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A direct interaction between the N-terminus of adenylyl cyclase AC 8 and the catalytic subunit of protein phosphatase 2A

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Running title page

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AC8, adenylyl cyclase 8; $[Ca^{2+}]_i$, intracellular calcium; CCE, capacitative Ca^{2+} entry; GFP, green fluorescent protein; GST, glutathione S-transferase; LTP, long-term potentiation; mAB, monoclonal antibody; MW, molecular weight; NR, NMDA receptor subunit; PKA, cAMP-dependent protein kinase; PSD, post-synaptic density; PP2A, protein phosphatase 2A; $PP2A_A$, PP2A scaffolding subunit A; $PP2A_B$, PP2A regulatory subunit B; $PP2A_C$, PP2A catalytic subunit; PKA, protein kinase A; SDS, sodium dodecyl sulphate; TRP, transient receptor potential; VGCC, voltage-gated calcium channel.

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ABSTRACT

Although protein scaffolding complexes compartmentalize protein kinase A (PKA) and phosphodiesterases to optimize cAMP signalling, adenylyl cyclases, the sources of cAMP, have been implicated in very few direct protein interactions. The N-termini of adenylyl cyclases are highly divergent, which hints at isoform-specific interactions. Indeed, the Ca^{2+}-sensitive adenylyl cyclase 8 (AC8) contains a Ca^{2+}/calmodulin binding site on the N-terminus that is essential for stimulation of activity by the capacitative entry of Ca^{2+} in the intact cell. Here, we have used the N-terminus of AC8 as a bait in a yeast 2-hybrid screen of a HEK 293 cell cDNA library, and identified the catalytic subunit of the serine/threonine protein phosphatase 2A ($PP2A_C$) as a binding partner. Confirming the highly specific nature of this novel interaction, GST fusion proteins containing the full length N-terminus of AC8 affinity-precipitated catalytically active $PP2A_C$ from both HEK293 and mouse forebrain membranes – the latter a normal source of AC8. The scaffolding subunit of PP2A ($PP2A_A$; 65kDa) was also precipitated by the N-terminus of AC8, indicating that AC8 may occur in a complex with the PP2A core dimer. Intriguingly, the interaction between the N-terminus of AC8 and $PP2A_C$ was antagonized by Ca^{2+}/calmodulin. However, $PP2A_C$ and Ca^{2+}/calmodulin did not share identical binding specificities in the N-terminus of AC8. PKA-mediated phosphorylation did not influence either calmodulin or $PP2A_C$ association with AC8. In addition, both $PP2A_C$ and AC8 occurred in lipid rafts. These findings are the first demonstration of an association between adenylyl cyclase and any downstream element of cAMP signaling.

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INTRODUCTION

Many of what have later turned out to be generalities in signal transduction were first established in cAMP signaling. Thus it is surprising that although phosphodiesterases and PKA have all been shown to be involved in scaffolding complexes, AC, the instigator of the pathway, has not been found until now to be directly associated with any downstream regulatory element. The organization of regulatory proteins into macromolecular complexes is now expected to be encountered as a device to optimize the efficiency of signal transduction - particularly between different regulatory pathways (Smith and Scott, 2002). The interaction between Ca^{2+}- and cAMP-regulated pathways occurs at multiple levels, with the earliest modulation occurring at the site of cAMP synthesis. Of the nine membrane bound isoforms of adenylyl cyclase (AC^{1}), five are either stimulated or inhibited by physiological increases in $[Ca^{2+}]_i$. AC1 and AC8 are activated by $[Ca^{2+}]_i$ in a calmodulin-dependent manner, while AC5 and AC6 are inhibited by Ca^{2+}, independently of calmodulin (Cooper, 2003). In the intact cell, Ca^{2+}-regulated adenylyl cyclases selectively respond to Ca^{2+} entry through either capacitative calcium entry (CCE) channels or voltage-gated calcium channels (VGCCs) (Fagan et al., 2000a). A growing body of evidence suggests that Ca^{2+}-sensitive adenylyl cyclases and CCE channels are functionally co-localized, (Chiono et al., 1995; Cooper et al., 1994; Fagan et al., 2000a; Fagan et al., 1996; Fagan et al., 1998; Smith et al., 2002) a situation which is reinforced by their compartmentalization in plasma membrane domains rich in cholesterol and sphingolipids, known as lipid rafts (Fagan et al., 2000b; Smith et al., 2002). The presence of Ca^{2+}-sensitive adenylyl cyclases in such domains may permit specific interactions with other signaling proteins present in rafts (Davare et al., 2001; Foster et al., 2003; Lavine et al., 2002). However, very few proteins have been identified that specifically interact with adenylyl cyclases.

AC8 is predominantly expressed in brain areas associated with learning and memory (Mons et al., 1998). Indeed, AC8 knockout mice display deficits in hippocampal LTP, a cellular correlate of memory formation, which involves dynamic alterations in $[Ca^{2+}]_i$ and cAMP (Frey et al., 1993; Poser and Storm, 2001; Wang et al., 2003). The molecular basis whereby a rise in $[Ca^{2+}]_i$ regulates AC8 activity is becoming clearer (Gu and Cooper, 1999). The N-terminus of AC8 contains an amphipathic alpha-helical

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calmodulin-binding domain, which is absolutely essential for stimulation by Ca^{2+} in the intact cell (Gu and Cooper, 1999; Smith et al., 2002). Deletion of the N-terminus results in an activity that, while still stimulable by Ca^{2+}/calmodulin *in vitro*, is no longer stimulable by CCE in the intact cell (Smith et al., 2002). The N-termini of all adenylyl cyclase isoforms are highly divergent and might be expected to function in isozyme-specific interactions. Indeed, recently, using a yeast two-hybrid approach, the N-terminus of AC6 was found to interact with snapin, a component of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attached protein receptors) complex. (Chou et al., 2004). Since we were convinced that the N-terminus of AC8 played a critical role in regulation in the intact cell, we adopted a yeast two-hybrid strategy to search for regulatory partners of AC8. We found that, in addition to associating with calmodulin, the N-terminus of AC8 interacts with the catalytic subunit of protein phosphatase 2A ($PP2A_C$). The N-terminus of AC8 pulled down $PP2A_C$, not only from HEK 293 cell lysates, but also from brain homogenates. Additionally, AC8 and $PP2A_C$ were both detected in lipid rafts, which suggests that PP2A may be situated to efficiently control downstream kinase activity, initiated by the Ca^{2+}-dependent activation of AC8. These findings identify a potentially important direct interaction between adenylyl cyclase and a downstream component of the cAMP signaling cascade.

