal integrity/rigidity, 2) restricting the effective sphere of influence of second

messenger/signaling systems, or 3) by altering the organization of second messenger/signaling components in relation to downstream components. The functional regulation of the membrane-cytoskeleton by type II tumor suppressors can be controlled at the transcriptional level as with SSeCKS/clone 72 in rat fibroblast transformation, or through post-translational mechanisms such as protein phosphorylation (94,100,162,164). Protein phosphorylation is emerging as an important post-translational mechanism of regulating membrane/cytoskeleton linking proteins.

The regulatory effects of phosphorylation on cytoskeletal interactions has been well studied both *in vitro* and *in vivo*. For instance, the actin filament bundling activity of MARCKS and GAP43 *in vitro* are both regulated by PKC phosphorylation (173). *In vivo,* activation of PKC induces alterations of actin and vinculin organization in green monkey kidney cells (174) and induces changes in the cytoskeleton of microglia cells coincident with phosphorylation of vimentin (175). *In vitro* and *in vivo* studies indicate that plectins interactions with lamin B and vimentin are regulated by PKA and PKC phosphorylations (176). Neurite outgrowth, a form of cell motility, is modulated by PKC and PKA (177). Microinjection of free PKA catalytic subunit into fibroblasts causes changes in the distribution of vimentin containing intermediate filaments similar to distributive changes observed during mitosis (178).

It is intriguing to think that gravin may be exerting its regulatory effect on cell adherence and mitosis by localizing PKA and PKC. These PKA and PKC phosphorylations of membrane-cytoskeletal components could cause changes in the cell similar to those discussed above. In addition, gravin contains potential membrane/cytoskeleton binding regions and is also regulated by phosphorylation and could thus be a substrate for the very kinases it localizes (166). The analysis of these membrane and cytoskeleton binding regions will be the focus of the next chapter.

CHAPTER THREE FIGURE LEGENDS

CHAPTER 3, FIGURE 1. The Sequence of Gravin. Sequence homology between gravin and SSeCKs/clone 72. Identical residues are indicated and amino acids are presented in the one letter code.

CHAPTER 3, FIGURE 2. Sequence comparison Gravin/AKAP 250 and identification of functional domains. **A)** Depicts the similarity between AKAP250, the original gravin fragment and SSeCKS/clone 72. The likely RII-binding site and prospective PKC-binding sequences are boxed. **B)** Sequence homology between gravin (residues 1540-1553) and the RII-binding sequences of two other AKAPs are indicated. Boxed areas represent conserved amino acids. **C)** A helical-wheel representation of gravin/AKAP 250 residues 1540-1553 drawn as an α-helix of 3.6 amino-acids per turn. The shaded area represents the hydrophobic residues and the boxed area represents the hydrophilic side-chains.

CHAPTER 3, FIGURE 3. Recombinant Gravin fragments specifically bind RIIα. Recombinant fragments of gravin were expressed in E. coli using the pET16b bacterial expression system and purified on Histag resin. **A)** The first and last residues of each fragment are indicated and the sequence of the putative RII-binding site is presented. **B)** Purified protein (10 μg) was separated by electrophoresis on 10% (w/v) SDS-PAGE and stained with Coomassie blue dye. Fragment sizes are indicated above each lane. **C)** Purified protein (1 μg) was separated by electrophoresis on 10% (w/v) SDS-PAGE, electrotransferred to PVDF membranes, and RII-binding proteins detected by a solid-phase binding assay using ^{32}P-radiolabelled RIIα as a probe in the presence **D)** or absence **C)** of excess (10 μM) gravin 1537-1563 peptide.

CHAPTER 3, FIGURE 4. Affinity measurement of Gravin/RIIα interaction. The binding affinity of RIIα 1-45 for the gravin 1526-1780 fragment was measured by surface plasmon resonance. The gravin fragment was immobilized on the surface of the IAsys (Fison) cuvette by a protocol described in the methods section and incubated with RIIα over a range of concentrations (25 to 150 nM). **A)** Extent measurements (measured in arc seconds) showing the binding profiles for selected concentrations of RII interacting with the immobilized gravin fragment. **B)** The measured on rates (M-1 sec-1) are plotted against RII 1-45 concentration.

CHAPTER 3, FIGURE 5. Gravin binds and inhibits PKC. Recombinant fragments of gravin were expressed and purified as described in the methods. **A)** The amino- and carboxyl-terminal residues of each fragment are indicated and the location of polybasic regions in the full-length gravin sequence is indicated by the black boxes. **B)** Purified protein (2 μg) was separated by electrophoresis on 10% (w/v) SDS-PAGE and stained with Coomassie blue. The fragments are indicated above each lane. **C)** Proteins (1μg) were electrophoresed, transferred to nitrocellulose and PKC-binding proteins were detected by a solid-phase binding assay using partially purified PKC as the probe as described in the methods. **D)** PKC-binding assay in the presence of excess (1 μM) AKAP79 32-51 peptide. **E)** The PKC inhibition profile of the gravin 265-556 fragment over a range of concentrations (0.1 - 10 μM) using the EGF receptor peptide as a substrate.

CHAPTER 3, FIGURE 6. Induction of Gravin expression in HEL cells. HEL cells were grown in RPMI 1640 medium with and without 40 nM PMA for 18 hours. The supernatants of control and treated lysates (25 μg) were separated by electrophoresis

on a 4-15% SDS-PAGE gradient gel and electrotransferred to nitrocellulose filters. The sample sources are indicated above each lane. **A**) Gravin was detected by western blot using affinity purified antibodies raised against residues 1130-1780 of the recombinant protein. **B**) PKC-binding proteins were detected by the overlay assay using a partially purified rabbit brain PKC preparation as a probe and a monoclonal antibody that recognizes both α and β PKC. **C**) RII-binding proteins were detected by the RII-overlay using rabbit anti-RII to detect immobilized RII. The migration position of gravin is indicated by an arrow.

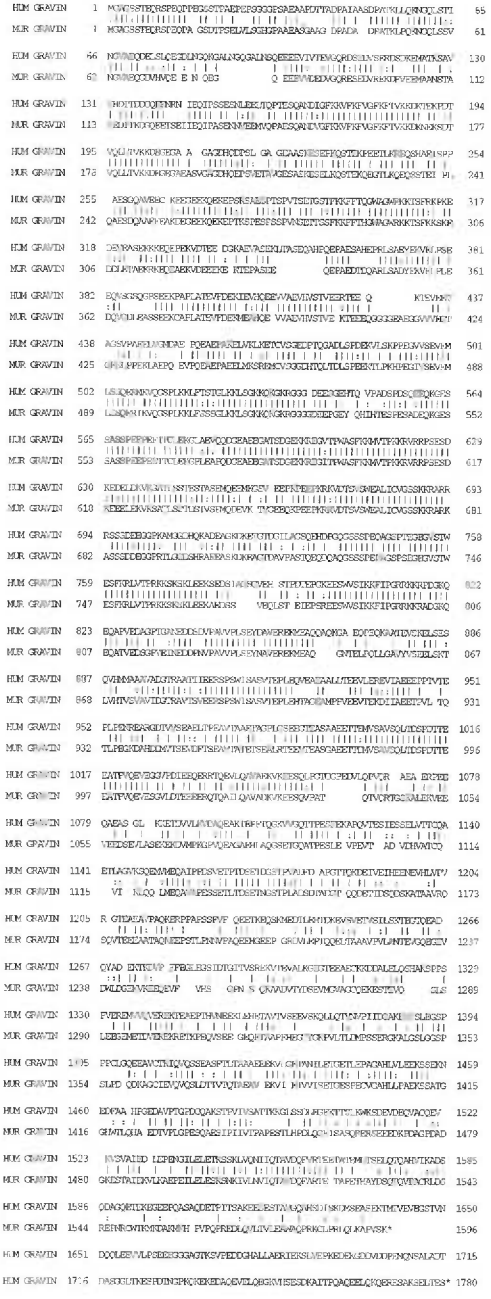
CHAPTER 3, FIGURE 7. Gravin in HEL cells. HEL cells were grown on coverslips in the presence of 40 nM PMA for 18 hr. Cells were fixed with 3.7% formaldehyde and permeabilized with 100% acetone at -20°C. **A & C**) The actin was stained with rhodamine phalloidin and **B&C**) gravin was stained with affinity purified antibodies (0.5 μg/ml) and detected with a fluorescein conjugated secondary antibody (1:100). **D**) Specific staining was not detected with preimmune serum. Fluorescence detection was conducted on a Lietz Fluovert-FU confocal photomicroscope with a 63/1.4 N.A. OEL PL lens.

CHAPTER 3, FIGURE 8. The subcellular distribution of Gravin. HEL cells were seeded on coverslips and grown in the presence of PMA for 18 hr stained with affinity purified gravin antibodies as described in the methods. Fluorescence detection was conducted on a Lietz Fluovert-FU confocal photomicroscope with a 63/1.4 N.A. OEL PL lens. Panels A-D portray 1 micron confocal sections through an individual cell.

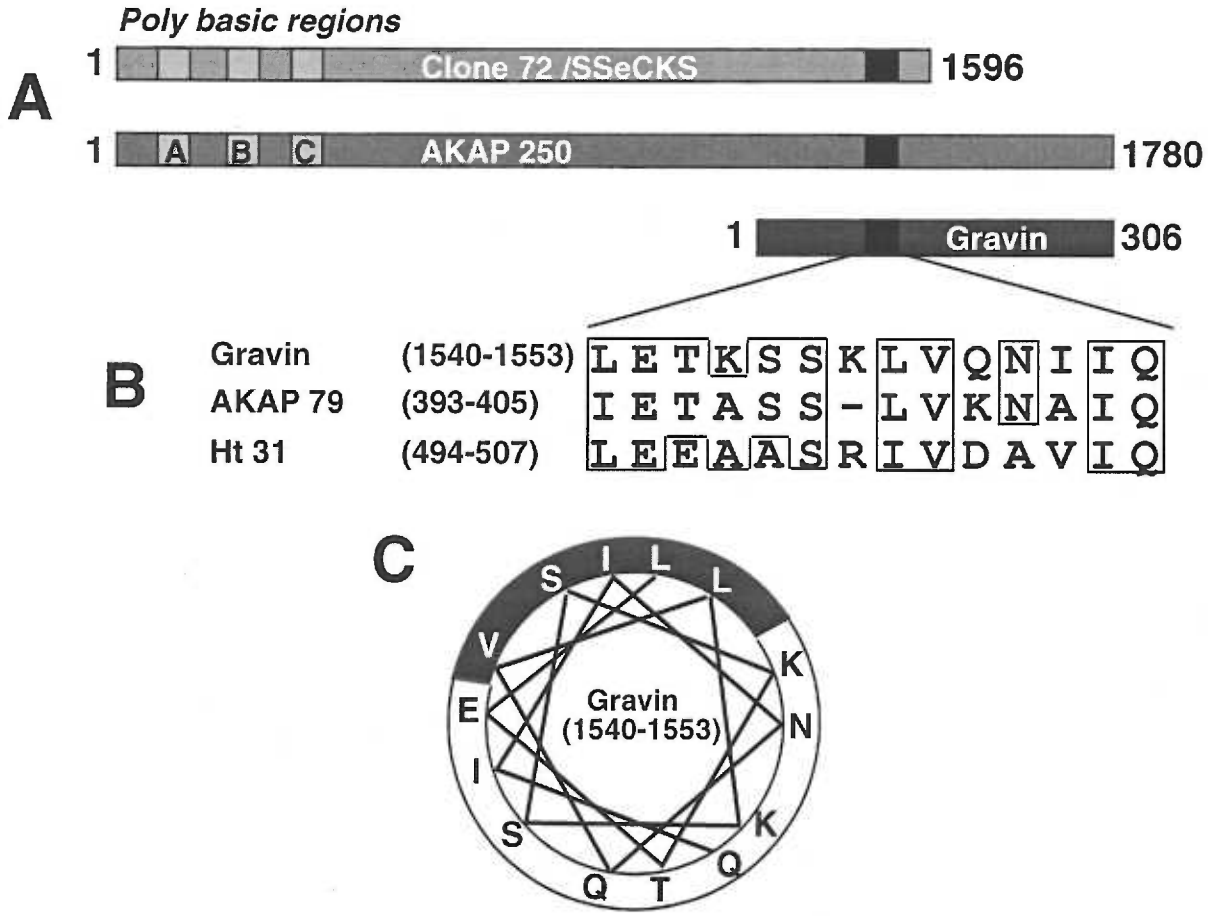
CHAPTER 3, FIGURE 9. Fluorescent detection of *in situ* RII-AKAP complexes. HEL cells were seeded on coverslips, grown in the presence of PMA for 18 hr, fixed with 3.7% formaldehyde and permeabilized with 100% acetone at -20°C. **A**) Gravin was detected immunochemically as described in the methods. **B**) Unoccupied RII-binding sites were detected by an *in situ* overlay procedure that is described in the experimental methods. Detection of anchored murine RIIα was achieved by indirect immunofluorescence using a Texas red conjugated secondary antibody. **C**) Double staining of RII and gravin was displayed by superimposing images from the same focal plane in panels A and B. **D**) Cross reaction of the anti-murine RII antibody with the endogenous human RII was analyzed.

