

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

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Background: Subcellular targeting of protein kinases and phosphatases provides a mechanism for co-localizing these enzymes with their preferred substrates. A recently identified mammalian scaffold protein, AKAP79, controls the location of two broad-specificity kinases and a phosphatase.

Results: We have identified and characterized another mammalian scaffold protein which coordinates the location of protein kinase A and protein kinase C. We isolated a cDNA encoding a 250 kDa A-kinase anchoring protein (AKAP) called gravin, which was originally identified as a cytoplasmic antigen recognized by myasthenia gravis sera. Sequence homology to proteins that are known to bind protein kinase C suggests that gravin also binds this kinase. Studies of binding *in vitro* show that residues 1526–1780 of gravin bind the regulatory subunit (RII) of protein kinase A with high affinity, and residues 265–556 bind protein kinase C. Gravin expression in human erythroleukemia cells can be induced with phorbol ester, resulting in the detection of a 250 kDa RII- and PKC-binding protein. Immunolocalization experiments show that gravin is concentrated at the cell periphery and is enriched in filopodia. Gravin staining is coincident with an AKAP detected by an *in situ* RII-overlay assay, and a PKA–gravin complex can be isolated from human erythroleukemia cells.

Conclusions: We present biochemical evidence that gravin forms part of a signaling scaffold, and propose that protein kinases A and C may participate in the coordination of signal transduction events in the filopodia of human erythroleukemia cells.

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Background

The intracellular transduction of signals from the plasma membrane to specific subcellular compartments is a complex and highly regulated series of events controlling essential physiological processes [1]. Perhaps the most graphic demonstration of the central place signaling pathways have in cellular homeostasis is provided by the many transforming oncogenes that encode mutated signal transduction components, such as low molecular weight GTP-binding proteins, protein kinases or phosphatases [2]. Now that many of these genes have been identified, research has begun to focus on how the enzymes they encode interact to control cellular events. A critical element is the subcellular location of each signaling enzyme [3]. For example, the correct intracellular targeting of kinases and phosphatases directs these enzymes to their preferred substrates and reduces indiscriminate background phosphorylation and dephosphorylation that would otherwise ensue upon basal activation [4].

Targeting of kinases and phosphatases is achieved through association with targeting proteins or subunits (see [3,5,6] for reviews). For example, tyrosine kinase and

tyrosine phosphatase activity can be coupled to downstream cytoplasmic enzymes through adaptor proteins that contain Src homology 2 (SH2) and SH3 domains [7]. Modular adaptor proteins, such as Grb2, p85, IRS-1, Crk and Nck, consist of a single SH2 domain that recognizes certain phosphotyrosyl residues on signaling enzymes, coupled to two SH3 domains that bind to a Pro–X–X–Pro motif (where X is any amino acid) on a separate set of target proteins [7]. Similarly, many phosphodiesterases, phospholipases, kinases, phosphatases and heterotrimeric G proteins are targeted by specific membrane-targeting motifs such as the LIM, C2, pleckstrin-homology and lipid-anchoring domains [8,9]. As a result of these interactions, signaling complexes assemble around receptor kinases or scaffold proteins to mediate cellular processes including growth factor signaling events, insulin action and immune-cell function.

Until recently, kinases and phosphatases stimulated by second messengers were thought to be localized through association with individual targeting proteins. For example, three classes of phosphatase-targeting subunits have been identified which are specific for either protein

phosphatase-1 (PP-1) [5,10], PP-2A [11,12] or PP-2B [13,14]. Likewise, three classes of protein responsible for targeting protein kinase C (PKC) have been identified [6,15,16], whereas compartmentalization of the cAMP-dependent protein kinase (protein kinase A, PKA) is achieved through interaction of its regulatory (R) subunits with a functionally related family of 30 or so A-kinase-anchoring proteins, called AKAPs (reviewed in [17,18]). On the basis of these observations, we have proposed that anchoring proteins confer specificity on serine/threonine kinases and phosphatases, by directing them to discrete subcellular sites where they have restricted access to certain substrates and are optimally positioned to respond to fluctuations in the levels of second messengers [3].

A variation on this theme is the recent identification of multivalent binding proteins that coordinate the location of serine/threonine kinase and phosphatase signaling complexes [19]. For example, the pheromone-mediated mating response in yeast is initiated through a G-protein-linked receptor that activates a yeast homologue of the mammalian mitogen-activated protein kinase (MAP kinase) cascade [20]. This process proceeds efficiently because each enzyme in the cascade is associated with a scaffold protein called Ste5 [21–23]. 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 [20]. Another example of a multivalent binding protein is AKAP79, which targets PKA, PKC and PP-2B at the postsynaptic densities of mammalian synapses [24]. The structure of AKAP79 is modular and resembles that of Ste5, in that deletion analysis, peptide studies and co-precipitation techniques have demonstrated that each anchored enzyme binds to a distinct region of the anchoring protein [14,24]. Targeting of the AKAP79 signaling complex to the postsynaptic densities suggests a model for reversible phosphorylation in that the opposing activities of kinase and phosphatase are co-localized by a common anchoring protein [25].

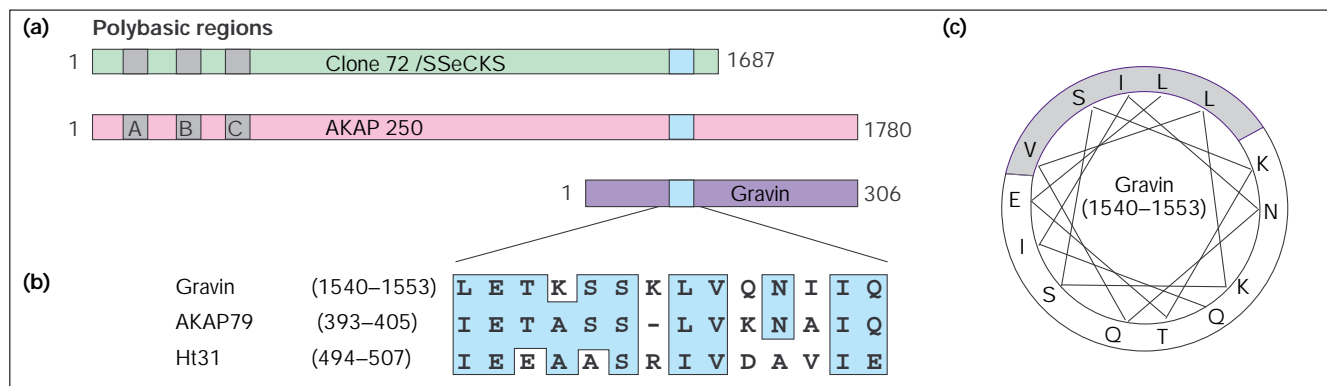
In this report, we describe the cloning and characterization of a second multivalent kinase-scaffold protein called gravin. A fragment of gravin was originally identified as a cytoplasmic antigen recognized by sera from patients with myasthenia gravis [26]. We now show that distinct regions of gravin show homology to AKAPs and proteins that are substrates for, and/or bind to, PKC. Binding studies *in vitro* have mapped the binding sites for both A and C kinases,

Figure 1

The sequence of gravin. (a) The deduced amino-acid sequence of the cDNA encoding gravin. (b) Sequence homology between gravin and SSeCKs/clone 72. Identical residues are indicated by bars, and amino acids are presented in the one letter code. The Genbank accession number for the gravin sequence is U81607.