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MATERIALS AND METHODS

Materials. Monoclonal flotillin and PP2A catalytic subunit antibodies were obtained from BD Transduction Labs (Erembodegem, Belgium). Monoclonal calmodulin antibody was from Upstate (Lake Placid, NY). Polyclonal PP2A structural subunit A and the polyclonal pan-PP2A$_B$ antibody were a gift from Dr. Brian Wadzinski (Department of Pharmacology, Vanderbilt Univ. Nashville, Tennessee). Polyclonal Gαs-olf and PKAα catalytic subunit (C-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Amersham Biosciences (Little Chalfont, UK) and horseradish peroxidase-conjugated goat anti-mouse IgG conjugated was from Promega (Madison, USA). Paraformaldehyde was obtained from TAAB Laboratories (Aldermaston, UK). Tissue culture media and mammalian protease inhibitor cocktail was purchased from Sigma (Poole, UK). His-Select™ cobalt affinity gel was from Sigma and glutathione-Sepharose was from Amersham Biosciences.

Yeast 2-Hybrid System. The N-terminus of rat AC8 (residues 1-179) was PCR amplified from a plasmid containing full-length AC8 (construct 8Nt). The following oligonucleotide primers were used: 5'-ccg aat tca tgg aac tct cgg atg tgc act gcc tta g-3' (primer r_AC8-Nt-F), and 5'-atg gat ccc tcc gat ttg cgc ctc tgg ccc agg aa-3' (primer r_AC8-Nt-R). The resulting PCR product was digested with *Eco*RI and *Bam*HI restriction enzymes and subcloned between the *EcoR*I and *BamH*I sites of plasmid pGBK-T7 (BD Biosciences). The PCR-amplified insert (pGBK-8Nt) was sequenced to check for errors, and then the vectors were tested for self-activation in the yeast 2-hybrid system alongside the empty transcription-activation domain vector pGAD-T7 (BD Biosciences). Plasmids pGBK-8Nt failed to self-activate. The AC8 N-terminus-encoding the DNA fragment was also subcloned between the *Eco*R1 and *Xho*I sites of plasmid pGAD-T7 (generating pGAD-8Nt1) to allow interactions detected in the screen to be confirmed in both directions. Yeast strain AH109 (genotype: *MAT***a** *trp1*-901 *leu2*-3,112 *ura3*-52 *his3*-200 *gal4*Δ*gal80*Δ *LYS2*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 MEL1 GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2*, *URA3*::*MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*), a human kidney cDNA library (with 2.5x10^6

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independent clones and average cDNA size of 1.5 kb), as the source of cDNA and plasmids used in this study were obtained from BD Biosciences as part of their Matchmaker 3 system. For the stringent selection of interacting clones, media lacking histidine, leucine, tryptophan and adenine were used.

Production and Expression of Recombinant Proteins. The N-terminus of rat adenylyl cyclase 8 (AC8) was PCR amplified from a plasmid containing full-length AC8, from residues 1-179 (construct 8Nt). The following oligonucleotide primers were used: 5'-ccg aat tca tgg aac tct cgg atg tgc act gcc tta g-3' and 5'-cgc gtc gac tta ctc cga ttt gcg cct ctg g-3'. The resulting PCR product was digested with *Eco*RI and *Sal*I restriction enzymes and subcloned between the *EcoR*I and *Sal*I sites of plasmid pGEX4T (Amersham Biosciences) to produce a fusion protein between Glutathione-S-Transferase (GST) and NtAC8. The C-terminus of rat AC8 was PCR amplified from a plasmid containing full-length AC8, from residues 1106-1248 (construct C2bAC8). The following oligonucleotide primers were used: 5'-gcg agc tcg aca ttt ggg gta aaa ctg-3' and 5'-acg cgt cga ctt atg gca aat cgg att tg-3'. The resulting PCR product was digested with *Sac*I and *Sal*I restriction enzymes and sub-cloned between the *Sac*I and *Sal*I sites of plasmid pQE30 (Qiagen), to produce a fusion protein containing 6xHistidines at the Nt of C2bAC8. Both fusion proteins were expressed in *E. coli* XL10 Gold, and purified either on glutathione-Sepharose (GST-NtAC8) or cobalt affinity gel (His-C2bAC8).

Preparation of HEK293 Crude Membranes – HEK293 cells were detached with PBS containing 0.03% EDTA and centrifuged at 195 x g for 5min. The supernatant was removed and the pellet re-suspended in hypotonic lysis buffer (10mM Tris, 1mM EDTA, 1mM EGTA, protease inhibitors, pH 8.0). After 10 min cells were homogenized at 4^0C by 50 strokes in a tight fitting Dounce homogenizer followed by centrifugation (195 x g at 4^oC for 5 min). The supernatant was centrifuged at 17, 257 x g, (15min, 4^oC). The supernatant was removed and the pellet, representing crude membranes, was collected.

Preparation of Mouse Forebrain Crude Membranes. Adult mouse forebrains were dissected into cold HB buffer (50mM Tris, 1mM $MgCl_2$, 1mM EDTA, 1mM 4-(2-

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aminoethyl) benzenesulfonyl fluoride, 1mM benzamidine, 1µg DNAase, pH 7.4) and lysed by passing through a 0.22 gauge needle 20 times. After further centrifugation (195 x g at 4°C for 5 min) and dissociation, the lysate was centrifuged at 17, 257 x g, (15min, 4°C). The supernatant was removed and the pellet, representing crude membranes was collected.