CHAPTER 3, FIGURE 10. Immunoprecipitation of the Gravin signaling complex. Supernatants of HEL cell lysates grown in the presence of 40 nM PMA were incubated with 15 μg of anti-gravin antibodies or 15 μg of preimmune IgG. Immunoprecipitations were performed as described in the experimental methods. Control and immunoprecipitated fractions (5 μg) were separated by electrophoresis on a 4-15% SDS PAGE gradient gel and electrotransferred to nitrocellulose filters. The sample sources are indicated above each lane. **A**) Detection of gravin by western blot using affinity purified gravin antibodies. **B**) Detection of PKC-binding proteins by the overlay procedure. **C**) Detection of RII-binding proteins by overlay using affinity purified antibodies to detect the immobilized murine RIIα. **D**) Catalytic subunit of PKA eluted with 1mM cAMP from the immunecomplexes detected using affinity purified antibodies to the C subunit of PKA.

CHAPTER 3, FIGURE 11. Co-purification of the Gravin-RII complex. A) The R subunits of PKA were purified from HEL cell lysates by affinity chromatography with cAMP-agarose. **B**) Gravin was detected immunochemically in fraction eluted from the column in buffer containing 75 mM cAMP.



CHAPTER 3, FIGURE 1



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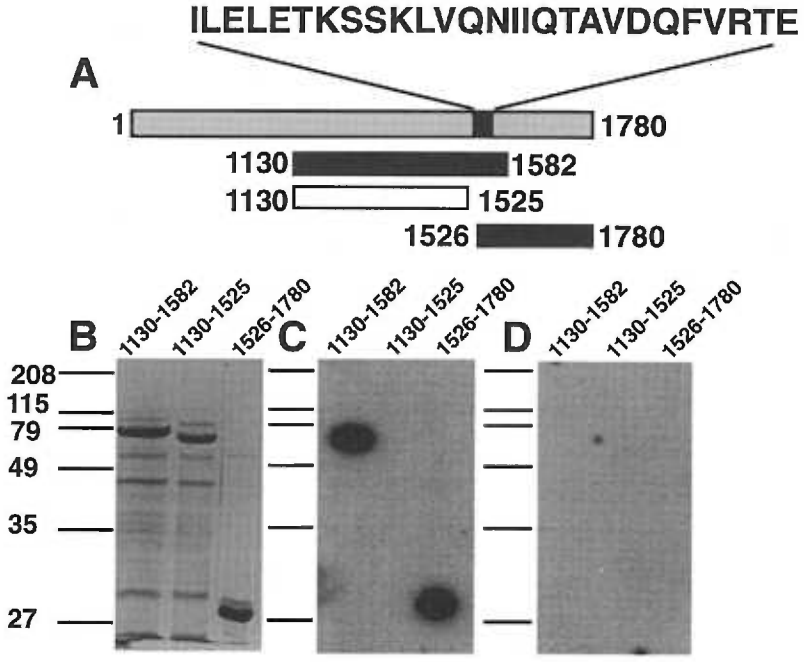
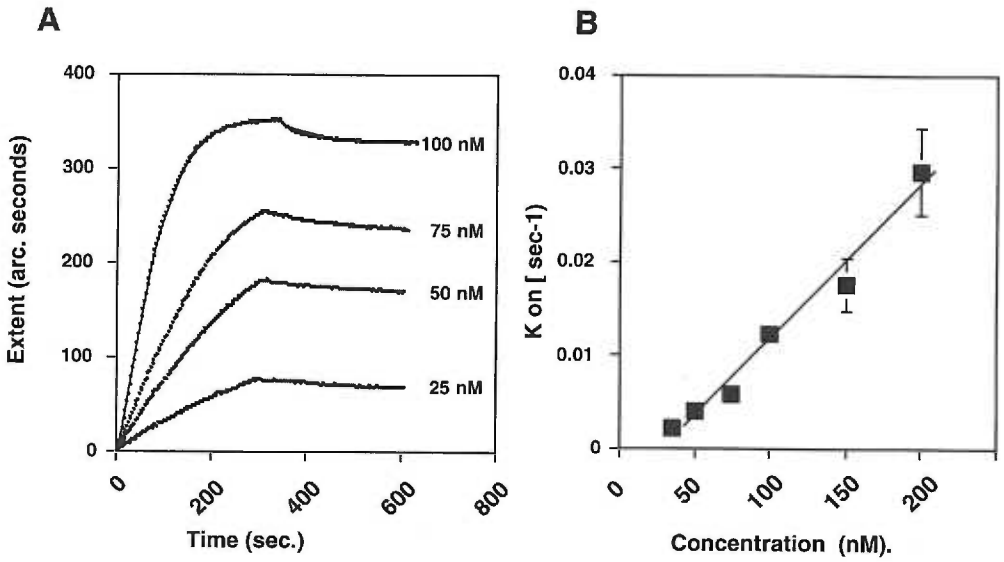


CHAPTER 3, FIGURE 3



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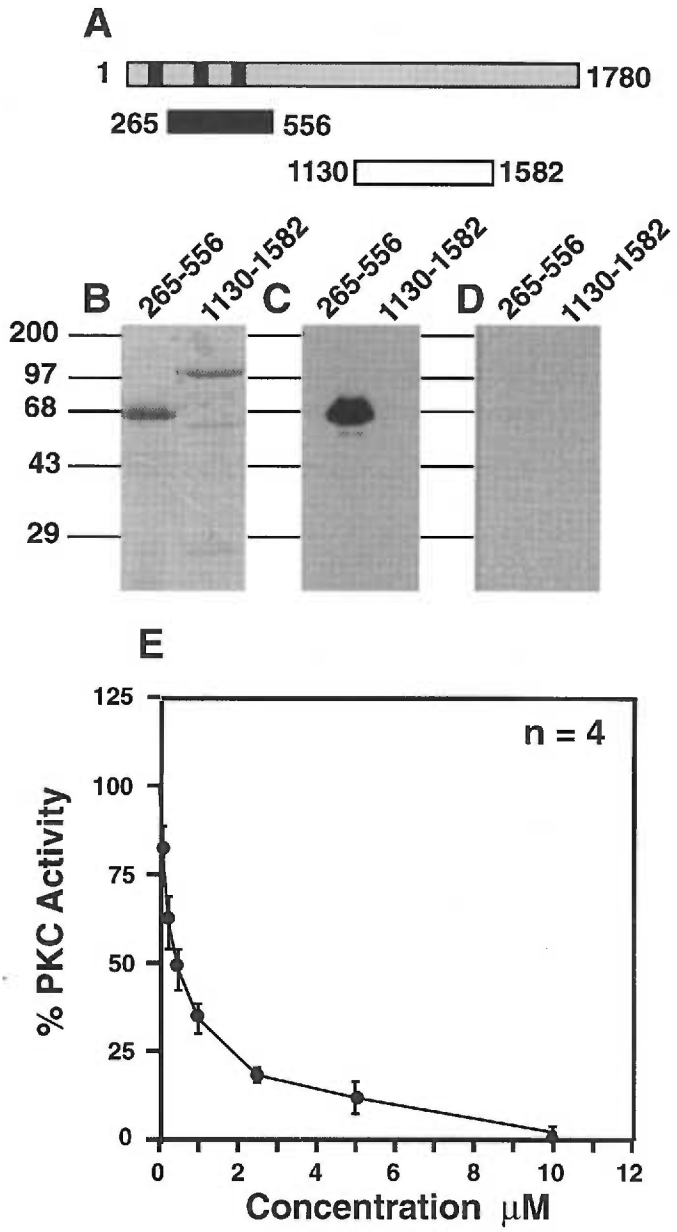
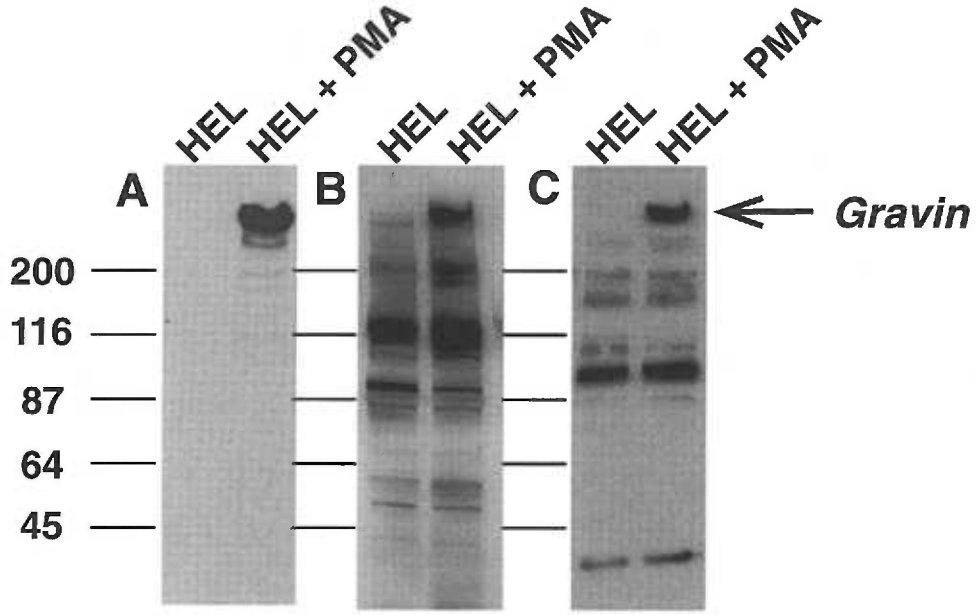