(a)	
1	MGAGSSTEQRSPPEPPGSSSTPAEPEPSGGGSAEAAAPDTTADPAIAASD 50
51	PATKLLQKNGQLSTINGVAEQDELSLQEGDLNGQKALNQQGALNSQEE 100
101	EVIVTEVGGQDSEVDVSRSDKEMATKSAVVHDIIDDQEQENRNIEQLT 150
151	SESNLEELTQPTSEQANDIGFKKVFVKFGKFTVKKDKTEKPDVQLTV 200
201	KKDGGGGAAGDQDPFSLGAEASKESEPKQSETEKPEETLKRQSHAE 250
251	TSPPAEAGQAVECEKEEKEKPKSASRSTPSVFTSTGTEKPF 300
301	QGWAGWRKKTSPRKPEDDVEASEKKEQEPQKVDTEKDKGAEVASEKL 350
351	ASEQHPQPEVPSAHEPRLSAEYKVELPSEVEQVSGSSEKPAPLAT 400
401	EFVDEKIEVHQEVVAEVHVSTVEERTEKQTEEVEETAGSVPAAELVMD 450
451	AEPQEAEPKELVVKLETQVSGEDTQAGDLSDFDEKVLSPKPPGVVSE 500
501	MLSSQERMKVQGSPLKLLFTSTGLKLLSGKKGKGRGGGDEESEHTQV 550
551	PADSPDSQEEQKSSASSPPEPEIITCLEKGLAEVQDQGEAEAGATSDG 600
601	EKKREGVTPWASFKKMTVPKRRVRSSEKDELDLKVKSATLSSTESTA 650
651	SEMQEEMKGSVSEPKPEPKRKRVDTSVSWALLCVGSSKRRARRRSSDE 700
701	EGGPKAMGDHQKADAEAGDKETGTDGILAGSGEHDGQGGSSPEQAQSP 750
751	TEGEGVSTWESFKRLVTPRKKSKSLEEKSEDSIAGSGVHSTPDTPEPK 800
801	EESWSISKFIPGRKKRFDGKQEQAVEDAGTGANEDSDVPAVFLS 850
851	EYDAVERKMEQQAQKGAEQEQAATEVSKLESESQVHMMAAVADGT 900
901	RAATIIEERSPSWISASVTEPLEQVEAAEALLTEVLEEREVIAEEPTV 950
951	TEPLPNNREARGDVTVSEAELETPAATAAGPLGSEEGTEASAAPT 1000
1001	EMVSAVSLQDSDPTTEEATPVQVEGGVDPDIEEQERTQEVQLQVAEKK 1050
1051	KEESQLPGTGGPEVLDLPQVQRAEAERPEQAEASGLKKEVDVVLQDAQE 1100
1101	AKTEPPTQGVVGGTTPESFEKAPQVTESEISSLVTTCAQATLQAGVKSQ 1150
1151	EMVMEQAIPPDSVETPTDSETDGTVPADPAFGPTQKDIIVEIHEENEV 1200
1201	HLVVRGTAEAAVPAQKERPPAPSPFVQEEETKEQSKMEDTLEHDKVE 1250
1251	VETVLSLSTEGTQADQVADEKTKDVPFPEGLGSDITGIVTSREKVT 1300
1301	VALKGEGETEAECKDDALELQSHAKSPVSPVEREMVQVVEREKTEAPT 1350
1351	HYNEEKLEHETAVTVSEVSKQLLQTVNVPIDGAKVEVSESLGSPPPCLG 1400
1401	QEAIVCTKIQVQSSSEFTLTAABEELVGETANILETGETLEPAGHL 1450
1451	VLEKSKSEKNEFPAHGDVAVPTGPDCQAKSFPVIVSATTKGLSSDLE 1500
1501	QKETTSLKWKSDVEQVACQEVKVSVAIDLEPENGILLETKSKVLQV 1550
1551	NIQTVADQVFRTEETATEMLTSELQQAAMHKADSDAQGETEKEGEP 1600
1601	QASQDETPITSAKBESESTAVGQAHSDDISKDMSASAKMTVEVGGST 1650
1651	NDQQLEEVVLPSEEGGAGTKSVPEDDGHALLAERIKSLSVPEKEDKGG 1700
1701	DDVDDPNQNSALADDTASGGLTKESPDTPNGKQKEDAKEDAQVELQEGKV 1750
1751	HSESDKAITPQAQEEELQKQERESAKSELTES 1781
(b)	
HUM GRAVIN	1 MGAGSSTEQRSPPEPPGSSSTPAEPEPSGGGSAEAAAPDTTADPAIAASD PATKLLQKNGQLSTI 65
CLONE 72	1 MGAGSSTEQRSPPEPPGSSSTPAEPEPSGGGSAEAAAPDTTADPAIAASD PATKLLQKNGQLSTI 61
HUM GRAVIN	66 NGVAEQDSELSQEGDLNGQKALNQQGALNSQEEIIVTEVGGQDSEVDVSRSDKEMATKSAVVHDI 130
CLONE 72	62 NGVAEQDSELSQEGDLNGQKALNQQGALNSQEEIIVTEVGGQDSEVDVSRSDKEMATKSAVVHDI 112
HUM GRAVIN	131 VHDITDDGQENRN IEQIPSSSENLLELTPPESQANDIGFKKVFVKFGKFTVKKDKTEKPD 174
CLONE 72	131 VHDITDDGQENRN IEQIPSSSENLLELTPPESQANDIGFKKVFVKFGKFTVKKDKTEKPD 197
HUM GRAVIN	195 VQLLVTKKDEGGA A GAGHDQPSL GA GEASKESEFQSTKPEETLKRQSHAEISFP 254
CLONE 72	178 VQLLVTKKDEGGA RASVAGDHPQESVETAVGAEASKESEFQSTKPEETLKRQSHAEISFP 241
HUM GRAVIN	255 AESQAVEEC KEKREKQEKPKSASRSTPSVFTSTGTEKPF 317
CLONE 72	242 QAESDQAAREEAKDEEKEKQEKPKSASRSTPSVFTSTGTEKPF 306
HUM GRAVIN	318 DEVEASEKKEQEPKVDTEE DOKAVEASEKLTASQAIHQPARASHEPRLSAEYKVELPSE 361
CLONE 72	306 DDLETAKRKEQEAQKVDDEEKE KTEFASPE QEPARTDQARLSADYVLEKLE 381
HUM GRAVIN	382 EQVSGSGPSEKPAPLATEVDFEKEVHQEVVAEVHVSTVEERTEE Q KTEVET 437
CLONE 72	362 DQVDELEASSEKCAPLATEVDFEKEVHQEVVAEVHVSTVEERTEE KTEEQQGGGAEAGVVGST 424
HUM GRAVIN	438 AGSVPAEELVMDAR PQEAEPKELVVKLETQVSGEDTQAGDLSDFDEKVLSPKPPGVVSEVM 501
CLONE 72	425 GESLPEKLAEPQ EVQQAELPAEELVMDAR PQEAEPKELVVKLETQVSGEDTQAGDLSDFDEKVLSPKPPGVVSEVM 488