AC8 N-terminal and C-terminal Affinity Precipitation. Isolated crude membranes were solubilized in 2% SDS solubilization buffer (50mM Tris, 150mM NaCl, 1mM EGTA, 1mM EDTA, protease inhibitors, pH7.4) (Leonard et al., 1998) followed by centrifugation (17, 257 x g at 4°C for 15min). The supernatant was diluted (1:20) in binding buffer (50 mm phosphate buffer pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 1 mM EDTA, 1mM EGTA) and pre-cleared for 30 min or more with 5µL of a 50% suspension of GST glutathione-Sepharose. GST-NtAC8 glutathione-Sepharose that had been washed 3 times in dilution buffer was added to the pre-cleared sample and rotated for 3 h at 4°C. Alternatively, for *in vitro* pull downs, GST or cobalt affinity gels were used as controls, as noted in the figure legends. The Sepharose/cobalt beads were collected and washed four times in ice cold binding buffer, re-suspended in 2X boiling buffer (final concentration: 125mM Tris, 300mM dithiothreitol, 20% glycerol and 0.004% bromophenol blue, pH 6.8) and heated at 100°C for 5 min. Supernatant was collected and samples were stored at –80°C. The results shown are representative of at least five experiments with similar results.

Phosphorylation Assay. GST-NtAC8, His-C2bAC8, or myelin basic protein, as positive control), were incubated with either a cell-free extract of forskolin (20µM) and prostaglandin E_1 (100nM) induced HEK293 cells, or the catalytic subunit of PKA (Sigma) in phosphorylation buffer incubated with gamma [^{32}P]ATP (Amersham) (10 µCi) [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 10 µM ATP, 2 mM dithiothreitol, 0.01% Triton X-100, 20 µg/ml aprotonin, 20 µg/ml leupeptin, and 10 µg/ml pepstatin A], with or without six units of PKA (Sigma) at 30 °C for 20 min. Following separation by SDS-PAGE, phosphorylated proteins were detected by autoradiography.

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Dephosphorylation Assay.: 250µg of myelin basic protein was phosphorylated as described above with 24 units of PKA in phosphorylation buffer incubated with gamma [^{32}P]ATP. The reaction was terminated by filtration in a microcon YM-3 filter (Millipore) and washed with 500 µl of 20mM TrisHCl, 140mM NaCl pH 7.6. The phosphorylated myelin basic protein was re-suspended in 100µl of 12.5mM TrisHCl, 25µM $CaCl_2$, pH 7.6 and 25µl fractions were mixed with the resultant affinity precipitates (GST or GST-NtAC8) from Triton X-100 solubilised HEK293 cells. After incubation (30 min at 30^{o}C) the reaction was terminated by the addition of 6µl of 5x boiling buffer and heated at 100^{o}C for 5 min. Samples were run on a 13% SDS-PAGE gel and exposed to Hyperfilm film for 17 hours

Immunofluorescence. Rat hippocampal neurons were cultured as described (Chawla et al., 2003)and transfected with N-terminally tagged GFP-AC8 (0.4µg of cDNA) after 8 DIV using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty eight hours after transfection, neurons were washed with phosphate-buffered saline (PBS: 12.1mM Na_2HPO_4, 4mM KH_2PO_4 and 130mM NaCl, pH 7.4) and fixed using 4% paraformaldehyde (1h, 20^{o}C). Alternatively, prior to fixing, neurons were pre-permeabilized in 0.5% Triton X-100 at 4^{o}C for 30 mins (Hering et al., 2003). Coverslips were mounted in Antifade (Molecular Probes) according to the manufacturer's procedures and the cells were visualized on a Zeiss Axiovert LSM510 confocal microscope, using 40X and 63X oil immersion objectives. The scale bar in all images represents 10µM.

Detergent Resistant Membrane Preparation. Mouse forebrain crude membranes were incubated with cold Triton X-100 in TNE buffer (150 mM NaCl, 5mM EDTA, 25 mM Tris) containing 0.1M sodium carbonate pH11 (Ostermeyer et al., 1999). The suspension was transferred to a Dounce homogenizer and homogenized with 20 strokes and left on ice for 30 min. The homogenate was adjusted to 40% sucrose, by the addition of 60% sucrose in TNE buffer (pH7.4). The extract was placed below a 5% and 30% discontinuous sucrose gradient prepared in cold TNE buffer and centrifuged in a Beckman SW55 rotor at 24,000 rpm for 16 hr at 4^{o}C. Fractions (10 x 0.5ml) were

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collected from the top of the gradient. The isolated fractions were diluted in TNE buffer and centrifuged in a Beckman SW55 rotor at 50, 000 rpm for 1 hr at 4^{o}C. The pelleted membranes were re-suspended in 1% SDS. Finally, samples were suspended in boiling buffer heated to 100^{o}C for 5 min and stored at – 80^{o}C.

Immunoblotting - Proteins were resolved using SDS-PAGE 7.5% and 12% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was incubated in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl, [TBS]) containing 5% skimmed milk powder, for 30 min, followed by two 10 min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Membranes were incubated overnight at room temperature with anti-PP2A$_C$ mAb (1:5,000), anti-PKAcat pAb (1:1,000), anti-flotillin mAb (1:5,000), anti-calmodulin mAb (1:5,000), mAb anti Gαs-olf (1:1,000), in TTBS containing 1% skimmed milk powder (antibody buffer). The membranes were washed (2 × 10 min) in TTBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000 dilution of stock) or goat anti-mouse IgG conjugated to horseradish peroxidase (1:3,000) in antibody buffer for 1 hr. Finally, the membranes were washed in TTBS (2 × 10 min), rinsed in TBS and treated with enhanced chemiluminescence plus (ECL) reagent and exposed to Hyperfilm (Amersham Biosciences).

