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Identification of an IQGAP1/AKAP79 Complex in β-Cells

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Abstract IQGAP1, is a recently discovered scaffold protein proposed to regulate membrane cytoskeleton events through protein–protein interactions with F-actin, E-cadherin, β-catenin, and CLIP170. The binding of IQGAP1 to its partners is regulated by calcium/calmodulin (Ca^{++}/CaM) and the small molecular weight guanine nucleotide triphosphatases (GTPases), Cdc42, and Rac1. Here we identify a novel IQGAP1 scaffolding function by isolating the cyclic AMP dependent kinase (PKA) with IQGAP1. IQGAP1 was co-purified with PKA using 5′-cyclic AMP (cAMP) affinity chromatography and PKA activity was co-immunoprecipitated with IQGAP1 using an anti-IQGAP1 antibody. The association of IQGAP1 with PKA was shown to occur through a direct interaction between A kinase anchoring protein 79 (AKAP79) and the carboxyl-terminal domain of IQGAP1. This suggests that cAMP/PKA may be coupled with Ca^{++}/CaM and GTPases through an IQGAP1/AKAP79 complex. J. Cell. Biochem. 90: 97–108, 2003.

Key words: β-cells; Rho family; GTPases; cAMP/PKA; IQGAP; signal transduct

Cells are no longer thought of as bags of enzymes, instead a picture of a highly organized cellular architecture with tightly co-ordinated signaling complexes is emergent [Alberts, 1998]. A key component to this co-ordination is the organization of multiple signaling enzymes and scaffolding proteins into discrete signaling units or what we call signal transduction organizing complexes (STOCs) [Jordan et al., 2000; Bhalla and Iyengar, 2001; Smith and Scott, 2002]. These STOCs enhance the kinetics and ensure the specificity of a particular signal [Ferrell, 1998, 2000]. Many scaffold proteins co-ordinate serial signals initiated by a single type of ligand [Pawson and Scott, 1997]. It is now apparent that scaffolding proteins exist that integrate parallel signaling pathways initiated by different ligands [Lester and Scott, 1997; Pawson and Scott, 1997; Tavalin et al., 1999]. The integration of parallel signaling pathways into a co-ordinated message is particularly important given the numerous extracellular cues a cell receives [Bornfeldt and Krebs, 1999; Dumont et al., 2001]. In this report, we describe a multiprotein STOC that may serve to integrate Rho family guanine nucleotide triphosphatase (GTPase), calcium/calmodulin (Ca^{++}/CaM), and the 5′-cyclic AMP/cyclic AMP dependent kinase (cAMP/PKA) signaling pathways.

The Rho family of small molecular weight GTPases are molecular switches activated by extracellular stimuli including G-protein coupled receptors (GPCRs) and growth factor receptors [Mackay and Hall, 1998]. The Rho family consists of Rho, Rac, and Cdc42, and is known to induce formation of stress fibers, lamellipodia, or filopodia, respectively [Ridley and Hall, 1992a,b; Ridley et al., 1992; Nobes and Hall, 1995a,b]. These changes in the cellular architecture are through effects on the actin cytoskeletal network and are manifested through interactions of the Rho family with a

Abbreviations used: AKAP, A kinase anchoring protein; CaM, calmodulin; cAMP, 5′-cyclic AMP; GTPase, guanine nucleotide triphosphatase; HT31, human thyroid AKAP; MAPK, mitogen activated protein kinase; PKA, cyclic AMP dependent kinase; RII, type 2 regulatory subunit of PKA; RII-OL, PKA regulatory subunit overlay; STOC, signal transduction organizing complex.

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variety of effector proteins [Fukata et al., 1999b; Hall and Nobes, 2000; Price and Collard, 2001].

One important effector for Cdc42 and Rac1 is IQGAP1 [Hart et al., 1996; Kuroda et al., 1996]. In addition to being a Cdc42/Rac1 effector, IQGAP1 also co-ordinates Ca^{++}/CaM signaling events [Hart et al., 1996; Ho et al., 1999] IQGAP1 has been shown to bind to F-actin [Bashour et al., 1997; Erickson et al., 1997; Fukata et al., 1997], E-cadherin [Kuroda et al., 1998, 1999], β-catenin [Kuroda et al., 1998, 1999], and CLIP170 [Fukata et al., 2002]. These interactions are modulated by Cdc42/Rac1 [Erickson et al., 1997; Kuroda et al., 1998; Fukata et al., 1999a; Ho et al., 1999], whereas Ca^{++}/CaM is known to modulate IQGAP1's interaction with F-actin and E-cadherin [Joyal et al., 1997; Ho et al., 1999; Li et al., 1999]. This has lead to the hypothesis that IQGAP1 functions as a signaling scaffold that co-ordinates GTPase and calcium signaling events resulting in alterations in cytoskeletal and cell adhesion characteristics [Kuroda et al., 1998; Ho et al., 1999].

The cAMP signaling pathway is also known to affect the actin cytoskeletal network and modulate cellular events involving the actin cytoskeleton, including insulin secretion [Lester et al., 1997; Diviani and Scott, 2001; Wilson et al., 2001]. The PKA is localized near key substrates through interactions with A kinase anchoring proteins (AKAPs) [Lester and Scott, 1997; Pawson and Scott, 1997]. One of the most characterized AKAPs is AKAP79 [Dodge and Scott, 2000]. In addition to anchoring PKA, AKAP79 targets protein phosphatase 2B (PP-2B), and protein kinase C (PKC) to membrane regions [Klauck et al., 1996]. AKAP79 also participates in macromolecular assemblies through a direct protein–protein interaction with the β_2-adrenergic receptor and WAVE-1, and indirectly with the AMPA glutamate receptor (AMPA GluR1) [Tavalin et al., 1999; Colledge et al., 2000; Fraser et al., 2000; Westphal et al., 2000; Cong et al., 2001]. Thus, like IQGAP1, AKAP79 serves as a signaling scaffold by co-ordinating the activities of diverse signaling pathways, through either direct interactions with signaling enzymes or incorporation into macromolecular STOCs.