CHAPTER 3, FIGURE 5



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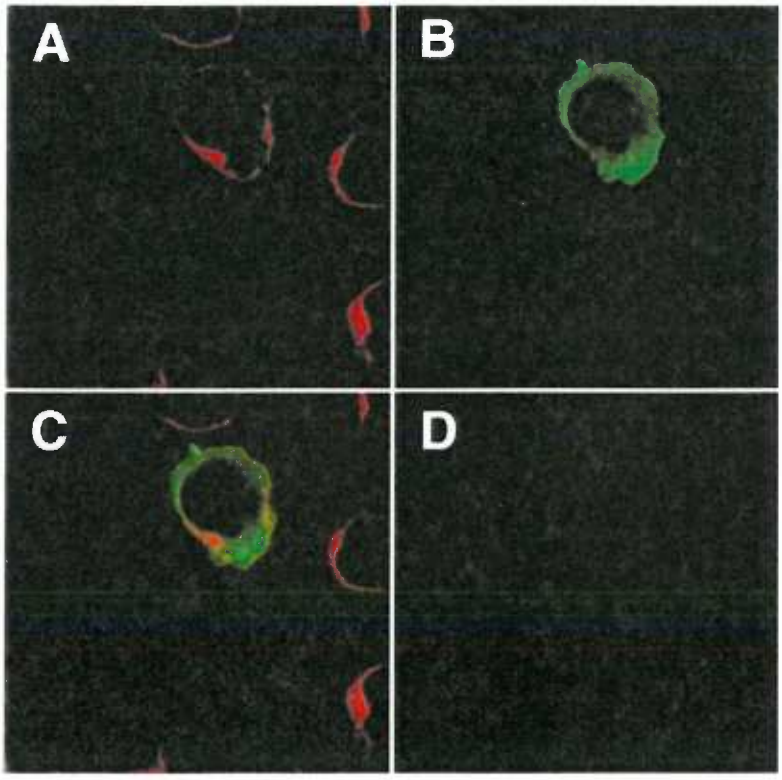
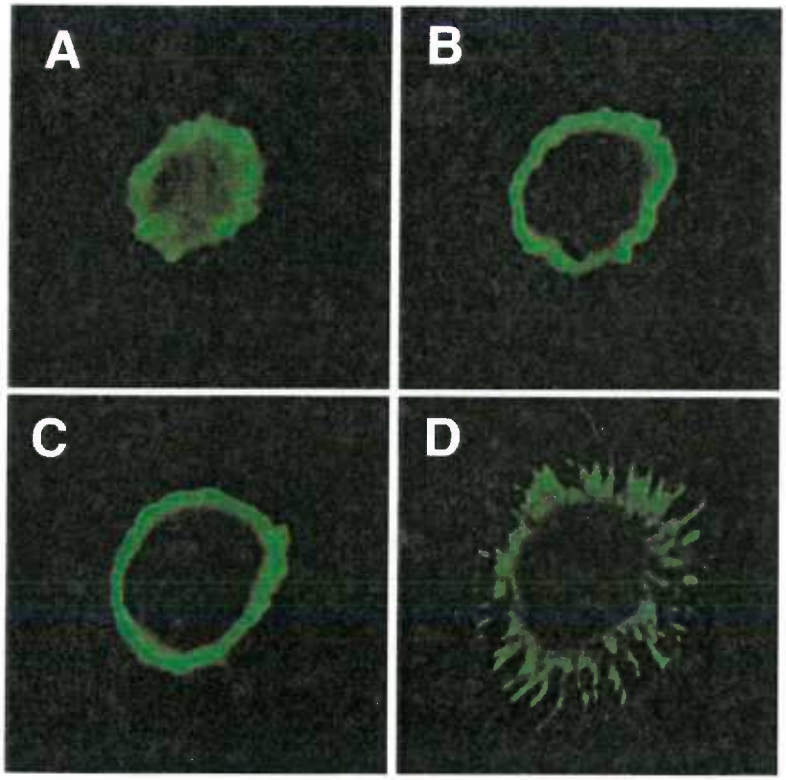
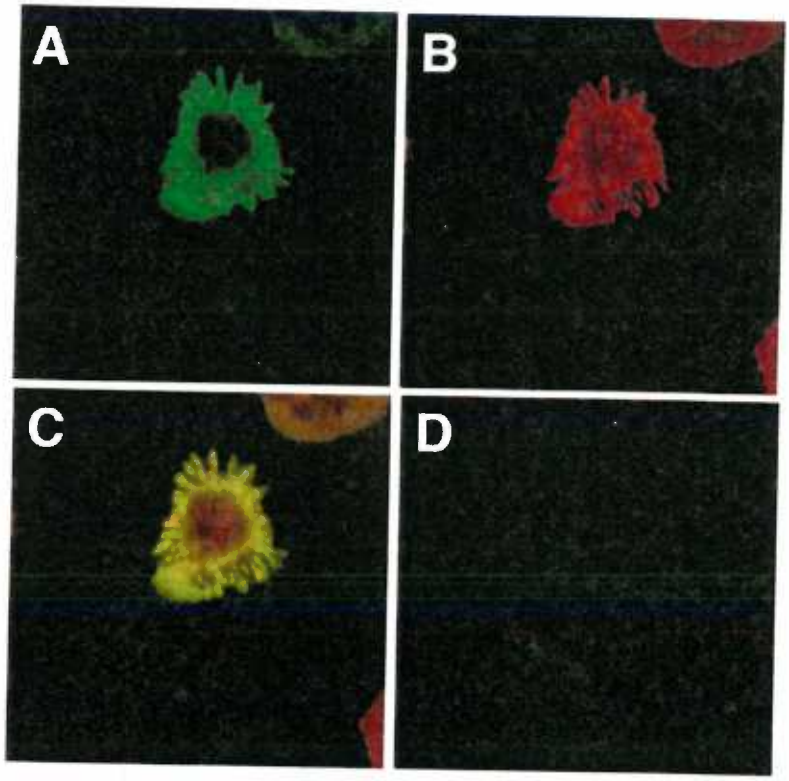


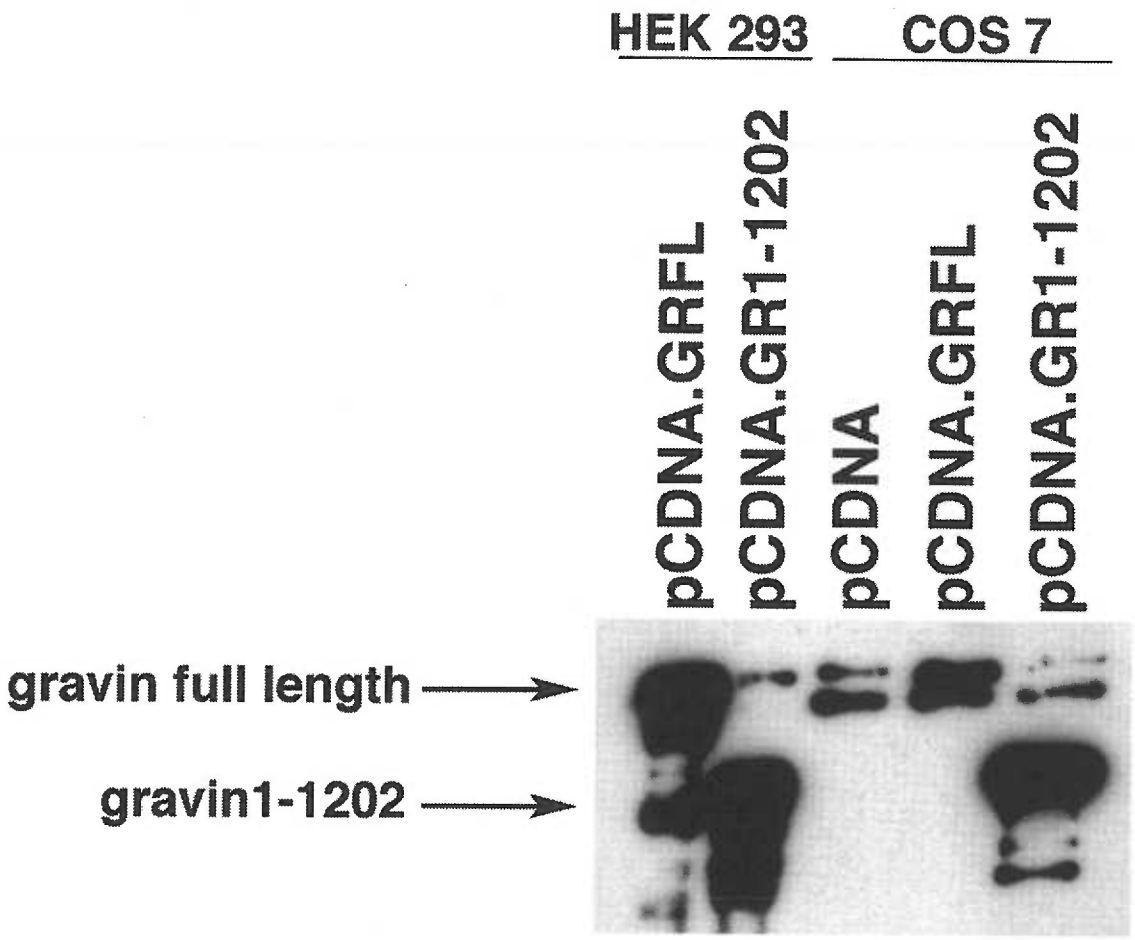
CHAPTER 3, FIGURE 7



CHAPTER 3, FIGURE 8



CHAPTER 3, FIGURE 9



CHAPTER 3 FIGURE 12

CHAPTER FOUR

Characterization of the amino terminal polybasic regions of gravin for PIP2 and F-actin binding and their role in subcellular targeting in gravin.

INTRODUCTION

Several studies have suggested that PKA is not randomly distributed throughout the cell (39,179). Differences in particulate versus soluble ratios of the RII subunit have been observed in different cell or tissue types (34,41,180,181); additionally, changes in these fractionation ratios following stimuli of some cell types has been observed (182). Furthermore, immunocytological studies of the subcellular RII distribution have shown distinct spatial (40,41) and temporal (65) subcellular distributions in different cell or tissue types. These independent observations have culminated in the identification of a class of proteins, *A kinase anchoring proteins* (AKAPs). AKAPs are proposed to control the subcellular distribution of RII (136). Many such proteins have been identified, their genes cloned, and their interaction with RII characterized (42,43,51,65,70,71,73,79,183).

The binding of RII to the AKAP determines the subcellular distribution of the type II PKA holoenzyme. However, it should be noted that all cell types examined to date have more than one AKAP and not all the RII binding sites of any particular AKAP within a cell will be occupied (136). Consequently, the composite subcellular distribution of RII in a cell is determined by the variety of AKAPs expressed and the population of RII binding sites occupied within any one AKAP type. Precisely which RII binding sites are occupied within any particular AKAP type and how this is regulated is not well understood (136).

Inherent in the anchoring hypothesis is that the AKAP must itself be localized within the cell (136). The subcellular locales of many AKAPs have been identified. For instance, AKAP95, is localized to the nucleus (65), mAKAP to the endoplasmic/sarcoplasmic reticulum (67), AKAP84 to mitochondrion (70), AKAP85 to golgi structures (81), and AKAP350 to the centrosome (82). These subcellular distributions are proposed to be determined through a targeting domain contained within the AKAP protein (136). Interaction(s) with other biomolecules by the AKAP targeting domain(s) will determine the

subcellular distribution of AKAPs, and therefore the subcellular distribution of the bound type II PKA. Several targeting domains on AKAPs have been determined. MAP 2 is known to be targeted to microtubules through its microtubule binding region (MTBR) (184), AKAP 79 is targeted to the plasma membrane by the interaction of three amino terminal basic rich regions with acidic phospholipids (57), and AKAP 18 is targeted to the plasma membrane through three acylation sites in its amino terminus (79).

We recently cloned an AKAP called gravin (183). Immunocytochemical analyses of the subcellular distribution of gravin in human erythroleukemic cells (HEL) a macrophage cell type, COS7, and human embryonic kidney (HEK293) cell lines indicates gravin to have a complex cellular distribution and is localized to multiple locations throughout a single cell. Gravin is part of the cortical cytoskeleton, extending into the lamellapodia, filopodia, membrane ruffles, and microvillar structures. Gravin is also concentrated in a perinuclear fashion, with sparse non-uniform cytoplasmic and occasional stress fiber staining. In addition, gravin expression levels and subcellular distribution appear to change during the cell in relation to the cell cycle and in response to various external stimuli (166).