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RESULTS

The N-terminus of AC8 interacts specifically with PP2A catalytic subunit. AC8 lacking the N-terminus is insensitive to regulation by Ca^{2+} in the intact cell, despite being targeted to lipid rafts, which is a property that is essential for regulation by CCE (Smith et al., 2002). However, AC8 is fully stimulated by Ca^{2+}/calmodulin *in vitro* (Smith et al., 2002). This observation suggests that the N-terminus of AC8 might engage with elements of the CCE regulatory apparatus. To determine whether such proteins would interact with the N-terminus of AC8, amino acids 1-179 (i.e. the full length N-terminus) was cloned into a bait vector to screen a HEK293 cDNA library using the yeast 2-hybrid system. The first screen identified 77 colonies that survived on synthetic media lacking histidine. The plasmids were recovered into *E. coli* and sequenced. Duplicate clones and common false positives (transcription factors and 'housekeeping' genes, such as those involved in metabolism) were discarded, leaving a pool of 22 genes of potential interest. The bait and prey vectors were reversed and tested positive in every case. Of these positive interacting plasmids, the one with most potential interest was a peptide sequence (S^{120} - to L^{309}) corresponding to the C-terminus of the α- catalytic subunit of the serine/threonine protein phosphatase 2A ($PP2A_C$). Other proteins that may yet be of some interest included a G protein - Gβ2, a laminin receptor and a WD repeat domain protein; the others were rather obscure.

The finding that PP2Ac interacted with the N-terminus of the Ca^{2+}/calmodulin-stimulable AC8 was deemed extremely interesting since PP2Ac participates in a number of signaling complexes (Boudreau et al., 2002; Lebrin et al., 1999). Specifically, PP2Ac interacts with the C-terminal region of the α_{-1C} L-type VGCC, which is known to be in a functional signaling complex in the rat forebrain with the β2-adrenergic receptor and an unidentified adenylyl cyclase isoform (Davare et al., 2001; Davare et al., 2000). No obvious candidates that might have been involved in regulating CCE were identified; therefore further investigations centered on the association of the N-terminus with $PP2A_C$.

PP2Ac is a member of the PPP family of serine/threonine phosphatases, which have conserved catalytic regions. Evidence from the crystal structure of the PPP family member PP1c suggests that regulation, either through post-translational modification or

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binding proteins, occurs at the C-terminus (Barford et al., 1998; Egloff et al., 1997). In an effort to narrow down more precisely the domain of PP2Ac that interacted with the N-terminus of AC8, two halves of the positive interacting PP2Ac fragment (S^{120} - R^{215}) and (R^{215} - L^{309}) were expressed as histidine fusion proteins in *E. Coli*. However, the peptide fragments were only present as an aggregated form within inclusion bodies, preventing further binding studies.

AC8 is predominantly neuronal in expression, with the highest levels of mRNA detected in the cortex, hippocampus and cerebellum (Cali et al., 1994). Therefore solubilized mouse cortical membranes were used to confirm the selectivity of the interaction between the N-terminus of AC8 and $PP2A_C$, using a GST recombinant fusion protein containing the full length N-terminus of AC8 (GST-NtAC8). Initial GST pull-downs were carried out on cortical membranes solubilized with 1%NP40. Incubating GST or GST-NtAC8 with solubilized mouse forebrain membranes demonstrated that the full length, native $PP2A_C$ (36 kDa) was affinity precipitated by GST-NtAC8, compared to a far less extent by a gross excess of GST (Fig. 1A).

Other solubilization procedures were also investigated; GST-NtAC8 additionally affinity precipitated $PP2A_C$ from whole cell HEK293 lysates solubilized in 1% Triton X-100 (data not shown). The post-synaptic density (PSD) is the proposed localization of AC8 (Mons et al., 1995; Wang et al., 2003) and proteins in this domain are not solubilized by NP40 or Triton X-100 (Lau et al., 1996). Therefore we used 2% SDS, which solubilizes PSD proteins and enables the co-immunoprecipitation of interacting proteins in the PSD, as a means of investigating whether any additional proteins interacted with GST-NtAC8 (Lau et al., 1996; Mehta et al., 2001). The dilution of SDS and addition of Triton X-100 reduces the potential of SDS to dissolve or denature proteins (Leonard et al., 1998). However, we observed no differences in the protein profile between pull-downs using cortical membranes solubilized with either SDS or NP40, as determined by in-gel silver nitrate staining (data not shown). There was a consistent reduction in non-specific binding of PP2Ac to GST when membranes were solubilized with SDS and therefore we opted for SDS solubilization for further studies (Fig. 1B).

GST-NtAC8 also affinity precipitated $PP2A_C$ from solubilized HEK293 membranes (Fig. 1C). We believe *in vitro* GST pull-downs to be a more accurate method than co-

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immunoprecipitation experiments to ascertain genuine interactions involving AC8 and native $PP2A_C$. This is because it is extremely difficult to solubilize adenylyl cyclases to any degree of relative purity. Just how difficult this can be, is clearly demonstrated with Western blots of adenylyl cyclases which typically run at a much higher than expected molecular weight. Indeed genuine interactions between adenylyl cyclase and interacting proteins are thought to be lost during solubilization procedures (Chou et al, 2004).

Taken together, the results obtained with the yeast 2-hybrid analysis, which depend upon correctly folded protein fragments for the detection of positive interactions and results from the GST-NtAC8 pull-down experiments, which examine interactions with full length native $PP2A_C$, clearly demonstrate that the N-terminus of AC8 can efficiently interact with native PP2A catalytic subunit, even in the presence of a highly diverse range of membrane proteins.