HUM GRAVIN	502 LSSQERMKVQGSPLKLLFTSTGLKLLSGKKGKGRGGGDEESEHTQV FADSPDSQEEQKSSASSPPEPEIITCLEKGLAEVQDQGEAEAGATSDG 564
CLONE 72	489 LSSQERMKVQGSPLKLLFTSTGLKLLSGKKGKGRGGGDEESEHTQV FADSPDSQEEQKSSASSPPEPEIITCLEKGLAEVQDQGEAEAGATSDG 552
HUM GRAVIN	565 SASSPEPEIITCLEKGLAEVQDQGEAEAGATSDG EKKREGVTPWASFKKMTVPKRRVRSSEKDELDLKVKSATLSSTESTA 629
CLONE 72	553 SASSPEPEIITCLEKGLAEVQDQGEAEAGATSDG EKKREGVTPWASFKKMTVPKRRVRSSEKDELDLKVKSATLSSTESTA 617
HUM GRAVIN	630 KEDELKVKSATLSSTESTASEMDEKMGV EEPKPEPKRKRVDTSVSWALLCVGSSKRRARR 693
CLONE 72	618 KEDELKVKSATLSSTESTASEMDEKMGV EEPKPEPKRKRVDTSVSWALLCVGSSKRRARR 681
HUM GRAVIN	694 RSSDDEGGPKAMGDHQKADAEAGDKETGTDGILAGSGEHDGQGGSSPEQAQSPTEGEGVST 758
CLONE 72	682 ASSDDEGGPRTLQGDHRAEASKKEAGTDVAVSTQDQQAQSSPEQAQSPTEGEGVST 746
HUM GRAVIN	759 EEFKLVTPRKKSKSLEEKSEDSIAGSGVHSTPDTPEKERTQEVQLQVAEKK 822
CLONE 72	747 EEFKLVTPRKKSKSLEEKSEDSIAGSGVHSTPDTPEKERTQEVQLQVAEKK 806
HUM GRAVIN	823 EQATVEDAGPTGANEDSDVPAVFLSEYDAVERKMEQQAQKGA EQEQAATEVSKLESES 886
CLONE 72	807 EQATVEDAGPTGANEDSDVPAVFLSEYDAVERKMEQQAQKGA EQEQAATEVSKLESES 874
HUM GRAVIN	887 QVHMMAAVADGTRAAITIEERSPSWISASVTEPLEQVEAAEALLTEVLEEREVIAEEPTVTE 951
CLONE 72	868 LVHTSVAVIDGTRAVTSVEERSPSWISASVTEPLEQVEAAEALLTEVLEEREVIAEEPTVTE 931
HUM GRAVIN	952 PLPNNREARGDVTVSEAELETPAATAAGPLGSEEGTEASAAPTTEVMSVQSLQDSDPTTE 996
CLONE 72	932 PLPNNREARGDVTVSEAELETPAATAAGPLGSEEGTEASAAPTTEVMSVQSLQDSDPTTE 916
HUM GRAVIN	1017 EATPVQVEGGVDPDIEEQERTQEVQLQVAEKKESQLPQGGVDPVLPQVOR AEA ERPEE 1078
CLONE 72	997 EATPVQVEGGVDPDIEEQERTQEVQLQVAEKKESQLPQGGVDPVLPQVOR AEA ERPEE 1054
HUM GRAVIN	1079 QAES GL KRETDVVKVDAQEAETPTTQGVVGGTTPESFEKAPQVTESEISSLVTTCAQ 1140
CLONE 72	1055 VEEDSEVLASEKEDVMPKQVQEAQALQAGSTGQATPESLE VPEVT ADVDHATVQ 1114
HUM GRAVIN	1141 ETLGAVKQSMVMQAIIPDSVETPTDSETDGTVPADPAFGPTQKDIIVEIHEENKHLVSP 1204
CLONE 72	1115 VI KLQQ LMEQAVAPSESELTDSHTNGTFTLADSTADGQDQDITDSDQSKATAAVRQ 1173
HUM GRAVIN	1205 R GTEARVAEPQERPPAPSPFVQ EETKEQSKMEDTLEHDKVEVSEVTSISLSTEGTQAD 1267
CLONE 72	1174 SQVTEEAATAQERPESTLNNVPAQEHEGPEEPG DYLEPTQQELTAAVPLAMTEVGGQEVQ 1236
HUM GRAVIN	1267 QYAD ETKTQVDF PEGLESDITGIVTSREKVTVALEKGETEAEACKDDALELQSHAKSPFS 1329
CLONE 72	1238 DWLDEKVKKEQEVV VHS GPN S QKAADVTYDSEVMGAGQEKESTEVQ SLS 1289
HUM GRAVIN	1330 PVEREMVQVVEREKTEARPTHNEEKLEHETAVTVSEVSKQLLQTVNVPIDGAKVEVSESLGSP 1394
CLONE 72	1290 LEGEMETDVEKREKTEKPEQVSEEE EQTAARPEERTGTYGKPVLLDMPSSERGLKGLSGSP 1353
HUM GRAVIN	1395 PFCLEQREAVCKTIQVQSSSEFTLTAABEELVGETANILETGETLEPAGHLVLEKSKVLQV 1459
CLONE 72	1354 SLPD QKAGCIEVQVQSLDITVTTQARA AVKVI ETVVISTEESPEKCAHLLPAKSSATG 1415
HUM GRAVIN	1460 EDFAAHPGDEAVPTGPDCAQKSTPVIYSATTKGLSSDLEKETTSLKWKSDVEQVACQEV 1522
CLONE 72	1416 GHWTLQHAEDTVLPQESQAESIPIVTPAPESTLHPDQGEISASQSRSEKEDKDPAGDPA 1479
HUM GRAVIN	1523 KVSVAIED LEFENGILELTKSKLQNIQTAVDQVFRTEETATEMLTSELQQAAMHKADS 1585
CLONE 72	1480 GKESTALEKVAEPELELESKNVILNVIQTAVDQVFRTEETATEMLTSELQQAAMHKADS 1544
HUM GRAVIN	1586 QDAGQTEKKEGEPQASQDETPITSAKBESESTAVGQAHSDDISKDMSASAKMTVEVGGSTV 1650
CLONE 72	1545 QGQQLMIDNESQDETPITSAKBESESTAVGQAHSDDISKDMSASAKMTVEVGGSTV 1609
HUM GRAVIN	1651 DQQLLEVVLPSEEGGAGTKSVPEDDGHALLAERIKSLSVPEKEDKGDVDDPNQNSALADT 1714
CLONE 72	1610 QKHAADGQQLSQAQAEASAGNLNTEKSPDTPNGKQKEDAKEDAQVELQEGKV 1675
HUM GRAVIN	1716 DASGGLTKESPDTPNGKQKEDAKEDAQVELQEGKVHSESDKAITPQAQEEELQKQERESAKSELTES* 1780
CLONE 72	1675 EEDLQEPKGLARS* 1687