Biochemical evidence suggests some possible targeting motifs in the primary sequence of gravin. Gravin is approximately 25% acidic in amino acid composition. Six clusters of polybasic residues (Fig. 1, designated A-F) in the amino terminus are identifiable within this acidic background. Homology of several of these polybasic clusters with targeting regions from other proteins suggest that the polybasic regions may be involved in the targeting of gravin. Residues 165-183 of gravin (region A) share 45% identity with the effector region of MARCKS (residues 156-173), including conservation of key lysine and phenylalanine residues (108) (Fig. 2A). Additionally, gravin has three internal repeats, residues 609-621, 758-770, 803-815 (regions D, E, F) that are homologous with residues 79-91 of AKAP79, residues 77-89 of growth-associated protein (GAP-43), and residues

32-43 of neurogranin (Ng) (57) (Fig. 2B). These regions in MARCKS, AKAP79, GAP-43, and Ng contain lipid and/or actin binding activities and effect the subcellular distribution of the full length protein (57,108,185,186). Thus, it seems reasonable that the homologous regions of gravin may have similar binding activities and effect a subcellular distribution respective of these binding activities. In addition, gravin contains a consensus myristoylation signal that may also contribute to its localization.

MARCKS is targeted to the membrane cytoskeleton through the interaction of its effector domain with actin and acidic phospholipids (173,187). Phosphorylation of the MARCKS effector domain by PKC disrupts the binding to actin and acidic phospholipids and leads to a redistribution of MARCKS to a perinuclear region (173,188,189). In addition, the interaction of MARCKS with actin and acidic phospholipids is blocked by the binding of calmodulin (CaM) to the effector domain (173,188). CaM also inhibits the phosphorylation of the MARCKS effector domain by PKC (167). The homologous region of gravin will be examined for its ability to exhibit similar biochemical and cellular targeting properties.

Gravin also has three internal polybasic repeats which have extensive homology with a region in AKAP79, GAP 43 and Ng (57). This region in AKAP 79 has been shown to bind to acidic phospholipids (57). In particular, this region binds phosphatidylinositol-4,5-bisphosphate (PIP_2) and this interaction is implicated in the targeting of AKAP79 to the cell membrane (57). Regulation of the binding of AKAP 79 to PIP_2 by PKC phosphorylation and CaM binding, as measured by the release of AKAP 79 from subcellular membrane fractions, is similar to that observed with MARCKS (57,188).

GAP-43 and Ng are both neuronal proteins that associate with the plasma membrane (185,190). These proteins share a common property of binding CaM with higher affinity

in the absence of calcium than in the presence of calcium (191). This is in stark contrast to the observed behavior of the polybasic region of MARCKS (192). However, like MARCKS, phosphorylation by PKC releases the binding of CaM to GAP-43 and Ng (191,193). In addition, the polybasic CaM binding region of GAP-43 binds actin filaments (194). The F-actin binding activity of the polybasic region of GAP-43 is regulated by PKC phosphorylation (194). However, like GAP-43 CaM binding, the regulation is in stark contrast to that seen with MARCKS. Phosphorylation of MARCKS relieves actin binding, whereas phosphorylation of GAP-43 stimulates actin binding (194).

The aim of the present study was to identify both the targeting regions on gravin and the biomolecules these regions bind. A deletion analysis approach was used to identify minimal regions of gravin that have wildtype targeting. Additionally, regions A and D-F were analyzed for proposed binding functions predicted from their homologies with other membrane-cytoskeleton proteins. The contribution of these regions to the targeting of gravin was also analyzed. Specifically, these two regions were assayed for actin and acidic phospholipid binding activities. These regions were also heterologously expressed using either a myc epitope tag or as a GFP fusion to determine if they co-distributed to the actin based membrane-cytoskeleton in a cellular background. Actin binding activity was not detected using either A or D-F in a co-sedimentation assay. However binding of gravin polybasic regions, A-F, D-F, and B-C to acidic phospholipids in a co-sedimentation assay was detected. Immunocytochemical analysis identified the amino terminal half containing the polybasic regions as sufficient for wildtype targeting, but no subregion within this larger region could be attributed to a dominant role in the wildtype distribution pattern. In conclusion, we have identified that gravin's amino terminal polybasic regions contribute to its membrane cytoskeleton distribution; however we were not able to assess any single polybasic region as sufficient on its own to target gravin to cytoskeletal and membrane regions.

MATERIALS AND METHODS

Generation of gravin expression constructs. Constructs for procaryotic expression were subcloned into pET21D. This vector contains a T7 tag that allows for detection of recombinant proteins by a commercially available monoclonal antibody when a gravin specific antibody is not available. A 2532 b.p. fragment, encompassing domains A-F (residues 1-844) was amplified by PCR using primers 21D5'-1 and 21D3'-844, digested with BamH1 and Xho1, and subcloned into BamH1 and Xho1 digested pET21D (21D5'-1:CGAGCGGATCCGCGCCGGGAGCTCC, 21D3'-844: CTCGTCCTCGAGGGCCGGGACATCAGA). A 708 b.p. fragment representing domain A (residues 9-245) was amplified by PCR using the primer pair 21D5'-9 and 21D3'-245, digested with BamH1, and subcloned into BamH1 digested pET21D (21D5'-9: GCGGATCCAGCGCAGCCCGGAGCAGC, 21D3'-245: GCGGATCCTCACGCTTCAGGGTC). A 771 b.p. fragment encompassing domains D-F was amplified by PCR using primers 21D5'-587 and 21D3'-844, digested with BamH1 and Xho1, and subcloned into BamH1 and Xho1 digested pET21D (21D5'-587: GCACTAGGATCCAGGATGGGGAAGCTG). Eucaryotic constructs were subcloned into either pCDNA3.1, pCDNA3.1/MycHis or pEGFP vectors. A 5682 b.p. EcoR1 and Xba1 restriction fragment containing a full length gravin ORF was subcloned into EcoR1 and Xba1 digested pCDNA3.1 and pEGFP(N1). A 3797 b.p. EcoR1 and Kpn1 restriction fragment representing domains A-F (residues 1-1202) was subcloned into EcoR1 and Kpn1 digested pCDNA3.1(-)A/MycHis and pEGFP(N1). A 734 b.p. fragment encompassing domain A (residues 1-245) was amplified by PCR using the primer pair MH5'-1 and 21D3'-245 (MH5'-1: CAGCGAATTCGGAGGAGCCATGGGCGCCGGGAGCTCCACCGAG), digested with EcoR1 and BamH1, and subcloned into EcoR1 and BamH1 digested pCDNA3.1(-)A/MycHis vector. A 768 b.p. fragment containing domains D-F (residues 591-844) was

amplified by PCR using the primer pairs MH5'-591 and 21D3'-844 (MH5'-591: CAGGGAATTCGGAGGAGCCATGGCTGAAGAAGGAGCTACTTCC), digested with EcoR1 and Xho1, and subcloned into a EcoR1 and Xho1 digested pCDNA3.1(+)A/MycHis vector. All vectors were sequenced using an ABI automatic sequencer.

Procaryotic expression and purification of recombinant proteins. Procaryotic expression constructs were transformed into BL21(DE3)pLysS *E. coli* strain. A 1:50 dilution of an overnight culture was made into 1-2 liters of LB broth. This was grown to O.D. 0.3, IPTG was added to 1 mM to induce expression of the protein, and the bacteria were grown an additional 3-4 hours. The bacteria were collected, pelleted by centrifugation, and resuspended in 1X BB (20 mM HEPES 7.4, 500 mM NaCl, 5 mM imidazole). The protease inhibitors AEBSF, leupeptin/pepstatin, and benzamidine were added and the bacteria suspension lysed by sonication. The lysate was cleared by centrifugation at 15,000 x g, the supernatant collected, and then filtered through a 0.2 μM syringe filter. The filtered supernatant was added to a pre-equilibrated and charged Ni column. The column was then washed with 10 column volumes of 1X binding buffer (1X BB), 10 column volumes of 1X wash buffer low (1X BB + 60 mM imidazole), 10 column volumes of 1X wash buffer high (1X BB + 100 mM imidazole), then eluted in 10 column volumes (10 mls) in 2 ml increments with 1X elution buffer (1X BB + 500 mM imidazole). The buffer ot the eluate was exchanged either by extensive dialysis, or under nitrogen pressure in an amicon concentrator. Protein sample concentrations were then determined by the Lowry method, and checked for purity by coomassie stain and western analysis.

Actin binding assay. Powdered monomeric actin (G-actin) was resuspended in G-buffer (10 mM Tris 7.4, 0.2mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT). The monomeric actin was polymerized to filamentous actin (F-actin) by the addition of KCl to 100 mM and $MgCl_2$ to

2 mM (F-buffer) and incubating the solution for 30 minutes at 25°C. This solution was centrifuged at 40,000 x g for 30 minutes in a F-20/micro rotor using a Sorvall RC-26 centrifuge to pellet the F-actin away from the G-actin. The supernatant was removed and the pellet resuspended in 150 µl of F-buffer. The co-sedimentation assay was performed for region A in 100 µl with 5 µM actin and 5 µM gravin protein concentrations with the appropriate buffer (G or F buffer). Region D-F was assayed in 100 µl with 5 µM gravin protein and either 0, 0.5, 5, or 50 µg. The samples were centrifuged at 40,000 x g for 30 minutes in a F-20/micro rotor using a Sorvall RC-26 centrifuge. The supernatant, containing unbound gravin fragments, and the pellet fractions, containing F-actin and bound gravin fragments, were run on SDS-PAGE and analyzed by coomassie or by immunodetection following transfer to a nylon support.

Lipid binding assay. Chloroform lipid solutions were mixed in a glass test tube at fixed molar ratios of phosphatidylcholine (PC) and phosphatidylinositol-4,5-bisphosphate (PIP_2) then dried under a nitrogen stream. The lipid was air dried in a fumehood for an additional two hours to ensure that all the chloroform had evaporated. The lipid was then resuspended to a concentration of 5 mM in a sucrose dense solution (170 mM sucrose, 20 mM HEPES 7.4) by vortexing. This solution was left to sit for 15 minutes at 25°C, followed by five cycles of freeze/thaw in liquid nitrogen. These vesicles were then stored at -20°C until used. The dense sucrose loaded vesicles were thawed on ice and extruded through two 100 nm pore polycarbonate filters using a microextruder (Liposofast, Avestin) to form large unilamellar vesicles (LUVs). The LUVs were then washed with KH buffer (20 mM HEPES 7.4, 100 mM KCl), pelleted at 100,000 x g in a TLA 45 rotor using a Beckman Optima TL centrifuge for 30 minutes at 4°C, and resuspended at a concentration of 1 mM in KH buffer. Binding assays were performed using 10 µL of this lipid solution ([100 µM lipid]), mixed with approximately 1 µg of test protein ($[100\ nM - 1\ \mu M]_F$) in a total volume of 100 µl of assay buffer (20 mM HEPES 7.4, 100 mM KCl, 0.3 mg/ml

BSA, 1 mM DTT). The binding mixture was centrifuged at 100,000 x g in a TLA 45 rotor using a Beckman Optima TL centrifuge for 30 minutes at 4°C. 80 µl was removed as the supernatant containing any unbound protein and the remaining 20 µl, the pellet, contained the LUVs and bound proteins. Both fractions were brought up to 100 µl with assay buffer, SDS-PAGE loading buffer added, and the distribution of gravin protein between supernatant and pellet fractions assayed by 4-15% SDS-PAGE, electrotransfer to a nylon support, and immunodetection using the T7 monoclonal antibody.

Cell culture transfection and immunocytochemistry. Cos 7 cells were seeded onto glass coverslips. 24-48 hours later they were transfected by calcium phosphate precipitation of gravin expression constructs overnight under 5% CO_2 at 37°C. The cells were washed with PBS, fed with growth medium (DMEM, 10% FCS, 1% pen/strep) and grown an additional 48 hours. The coverslips were then washed twice with PBS, fixed with 3.7% formaldehyde/PBS for 5-10 minutes and permeabilized with -20°C acetone for 1 minute. The coverslips were blocked for 30 minutes with a 0.1% BSA/PBS solution at 25°C, washed with PBS, incubated with the primary antibody for 30 minutes in a 0.1%BSA/PBS solution at 25°C (this step omitted for GFP transfectants), washed with PBS, followed by a 30 minute incubation with a FITC conjugated secondary antibody (except for GFP transfectants) and rhodamine-phalloidin for 30 minutes in a 0.1%BSA/PBS solution at 25°C, and a final PBS wash. The coverslips were mounted on glass slides using the Prolong Antifade Kit (Molecular Probes, Eugene, OR). Indirect immunofluorescent staining or intrinsic GFP staining was detected by fluorescent microscopy or laser-scanning confocal microscopy.