The N-terminus of AC8 interacts with the PP2A core enzyme. The core enzyme of PP2A is a dimer consisting of the catalytic subunit and a scaffolding subunit of 65 kDa ($PR2A_A$), referred to as $PP2A_D$ (Janssens and Goris, 2001). A third regulatory B subunit, of which there are four separate families, can associate with the core enzyme to target the trimeric holoenzyme to specific sub-cellular locations. However, the core enzyme is functional without the B subunit (Kremmer et al., 1997). To determine whether the N-terminus of AC8 associated with the PP2A core enzyme complex, GST-NtAC8 affinity precipitates were probed with antibodies to both $PP2A_C$ and $PP2A_A$. GST-NtAC8 pulled down both $PP2A_A$ (Fig. 2A) and $PP2A_C$ (Fig. 2B) from the same solubilized brain membrane preparation, which demonstrated that the N-terminus of AC8 interacts with the PP2A core enzyme complex. However, no immunoreactivity against the B subunit was detected from aliquots that were positive for the core enzyme (data not shown).

The N-terminus of AC8 interacts with active $PP2A_C$. The PP2A enzyme complex interacts with a wide variety of proteins, suggesting that it may act as a scaffolding protein, in addition to its well defined role as a Ser/Thr phosphatase (Boudreau et al., 2002; Chan and Sucher, 2001; Davare et al., 2000; Hsu et al., 1999). Therefore, we felt that it was important to ask whether the fraction of $PP2A_C$ that interacts with the N-

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terminus of AC8 is catalytically active, by examining its ability to dephosphorylate myelin basic protein. HEK293 cells solubilized in 1% Triton X-100 were incubated with GST or GST-NtAC8 and the resultant pull-downs were incubated with PKA-phosphorylated myelin basic protein. There was a clear reduction in the phosphorylation state of myelin basic protein following incubation with GST-NtAC8 pull-downs, as compared to GST pull-down controls (Fig. 3). These results clearly show that the N-terminus of AC8 interacts with catalytically active $PP2A_C$.

Mutually Exclusive Binding of Calmodulin and $PP2A_C$ to the N-terminus of AC8. To further address the nature of the association between catalytically active $PP2A_C$ and the N-terminus of AC8, we investigated the putative regulation of the interaction. The N-terminus of AC8 contains a calmodulin binding site (Gu and Cooper, 1999) that is absolutely necessary for efficient stimulation by CCE in the intact cell (Smith et al., 2002). As expected, exogenous calmodulin was precipitated by GST-NtAC8 *in vitro,* but only weakly by a great excess of GST (Fig. 4A). We therefore asked whether endogenous PP2A could bind to the N-terminus of AC8 in the presence of added calmodulin. The preparations of solubilized brain membranes used in our experiments would reduce Ca^{2+} to very low levels, since they contain the chelators EGTA and EDTA (at 1mM), to prevent protease activation. As an initial exploration of the effects of Ca^{2+}/calmodulin on the ability of GST-NtAC8 to interact with PP2A, EGTA and EDTA were omitted, which slightly reduced the affinity precipitation of $PP2A_C$ (Fig. 4B). Inclusion of 20μM Ca^{2+} further reduced the ability of GST-NtAC8 to interact with $PP2A_C$. The inclusion of 0.5μM exogenous calmodulin and 20μM Ca^{2+} eliminated any detectable interaction between GST-NtAC8 and $PP2A_C$. At this concentration of exogenous calmodulin, GST-NtAC8 bound preferentially to calmodulin (Fig. 4C). Therefore, the binding of $PP2A_C$ and calmodulin to the N-terminus of AC8 is mutually exclusive.

The amino acids in the N-terminus of AC8 that are critical for calmodulin binding are not critical for $PP2A_C$ binding. Point mutations in the helical calmodulin binding domain between amino acids 34 and 51 in the N-terminus of AC8 prevent Ca^{2+}-stimulation of AC8 in the whole cell (Smith et al., 2002). We generated a GST

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recombinant fusion protein containing the full length N-terminus of AC8 with the same six critical amino acids mutated to alanine (GST-Nt8M34). To verify that this prevented calmodulin binding, GST-Nt8M34 along with GST and GST-NtAC8 were incubated in the presence of exogenous Ca^{2+} and calmodulin *in vitro*. GST-Nt8M34 clearly showed no interaction with calmodulin as compared to GST-NtAC8 (Fig. 5A). To determine the relative amounts of fusion proteins present in the individual incubations the blot was stripped and re-probed for GST immunoreactivity (Fig. 5A, lower blot). Densitometric analysis of this immunoblot demonstrated that for equal levels of GST-fusion protein, GST-Nt8M34 is unable to interact with calmodulin as compared to GST-NtAC8 (Fig. 5A). Therefore, GST-Nt8M34, which is unable to bind calmodulin *in vitro*, was examined for its ability to interact with endogenous $PP2A_C$. Solubilized mouse forebrain membranes were pre-cleared with an excess of GST and divided into two aliquots. GST-NtAC8 was added to one and GST-Nt8M34 to the other. Equal volumes from the resulting pull down were compared for $PP2A_C$ immunoreactivity. Both GST-Nt8M34 and GST-NtAC8 could clearly affinity precipitate $PP2A_C$ (Fig. 5B). This demonstrates that the amino acids critical for binding calmodulin are not critical for binding $PP2A_C$. Therefore, if GST-Nt8M34 does not bind calmodulin then the addition of exogenous calmodulin to solubilized mouse forebrain membranes would not be expected to prevent interaction of GST-Nt8M34 with $PP2A_C$. Indeed, including 0.5μM calmodulin and 20μM Ca^{2+} in the pull down assay did not prevent the affinity precipitation of $PP2A_C$ by GST-Nt8M34 (Fig. 5C).

Taken together, these results demonstrate that calmodulin and $PP2A_C$ have overlapping binding domains on the N-terminus of AC8, but the precise amino acids that are essential for binding calmodulin are not essential for binding $PP2A_C$.