The focus of this paper is the identification of a STOC, consisting of IQGAP1 and AKAP79. This finding further defines the extent of crosstalk between the cAMP and small molecular weight GTPase signaling pathways as has been documented in several instances [Westphal et al., 2000; Feoktistov et al., 2000; O'Connor and Mercurio, 2001; Whittard and Akiyama, 2001]. We demonstrate that this STOC exists through direct interaction between the carboxyl terminus of IQGAP1 with AKAP79. The possible ramifications of this interaction for signaling, with particular emphasis on pancreatic β-cell development and function, are discussed.

MATERIALS AND METHODS

Materials

Protein A sepharose, cAMP agarose, CaM agarose, and collagenase are purchased from Sigma (St. Louis, MO). Glutathione resin is purchased from Pharmacia (Piscataway, NJ), and nickel resin is purchased from Novagen (Madison, WI). All tissue culture reagents are from Invitrogen (Carlsbad, CA). Fluorescent secondary antibodies are from Jackson ImmunoResearch (West Grove, PA) and HRP conjugated secondary antibodies were from Amersham (Piscataway, NJ). Rabbit anti-IQGAP1 is a kind gift from Dr. George S. Bloom (University of Virginia), mouse anti-IQGAP1 and anti-AKAP79 are from BD Transduction Labs (San Diego, CA). The human thyroid AKAP (HT31) peptide, encompassing residues 493–515 of the HT-31 protein, is synthesized and purified by Macromolecular Resources (Colorado State University, Fort Collins, CO) [Lester et al., 1996].

Isolation of Islets and β-Cell Culture

Islets are isolated from human and rat pancreata by collagenase digestion as described [Lester et al., 1997]. The transformed β-cell line, RINm5F, is cultured as previously described [Lester et al., 1997].

CaM Affinity Purification

RINm5F cells were grown to 80% confluency on 150-mm tissue culture plates. The cells are scraped off the plate, washed, and lysed in a 1% Triton lysis buffer (LB, 10 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM KCl, 1% Tx-100) containing protease inhibitors. Lysates are kept on ice for 30 min, and then spun at 40,000*g* for 20 min. The supernatant is collected and mixed with calmodulin agarose at 4°C for 16 h. The resin is then collected, washed, and eluted with EDTA/SDS.

The eluate is separated by SDS–PAGE. The gel is stained with Coomassie blue and the band cut out for microsequencing. The purified band was sent to the Harvard Peptide Sequencing Core (see http://golgi.harvard.edu/microchem, for detailed technical information).

cAMP Affinity Purification

cAMP purification of type 2 regulatory subunit of PKA (RII) binding complexes is accomplished as described [Nauert et al., 1997]. Briefly, RINm5F cells are grown in 10-cm dishes to 90% confluency. The cells are lysed in 1% LB containing 1% Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM IBMX, and protease inhibitors by incubating on ice for 30 min. The lysates are clarified by centrifugation at 12,000 RPM using a bench top microcentrifuge, added to cAMP resin pre-equilibrated in 1% LB, and incubated at 4°C for 2 h. The cAMP resin is then sequentially washed, twice in 1% LB, once in 1% LB plus 1 M NaCl, and finally in 1% LB. Bound proteins are eluted with SDS sample loading buffer.

PKA Activity Measurements

PKA activity is measured as described [Lester et al., 1997, 2001]. The catalytic subunit of PKA is eluted from the IQGAP1 immunoprecipitates using 75-mM cAMP. The assay is carried using a standard filter paper assay according to Corbin and Reimann [1974] using Kemptide (LRRASLG) as the substrate. To determine the specific activity in μmol/min/mg, the immunoprecipitates are then boiled in SDS to elute any remaining proteins and the concentration of the immunoprecipitates determined using the Bio-Rad DC Protein Assay Kit. Specific activity is then determined by subtracting out residual kinase activity not blocked by the PKA specific inhibitor PKI [Lester et al., 1997].

Solid Phase Overlays and Immunoblots

Immunoblots and RII overlays (PKA regulatory subunit overlay, RII-OLs) are performed as described [Lester et al., 1997]. Briefly, purified proteins or cell lysates are separated by SDS–PAGE and electro-transferred to PVDF (Immobilon) membranes. The membranes are blocked in blotto, and incubated with either a primary antibody to a specific antigen or with ^{32}P radiolabeled RII. To detect bound primary antibody, a HRP conjugated secondary antibody (Jackson Labs) is used with a peroxidase substrate chemiluminescent developing solution (ECL, Pierce, Rockford, IL). RII-OLs are washed and directly exposed to Kodak X-OMAT film overnight.

Full-length IQGAP1 purified from SF9 insect cells extracts by nickel affinity chromatography is dot blotted onto nylon membranes. Full-length AKAP79 expressed and purified from *E. coli* is then incubated with the blot. The blot is washed with TTBS and probed with anti-AKAP79 antibody. HRP conjugated secondary is used to visualize the anti-AKAP79 antibody by chemiluminescence.

Immunocytochemistry

Purified islets are dispersed using trypsin and replated onto coverslips. The dispersed islets or RINm5F cells are grown on coverslips, then fixed with 3.7% formaldehyde in PBS for 10 min at room temperature (RT), permeabilized with −20°C acetone for 1 min, and then blocked in 1% BSA in PBS. The cells are then incubated with a mouse anti-AKAP79, mouse anti-IQGAP1, or rabbit anti-IQGAP1 for 1 h at RT. The cells were washed and then incubated with FITC anti-mouse and Texas Red anti-rabbit secondary antibodies. The secondary antibodies were washed off and the coverslips treated with Slofade (Molecular Probes, Eugene, OR). The images are obtained using a Bio-Rad confocal or Nikon immunofluorescent microscope and processed using Adobe Photoshop.

Immunoprecipitations

Immunoprecipitations are conducted as described [Nauert et al., 1997]. Cells are lysed in 1% LB as described above, and incubated with 2–5 μg of affinity purified polyclonal anti-IQGAP1 for 2 h at 4°C. The lysate/antibody solution is added to protein A sepharose beads that had been pre-equilibrated in 1% LB for an additional two hours at 4°C. The protein A beads are washed and precipitated proteins eluted with SDS sample buffer and the eluates analyzed by immunoblotting following separation by SDS–PAGE.