Figure 2



Sequence comparison of gravin and AKAP250, and identification of functional domains. (a) The similarities between AKAP250, the original gravin fragment and SSeCKS/clone 72. The likely RII-binding site is shown in light blue 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. Light blue highlighted areas represent conserved amino acids. (c) A helical-wheel representation of gravin/AKAP250 residues 1540–1553, drawn as an α -helix of 3.6 amino acids per turn. The shaded area represents the hydrophobic residues and the white area the hydrophilic side-chains.

and co-purification experiments have allowed us to propose that gravin may function as a scaffold protein that coordinates the intracellular locations of PKA and PKC.

Results

Isolation of gravin clones

In order to isolate cDNAs encoding proteins that potentially bind to the type II regulatory subunit of PKA, RII, a human fetal brain cDNA library was screened using a modified overlay procedure, with radiolabeled RII α as a probe [27]. Eight RII-binding clones were identified and plaque-purified, and the ends of each cDNA insert were sequenced. Two of the clones represented known sequences. One matched MAP2, a previously identified AKAP [28]. 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 cDNA library with serum from a myasthenia gravis patient [26]. The other six clones had no homology to any sequence in Genbank.

Further sequencing of clone HF 9 showed that the cDNA was 3 023 base-pairs (bp) in length and encoded a continuous open reading frame of 651 amino acids. Northern-blot analysis using a 32 P-labelled random-primed 1 676 bp *EcoRI-SpeI* fragment of HF 9 as a probe indicated that gravin mRNA is selectively expressed in certain human tissues. Two predominant mRNA species of 8.4 kilobases (kb) and 6.7 kb were detected in all tissues but predominated in liver, brain and lung, whereas an additional 5.5 kb mRNA was detected in brain (data not shown). Given the relatively large sizes of all the gravin mRNAs, we concluded that the HF 9 clone represents a partial transcript. The 1 676 bp fragment was therefore used to further screen

the human fetal brain cDNA library for more complete transcripts; five additional clones were obtained that yielded an additional 600 bp of coding region. As an alternative strategy, a human heart cDNA library was screened with the same 1 676 bp HF 9 fragment. Of the five hybridizing clones isolated, the longest clone contained a 4 216 bp insert which overlapped with the 5' end of the original clone. This provided a contiguous sequence of 6 605 bp which is predicted to encode a protein of 1 780 amino acids (Fig. 1a).

On the basis of our interaction-cloning strategy, we postulated that the last 651 amino acids of the gravin protein contain a binding site responsible for association with the type II regulatory subunit of PKA [17]. We have previously shown that regions of conserved secondary structure which are likely to include amphipathic α helices are responsible for binding to RII [29,30]. Residues 1540–1553 of gravin fulfil these criteria (Fig. 2); this region shows sequence identity with corresponding regions in other AKAPs (Fig. 2b), and a helical-wheel plot suggests that there is a segregation of hydrophobic and hydrophilic side-chains which would be compatible with the formation of an amphipathic helix (Fig. 2c).

Surprisingly, further sequence analysis revealed another potential function of gravin (Fig. 2a). A search of the nucleotide database using the complete gravin sequence showed that the first 1 000 residues are 69% identical to a murine mitogenic regulatory gene, SSeCKS [31], which is also called clone 72 and which has recently been shown to be a PKC substrate/binding protein (Fig. 1b) [32]. We therefore postulate that gravin may be a kinase-scaffold protein that binds both PKA and PKC.

Gravin is an AKAP

On the basis of our previous structure–function studies on AKAPs [29,30,33–35], we proposed that residues 1540–1553 of gravin were likely to comprise the RII-binding site (Fig. 2b,c). In order to test this hypothesis, a set of recombinant fragments encompassing this region (Fig. 3a) was generated using the polymerase chain reaction (PCR) and expressed in *Escherichia coli* using the pET16b His-tag bacterial expression/affinity purification system (Fig. 3b). A 452-residue fragment encompassing residues 1130–1582 bound ^{32}P -radiolabeled RII α in the overlay, whereas a smaller fragment, residues 1130–1525, which lacks the proposed RII-binding region, was unable to bind RII α (Fig. 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 (Fig. 3c), and a synthetic peptide covering residues 1537–1563 blocked all RII-binding in the overlay (Fig. 3d). In addition, the anchoring inhibitor peptide Ht31(493–515), which is a competitive inhibitor of RII/AKAP interactions [29,36], 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 that 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 a recombinant RII α fragment was measured by surface plasmon resonance. The binding properties of the immobilized gravin fragment were measured over a range of concentrations of RII α 1–45 peptide from 25 to 150 nM (Fig. 4a). Uniform first-order binding was recorded with a K_{ass} of $16\,0006 \pm 700\ \text{M}^{-1}\ \text{sec}^{-1}$ ($n = 3$) and with a K_{dis} of $0.016 \pm 0.001\ \text{M}^{-1}$ ($n = 3$; Fig. 4b).

Figure 4

Affinity measurement of the gravin–RII α interaction. The binding affinity of a recombinant RII α fragment (residues 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 as described in Materials and methods, and was incubated with the RII α fragment over a range of concentrations (25–150 nM). (a) Extent measurements (in arc seconds) showing the binding profiles for selected concentrations of RII interacting with the immobilized gravin fragment. (b) The measured on-rates ($\text{M}^{-1}\ \text{sec}^{-1}$) are plotted against concentration of RII 1–45 peptide.

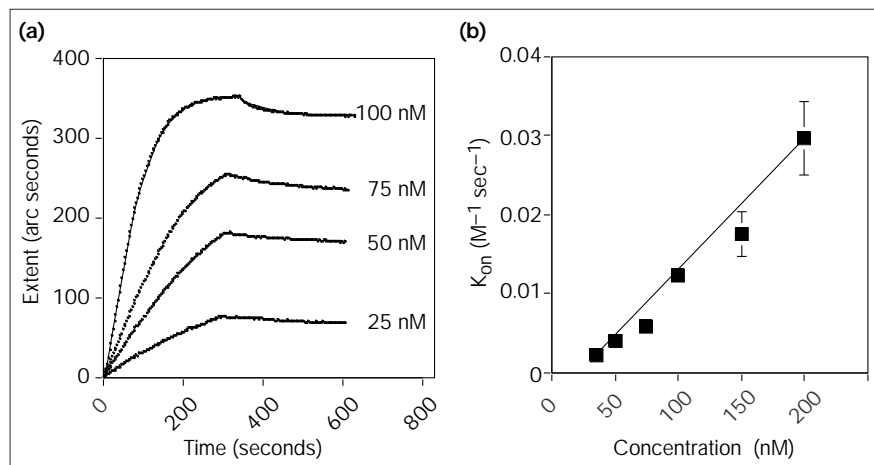
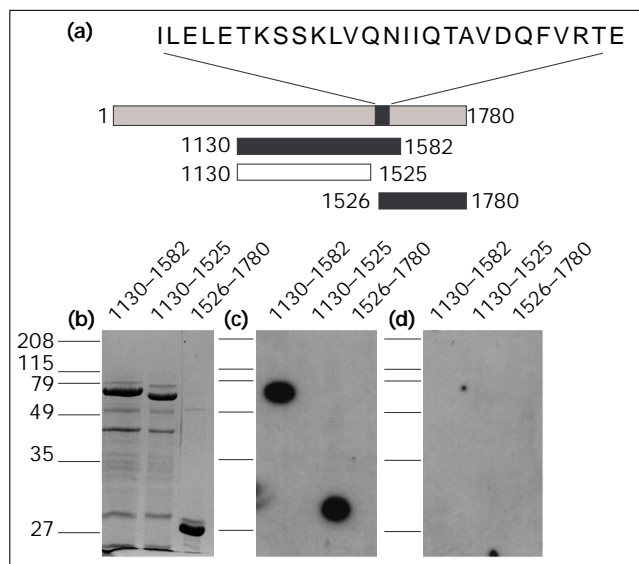