Calmodulin binding to AC8 is not regulated by phosphorylation. The ability of calmodulin to bind to its target sequence is regulated by phosphorylation in a wide range of proteins (Enyedi et al., 1997; Hofmann et al., 1994; Turner et al., 2004; Williams and Coluccio, 1995). For instance, endothelial nitric oxide synthase (eNOS) which contains an alpha-helical calmodulin binding domain similar to that present on the N-terminus of AC8, contains a threonine residue, which, upon phosphorylation, prevents calmodulin

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binding (Fleming et al., 2001). Additionally, this site is dephosphorylated by PP2A and this reversible phosphorylation regulates Ca^{2+}/calmodulin stimulated eNOS activity (Fleming et al., 2001; Greif et al., 2002). AC8 contains two calmodulin binding domains, both of which are essential for *in vivo* stimulation, (Gu and Cooper, 1999) therefore the possibility can be considered that reversible phosphorylation of either site could regulate calmodulin binding and hence, activity. There are putative PKA-phosphorylation sites at positions 46 and 66 in the N-terminus and 1156 and 1164 in the C-terminus. The second calmodulin-binding domain of AC8 is an IQ motif that is situated in the C2b domain. We generated recombinant C2b domain protein containing a hexa-his tag at the N-terminus (His-C2bAC8) to determine whether this would bind calmodulin in an *in vitro* assay, as a prelude to conducting phosphorylation experiments. Incubating His-C2bAc8 with exogenous Ca^{2+} and calmodulin *in vitro*, showed that the C2b domain specifically bound calmodulin (Fig. 6).

Therefore, we asked whether putative phosphorylation of the N-terminus or C2b calmodulin binding domains could regulate calmodulin association and thereby provide a rationale for the interaction with PP2A. Incubating GST-NtAC8 or His-C2bAC8 with either the catalytic subunit of PKA (PKAcat) or a lysate prepared from forskolin- and prostaglandin E_1-stimulated HEK293 cells, failed to demonstrate any ability of either domain to be phosphorylated. Myelin basic protein used as a positive control, was phosphorylated by both PKAcat and the stimulated HEK293 lysate (data not shown). In a further experiment, mutating serine-66 (a putative PKA phosphorylation site in the N-terminus, immediately upstream of one calmodulin binding domain) to aspartate did not prevent calmodulin association (data not shown). We conclude that neither of the calmodulin binding domains of AC8 is directly regulated by PKA-mediated phosphorylation – and by implication, of course, that it is not a substrate for PP2A.

Lipid Raft Localization of AC8 and $PP2A_C$. If reversible phosphorylation of AC8 is not a major regulatory influence on calmodulin binding, the association of AC8 with PP2A might represent a novel mechanism for regulation and/or localization of the phosphatase.

When heterologously expressed in non-excitable cells, AC8 occurs in lipid rafts. This

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specific membrane compartmentalization is essential for regulating AC8 activity *in vivo* (Smith et al., 2002). In the whole animal, AC8 is largely confined to the brain, and we therefore examined whether in primary hippocampal neurons a similar membrane compartmentalization occurs to that observed in non-neuronal cells. Hippocampal neurons that were transfected with AC8 tagged with GFP at the N-terminus (GFP-AC8) displayed predominant plasma membrane labeling at the soma, with fluorescence extending into the dendritic network (Fig. 7B). Higher magnification of axons demonstrated a clear plasma membrane localization of GFP-AC8 (Fig. 7B insert). This was in obvious contrast to neurons transfected with GFP alone, where fluorescence was clearly intracellular in the soma and confined to intracellular puncta throughout the processes (Fig. 7A and insert).

To examine the plasma membrane domain in which GFP-AC8 resides, neurons were pre-permeabilized with Triton X-100 at 4^{o}C, prior to fixation. This procedure has demonstrated the lipid raft targeting of the calmodulin binding protein GAP-43/neuromodulin in PC12 cells (Arni et al., 1998). Additionally, with the use of specific fluorophore lipid markers, cold Triton X-100 preferentially solubilizes unsaturated lipids in primary hippocampal neurons, leaving a subset of less soluble cholesterol/sphingolipid rich domains at the plasma membrane (Hering et al., 2003). Confocal imaging revealed that GFP-AC8 was localized in detergent-resistant membrane domains along the dendrites of positively labeled neurons (Fig. 7C and insert). There was no detectable fluorescence from pre-permeabilized GFP control neurons (data not shown) - most likely because the soluble GFP protein was washed out. It should be noted that pre-permeabilization with Triton X-100 would not be expected to remove non-raft plasma membrane proteins associated with the cytoskeleton. However, AC8 also localizes to cholesterol-rich membranes in HEK293 cells and its regulation by CCE is dependent on the presence of membrane cholesterol.

This result clearly suggests that, as with non-excitable cells, AC8 resides in lipid rafts in primary neurons. A component of cellular PP2A might be expected to occur in lipid rafts to permit interaction with the N-terminus of AC8. PP2A is a highly abundant protein (Goldberg, 1999)and incubating hippocampal neurons with an antibody raised against $PP2A_C$ demonstrated strong labeling throughout the entire neuronal structure, with no

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clear specific compartmentalization (data not shown). In order to identify whether $PP2A_C$ was found in lipid rafts, a clearer method for the separation of cellular compartments was required. Lipid rafts resist extraction with cold Triton X-100 and have a higher lipid to protein ratio, which permits their isolation due to their increased buoyancy in sucrose density gradients (Pike et al., 2002).