RESULTS

Immunocytochemistry of Cos cells. Based on several lines of evidence, gravin has been proposed to link membrane and cytoskeletal events in the cell (88,90,163,166,183). It is expected that gravin would need to interact with membrane, cytoskeletal components or both to effect changes in the membrane/cytoskeleton. Reports of the endogenous cellular distribution of gravin/SSeCKS/clone72 shows gravin to have a complex cellular distribution, being localized to multiple locations throughout the cell (88,90,166,183). Gravin has been shown to be in regions of the cell containing the cortical cytoskeleton, extending into lamellapodia, filopodia, membrane ruffles, and microvilli, at focal adhesion sites, as well as a non-uniform distribution throughout the cytoplasm (88,90,166,183). Moreover, some cells showed a concentration of gravin at a perinuclear region (166). In addition, gravin expression and cellular distribution changes in accordance with the status of the cell in relation to the cell cycle and external stimuli respectively (166,172).

Results from the heterologous expression of full length gravin alone or as a GFP fusion protein in Cos 7 cells affirm results obtained from staining of endogenous protein (Fig. 3 and 4). We detected gravin in regions of the cell containing the cortical cytoskeleton, extending into lamellapodia, filopodia, membrane ruffles, and microvilli, as well as staining in the cytoplasm. Co-staining with rhodamine-phalloidin to highlight actin filaments indicated a restricted co-distribution with gravin (Fig. 3C, 4). The co-distribution of gravin with actin was limited to regions of the cortical cytoskeleton including microvillar, filopodial, and lamellapodial structures (Fig. 3C, 4). Gravin was infrequently co-distributed with along stress fibers and at focal contacts (Fig. 4). A fragment of gravin containing all six polybasic regions, residues 1-1292, gave staining comparable to endogenous staining patterns (data not shown). However, expression of region A and region D-F as myc epitope tagged fragments were not interpretable as both constructs were nonspecifically targeted to the nucleus (data not shown).

Acidic phospholipid binding activity. As mentioned above, a number of membrane/cytoskeleton linking proteins contain lipid and actin binding regions, or at least one multifunctional region that can bind both (95,108,128,185,194,195,196). A subgroup of these proteins share a region of similar biochemical composition that is short in length (~20 residues) and rich in basic residues. MARCKS, GAP-43, and ezrin are examples of these types of proteins (108,185,194,195,196). A co-sedimentation assay has been used to analyze the lipid binding activity of some of these proteins as well as functionally different molecules. The assay is performed using large unilamellar vesicles (LUV) of increasing mol percent of a test lipid, PIP_2 in this case, in a PC background (57). The LUVs are loaded with sucrose to make them dense, so they may be pelleted at 100,000 x g. Proteins are mixed with the LUVs, and proteins that bind to PIP_2 will pellet with the sucrose dense LUV upon centrifugation at 100,000 x g (57).

As mentioned above, gravin contains multiple regions with similarities to demonstrated lipid binding regions. Some of these similarities are based on sequence homology (A,D-F), while others are based on a more general biochemical resemblance (B,C). Region A is homologous to the effector region on MARCKS (Fig. 2A) (108,197). This region has been shown to bind preferentially to acidic phospholipids. The three repeats of gravin, D-F, have homology to the phospholipid binding region on AKAP79, GAP-43, Ng (Fig. 2B) (57,185,186,198). These regions, like the region found in MARCKS, also preferentially bind acidic phospholipids (57,185,186,198). Regions B and C of gravin, the PKC binding region of gravin, though they do not have homology with any known phospholipid binding regions, also are rich in basic residues (183). These regions may also participate in lipid binding.

Increasing concentrations of PIP_2, from 0 to 10 mol percent, in a PC background were used to test which if any of the polybasic regions of gravin may bind to phospholipid

membranes. A polypeptide containing all six polybasic regions (A-F) was tested for binding to PIP2. The A-F polypeptide was found to associate with PIP2 as determined by its PIP2 dependent re-distribution to the pellet fraction upon centrifugation (Fig. 5A). Fragments representing A, B-C, and the D-F regions of gravin were then tested for lipid binding activity in the same assay. Region A did not show any re-distribution with increasing PIP2 mol percent (data not shown). However region B-C and region D-F both mimicked the PIP2 binding activity of the A-F polypeptide (Fig. 5B, 5C). These regions of gravin pelleted at about 5 mol % PIP2, which is consistent with what has been observed for the association of AKAP79 polybasic regions with PIP2 and is within the physiological concentrations of PIP2 found in cells (57)

Actin binding assay. Many proteins that are proposed to link the cytoskeleton and membrane have been identified as containing an actin, lipid or multifunctional region that binds both (108,128,194,199,200,201). A subgroup of these actin binding regions are short (~20 residues) and rich in basic residues (108,194,200). One experimental approach used to assess actin binding activity of membrane/cytoskeleton linking proteins has been a co-sedimentation assay (112,173,194,199,201,202). F-actin and proteins bound to F-actin can be purified away from other proteins by pelleting the complex by centrifugation at $\geq$ 40,000 x g. Gravin contains six basic rich regions in the amino terminus that are potential actin binding domains. Four of these clusters (A,D,E,F) have homology with proteins with demonstrated actin and lipid binding regions (173,194). Region A has homology with the effector region of MARCKS (Fig. 2A), and three repeats D-F have homology with the actin binding region of GAP-43 (Fig 2B) (173,194). The other two polybasic regions, B and C, though they have a similar cluster of basic residues they do not have any homology with known lipid and actin binding. Recombinant gravin polypeptides of A and D-F were assayed for actin binding activity by a co-sedimentation assay. Neither of these polypeptides showed any F-actin binding activity using this assay (Fig. 6).

DISCUSSION

Observations surrounding the cell type, subcellular distribution, and protein homologies suggest that gravin plays a role in modulating membrane cytoskeleton events (88,90,183). Furthermore, six regions in the amino terminus of gravin that are rich in basic residues share similarities with known actin/lipid binding domains and may be involved in regulating membrane cytoskeleton events. We have used a combination of immunocytochemistry and *in vitro* actin binding and lipid binding assays, to assess possible contributions from the amino terminal polybasic regions in targeting of gravin and mediating changes in membrane cytoskeleton dynamics.

Heterologous expression of full length gravin constructs, as detected by indirect immunofluorescence through anti-gravin antibodies and by direct fluorescence of a GFP fusion protein, has shown a cellular distribution pattern corresponding to the distribution of endogenous gravin. Endogenous gravin exhibits some cytoplasmic distribution, is concentrated at a perinuclear region, and was occasionally found along stress fibers and at focal adhesion sites (166). In addition, gravin is found concentrated at the cortical cytoskeleton, including lamellapodia, filopodia, membrane ruffles, and microvilli, all specialized extensions of the cell cortex. The cell cortex consists of the plasma membrane and the underlying cortical cytoskeleton made up of F-actin microfilaments. The cortical cytoskeleton is an actin based structure that provides organization and mechanical integrity to the membrane, but is plastic enough to accommodate the dynamic processes of endocytosis, exocytosis, motility, cell growth and differentiation, among other processes involving changes in the cortex of the cell (92,93,94,95,96). Gravin's staining pattern is consistent with previous observations that led to the proposal that gravin regulates membrane cytoskeleton events (88,90,183).

Co-staining with rhodamine phalloidin to highlight F-actin indicated some overlapping distribution with gravin at the cell cortex, in cell extensions, and areas of cell attachment to substrata. The cortical staining of gravin at the cell edge forms a submembranous patchwork, whereas actin staining tended to be found uniformly around the whole cell and was also denser and thicker. This aspect of gravin staining is reminiscent of membranous patchwork staining observed for calpactin and merlin, two other phospholipid- and actin-binding proteins (203,204,205,206). Gravin is also concentrated in cell extensions such as filopodia, lamellapodia, membrane ruffles, and microvilli. This aspect of gravin distribution is reminiscent of the distribution observed for merlin, particularly in membrane ruffles, and of a carboxy-terminal actin binding fragment of ezrin and villin, particularly in the microvilli (93,195,204,206,207,208). Filopodia, lamellapodia, membrane ruffles, and microvilli are all highly plastic, actin rich structures that require a highly coordinated, regulated membrane cytoskeleton (96). The co-distribution of gravin with cortical actin, particularly in the cell extensions, correlates well with the proposed role of gravin as a membrane-cytoskeleton protein, a subcellular region where gravin may interact with both actin and phospholipid. The limited co-distribution of gravin with actin at stress fibers and focal adhesion sites is also in agreement with the proposed actin and phospholipid binding properties of gravin (94,209).

However, cytoplasmic and perinuclear staining of gravin was not coincident with actin staining. This may be due to alternative functions of gravin not related to regulating membrane cytoskeleton plasticity. Microfilaments are known to have associated motors that may play a role in transport of biomolecules or membranous structures (210). It is interesting to speculate that the proposed membrane binding domains of gravin may be involved in the binding of membranous vesicles and their shuttling between the Golgi, a perinuclear structure, and the cortex of the cell. For example, endocytic vesicles have been shown to accumulate in the Golgi (211). The membrane-cytoskeleton protein GAP-43 also

exhibits a Golgi distribution in early stage primary hippocampal neurons and in Cos 7 cells (212). It has been suggested that GAP-43 might achieve its membrane localization through binding and co-transport with other membrane targeted molecules in the Golgi (212). Alternatively, many of the actin- and lipid-binding activities of membrane-cytoskeleton proteins containing polybasic actin/lipid binding domains are regulated by phosphorylation (57,173,188,191,193). Phosphorylation of MARCKS in the polybasic domain induces its migration from the cell cortex to a perinuclear region (189). SSeCKS has been shown to be phosphorylated *in vitro* by PKC in polybasic domains B, C, D, and E (166). The perinuclear distribution of gravin may represent a phosphorylated species that has been induced to migrate as seen with MARCKS (57,173,188,189,191,193). This regulated movement may not be mutually exclusive with possible shuttling functions described above.

Immunocytochemical analysis of a deletion construct of gravin, residues 1-1202, had a cellular distribution comparable to full length gravin. Like full length gravin this construct co-distributes with actin in areas of the cellular cortex associated with membrane events requiring a plastic membrane-cytoskeleton (92,93,94,95,96). This construct contains the six polybasic regions in the amino terminus of gravin that share similarities with actin/lipid binding regions of other proteins. The subcellular distribution of this fragment suggests that the polybasic regions target gravin and may regulate membrane-cytoskeleton events. Four of the six polybasic regions show extensive homology with known actin binding regions. Region A has homology with the effector region of MARCKS, and regions D-F have homology with the phospholipid- and actin-binding domain of GAP-43 (173). Immunocytochemical analyses of regions A and D-F expressed as myc epitope fusions were tested to assess the individual contribution of these two regions to aspects of gravin co-staining with actin (e.g. cell edge versus ruffle versus stress fiber). Both of these regions of gravin were however targeted to the nucleus.