Figure 3

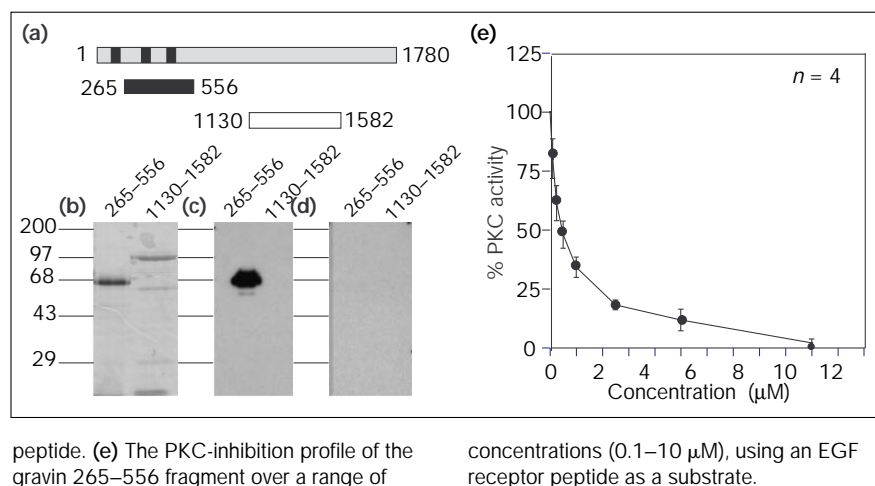


Recombinant gravin fragments bind specifically to RII α . Recombinant fragments of gravin were expressed in *E. coli* using the pET16b bacterial expression system and purified on His-tag resin. (a) The first and last residues of each fragment are indicated and the sequence of the putative RII-binding site is presented. (b) Purified protein (10 μg) was separated by electrophoresis on 10% (w/v) SDS–polyacrylamide gels, and gels were stained with Coomassie blue. Fragment sizes are indicated above each lane. (c,d) Proteins (2 μg) were separated as above and electrotransferred to PVDF membranes. RII-binding proteins were detected by a solid-phase binding assay using ^{32}P -radiolabeled RII α as a probe in the absence (c) or presence (d) of excess (10 μM) gravin 1537–1563 peptide.

These values were used to calculate a dissociation constant (K_{D}) of 100 nM ($n = 3$) for the RII–gravin fragment interaction (Fig. 4b). The nanomolar binding constant for RII and gravin is consistent with the notion that the proteins may associate *in vivo*.

Figure 5

Gravin binds to, and inhibits, PKC. Recombinant fragments of gravin were expressed and purified as described in Materials and methods. (a) The amino- and carboxy-terminal residues of each fragment are indicated, and the locations of polybasic regions in the full-length gravin sequence are indicated by black boxes. (b) Purified protein (2 μ g) was separated by electrophoresis on 10% (w/v) SDS-polyacrylamide gels and stained with Coomassie blue. The fragment sizes are indicated above each lane. (c) Proteins (1 μ g) 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 Materials and methods. (d) PKC-binding assay in the presence of excess (1 μ M) AKAP79 32–51



Gravin is a PKC-binding protein

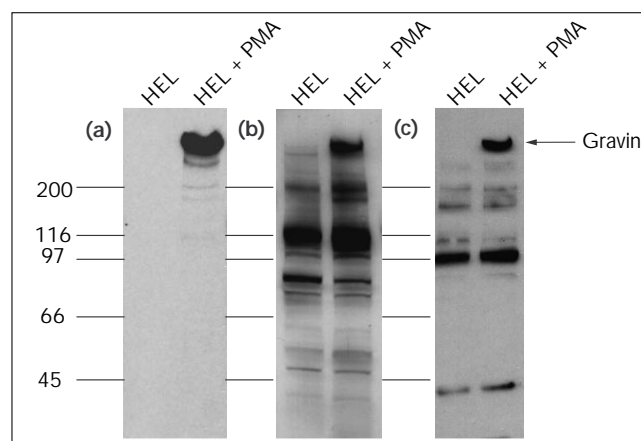
On the basis of sequence homology to SSeCKS/clone 72 (Fig. 2a), we postulated that the amino-terminal regions of gravin may bind PKC. Therefore, a 290 amino-acid fragment corresponding to residues 265–556 of gravin was expressed in *E. coli* (Fig. 5a). The affinity-purified gravin 265–556 fragment bound PKC in a phosphatidylserine-dependent manner, as assessed by an overlay assay (Fig. 5b,c), whereas a carboxy-terminal fragment, residues 1130–1582, did not (Fig. 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 phosphatidylserine (data not shown), which confirms other reports that phospholipid is cofactor in the PKC-binding-protein complex [15,32]. The excessive refractive properties of the phosphatidylserine moiety prevented affinity measurements of the PKC–gravin interaction by surface plasmon resonance.

It has been suggested that polybasic regions participate in formation of a phospholipid bridge between PKC and its binding proteins [15,32]. We have shown that a polybasic region on another scaffold protein, AKAP79, forms the PKC-binding site [24]. Because the AKAP79 32–51 peptide also blocked the binding of PKC to gravin (Fig. 5d), it seems that both anchoring proteins bind to a similar site on PKC. Additional evidence of similarity between gravin and AKAP79 came from the ability of the gravin 265–556 fragment to inhibit PKC activity towards a peptide substrate with a half-maximal inhibitory concentration (IC_{50}) of $0.50 \pm 0.12 \mu$ M ($n = 4$; Fig. 5e), whereas the RII-binding peptide did not inhibit PKC (data not shown). There are two polybasic regions in the gravin 265–556 fragment, located between residues 295–316 and 514–536 (Fig. 2a). Interestingly, peptides made from sequences within either polybasic region blocked PKC–gravin interactions as assessed by the overlay (data not shown). Collectively,

these experiments show that PKC binds gravin *in vitro* at one or more polybasic sites located between residues 265 and 556 of gravin. 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 [26,37]. An immunochemical survey of cell lines indicated that gravin was expressed in MG-63

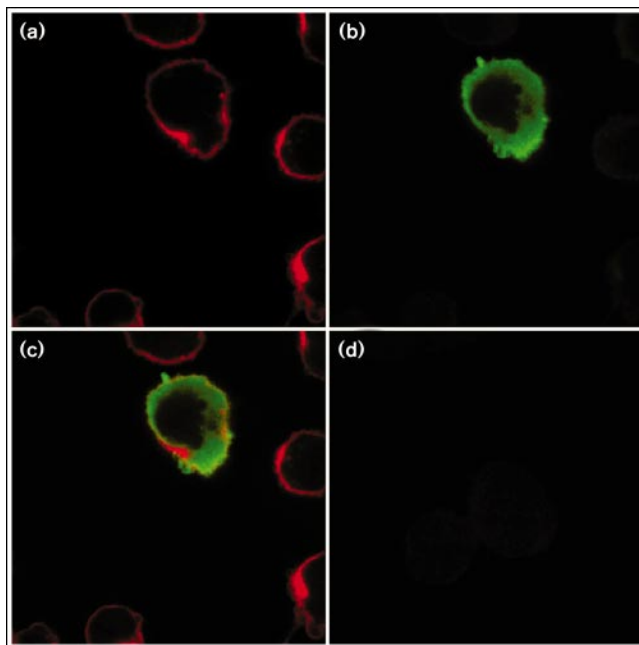
Figure 6

Induction of gravin expression in HEL cells. HEL cells were grown with or without 40 nM PMA for 18 h. The supernatants of control and treated lysates (25 μ g) were separated by electrophoresis on a 4–15% SDS-polyacrylamide 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 antibodies to detect immobilized RII; the migration position of gravin is indicated.

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 [38]. One hallmark of this process is the robust induction of gravin [26]. We therefore decided to examine the profile of PKA- and PKC-binding proteins in HEL cells after prolonged exposure to phorbol esters. HEL cells were grown in the presence of 40 nM phorbol myristyl acetate (PMA) for 18 hours and extracts from control and treated cells were western blotted with an affinity-purified antibody raised against residues 1130–1780 of gravin. PMA treatment caused induction of a 250 kDa protein that specifically reacted with anti-gravin antibodies (Fig. 6a). Subsequent overlay assays demonstrated that PMA treatment induced the expression of a 250 kDa PKC-binding protein (Fig. 6b) and an RII-binding protein of the same size (Fig. 6c). These results confirm that phorbol ester treatment induces gravin expression in HEL cells and suggest that native gravin binds PKA and PKC *in vitro*.