Mouse brain membranes were fractionated at an optimized detergent to protein ratio as described in "Experimental Procedures". Sodium carbonate was included in the extraction procedure to reduce binding of raft associated proteins to high density Triton-insoluble material that pellets during sucrose centrifugation, thus retaining a greater fraction of genuine raft proteins in the buoyant fraction (Arni et al., 1998). Flotillin immunoreactivity was used to identify the lipid raft fraction (Lang et al., 1998). At low detergent to protein ratios, flotillin was distributed in both the raft and non-raft fractions, whilst at ratios of 20:1 and greater, flotillin immunoreactivity was only observed in the pellet, demonstrating complete membrane solubilization (data not shown) (Lang et al., 1998). At a 10:1 detergent to protein ratio, flotillin was clearly enriched in fraction 4, which corresponded to 20% sucrose (Fig. 7D). Gαs-olf was also enriched in the light membrane fraction (Fig. 7D) (Rybin et al., 2000). As both of these lipid raft markers were clearly enriched in the buoyant membrane fraction, this indicated a high degree of separation between lipid rafts and bulk membranes. Lipid rafts constitute only a small amount of total protein, with the bulk of the protein content residing in fractions 6-10 (approx. 80% of total protein; data not shown). The catalytic subunit of PKA was present in lipid rafts (fraction 4), as well as the bulk membrane, corresponding to fraction 7 (~ 35% sucrose) (Fig. 7D) (Razani et al., 1999). This clear separation of lipid rafts, allowed us to search for PP2A. Immunoblotting with an antibody raised against $PP2A_C$ demonstrated $PP2A_C$ in lipid rafts (Fig. 7D). However, as might be expected for a highly expressed protein with diverse regulatory subunits, $PP2A_C$ immunoreactivity was also present in the bulk membrane and particulate fractions. Importantly, however, a fraction of $PP2A_C$ occurs in an environment that would permit interaction with AC8.

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DISCUSSION

When heterologously expressed in non-excitable cells, AC8 is exclusively regulated by CCE (Smith et al., 2002). This observation, along with other data has led to the suggestion that AC8 and CCE channels reside in close proximity at the plasma membrane (Chiono et al., 1995; Cooper et al., 1994; Fagan et al., 2000a; Fagan et al., 1996; Fagan et al., 1998; Murthy and Makhlouf, 1998; Smith et al., 2002; Watson et al., 2000). The targeting of AC8 to specialized domains of the PM, enriched in cholesterol and sphingolipids, termed lipid rafts, is essential, but not sufficient, for enabling regulation by CCE (Smith et al., 2002). The molecular identity of CCE channels remains uncertain, although mammalian homologues of the *Drosophila melanogaster* transient receptor potential (TRP) proteins are putative candidates (Putney and McKay, 1999). Of the TRP proteins, TRP1, at least, is enriched in lipid rafts (Lockwich et al., 2000). An N-terminally truncated form of AC8 is unresponsive to CCE regulation, although the enzyme remains localized in lipid rafts and is fully stimulated by Ca^{2+}/calmodulin *in vitro* (Smith et al., 2002). This led us to consider that the N-terminus of AC8 might associate with elements of the cellular CCE apparatus.

Consequently, in this study we used the N-terminus of AC8 in a yeast 2-hybrid screen of an HEK293 cDNA library to try to identify interacting proteins that might contribute to CCE regulation of AC8. It turned out that none of the positive candidates identified were proteins that might obviously be involved in regulating CCE. However, the catalytic subunit of PP2A emerged as one compelling interacting protein. The failure to find more obvious candidates in this screen does not exclude the possibility that the N-terminus targets AC8 directly to elements of the CCE apparatus, although it suggests that more indirect interactions should be countenanced.

Extending and confirming the interaction identified by the yeast 2-hybrid screen, an N-terminal AC8 GST-fusion protein affinity precipitated the full length $PP2A_C$ from both brain – the natural source of AC8 - and HEK293 membranes. This underlined the highly specific nature of the interaction between $PP2A_C$ and the N-terminus of AC8, against a large background of other proteins.

PP2A is one of the four major types of Ser/Thr protein phosphatase and associates

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in vivo with a scaffolding subunit of 65kDa ($PP2A_A$). This core dimer can further associate with one of a class of regulatory B subunits, ranging in molecular weight from 55 - 130 kDa. The B subunits apparently play a role in specifying substrate selection and sub-cellular location (Goldberg, 1999; Janssens and Goris, 2001). We identified $PP2A_A$ in pull downs that were positive for $PP2A_C$, which suggested that the N-terminus of AC8 interacted with the PP2A core dimer. However the possibility cannot be ruled out that $PP2A_A$ and $PP2A_C$ interact at individual sites on the N-terminus of AC8. We did not detect any B subunits, when immunoblots were probed with a pan-specific $PP2A_B$ antibody. This may indicate that the core dimer preferentially associates with the N-terminus of AC8, or that $PP2A_B$ was not present in sufficient quantity to allow signal detection by ECL on immunoblotting with a pan-$PP2A_B$ antibody.

Although the regulation of PP2A is thought to occur through the A and B subunits, additional proteins that bind specifically to the PP2A catalytic subunit have been identified. Axin, a component of the Wnt signal transduction system, PKC-α, the $\alpha 1_c$ subunit of the L-type VGCC and the NR3A subunit of the NMDA receptor, all display a specific interaction with $PP2A_C$ (Boudreau et al., 2002; Chan and Sucher, 2001; Davare et al., 2000; Hsu et al., 1999). Such observations suggest that the catalytic subunit may itself confer a degree of regulation to PP2A activity. Among proteins shown to bind to $PP2A_C$, there appears to be no immediately obvious motif responsible for $PP2A_C$ association. Indeed, it is possible that $PP2A_C$ associates with low stringency to its respective binding sites (Ma and Sucher, 2004).

To begin exploring the putative physiological meaning of the interaction between $PP2A_C$ and the N-terminus of AC8, we investigated whether $PP2A_C$ was catalytically active. Indeed, the fraction of $PP2A_C$ that associated with the N-terminus of AC8 dephosphorylated myelin basic protein that had been phosphorylated by PKA. This finding of an interaction between AC8 and catalytically active $PP2A_C$ opens up a very interesting possibility and a means of drawing together previous speculations that AC, VGCCs, PKA, β2-adrenergic receptors and PP2A might form a regulatory complex (Davare et al., 2001).