The mislocalization of the A and D-F fragments is explainable since many proteins targeted to the nucleus contain a bipartite basic nuclear localization signal (NLS) that is similar to the polybasic regions (A, D, E, and F) of gravin. Similar polybasic regions, such as in MARCKS, AKAP79, N-WASP and plectin, exhibit false nuclear targeting when expressed as a fragment of the full length (128,213). These pseudo-NLS must be masked or prevented from functioning as NLS in the full length since these proteins are not ordinarily found in the nucleus. Other explanations of physiological importance must however be considered. First, as detected by immunocytochemistry, gravin is occasionally targeted to a perinuclear region and phorbol ester treatment of rat fibroblasts can induce the cortically localized SSeCKS to move to a perinuclear region (166). This movement to a perinuclear region may occur through the binding of gravin's polybasic regions by components of the nuclear shuttling apparatus. Alternatively, it has been suggested that the NLS of cytoplasmic proteins may act as a sink for NLS-binding proteins (NLSBP) (213). Thus, the polybasic regions of gravine may have an active role as regulators of the transport of cytosolic proteins to the nucleus. However, the contribution of these two regions (A, D-F) in targeting gravin to actin based structures of the cell remains uncertain.

Several groups have proposed that gravin performs a regulatory role at the membrane cytoskeleton (163,183). The immunocytochemically detected subcellular distribution of gravin is consistent with this proposed function (166,183). Gravin is proposed to effect its regulatory role through interactions with acidic phospholipids and F-actin via amino terminal polybasic domains. *In vitro* binding analyses were performed to assess the ability of these regions to bind acidic phospholipid and F-actin.

Using a co-sedimentation assay, a bacterially expressed 844 residue polypeptide encompassing polybasic regions A-F was shown to bind to LUVs in a PIP_2 dependent

manner . Based on homology to acidic phospholipid binding regions of AKAP79, GAP-43, and MARCKS, regions A and D-F were considered to be the source of this binding activity (185,187,188,198). When these regions were individually tested D-F bound the LUVs in a PIP_2 dependent manner, whereas region A failed to bind. Regions B-C, though not having homology with known lipid binding domains, did share an overall biochemical similarity with the AKAP79, MARCKS, and GAP-43 polybasic regions. These regions were also tested and did exhibit binding to the LUVs in a PIP2 dependent manner. A similar result was obtained with AKAP79 (57). AKAP79 has three polybasic regions in its amino terminus but only one polybasic region has homology with regions of other proteins known to possess lipid binding activity (57). Yet the other two regions contributed comparably to binding PIP2 loaded vesicles as the GAP-43 homology region (57). These studies suggest that at least five out of the six polybasic regions in the amino terminus of gravin may contribute to an interaction with membranes through binding of acidic phospholipids. These interactions may contribute in part to the localization of gravin to cortical regions and also to the Golgi in the cell. In addition to targeting of gravin to membranes this interaction may be playing an active role in regulating membrane and cytoskeletal architecture. The MARCKS lipid binding region has been shown to organize PS and PIP2 into lateral domains on vesicles (214,215). This type of lateral localization of the membrane could serve to facilitate coordination of signaling activators (PIP2), substrate (MARCKS), and enzyme (PKC) (214,215). An additional level of regulation in this type of organizational system may take place as several PIP_2 binding regions have also been shown to inhibit PIP_2 specific PLC (197). PLC catalyzes the release of DAG and IP_3, both upstream activators of PKC (171). Thus gravin may form a coordinated signaling unit by localizing PKC to lateral domains of PIP_2 and PS formed by the amino terminal polybasic regions of gravin (183).

PIP2 is also an important regulator of cytoskeletal reorganization. The binding of PIP2 by a variety of actin binding proteins regulates the filamentous state of actin in the cell (96,128). For example, ezrin/radixin/noesin (ERM), a family of membrane-cytoskeleton linkers, anchors F-actin to the membrane by the simultaneous binding of F-actin, and a PIP_2 dependent interaction with transmembrane proteins (CD44,CD43,ICAM-2) (93,96,195,216). Gravin may be another example of an actin binding protein that utilizes PIP2 to modulate cytoskeletal architecture. However, analysis of gravin in an actin co-sedimentation/binding assay showed no actin binding capacity for region A or a polypeptide containing regions D-F. Thus, either the polybasic domains of gravin do not bind actin, or the negative result was due to a technical shortcoming and gravin does bind actin. The latter explanation is preferred for several reasons. First, in reports of actin binding by MARCKS, GAP-43, ezrin, and calpactin using this co-sedimentation assay, the test proteins were purified from eucaryotic sources (194,201,203,217). Bacterially expressed proteins, such as the ones used in my experiments, may not be adequate due to improper folding or lack of co- or post-translational modification. For instance, the binding of actin by GAP-43 is dependent on phosphorylation (194). Secondly, the gravin-actin interaction may be of lower affinity compared to other proteins assayed in this way. Lower affinity actin binding protein interactions may be tested by centrifugation at a lower g-force. F-actin will not pellet at 10,000 x g. However, if the F-actin has been bundled by an actin bundling protein it will pellet at 10,000 x g . Thirdly, the actin binding site(s) of gravin may be regulated by autoinhibiton, similar to those of ezrin and vinculin. The actin binding site of ezrin and vinculin are masked, or inhibited, through an intramolecular interaction (93,113,218). Monomeric ezrin is held in a "dormant" state through an intramolecular interaction of its N-terminal ERM Association Domain (N-ERMAD) and a C-terminal ERM Association Domain (C-ERMAD) (93,218). The "activation" mechanism of ezrin is unclear, but may involve tyrosine phosphorylation, as EGF treatment of A431 stimulates the tyrosine phosphorylation and subsequent oligomerization of ezrin,

unmasking the actin binding site (93,196). Vinculin, like ezrin, is held in a "dormant" state through a head-tail intramolecular interaction (113). The binding of PIP2 by vinculin dissociates the head-tail interaction, unmasking the talin- and actin-binding sites (199). An expanded examination of gravin fragments or the use of a peptide may highlight any intramolecular inhibition of F-actin binding occuring within gravin. The addition of co-factors, such as calcium or PIP_2 in the assay may also help to overcome any autoinhibitory effects (199,203). Lastly, the interaction of ezrin with α–actin may be of a low affinity and can be abolished at physiological salt concentrations (150 mM) (200). This led to the discovery that ezrin may have a specificity for the β– and γ–actin isoforms (201). It may be that gravin has an isoform specificity for actin, as α–actin was the only isoform assayed.

In conclusion, immunocytochemical analyses of gravin suggest that the polybasic regions in the amino terminus are sufficient for the wildtype targeting of gravin in Cos 7 cells. This cortical targeting is consistent with the suggested regulatory role of gravin in the membrane cytoskeleton of the cell (88,90,166,183). *In vitro* binding analyses were performed with PIP2 and actin to resolve the contribution of these molecules to the intracellular distribution of gravin and their influence on the regulatory role of gravin at the membrane cytoskeleton. The lipid binding assays suggest that gravin binds to acidic phospholipids, whereas gravin showed no actin binding activity. Thus the distribution of gravin and its regulatory effect on the membrane cytoskeleton may be restricted to the binding to acidic phospholipids. However for the reasons discussed above, the influence of actin binding on gravin distribution and membrane-cytoskeleton regulation can not be ruled out. The localization and function of gravin at the cortex of the cell is affected by phosphorylation as the corresponding basic regions of SSeCKS have been shown to be phosphorylated *in vitro* (166). This may account for the observed cytoplasmic and Golgi distribution. Conversely, the Golgi staining could be through the tethering gravin to the Golgi as well as

the plasma membrane through the lipid binding domains of gravin. Given the translocation of SSeCKS from the cortex to a Golgi/perinuclear distribution following stimulation of fibroblasts with phorbol ester and the observed Golgi staining of gravin, the two diverse staining patterns (cortical vs. Golgi) may reflect two ends of a functional pathway of shuttling lipid based vesicular structures between the Golgi and the plasma membrane.

CHAPTER FOUR FIGURE LEGENDS

CHAPTER 4, FIGURE 1 Diagram Of Potential Targeting Regions In Gravin.

CHAPTER 4, FIGURE 2 Homology Alignments Of Conserved Polybasic Regions In Gravin. A) Alignment of gravin and MARCKS. Identical residues are boxed. **B)** Alignment of three repeats of gravin with AKAP79 (and homologs), GAP-43, and Ng. Boxed are identical or conserved residues.

CHAPTER 4, FIGURE 3 Indirect Immunofluorescent Detection Of Full Length Gravin In COS 7 Cells. Cos 7 cells grown on coverslips, were transfected with a full length gravin cDNA (pCDNA.GRFL), fixed, permeabilized, and incubated with a gravin specific antibody. They were subsequently incubated with FITC conjugated secondary antibody and rhodamine-phalloidin and the coverslips mounted on slides. Gravin and actin localization was viewed by indirect immunofluorescence. **A)** Cos 7 cell showing example of perinuclear/Golgi distribution and membrane ruffle gravin distribution. **B)** Cos 7 cell showing example of cortical and membrane ruffle gravin distribution. **C)** Cos 7 cell showing co-distribution of gravin and actin. Gravin and actin show no co-distribution at the Golgi. There is some overlapping distribution at the cortical cytoskeleton. A microvillar structure is identified to demonstrate the co-distribution of gravin and actin in these small cell extensions.

CHAPTER 4, FIGURE 4 Direct Immunfluorescent Detection Of Full Length Gravin In COS 7 Cells. Cos 7 cells grown on coverslips, were transfected with a full length gravin cDNA (pEGFP.GRFL), fixed and permeabilized. They were subsequently incubated with rhodamine-phalloidin and the coverslips mounted on slides. Gravin localization was viewed by direct fluorescence of the GFP moiety and actin by indirect immunofluorescence. **A)** Cell showing co-localization of gravin and actin at microvillar and stress fiber structures. **B)** Cell showing co-localization of gravin and actin at stress fibers, membrane ruffles, and microvillar structures. Note the absence of co-localization of gravin and actin at the Golgi.

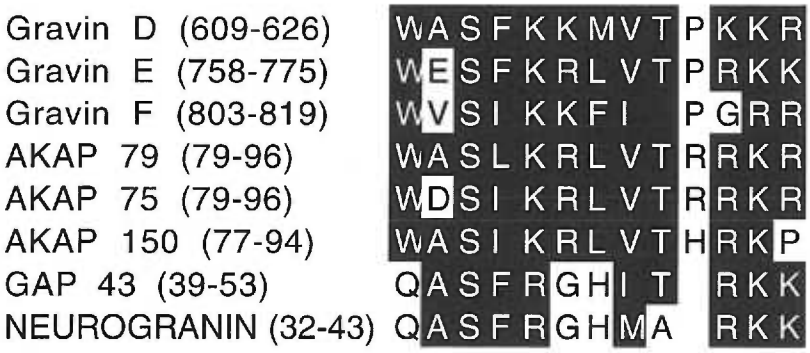
CHAPTER 4, FIGURE 5 Detection Of Binding Of Recombinant Gravin Fragments Of Phosphatidylinositol 4,5-Bisphosphate By C0-Sedimentation Assay. Large unilamellar vesicles (LUV) of increasing phosphatidylinositol-4,5-bisphosphate (PIP2) mol percent in a phostphatidylcholine background were loaded with a dense sucrose solution. LUVs were mixed with the test proteins and centrifuged. The sucrose dense vesicles and protein would pellet. Any protein found to shift its distribution to the pellet in correlation with increasing PIP2 amounts gave an indication of PIP2 binding. **A)** Test of a gravin fragment containing all six polybasic regions (A-F). **B)** Test of a gravin fragment containing the AKAP79/GAP-43/Ng repeats (D-F). **C)** Test of PKC binding region containing two polybasic regions (BC). *N.L.* refers to a control tube that contained the test protein and no lipid.

CHAPTER 4, FIGURE 6 Actin Co-Sedimentation Assay. F-actin and recombinant gravin fragments. This solution was centrifuged to pellet the F-actin.