Recombinant Expression of Proteins

Recombinant fragments representing overlapping fragments of IQGAP1 were constructed by PCR. Sense and anti-sense oligos were synthesized and used to amplify fragments of IQGAP1 by PCR. The oligo sets used were: (1) IQGAP1-CHD 5′-cgggatccctgtccgccatgtccgccgc-

agacgag-3′ and 5′-gccgctcgaggcaggcatctgg-3′, (2) IQGAP1-NTR/WW 5′-cgggatccctgtccgccatggttg-acttcacagaag-3′ and 5′-gccgctcgagctggatctcctccc-3′, (3) IQGAP1-IQR 5′-cgggatccctgtccgccatgctg-gagacccaggaa-3′ and 5′-gccgctcgagagacctcccttc-3′, (4) IQGAP1-GRD_L 5′-cgggatccctgtccgccatga-cgttgcaggatgtg-3′ and 5′-gccgctcgagtggattggatc-3′, (5) IQGAP1-GRD_S 5′-cgggatccctgtccgccatggt-cagctctgtgg-3′ and 5′-gccgctcgagtggattggatc-3′, (6) IQGAP1-CT 5′-cgggatccctgtccgccatgaccca-cactctcc-3′ and 5′-gccgctcgagcttcccgtagaac-3′. The resulting amplified fragments are subcloned into the pGEX4T3 vector (Pharmacia). The inserts are analyzed by restriction digest and sequencing. These constructs are expressed in the BL21 (DE3) strain of *E. coli* and the recombinant protein purified over a glutathione resin (Pharmacia). The resin is washed and the bound protein eluted by glutathione. Full-length histidine tagged IQGAP1 is recombinantly expressed using the baculovirus/SF9 system. The baculovirus His_6-tagged full-length IQGAP1 construct is a gift of Dr. George S. Bloom. The SF9 cells are infected with the IQGAP1-baculovirus, lysed by sonication and His_6-tagged full-length IQGAP1 purified by conventional nickel affinity chromatography.

Recombinant Binding Assays

The GST pull down experiments are performed by mixing 130 ng of IQGAP1–GST proteins with glutathione beads for 30 min at 4°C. Purified AKAP79 protein, 1.4 μg is added for 30 min and then spun down, washed, and eluted with SDS sample buffer. The eluate is analyzed by SDS–PAGE and immunoblotting, using anti-GST, anti-AKAP79, and HRP anti-mouse Ig secondary antibodies.

Statistical Analysis

Statistical assessment of means and standard deviation is performed using Prism Software. All tests are two-tailed, with a statistical significance at $P < 0.05$.

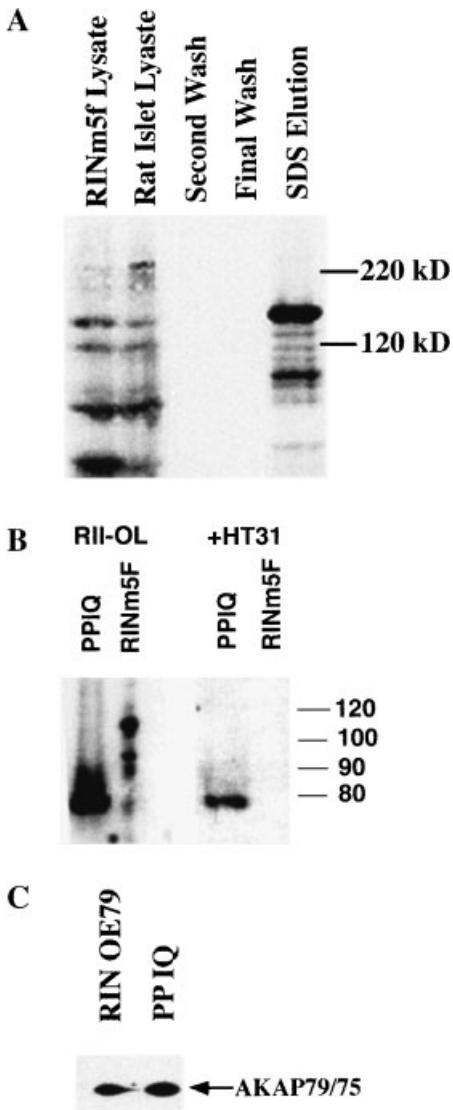
RESULTS

Identification of an IQGAP1/AKAP79 Complex From β-Cells

Our previous work with pancreatic β-cells indicated an important role for cAMP and Ca^{++}/CaM signaling pathways in regulation of insulin secretion [Lester et al., 1997, 2001]. We, therefore, wanted to identify any CaM binding AKAPs in β-cells that could co-ordinate these two signaling pathways. Coomassie staining of the elution from a CaM affinity purification of RINm5F cell extract indicated a predominant CaM binding protein that migrates at approximately 150–170 kDa. To identify the presence of any AKAPs in the elution, we probed the CaM purified proteins with radiolabeled RII in an RII overlay (RII OL) assay. Critical to the original identification and characterization of the AKAP family, the RII overlay assay relies on the limited refolding of a key amphipathic helix of the AKAP following transfer to the membrane [Carr and Scott, 1992; Hausken et al., 1998; Newlon et al., 2001]. In fact anchoring inhibitor peptides, such as the HT31 peptide, generated to this region are able to disrupt RII/AKAP interactions both in vitro and in vivo [Lester et al., 1997; Hausken et al., 1998]. An RII overlay performed on the CaM agarose elution indicates an AKAP of approximately the same molecular weight as the band observed on a Coomassie gel (Fig. 1A). To determine the identity of this protein, the Coomassie-stained band was cut out and sent for peptide sequencing. Peptide sequencing of this band identified three possible proteins, spectrin, myosin VI, and IQGAP1 as the band on the gel. Initially, spectrin and myosin VI were investigated. However, neither spectrin nor myosin VI were able to bind RII by RII-OL or surface plasmon resonance (SPR) (data not shown), therefore, we investigated the RII binding capacity of IQGAP1.