Concomitant with the macrophage-like shift in response to PMA, HEL cells become more adherent and display a

Figure 7



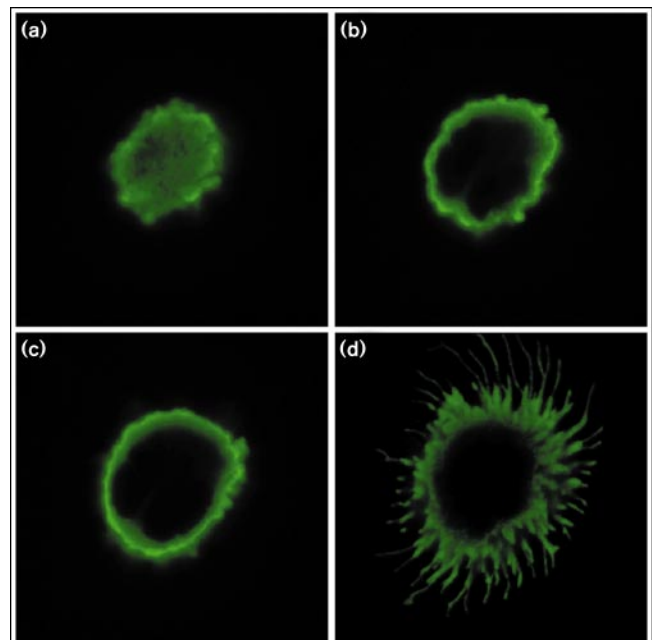
Gravin in HEL cells. HEL cells were grown on coverslips in the presence of 40 nM PMA for 18 h. Cells were fixed with 3.7 % formaldehyde and permeabilized with 100 % acetone at -20°C . (a,c) The actin was stained with rhodamine phalloidin and (b,c) gravin was stained with affinity-purified antibodies ($0.5\ \mu\text{g}\ \text{mL}^{-1}$) and detected with a fluorescein-conjugated secondary antibody (1:100). (d) No specific staining was detected with preimmune serum. Fluorescence detection was by a Lietz Fluovert-FU confocal photomicroscope with a 63/1.4 N.A. OEL PL lens.

considerable cytoplasmic spread [38]. 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, the actin in phorbol ester-treated HEL cells was stained with rhodamine phalloidin. All of the cells displayed a concentration of actin in the periphery (Fig. 7a). In contrast, gravin staining was predominantly cytoplasmic and only a subset of the cells (approximately 25 %) expressed large quantities of gravin (Fig. 7b). Variable levels of gravin expression were not unexpected, as HEL cells are a heterogeneous population at different stages of differentiation [38]. Superimposition of both images shows that gravin and actin exhibit distinct but overlapping subcellular locations (Fig. 7c). Control experiments in which cells were stained with preimmune serum were negative (Fig. 7d). More detailed confocal analysis of HEL cells showed gravin staining toward the periphery of the cell and enriched in filopodia at the adherent surface (Fig. 8). These findings are consistent with the notion that gravin functions in some capacity to enhance HEL cell adhesion to the substratum.

Dissection of the gravin signaling complex

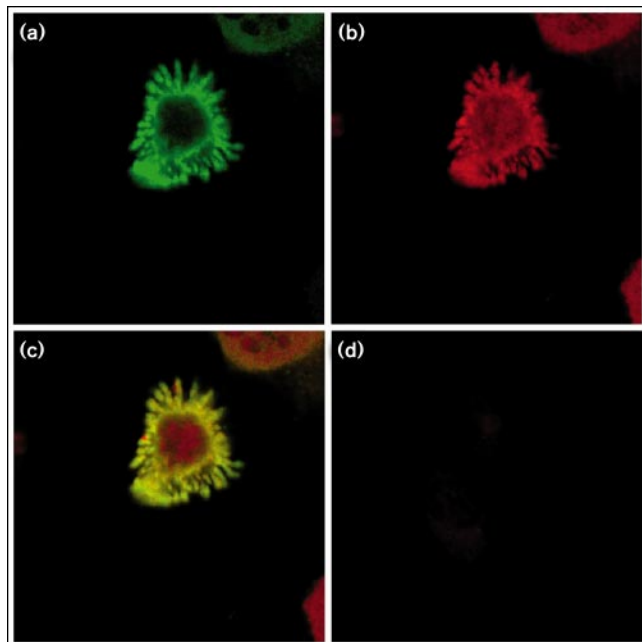
Binding studies *in vitro* suggested that gravin is a kinase-scaffold protein, so we initiated co-localization experiments

Figure 8



The subcellular distribution of gravin. HEL cells were seeded on coverslips and grown in the presence of PMA, then stained with affinity-purified gravin antibodies and the fluorescence detected as described for Figure 7. The panels depict sequential $1\ \mu\text{m}$ confocal sections through an individual cell.

Figure 9

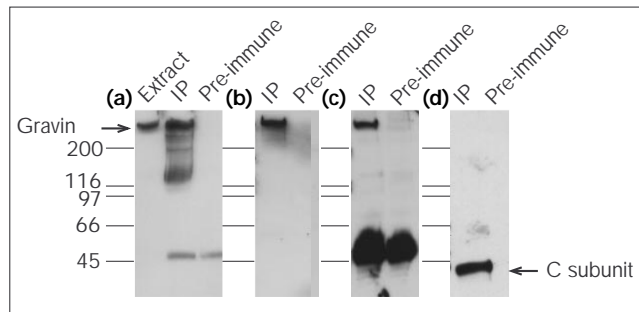


Fluorescent detection *in situ* of RII-AKAP complexes. HEL cells were seeded on coverslips, grown in the presence of PMA, fixed and permeabilized as described for Figure 7. (a) Gravin was detected immunochemically as described in Materials and methods. (b) Unoccupied RII-binding sites were detected by an *in situ* overlay procedure described in the Materials and methods. Detection of anchored murine RII α was 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) There was no cross reaction of the anti-murine RII antibody with the endogenous human RII.

to determine whether a gravin signaling complex could be detected in HEL cells. Fixed and permeabilized cells pre-treated with PMA were overlaid with recombinant RII α (Fig. 9). RII binding *in situ* was detected with antibodies that specifically recognize murine RII (Fig. 9b) and mimicked the staining pattern for gravin (Fig. 9a,c). As control experiments confirmed that the anti-murine RII antibodies did not detect the endogenous human RII (Fig. 9d), we conclude that the increased RII staining was due to direct association with gravin. This conclusion is supported by additional control experiments showing that *in situ* RII binding was blocked by incubation with the Ht31 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 anti-gravin antibodies specifically isolated a 250 kDa protein that could be faintly detected when gels were stained with Coomassie blue

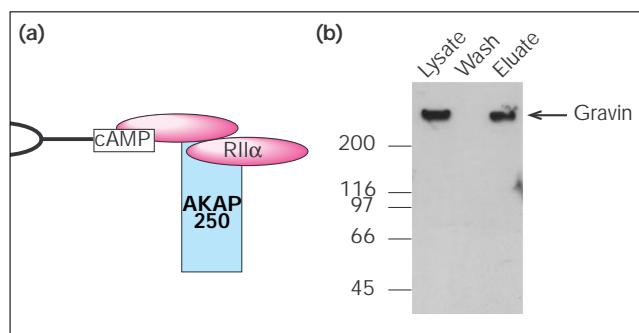
Figure 10



Immunoprecipitation of the gravin signaling complex. Supernatants of HEL cell lysates grown in the presence of 40 nM PMA were incubated with 15 μ g of anti-gravin antibodies or 15 μ g of preimmune IgG. Immunoprecipitations were performed as described in the Materials and methods. Control and immunoprecipitated fractions (5 μ g) were separated by electrophoresis on a 4–15 % SDS-polyacrylamide 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 RII α . (d) Catalytic subunit of PKA eluted with 1 mM cAMP from the immune complexes detected using affinity-purified antibodies to the C subunit of PKA.