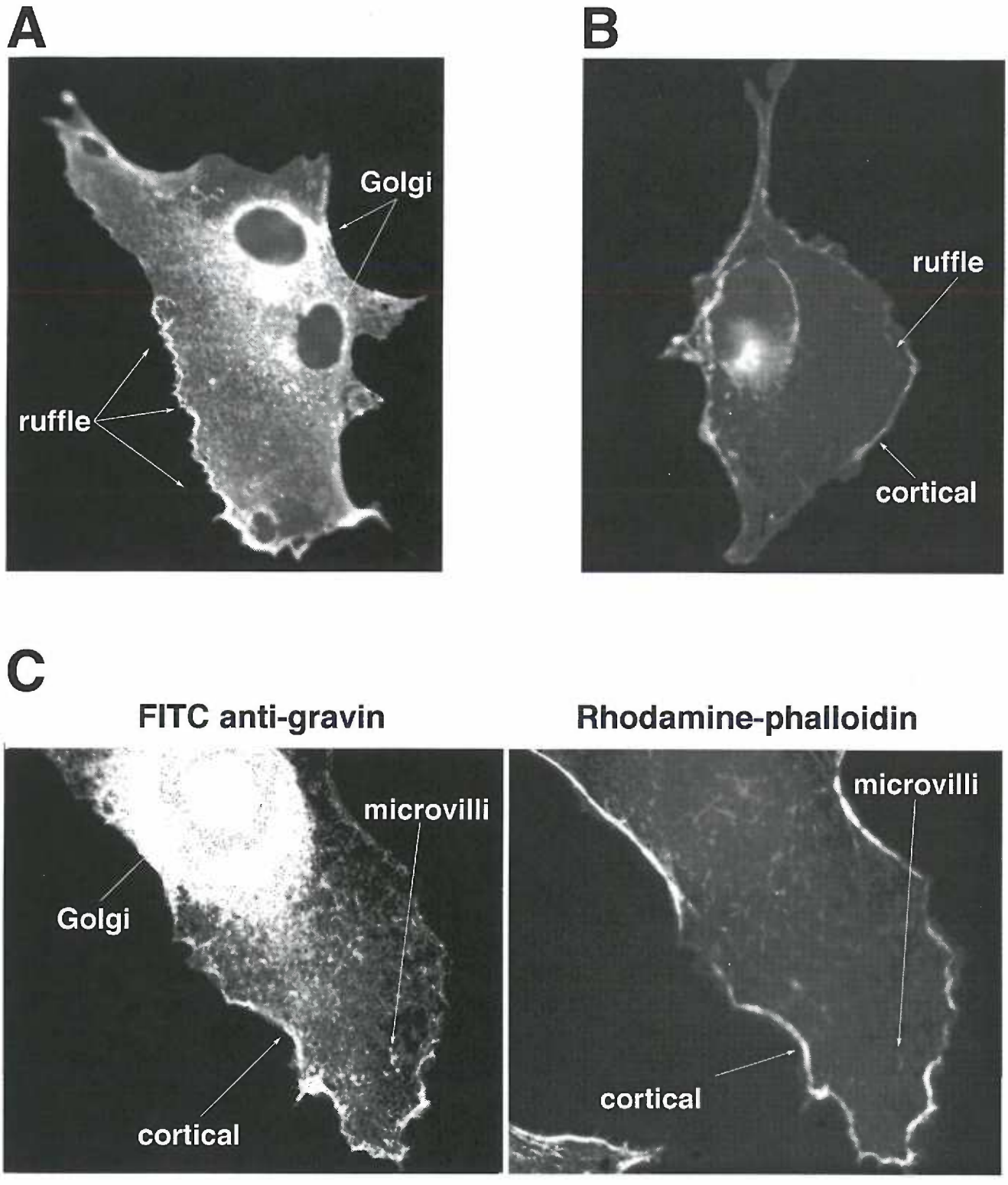
Proteins that bind or bundle actin will co-sediment with the F-actin. G-actin or no actin were used to assess how much of test protein sedimented on its own. A) Analysis of region A, the MARCKS homology region. 5 micromolar actin and protein were mixed, centrifuged, and the supernatants and pellets analyzed by immunodetection using a T7 monoclonal antibody. B) Analysis of the three repeats (D-F) that have homology with AKAP79, GAP-43, and Ng. In this experiment increasing amounts of either G- or F-actin were mixed with 5 micromolar gravin D-F, the sample centrifuged to pellet only F-actin and bound proteins. The pellets were analyzed by immunoblot using the T7 monoclonal antibody.

A

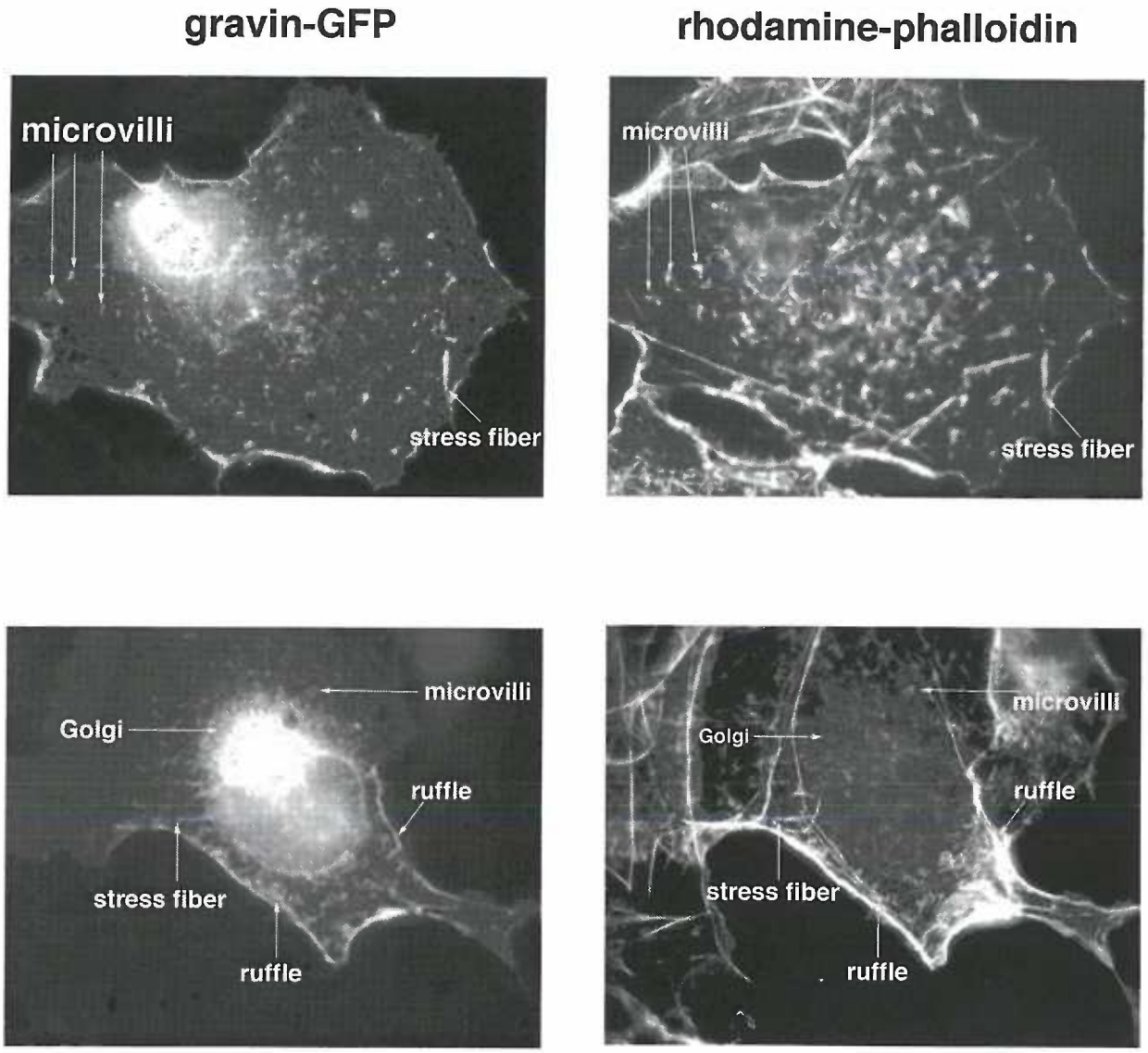
B



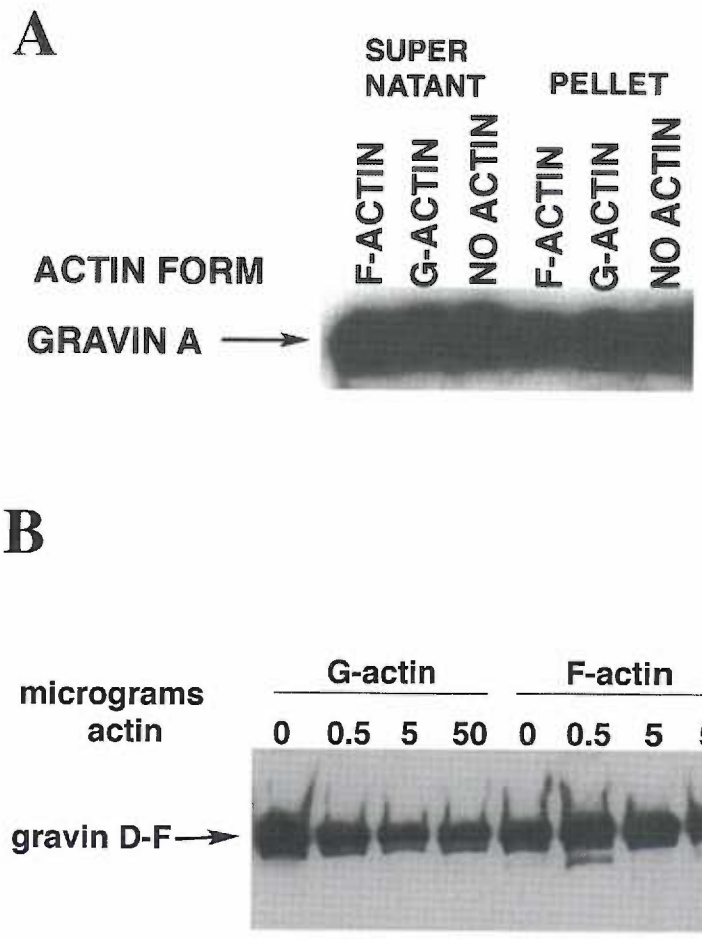
CHAPTER 4, FIGURE 2



CHAPTER 4, FIGURE 3



CHAPTER 4, FIGURE 4



CHAPTER 4, FIGURE 6

CHAPTER FIVE

Discussion and References

DISCUSSION

We have identified a novel AKAP, gravin, that is associated with myasthenia gravis, an autoimmune disease of the neuromuscular junction, where the nicotinic acetylcholine receptor is the primary autoantigen (85). A search for additional autoantigens associated with the neuromuscular junction was performed by screening an expression cDNA library with anti-sera from patients with myasthenia gravis. This led to the identification of a previously unidentified cytoplasmic protein that was given the name gravin (88). Gravin expression was found to be specific to adhesive tissue culture cells (88). Additionally, an immunohistochemical tissue survey found gravin expressed in fibroblasts, neurons, and neural crest derived cells, all cells that are migratory or are developmentally derived from migratory cells (90). An immunocytochemical analysis of gravin subcellular distribution suggests gravin is a part of the cortical cytoskeleton by immunocytochemistry (88). The sum of these observations suggest that gravin may modulate cell motility and adhesion by regulating the membrane cytoskeleton.

We have furthered the understanding of gravin by showing that gravin functions as an AKAP by binding PKA. We have additonally been able to show that gravin functions as a signal transduction organizing complex or scaffolding protein which also binds PKC (183). The primary sequence comprising the RII binding domain of gravin was identified by assaying a series of recombinant fragments in the overlay assay (183). This data was confirmed when a peptide representing the primary sequence of the predicted RII binding domain was synthesized and shown to be able to block the binding of RII to gravin in the overlay (183). A PKA-gravin complex was shown to be present in cells through two independent methods of co-purification. Gravin co-purified with RII by cAMP-agarose chromatography. Secondly, PKA was found in an immunoprecipitation of gravin An overlay technique was used to demonstrate the binding of PKC to gravin and to identify the

region of gravin that binds PKC. A kinetic analysis also demonstrated that the PKC binding region of gravin also inhibits PKC. The array of PKC isoforms that interact with gravin has yet to be determined and the presence of a gravin-PKC complex in cells has yet to be shown.