To determine if IQGAP1 is the AKAP purified by calmodulin affinity chromatography, we performed an RII overlay on a partially purified protein sample of IQGAP1 (PPIQ) from bovine adrenal tissue (gift of Dr. George Bloom). No RII binding protein at the molecular weight of IQGAP1 is observed (Fig. 1B) but a prominent RII binding protein at approximately 75 kDa (Fig. 1B) is identified. As described above, RII binding specificity in the RIIOL is typically confirmed by using the anchoring inhibitor peptide (HT31). As expected, in the presence of HT31 peptide no RII binding bands are observed in the RINm5f cell lane (Fig. 1B). A dramatic decrease in the RII binding signal is observed in the partially purified full-length native IQGAP1 lane (Fig. 1B). The residual signal is due to the large amount of AKAP79 present in the partially purified IQGAP1 lane and is consistent with previous experiments, we

have performed with several different AKAPs. Immunoblot analysis identifies this RII binding protein as AKAP75, the bovine homolog of rat AKAP150 and human AKAP79 (Fig. 1C). Since the partially purified IQGAP1 sample had been purified through three distinct chromatographic steps, we hypothesize that IQGAP1 and AKAP79 either directly interact or are part of a larger multiprotein complex. Retro-analysis of RINm5F cell lysates suggest that the AKAP eluted from the calmodulin agarose affinity purification (Fig. 1A) is most likely AKAP150, not IQGAP1. Thus, our discovery of the IQGAP1/AKAP79 complex is fortuitous in that the appearance of IQGAP1 amino acid sequence in our peptide analysis (Fig. 1) is probably due to a contaminating 150 kDa IQGAP1 breakdown product we commonly observe (see Fig. 5A).



IQGAP1 and AKAP79 Co-Localize to β-Cell Membranes

Our biochemical data suggest IQGAP1 and AKAP79 interact in β-cells. To confirm this and identify the subcellular distribution of each protein, we visualized AKAP79 and IQGAP1 by confocal microscopy in human islet cells. AKA79 is visualized using a FITC tagged anti-mouse secondary while IQGAP1 is visualized using a Texas Red tagged anti-rabbit secondary antibody. Areas of yellow color, seen around the periphery of the cells, indicate where the two proteins occupy the same focal plane (Fig. 2A). Furthermore, IQGAP1 and AKAP79 subcellular distribution is demonstrated using indirect immuofluorescence in the transformed rat β-cell line RINm5F (Fig. 2B). Here, heterologously expressed AKAP79 is seen in red, while endogenous IQGAP1 is seen in green. As observed in the islets, both proteins have predominant cellular staining at the periphery. This is consistent with previous observations from other

Fig. 1. Identification of an IQGAP1/A kinase anchoring protein 79 (AKAP79) complex. RINm5F cells were lysed in 1% Triton buffer and subjected to calmodulin (CaM) agarose affinity purification. After extensive washing of the CaM agarose column, bound proteins were eluted with SDS–PAGE sample buffer. **A**: An cyclic AMP dependent kinase (PKA) regulatory subunit overlay (RII-OL) of the CaM affinity purified sample was performed to identify possible CaM binding AKAPs. An islet lysate sample was run alongside the RINm5F sample lysate to demonstrate the similar AKAP population. The RII-OL indicates the enrichment of a ~150–170 kDa RII binding band in the elution lane that is not noticeably visible in the lysate, suggesting it is expressed at low levels in the cell. The ~150–170 kDa RII binding band in the elution lane was chosen for peptide sequencing. Peptide sequences for three proteins, spectrin, myosin VI, and IQGAP1 were obtained. Analyses for RII binding capability ruled out spectrin and myosin VI as AKAPs. **B**: RII-OL on a partially purified sample of IQGAP1 (PPIQ) isolated from bovine adrenal tissue obtained from George S. Bloom (University of Virginia). No RII binding band was identified at the expected 150–170 kDa size suggesting that IQGAP1 is not an AKAP. However, a co-purifying RII binding band at 75 kDa is identified. Incubation of 1 μM human thyroid AKAP (HT31) anchoring inhibitor peptide in the RII-OL blocked binding of RII to the AKAP on the blot consistent with the band being an AKAP. **C**: To identify the co-purifying AKAP in the IQGAP1 bovine adrenal sample we undertook a Western analysis using antibodies to known AKAPs. AKAP75 is a very well characterized bovine AKAP whose human and murine homolog are 79 and 150 kDa, respectively. Immunoblot analysis of the same partially purified bovine adrenal IQGAP1 sample using anti-AKAP75/79 antisera confirmed the identity of the co-purifying AKAP as AKAP75 (79/150). An AKAP79 over expressing RINm5F cell lysate was run as a control for AKAP79. Abbreviations used are: PPIQ for the partially purified adrenal IQGAP1 and RINOE79 for RINm5F cells overexpressing AKAP79.

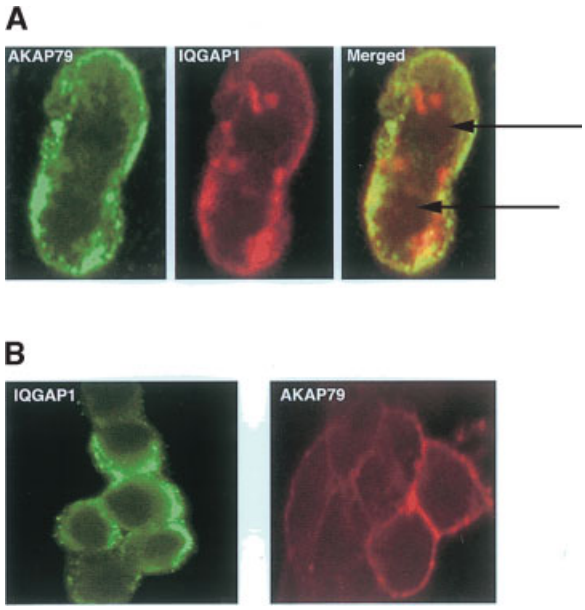


Fig. 2. Co-localization of IQGAP1 and AKAP79 in islets and RINm5F cells. **A**: IQGAP1 (red) and AKAP79 (green) have distinct yet overlapping patterns of distribution within the islet cells, consistent with the two proteins associating within the cell. Control experiments using FITC and Texas Red for IQGAP1 and AKAP79, respectively show similar results. The green and red images were obtained separately to minimize bleed through. Co-localization is seen as yellow in the micrograph. **B**: Individual staining of multiple AKAP79 overexpressing RINm5F cells for AKAP79 (red) or IQGAP1 (green). Consistent with the islet staining, both proteins are located primarily at the periphery of the cell.