(data not shown). This 250 kDa protein was present only in immunoprecipitates using the affinity-purified gravin antibodies and was not detected in control experiments using preimmune serum. Western blot and overlay assays confirmed that the 250 kDa protein was gravin (Fig. 10a), a PKC-binding protein (Fig. 10b) and an AKAP (Fig. 10c). Moreover, coprecipitation 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 (Fig. 10d). We were unable to detect PKA RII subunit in

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 a fraction eluted from the column in buffer containing 75 mM cAMP.

the immunoprecipitates because, with a molecular mass of 54 kDa, it migrates with the same mobility as the immunoglobulin heavy chain (Fig. 10c). However, the RII subunit–gravin complex was purified from PMA-induced HEL cell extracts by affinity chromatography on cAMP-agarose (Fig. 11a). After extensive washing in high-salt buffers, gravin was eluted from the affinity resin with 75 mM cAMP (Fig. 11b). Because free gravin does not react with 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, PKC was not detected with the gravin fraction by either method.

Discussion

In this report, we describe the cloning and characterization of a human kinase-anchoring protein called gravin. The carboxy-terminal fragment of gravin was originally identified as a cytoplasmic antigen recognized by myasthenia gravis sera [26]. We now show that the first half of the full-length gravin protein is homologous to SSeCKS/clone 72, a murine protein whose gene was independently cloned firstly because its mRNA product was transcriptionally suppressed by Src [31], and secondly because the product is a PKC substrate/binding protein [32]. Although they are clearly related, it is unclear whether gravin and SSeCKS/clone 72 represent human and murine homologs of the same protein. The first 1 000 residues of the two proteins share 69 % sequence identity, including selected regions which show homology to the PKC substrate proteins known as MARCKs [39]. However, the remainder of each protein sequence is distinct. Also, gravin is a protein of 1 780 amino acids which migrates with a mobility of 250 kDa, whereas SSeCKS/clone 72 is 1 687 residues and migrates at 207 kDa [31]. In addition, the identification of five prospective nuclear localization signals has led to the idea that SSeCKS is a nuclear protein [32], whereas our immunochemical data clearly shows that gravin is cytoplasmic and likely to be a cytoskeletal component. We propose that gravin and SSeCKS/clone 72 are members of an emerging class of mammalian scaffold proteins which contribute to the coordination and organization of signal transduction events by bringing kinases together [19].

We propose that residues 1537–1563 of gravin form the PKA-anchoring site, as a peptide made from this sequence is sufficient to block binding of the RII subunit of PKA in the overlay assay, and protein fragments containing this region bind RII with nanomolar affinities. Because this sequence is present in the carboxyl terminus of SSeCKS/clone 72, we predict that SSeCKS/clone 72 will be an AKAP also. Interestingly, this shared 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 [14,19,24].

The identification of a conserved RII-binding sequence in gravin, SSeCKS/clone 72 and AKAP79 was rather surprising as we have previously proposed that a lack of sequence identity between the AKAPs is due to a conservation of only secondary structure in the RII-binding motif [17,29]. Therefore, gravin, SSeCKS/clone 72 and AKAP79 may be members of a structurally related 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 classes of PKC-binding proteins have been identified by gel overlay and two-hybrid gene-interaction experiments (reviewed in [3,6]). PKC substrate/binding proteins [15] and ‘receptors for activated C-kinase’ (RACKs) [40] have been detected by gel overlay, whereas ‘proteins that interact with C-kinase’ (Picks) have been isolated in two-hybrid screens [16]. On the basis of the homology to SSeCKS/clone 72, we would propose that gravin is a PKC substrate/binding protein. Indeed, we have located a region of 290 amino acids that supports PKC-binding and blocks PKC activity *in vitro*. There is some ambiguity as to where the principal PKC-binding site(s) are located within this region, however. It has been suggested that phosphatidylserine supports a ternary complex of PKC and polybasic regions on the substrate/binding protein [41]. There are two polybasic regions in the gravin 265–556 fragment, both of which block PKC binding in the overlay assay (T.M.K. and J.D.S., unpublished observations). Furthermore, both polybasic regions (residues 295–316 and 514–536) resemble the PKC-binding site on AKAP79 which also blocks binding in the overlay assay and inhibits kinase activity [24]. At the moment, it is unclear how both regions participate in enzyme binding or whether there are multiple PKC-binding sites on gravin.

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, a distinguishing feature of substrate/binding proteins is that phosphorylation regulates their association with PKC [15]. This implies that substrate/binding proteins may interact with the kinase transiently, although how these proteins act to influence the subcellular location of PKC has yet to be precisely determined. Consequently, substrate/binding proteins may merely represent PKC substrates that release the enzyme slowly once the phosphotransfer reaction is complete [42]. The net effect of such a mechanism would be that substrate/binding proteins could deplete the pool of active PKC available for conventional substrates. This may explain why submicromolar concentrations of the gravin 265–556 fragment inhibit kinase activity in the presence of excess peptide substrate. A second possible explanation for our failure to detect the gravin–PKC

complex in cells is that the necessary inclusion of detergents in the cell permeabilization and extraction buffers used to isolate the complex may remove the phospholipid co-factor that cements the PKC–gravin interaction. A third possible explanation is that the prolonged exposure to phorbol esters which is necessary to induce high-level gravin expression in HEL cells also down-regulates PKC [43] and undoubtedly decreases the pool of enzyme available to associate with gravin. Nevertheless, the question of whether a pool of PKC is perpetually targeted in HEL cells through association with gravin or other substrate/binding proteins must remain open until there is definitive evidence that the complex is formed *in vivo*.

Evidence from two different models points to distinct, yet related, functions for a gravin signaling complex. A tissue survey has shown that gravin has a restricted cellular distribution and is predominantly expressed in fibroblasts, neurons and cells derived from the neural crest [37]. As each of these cell types participates in adherent, migratory or path-seeking functions, it was postulated that gravin might regulate membrane and/or cytoskeletal events [37]. This view has been further substantiated by our immunolocalization experiments which suggest that gravin concentrates PKA in the ruffles and filopodia of adherent HEL cells. In addition, a pair of reciprocal results point toward a role in cell adhesion. Phorbol ester-induced adhesion in HEL cells [26] is concomitant with increased gravin expression (Fig. 7), whereas loss of an adherent phenotype upon transformation of REF 52 fibroblasts with a derivative of simian virus 40 (SV40) is coincident with down-regulation of SSeCKS/clone 72 [32]. As phosphorylation events help to maintain the integrity of the membrane and its associated cytoskeleton, it is also tempting to speculate that anchoring of PKA and PKC by gravin may play a role in adherent processes.