The rat homolog of gravin was subsequently cloned independently by two groups, both groups investigating the progression of oncogenicity. One group was studying PKC binding proteins and had identified the major PKC binding protein in REF52 (rat embryonic fibroblasts) cells, whose expression decreased dramatically through a REF cell series that represented progressively transformed cellular phenotypes (163). They eventually cloned the cDNA for this PKC binding protein and refer to it as clone72 (163). The second group was studying rat 3T3 fibroblasts that were expressing a constituitively active form of src that conferred a transformed phenotype on the cell. Using a subtractive library approach they were able to identify several mRNA whose expression was decreased in the transformed phenotype. One of the decreased mRNA was referred to as *s*rc *s*uppr*e*ssed *C k*inase *s*ubstrate (SSeCKS) and is the rat homolog of gravin (162). In accordance with the tumor suppressor role identified by these two groups, gravin expression was also found to be regulated by culture confluency with elevated expression levels in areas of non-confluency, suggesting that gravin expression was regulated by contact inhibition (219). These studies suggest that gravin may be a type II tumor suppressor molecule. Type II tumor suppressors are generally cytoskeletal in nature and participate in the regulation of cellular adhesion and motility which is consistent with proposed cellular functions of gravin based on previous observations of the cellular and subcellular distribution of gravin (100,162,172).

Though several groups agree that gravin is part of the cortical cytoskeleton, gravin shows only a limited co-distribution with actin. In addition to cortical staining, gravin exhibits

prominent Golgi and sparse cytosolic distribution. Additionally, the relative proportion of gravin between these different cellular compartments varies from cell to cell and can also be induced to re-distribute in the cell. The observed mobility of gravin distribution may reflect a functional role of gravin to re-direct PKA or PKC, thereby restricting access to kinase activators or providing access to a new set of target proteins. Likewise, the mobile character of gravin distribution may alter the pools of PIP2, sets of actin filaments, or other binding partners gravin has access to.

The gravin polypeptide contains six polybasic regions that are proposed to bundle F-actin and/or bind to acidic phospholipids. In fact, one region of gravin has homology to the bifunctional actin/acidic phospholipid binding region of MARCKS and another three repeats of gravin have homology to singular regions in AKAP79, GAP43, and Ng that have acidic phospholipid binding and F-actin bundling functions as well as target their respective proteins to discrete subcellular targets (57,173,194). Thus, the polybasic regions of gravin may explain the observed co-distribution with F-actin. However, an attempt to identify the contribution of individual basic regions of gravin to the membrane and F-actin co-distribution of gravin was unsuccessful. The regions of gravin with homology to known membrane and/or F-actin binding capacity, when expressed on their own, failed to co-distribute with either membranes or F-actin, but instead were found in the nucleus. This however was not too surprising since these regions are rich in basic residues and resemble nuclear localization signals. Moreover, a complete analysis of the contribution of the polybasic domains to gravin distribution will be further complicated by the sheer numerical combinations of gravin polybasic domains that can be assembled for analysis. The contribution of the spacing of these polybasic domains and their arrangement in three dimensional space in relation to the native gravin protein may also have an acute affect on the binding activities and gravin localization by these regions. Furthermore, regions B-E of SSeCKS are phosphorylated *in vitro* by PKC (166). Phosphorylation of

the homologous regions in MARCKS, AKAP79, GAP43, and Ng has been shown to alter their F-actin and phospholipid binding activities (57,173,194). Thus the phosphorylation status of five polybasic domains in gravin must also be considered when analyzing their contribution to the subcellular targeting of gravin.

The polybasic regions were then tested to see if they would associate with acidic phospholipids and/or F-actin *in vitro*. We were not able to show an F-actin-gravin complex using an *in vitro* co-sedimentation assay. This result could be interpreted to mean that gravin does not bind F-actin or the result was a false negative. The latter explanation is preferred for several reasons discussed in the results section. Briefly, only one experimental approach was attempted using recombinant proteins. Enough evidence has accumulated to suggest that recombinant proteins may not function in this assay. This may be due to improper folding of the protein in a procaryotic system, or the possibility of a co-factor found in eucaryotic cells not being present. In addition, the phosphorylation status of the polypeptide may influence its F-actin binding activity. However, the homology of the polybasic regions of gravin to F-actin binding regions of several other proteins, coupled with the co-distribution of gravin and F-actin by immunocytochemistry, are motive enough to further pursue experiments to determine if gravin binds to F-actin. The use of eucaryotically expressed protein, analysis of phosphorylated forms, and a wider variety of assays (e.g. F-actin overlays, immunoprecipitation, light scattering, viscosity measurements) should clarify uncertainties concerning F-actin binding by gravin. Moreover, certain of these F-actin binding proteins require co-factors to promote binding or disengage an inhibitory component. This possibility must also be considered for analysis of F-actin binding by gravin.

However, binding of phosphorylated phosphoinositides by recombinant fragments of gravin was detected in a co-sedimentation assay. The co-sedimentation of a recombinant

fragment of gravin containing all six polybasic regions, A-F, with sucrose dense phospholipid vesicles corresponded with increasing PIP2 content of the vesicles. PIP2 binding activity could also be shown for recombinant fragments of gravin encompassing regions B-C, and D-F, whereas no PIP2 binding activity could be ascribed to region A alone. A recent finding showed that binding of acidic phospholipids by AKAP79 requires the presence of two polybasic regions (57). This suggests that the lack of PIP2-dependent association of the A region of gravin with phospholipid vesicles may be due to the insufficiency of a single polybasic region in binding to acidic phospholipid vesicles. Though gravin's PIP2 binding can be attributed to two composite regions, B-C and D-F, the contribution of individual polybasic regions to PIP2 binding by gravin remains to be elucidated. The regulation of PIP2 binding (e.g. phosphorylation, CaM) to gravin and its physiological significance are also yet to be determined.

To better understand the significance of gravin's scaffolding of PKA and PKC, and the effect of the polybasic domains to gravin's complex subcellular distribution, it will be important to elucidate a physiological role for gravin. This function should consider four possible roles for gravin in the cell; **1**) consequences of localization of PKA and PKC, **2**) the binding of PIP2, **3**) possible cytoskeletal interactions and **4**) cellular events involving perinuclear to cortical movements.

1) The localization and redistribution PKA and PKC would greatly alter their access to activators and set of substrates available for phosphorylation. With the understanding that adenylyl cyclase and cAMP phosphodiesterase are themselves localized and that this creates localized gradients of cAMP, it is readily apparent how the localization and redistribution of PKA by gravin could profoundly affect the set of substrates that are phosphorylated by gravin bound PKA. The activation of conventional PKC isoforms is dependent on its interaction with the membrane and the redistribuiton of this enzyme could alter its access to

membrane constituents and thus affect its acitivity and substrate selection. This is the broadest of concerns and may not be excluded from functional considerations involving the binding of PIP2, cytoskeletal elements, and the intracellular movements of gravin.

2) PIP2 is a key source of activators of PKC. An important function of gravin may be to coordinate the localization of PKC, its activators, and substrate through the binding of PIP2 through gravin's polybasic domains. Gravin binds to PIP2, is a PKC substrate, and may affect the localization of PKC. This is similar to what has been observed for the PKC substrate, MARCKS (214,215). MARCKS has been shown to co-localize with concentrations of acidic lipids, PS and PIP2, in lateral domains of membrane vesicles (214,215). This results in a concentration of kinase activators and substrate that could have a significant impact on the PKC signaling pathway. Alternatively, the induction of lateral membrane domains by MARCKS can also inhibit PLC. PLC releases IP3 and DAG, both activators of PKC, and by doing so inhibiting PKC activation. This would also have a significant impact on the PKC signaling pathway. So rather than being focal sites of PKC activation these lateral domains may be organized to restrict the influence of PKC activation on MARCKS and associated proteins. Gravin could act similary to MARCKS by clustering acidic phospholipids into lateral domains, possibly inhibiting PLC. Gravin has six polybasic regions that could potentially interact with PIP2 as discussed above and thus offers the possibility of coordinating a greater number or complexity of components.

3) Gravin may also be serving to regulate the higher order structure of the actin cytoskeleton through directly modulating network connections, the localization of PKA and PKC, or the recruitment of actin structures to areas rich in PIP2. The direct modulation of actin networks may be through the bundling of F-actin by gravin's polybasic regions. The localization of PKA and PKC to cytoskeletal structures may influence cytoskeletal dynamics as regulatory phosphorylations of cytoskeletal elements or enzymes that regulate

the actin network have been observed. The actin bundling activity of MARCKS is regulated by PKC phosphorylation, adducin phosphorylation by PKC inhibits the formation of adducin/spectrin/actin complexes, PKA phosphorylation of RhoA reduces binding and activation of ROKα by RhoA, and PKA phosphorylation of the head region of vimentin induces the dissociation of vimentin based intermediate filaments. As suggested above gravin may serve to form localized concentrations or lateral domains of PIP2 in the membrane. PIP2 has been shown to elicit changes to cytoskeletal structure through its direct interaction with PIP2 binding proteins. For example, the small GTPases Rac, Rho, and CDC42 have been implicated in the formation of lamellapodia, stress fibers, and filopodia respectively. GTP binding proteins become activated when in the GTP-bound state. The transition from the GDP-bound state to the GTP-bound state is catalyzed by guanine nucleotide exchange factors (GEFs). Many of these GEFs are activated by the binding of PIP2 to their PH domains. Thus, the concentrating of PIP2 into lateral domains by gravin may aid in the activation of these GEFs, leading to changes in the actin cytoskeleton.

4) The mobile nature of gravin detected by immunocytochemistry may be indicative of several functional roles of gravin in cells. The sole purpose of gravin's mobility may be in the retargeting of PKA and PKC to new discrete subcellular locations, or in the rearrangment of the actin cytoskeleton, with these events not being mutually exclusive. Alternatively, gravin may participate in the regulation of membrane trafficking of other molecules or membranous structures (220,221). This possibility is the most appealing as it would explain all proposed biochemical functions of gravin. Phosphorylation, PIP2, the actin cytoskeleton, and trafficking between a perinuclear location and the cortex of the cell are all important features of membrane trafficking (220,221).

SUMMARY AND CONCLUSIONS

This thesis has reported the cloning of a novel AKAP gravin that has more importantly been shown to be a second example of a scaffolding protein for broad specificity protein serine/threonine kinases (PKA and PKC). Gravin was also shown to bind to phosphorylated phosphoinositides through amino terminal polybasic domains that had homology to regions of proteins known to exhibit phosphoinositide binding. We have also shown by immunocytochemistry that gravin colocalizes with the cortical actin cytoskeleton and a perinuclear area resembling the Golgi. Furthermore, gravin's distribution appears to be dynamic as cytoplasmic staining between these two regions of the cell can also be identified, and activation of PKC in fibroblasts can trigger the redistribution of cortical SSeCKS to a perinuclear Golgi region. Futher experiments are needed to determine the contribution of individual polybasic domains to acidic phospholipid binding and specificity, as well as to the binding of F-actin. A more detailed immunocytochemical analysis should be performed to further define localization patterns of gravin in relation to 1) cell cycle, 2) culture conditions (e.g. confluency, serum condition, kinase inhibitors, ppase inhibitors), 3) expression levels, 4) effect of various cytoskeletal disrupters, and 5) the determination of gravin's cellular targets. This will enable a more detailed hypothesis concerning the cellular role of gravin's complex subcellular distribution.

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