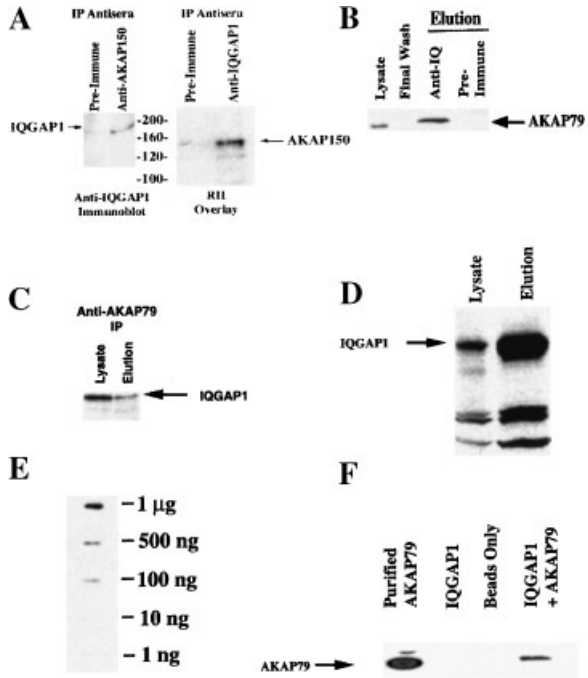


Fig. 3. Association of AKAP79/150 with IQGAP1 in rat β-cells and in vitro. **A**: On the **left**, IQGAP1 is detected by immunoblot of an AKAP150 immunoprecipitate but not in a pre-immune immunoprecipitate and on the **right**, AKAP150 is detected by immunoblot of an IQGAP1 immunoprecipitate but not in a pre-immune immunoprecipitate. **B**: AKAP79, from RINm5F cells over expressing human AKAP79, co-precipitates with IQGAP1 using an anti-IQGAP1 antibody. AKAP79 is identified in the eluate by immunoblotting with an AKAP79 specific antibody. **C**: Conversely, an IQGAP1 immunoblot of an AKAP79 immunoprecipitation shows the presence of IQGAP1. **D**: Immunoblot analysis demonstrating the enrichment of IQGAP1 following purification and concentration of the baculovirus purification of IQGAP1. **E**: Baculovirus expressed and purified full-length human IQGAP1 is slotted onto PVDF membranes at amounts of 1 μg, 500, 100, 10, and 1 ng. The membrane is incubated with purified AKAP79 (1 μg/ml or 12 nM) in Tris-buffered saline (TBS) for 30 min at 20°C. Bound AKAP79 is identified by immunoblotting. **F**: One microgram of baculovirus expressed and purified full-length IQGAP1 is incubated either alone (IQGAP1) or with purified AKAP79 (4 μg) (79 + IQGAP1). This represents a vast molar excess of AKAP79 over IQGAP1, explaining the much stronger band in the load as compared to the elution lane. IQGAP1 is then immunoprecipitated using anti-IQGAP1 polyclonal antisera. Control immunoprecipitations are done with AKAP79 plus anti-IQGAP1 and no IQGAP1 (beads) or with no AKAP79 in the precipitation. AKAP79 is detected by immunoblotting.

labs concerning IQGAP1's and AKAP79's subcellular distribution and biochemical function [Colledge et al., 2000; Mateer et al., 2002]. Cumulatively, these studies support an association between IQGAP1 and AKAP79 at the cell periphery.

Association of IQGAP1 and AKAP79 in Cells

Having identified AKAP75 in a partially purified sample of IQGAP1, we hypothesize that IQGAP1 participates in a complex with AKAP75/79/150. To demonstrate this complex in β-cells, we immunoprecipitated endogenous AKAP150 from RINm5F cells. We are able to detect IQGAP1 in AKAP150 immunoprecipitates but not in pre-immune elutions (Fig. 3A). Additionally, we are able to detect, by RII overlay, an AKAP at 150 kDa in IQGAP1 immunoprecipitates but not in immunoprecipitates of pre-immune sera (Fig. 3A). Notably, we only detect one prominent RII binding band in the immunoprecipitate. Due to excessive background reactivity of the anti-AKAP150 antibody with serum proteins we were unable to clearly identify this AKAP as AKAP150. To circumvent this problem we use RINm5F cells heterologously expressing human AKAP79 [Lester et al., 2001]. Using these cells we are able to detect AKAP79 in IQGAP1 immunoprecipitates, but not in immunoprecipitates using

pre-immune sera (Fig. 3B). Conversely, we are able to detect IQGAP1 in AKAP79 immunoprecipitates suggesting that AKAP79/150 interacts with IQGAP1 in β-cells (Fig. 3C). However, what remains to be determined is whether AKAP79 directly interacts with IQGAP1 or whether they are part of a larger protein complex.

Direct Binding of AKAP79 to IQGAP1

Having demonstrated that IQGAP1 exists in a STOC with AKAP79 in β-cells, we next examined whether this occurs through a direct or indirect interaction between IQGAP1 and AKAP79 using solid and solution phase binding assays. In a slot blot assay, using a partially purified baculovirus expressed full-length IQGAP1 immobilized on PVDF membranes with 12 nM AKAP79 as a probe, we demonstrate that AKAP79 binding to IQGAP1 can be detected at low levels ($\leq$100 ng of immobilized IQGAP1) (Fig. 3E). Control experiments using no protein or bovine serum albumin immobilized on PVDF showed no binding of AKAP79 to the filter (data not shown). We are also able to demonstrate an interaction between the partially purified baculovirus FL-IQGAP1 and AKAP79 in solution (Fig. 3F). AKAP79 is detected by immunoblot in the elution of an IQGAP1 immunoprecipitation when incubated with FL-IQGAP1 and anti-IQGAP1 antibody (IQGAP1 + AKAP79) but not with anti-IQGAP1 antibody and protein A agarose alone (beads only), or when no AKAP79 (IQGAP1) is included in the binding reaction (Fig. 3F). Thus, it appears that IQGAP1 and AKAP79 directly interact with each other, though we have not yet determined the actual affinity of the interaction. However, the full-length IQGAP1 protein purified from SF9 cells is only partially pure which brings up the possibility that an insect cell protein co-purifies with our recombinant IQGAP1 preparation and is responsible for the positive association of AKAP79 with IQGAP1 described above. To answer this question, we investigated the nature of the AKAP79/IQGAP1 interaction using bacterially expressed and purified samples of AKAP79 and overlapping recombinant fragments of IQGAP1.