Myasthenia gravis is a disease of neuromuscular transmission which is generally believed to be due to auto-antibodies to the nicotinic acetylcholine receptor. But myasthenics also frequently produce antibodies to cytoskeletal components that may be physically linked to the receptor [44]. Direct association between the acetylcholine receptor and gravin is unlikely, however, as gravin is not present at the neuromuscular junction [37]. Nevertheless, gravin's subcellular location in HEL cells is consistent with a role in the modulation of membrane–cytoskeletal events. It is noteworthy that gravin's proposed role in the coordination of PKA and PKC targeting to cytoskeletal components would be analogous to AKAP79's role in clustering PKA, PKC and PP2B at the postsynaptic density, which is a specialized structure of the dendritic cytoskeleton [14,24,36]. Future studies are planned to determine whether gravin interacts with additional membrane and/or cytoskeletal components and whether disruption of kinase anchoring affects cell shape and motility.

Acknowledgements

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Materials and methods

Cloning of gravin cDNAs

A 3 023 bp partial cDNA encoding an RII-binding protein was isolated by expression-cloning from a human fetal brain λ ZAP cDNA library using a modified form of the RII-overlay procedure as previously described [33]. Clones encoding full-length gravin were obtained by screening a human heart λ ZAP cDNA library using an α -[³²P]dCTP random-primed 1 676 bp *EcoRI*–*SpeI* fragment from the original clone as a probe. Nucleotide sequencing used the Sanger dideoxy method [45] or was with an Applied Biosystems sequencer (Foster City, California). The Genbank accession number for the gravin sequence is U81607.

Bacterial expression of recombinant proteins

A 1 953 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 the fragment using PCR with the original HF 9 clone as the template. Primers were synthesized that generated an *NdeI* site at the 5' end (CCGCCATGGTGCATATGTCCGAGTCCAGTGAGC) and a *BamHI* site at the 3' end (GGAGGATCCAGAGATTCTGTAGTTCTG) 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 (CCGCCATGGTGCATATGTCCGAGTCCAGTGAGC) 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 (CCGCCATGGTGCATATGTAGCAATTGAGGATTTAG) 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 original gravin cDNA using a 5' primer (CCGCCATGTGCATATGAAAGAGGAAGGAGAAGAG) which encoded an *NdeI* site and a 3' primer (GGAGGATCCAGAGCTGTCCGGAGAATCGGC) which encompassed a *BamHI* site.

Each gravin construct was transfected into *E. coli* and the recombinant Histag fusion proteins were induced using IPTG. Each recombinant protein was purified according to previously published methods [14]. The 1130–1780 gravin fragment was used for production of a polyclonal antibody (Bethyl Labs, Montgomery, Texas), hereafter referred to as R3698.

Surface plasmon resonance measurements

A recombinant fragment encompassing residues 1526–1780 of gravin was coupled to a carboxymethyl dextran IAsys cuvette using standard EDC/NHS coupling chemistry [46]. 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 μ g ml⁻¹) 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 1 M ethanolamine, pH 8.5 for 2 min at room temperature. After washing with PBST, a stable base-line was established for 10 min before data collection. All binding experiments were performed with a recombinant fragment of RII α (RII 1–45) which binds AKAPs with a similar affinity to that of the full-length protein. Previous experiments have indicated that release of RII α 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

of concentrations from 25 to 150 nM in volumes of 200 μ l. 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 over 3 sec 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 described previously [24,47]. Immunochemical detection of RII used an affinity-purified rabbit antibody to murine RII α . The PKC overlay was probed with a monoclonal antibody that recognizes both α and β PKC (Transduction Labs, Lexington, Kentucky). The catalytic subunit of PKA was detected using affinity-purified antibodies to an amino-terminal peptide of the human PKA C subunit (gift from Steven Pelech, Kinetek Biotechnology Corp, Vancouver, Canada).

PKC inhibition assay

PKC was assayed as described [48] in a reaction containing 40 mM HEPES (pH 7.5), 10 mM MgCl₂, 0.3 mM CaCl₂, 1 mM DTT, 100 μ M γ -[³²P]ATP (500 cpm pmol⁻¹), phosphatidylserine (20 μ g ml⁻¹), and epidermal growth factor receptor peptide (VRKRTLPLRL) as a substrate, at 30 °C for 10 min. PKC β II (20 ng μ l⁻¹) was diluted 1:10 in 20 mM Tris (pH 7.9), 1 mg ml⁻¹ bovine serum albumin (BSA) and 1 mM DTT. Inhibition constants (IC₅₀) were determined over an inhibition concentration range of 0.1–10 μ M 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 h. Cell lysates were prepared from either adherent cells grown in the presence of PMA, rinsed with PBS and scraped from the interior of 150 cm² 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 Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.25 % Triton X-100, 0.05 % Tween-20, 0.02 % Na₃N, 10 mM benzamidine, 2 μ g ml⁻¹ pepstatin, 2 μ g ml⁻¹ 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 \times g at 4 °C 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 h, rinsed with PBS, fixed in 3.7 % formaldehyde and extracted in –20 °C absolute acetone. Cells were rehydrated for 1 h in PBS plus 0.2 % BSA and then incubated with either affinity-purified anti-gravin antibody R3698 at 0.5 μ g ml⁻¹ or pre-immune IgG at 0.5 μ g ml⁻¹. After 1 h, the coverslips were carefully washed in PBS plus 0.2 % BSA and incubated with either a mixture of FITC-conjugated donkey anti-rabbit secondary antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and rhodamine-conjugated phalloidin (1 unit per coverslip; Molecular Probes, Eugene, Oregon) or secondary antibody alone. *In situ* RII overlays were performed essentially as described [34]. Prior to incubation with primary antibody, cells were incubated with 80 nM recombinant murine RII α for 2 h and unbound RII was removed by washing three times in PBS and 0.2 % BSA. The immobilized RII α was detected immunochemically with affinity-purified goat anti-murine RII (1 μ g ml⁻¹) and Texas red-conjugated donkey anti-goat secondary IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). 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 (200 μ l of 15 mg ml⁻¹), prepared as described above, were incubated with either 15 μ g of affinity-purified anti-gravin

antibodies, or 15 μ g of pre-immune IgG, at 4 °C for 18 h. Immune complexes were isolated by the addition of 200 μ l 10 % (v/v) protein A–Sepharose CL-4B (Sigma, St Louis, Missouri) 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 μ l 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–polyacrylamide gel, electroblotted onto nitrocellulose and the C subunit was detected, as previously described [33]. For the immunoprecipitation and detection of gravin, elution was accomplished by boiling the washed beads in SDS–PAGE sample buffer, followed by separation of proteins on a 4–15 % denaturing gel (5 μ g per lane), transfer to nitrocellulose and analysis by gravin western blot, PKC overlay or RII-overlay–western as described above and previously [24].

Copurification of gravin and RII on cAMP-sepharose

HEL cell lysates (400 μ l of 15 mg ml⁻¹, prepared as described above with the addition of 10 mM IBMX to the buffer), were incubated with 200 μ l packed cAMP-agarose (Sigma, St Louis, Missouri) which had been equilibrated in lysis buffer with 10 mM IBMX. The slurry was gently mixed for 18 h at 4 °C and then washed with 1.5 ml lysis buffer plus 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 0.5 ml 75 mM cAMP, 20 mM TrisHCl, pH 7.4, 150 mM NaCl for 30 min at room temperature. The final wash and the eluate were TCA-precipitated and the entire sample loaded into a single lane on a 4–15 % SDS–polyacrylamide gel. The separated proteins were blotted onto nitrocellulose and gravin was identified by western analysis as described above.

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