Identification of the Region of IQGAP1 That Interacts With AKAP79

To further characterize the IQGAP1/AKAP79 interaction, we identify the region of IQGAP1 responsible for the interaction with AKAP79 using a GST pulldown method. We subcloned overlapping fragments of IQGAP1, as diagrammed in Figure 4A, representing identified protein domains into a GST fusion vector. These fragments are expressed in *E. coli* and purified on glutathione resin. The identity of the purified GST fusion proteins is confirmed by immunoblot using an anti-GST antibody (Fig. 4B). The various IQGAP1 protein fragments are incubated with purified AKAP79 and pulled down with glutathione resin. AKAP79 is identified by immunoblotting (Fig. 4C). Only the carboxyl terminus of IQGAP1 (IQGAP1-CT) is found to pull down AKAP79. AKAP79 did not pull down with resin alone or with GST control protein (data not shown). The interaction of IQGAP1-CT with AKAP79 is inhibited by the addition of a separate but overlapping fragment of murine IQGAP1 that contained a histidine tag (K18, residues 1,056–1,657) (data not shown). These data further support a direct interaction between the C-terminus of IQGAP1 and AKAP79.

IQGAP1 Associates With PKA in Pancreatic β-Cells

Having observed that IQGAP1 and AKAP79 co-purify from bovine adrenal tissue (Fig. 1B,C), and directly interact (Figs. 3 and 4) we wanted to ascertain whether PKA is present in this ternary complex in pancreatic β-cells. Initally, PKA and associated binding proteins are isolated from human islet lysates by cAMP agarose affinity resin. The resin is then washed extensively and eluted with either cAMP (for kinase assays) or with SDS loading buffer (for immunoblots or RII overlays). Using this technique we are able to detect IQGAP1 in the eluate by immunoblotting (Fig. 5A). Conversely, we can co-purify PKA activity in IQGAP1 immunoprecipitates from RINm5F cell lysates (Fig. 5B). The specific PKA activity is 58× greater in the IQGAP1 IP than in the pre-immune IP (*, $P < 0.0004$) thus confirming an association between PKA and IQGAP1 in β-cells. Furthermore, the PKA activity is 14× greater in the IQGAP1 immunoprecipitate over the lysate, consistent with an IQGAP1 specific enrichment of kinase (**, $P = 0.004$). Importantly, treatment of IQGAP1 immunoprecipitates with the anchoring inhibitor peptide HT31 prevented the co-precipitation of PKA with IQGAP1 (***, $P = 0.031$). This indicates that the PKA activity that co-precipitates with IQGAP1 is associated

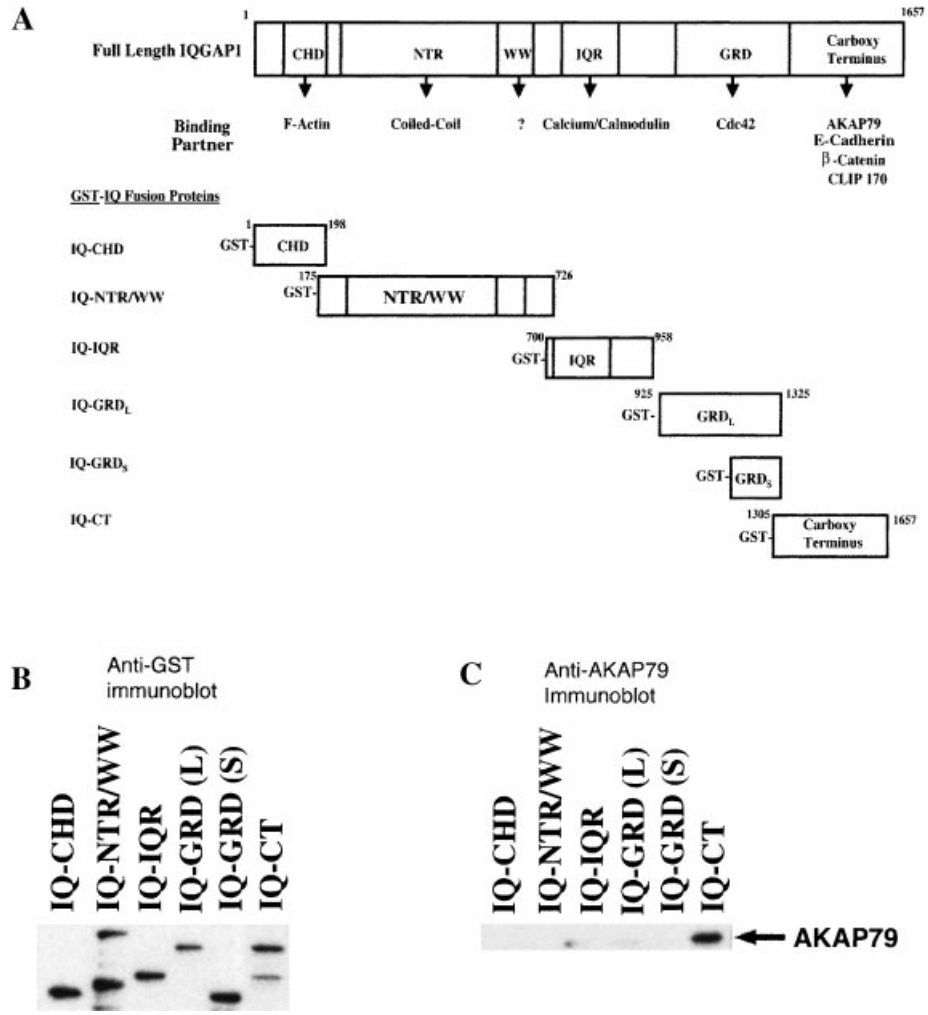


Fig. 4. Localization of the AKAP79 binding site to the carboxyl terminus of IQGAP1. **A**: Diagram of the recombinant fragments generated to identify the AKAP79 binding domain of IQGAP1. **B**: Anti-GST immunoblot of GST–IQGAP1 fusion proteins used in the binding experiments. The GST Western confirms the presence of similar amounts of GST fusion proteins in all lanes. **C**: Anti-AKAP79 immunoblot of the pulldown experiments. The blot shows AKAP79 only being found in the GST–IQGAP1-CT elution lane but not in the other lanes. Abbreviations used are: IQ-CHD is the calponin homology domain, IQ-NTR/WW is the N-terminal repeats, and WW domains, IQ-IQR is the IQ repeats, IQ-GRD$_L$ is the GTPase related domain based on homology of [Weissbach et al., 1994], IQ-GRD$_S$ is the GTPase related domain based on the homology of [Hart et al., 1996], and IQ-CT is the carboxy terminus of IQGAP1.

through an anchoring protein. Since we only detect one prominent RII binding protein, AKAP79/150, co-precipitating with IQGAP1 (Fig. 3A), we conclude that PKA associates with IQGAP1 through a direct interaction with AKAP79/150.

DISCUSSION

Our initial goal is the identification of novel signaling components in β-cells that co-ordinate Ca^{++}/CaM and cAMP signaling components into one STOC. We demonstrate that the multifunctional signaling scaffold, IQGAP1, forms a STOC with AKAP79, another multifunctional signaling scaffold protein. Given IQGAP1's and AKAP79's multiple protein–protein interactions, it is reasonable to hypothesize that the IQGAP1/AKAP79 STOC participates in many cellular signaling pathways. IQGAP1, in particular, has several biochemical features that may have a central role important for β-cell growth and function making it worthy of further investigation.

IQGAP1 serves as a signaling scaffold protein that co-ordinates Ca^{++}/CaM and Cdc42/Rac1

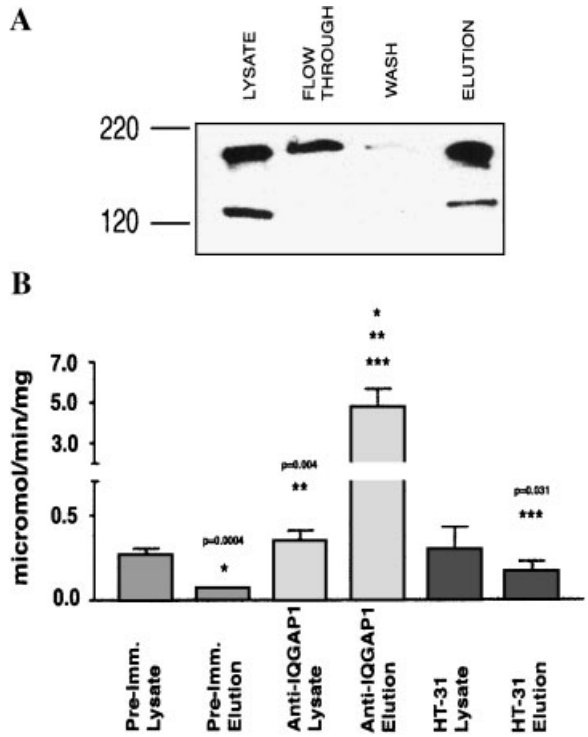


Fig. 5. IQGAP1 associates with PKA in β-cells. **A**: To determine whether PKA co-purifies with the IQGAP1/AKAP79/150 ternary complex from β-cells we undertook several lines of experimentation. The first methodology used is to co-purify IQGAP1 with PKA through cAMP affinity chromatography. Human islet lysates are incubated with cAMP sepharose. The lysate (Lys) and column fractions representing the flow through (FT), wash (W) and elution (El) are separated by SDS–PAGE. IQGAP1 is identified by immunoblotting. The lower band, ~150 kDa, represents a breakdown product of IQGAP1 we commonly observe using multiple IQGAP1 antibodies. **B**: RINm5F lysates are incubated with affinity purified anti-IQGAP1 antibody or an equal amount of pre-immune sera. The immunocomplex is precipitated on protein A sepharose. After washing, the catalytic subunit of PKA is eluted by incubating the columns in 75 mM cAMP, pH 7.4 for 15 min at 20°C. The cAMP eluted catalytic subunit activity is determined by a standard filter paper PKA assay. PKA activity in the IQGAP1 immunoprecipitate is 58-fold greater than in the pre-immune elution, 4.72 versus 0.081 μmol/mg/min, respectively (*, $P = 0.0004$). Additionally, the IP represents a 14-fold enrichment of kinase activity over the specific activity of the lysate, 4.72 versus 0.34 μmol/mg/min, respectively (**, $P = 0.004$), as expected with the specific precipitation of PKA with IQGAP1. Treatment of the immunoprecipitates with an anchoring inhibitor peptide to release anchored PKA results in the loss of immumoprecipitated PKA compared to non-treated IQGAP1 immunoprecipitates, 0.16 μmol/mg/min versus 4.72 μmol/mg/min respectively (***, $P = 0.031$). These data show that PKA is associated with IQGAP1 through an AKAP. Data shown are the mean of duplicates from four separate experiments as determined by two-tailed *t*-test using Prism software.

signaling pathways through several conserved protein–protein interaction domains [Ho et al., 1999]. IQGAP1 possesses a calponin homology domain (CHD) that binds and crosslinks actin filaments, six N-terminal repeats predicted to form a homomeric coiled-coil, a WW domain of unknown function, four IQGAP1 repeats involved in binding Ca^{++}/CaM, and a carboxyl terminal GAP related domain (GRD) [Hart et al., 1996; Kuroda et al., 1996; Bashour et al., 1997; Fukata et al., 1997]. Additionally, IQGAP1 binds to E-cadherin and β-catenin [Kuroda et al., 1998, 1999]. The interaction between IQGAP1 and E-cadherin/β-catenin results in a low affinity E-cadherin binding state and a reduction in cell–cell adhesion. Activated Cdc42 induces the dissociation of IQGAP1 from E-cadherin/β-catenin and the formation of a high affinity E-cadherin/β-catenin/α-catenin/F-actin complex [Kuroda et al., 1998, 1999]. Ca^{++}/CaM has an analogous regulatory role compared to Cdc42 in E-cadherin regulation [Li et al., 1999; Kim et al., 2000]. Consequently, IQGAP1 is thought to serve as a scaffold protein to integrate Cdc42/Rac1 and Ca^{++}/CaM signals into changes in the adhesive state of a cell.

The novel interaction between IQGAP1 and AKAP79 described here indicates that IQGAP1 may serve to integrate cAMP/PKA signals as well. Using a variety of co-purification and binding assays we have identified IQGAP1 as a major CaM binding protein in pancreatic β-cells that participates in a STOC that incorporates PKA. Moreover, we demonstrate that PKA and IQGAP1 can interact through AKAP79. Confocal microscopy of IQGAP1 and AKAP79 indicates co-localization at the cell membrane of pancreatic β-cells consistent with the known functions of IQGAP1 and AKAP79 in regulating membrane and cytoskeleton events.

The physiological role of this complex remains unknown. It is also unclear what regulates the association of IQGAP1 with AKAP79. However, crosstalk between the cAMP and small molecular weight GTPase signaling pathways has been documented. For example, the effect of PKA on Rap1 and the regulation of downstream mitogen activated protein kinase (MAPKs) in developmental progression are well documented [Cook and McCormick, 1993; Wu et al., 1993; Stork and Schmitt, 2002]. Rad1, a GTPase important for glucose transport in cultured muscle and fat cells, is phosphorylated by PKA. Furthermore, PKA causes a decrease in Rad1's affinity for GTP [Moyers et al., 1998].

Relevant to this paper, is the effect of cAMP on the Cdc42/Rac1 signaling pathways. Activation

of Rac1 in response to chemo-attractants or integrin clustering is regulated by PKA activity [O'Connor and Mercurio, 2001; Whittard and Akiyama, 2001]. Similarly, cAMP and PKA modulate the active state of Cdc42 in human mast cells [Feoktistov et al., 2000]. Both these events likely occur through PKA regulation of GTPase regulatory proteins, such as exchange factors or GTPase activating proteins.

Other GTPase effector proteins can also interact with PKA. WASP/WAVE was recently identified as an AKAP. WAVE-1 may function to recruit PKA and the abl kinase to sites of actin reorganization [Westphal et al., 2000]. Though the interaction between WASP/WAVE and PKA are similar to those described here, there are several poignant differences. IQGAP1 binds PKA indirectly via AKAP79, whereas WAVE-1 bound PKA directly. Also, IQGAP1 shows specificity for Cdc42, whereas WAVE-1 shows specificity for Rac1. Furthermore, AKAP79 also binds PKC and PP-2B, expanding the regulatory capacity of this STOC and further differentiating the signaling capacity of this complex from WAVE. Though WAVE-1 and IQGAP1/AKAP79 serve as scaffolds for similar signaling enzymes, their molecular organization and other known-binding partners suggest that they likely serve to generate very different changes in cellular physiology.

In this paper, we demonstrate a direct interaction between IQGAP1 and AKAP79 and have mapped the AKAP79 binding site on IQGAP1 to its carboxyl terminus. Interestingly, the carboxyl terminus of IQGAP1 directly interacts with CLIP170 and is involved in the regulation of IQGAP1's interaction with E-cadherin and β-catenin. It is not known whether AKAP79 binding competes with CLIP170 for the carboxyl terminus of IQGAP1. However, the fragment we identified as the AKAP79 binding site contained residues 1,305–1,657, which is larger than the region (residues 1,503–1,657) shown to bind CLIP170. Further studies are required to define the AKAP79 binding site on IQGAP1.

Several potential mechanisms could regulate the IQGAP1/AKAP79 interaction and are currently under investigation. It is critical to determine if Rac1/Cdc42 or Ca^{++}/CaM regulate the interaction of IQGAP1 with AKAP79. Initial studies in our lab indicate that Cdc42 does not compete with IQGAP1-CT for binding to AKAP79 (data not shown). Though the IQGAP1-CT fragment is necessary, it is not sufficient for high affinity binding of Cdc42 [Hart et al., 1996; Kuroda et al., 1996]. Therefore, to further investigate the role of Cdc42 in regulating the IQGAP1/AKAP79 interaction, we will need to test a larger fragment of IQGAP1. However, it remains possible that Cdc42 is necessary for the binding of full-length IQGAP1 to AKAP79. The AKAP79 binding site could be hidden through an intramolecular, a homomeric intermolecular, or a heteromeric intermolecular interaction. In this scenario, the IQGAP1-CT binding site would be not available for binding to AKAP79 in the full-length IQGAP1 until Cdc42 binding exposes the AKAP79 binding site. However, since we are able to immunoprecipitate purified AKAP79 with purified full-length IQGAP1 in vitro this may not be the case. It will, however, be necessary to investigate this possibility more thoroughly. Furthermore, the IQGAP1-CT region possesses a consensus PKA phosphorylation site, and can be phosphorylated by PKA in vitro (Lester, unpublished results). Whether phosphorylation of the carboxyl terminus of IQGAP1 occurs in vivo and how this might affect IQGAP1 function or localization is unknown. To date, we can state that IQGAP1-CT is sufficient to bind to AKAP79 independently of Cdc42 activity.

Our initial interest in IQGAP1 came during investigation of CaM binding proteins that may play a role in regulating insulin secretion. Though the IQGAP1/AKAP79 STOC function remains unknown, IQGAP1's known biochemical partners and function suggest it may regulate β-cell function and development. E-cadherin homodimeric interactions have been shown to be important in β-cell development and secretion [Rouiller et al., 1990, 1991; Dahl et al., 1996]. As well, both PKA and Cdc42/Rac1 have been shown to be important regulatory signaling components of the insulin secretion response [Kowluru et al., 1996, 1997; Lester et al., 1997]. We have been able to establish a shared mechanism between the cAMP signaling and Cdc42/Rac signaling pathways in the regulation of insulin secretion (Lester and Nauert, unpublished results). Therefore, IQGAP1 is ideally situated to have an important role in the coordination of the Cdc42/Rac, Ca^{++}/CaM, and cAMP signaling pathways in β-cell development and physiology. Studies are underway to investigate this.

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