Ca^{2+}/calmodulin binding to the single alpha helical domain in the N-terminus of AC8 is an essential step in the activation of AC8 by rises in $[Ca^{2+}]_i$ and is considered to

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occur on a one to one ratio (Gu and Cooper, 1999; Smith et al., 2002). Intriguingly, we found that the interaction of the AC8 N-terminus with $PP2A_C$ was prevented by Ca^{2+}/calmodulin, which suggests that PP2A also interacted with the N-terminus of AC8 on a one to one ratio. This competition between Ca^{2+}/calmodulin and $PP2A_C$ for the N-terminus of AC8 is comparable with the binding of PP2A to the autoregulatory domain of CaMKIV, which also occurs in a mutually exclusive manner with respect to Ca^{2+}/calmodulin (Anderson et al., 2004). However, there is no sequence homology between the autoregulatory domains of CaMKIV and the N-terminus of AC8. Although binding of $PP2A_C$ to the N-terminus of AC8 was prevented by Ca^{2+}/calmodulin, $PP2A_C$ was not binding to the identical amino acid sequence that bound Ca^{2+}/calmodulin, since mutations of amino acids within the alpha helical domain that were essential for Ca^{2+}/calmodulin association were not required for binding of $PP2A_C$. It is therefore likely that the respective domains overlap.

The association of calmodulin with its target sequence in proteins can often be regulated by dynamic phosphorylation of residues within or adjacent to the calmodulin binding domain (Black et al., 2004). When we explored the functional consequence of catalytically active PP2A association with AC8 in the context of regulating phosphorylation of the N-terminal or C2b calmodulin binding domains, we found that neither domain was phosphorylated by PKA, or a forskolin- and prostaglandin E_1-induced HEK293 cell lysate. This suggests that PKA-mediated phosphorylation is not involved in regulating calmodulin binding to AC8. However, this does not rule out the possibility that other sites on AC8 are regulated by phosphorylation, either by PKA or additional kinases. Consequently, PP2A in association with AC8, if not involved in the regulation of calmodulin binding to AC8, may be involved in regulating the phosphorylation status of proteins in the vicinity of AC8. For example, the association of PP2A with NR3A directly regulates the phosphorylation state, not of NR3A, but of the adjacent NR1 subunit (Chan and Sucher, 2001). In this context, both AC8 and $PP2A_C$ are present in lipid raft microdomains where $PP2A_C$ is positioned in an environment with other signaling molecules, including putative CCE channels and voltage gated calcium channels (Fagan et al., 2000a; Fagan et al., 2000b).

The association of $PP2A_C$ with the NR3A subunit of the NMDA receptor is

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disrupted by Ca^{2+} entry through the receptor ion channel; however the NR3A subunit has not been shown to bind calmodulin (Chan and Sucher, 2001). It is conceivable that a similar scenario occurs in relation to the association of the N-terminus of AC8 with PP2A. AC8 selectively responds to increases in $[Ca^{2+}]_i$ through either CCE channels or L-type VGCC. These modes of Ca^{2+} entry are thought to give rise to microdomains of elevated $[Ca^{2+}]_i$ in the vicinity of the Ca^{2+}-sensitive AC, an essential element for regulation within intact cells. Thus the high level of $[Ca^{2+}]_i$ at these sites would be expected to recruit calmodulin, which in turn may displace catalytically active $PP2A_C$ from the N-terminus of AC8, facilitating activation of the cyclase and the dephosphorylation of target proteins (Persechini and Cronk, 1999).

This study failed to detect any interaction between the N-terminus of AC8 and putative CCE channel proteins, despite the fact that the N-terminus is an essential component in enabling AC8 to respond to increases in $[Ca^{2+}]_i$ *in vivo*. Thus it would appear that the N-terminus of AC8 is not responsible for a direct association with CCE channels. Interestingly, however, the co-immunoprecipitation of an unidentified adenylyl cyclase isoform with an L-type VGCC has been described in rat forebrain membranes, which suggests a close association between endogenous Ca^{2+}-sensitive adenylyl cyclases and L-type VGCCs. This latter interaction formed part of a larger signaling complex, in which $PP2A_C$, the β2-adrenergic receptor, PKA and Gαs were all identified (Davare et al., 2001; Davare et al., 2000). Thus it is conceivable that the interaction of catalytically active $PP2A_C$ with AC8 may function as part of a larger signaling complex coordinating rises in $[Ca^{2+}]_i$ with the generation of cAMP, with concomitant alterations in the phosphorylation levels of key signaling intermediates by either Ca^{2+}- or cAMP-activated protein kinases. Despite the number of potential PP2A substrates, the *in vivo* regulation of PP2A by extracellular signals is not well understood; indeed its regulation may rely on protein-protein interactions that position PP2A to sites of required activity (Hsu et al., 1999; Sim and Scott, 1999).

AC8 links $[Ca^{2+}]_i$ rises to elevations in cAMP and, as such, is intimately involved in hippocampal LTP (Wang et al., 2003). This first demonstration of the association of an adenylyl cyclase with an active protein phosphatase provides a key intermediate in the organization of a dynamic signaling network. This may be particularly important in

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neuronal contexts, involving Ca^{2+}/calmodulin, L-type VGCC, PKA, AC8 and PP2A, where such signaling complexes may play an important role in regulating synaptic plasticity (Frey et al., 1993; Wang et al., 2003). Future experimental exploration of the physiological significance of the AC8/PP2A interaction described may be profitably considered in such neuronal contexts, where AC8 naturally occurs. FRET-studies, involving CFP- and YFP-labeled components, could provide a dynamic means for studying the interactions of the full-length proteins in living cells.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. The N-terminus of AC8 interacts with the catalytic subunit of protein phosphatase 2A. Mouse cortical membranes were solubilized by either (A) 1%NP40 or (B) 2% SDS, pre-cleared with an excess of GST and incubated with a GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads. The affinity precipitates were resolved by SDS-PAGE and were immunoblotted with an antibody raised against the catalytic subunit of protein phosphatase 2A (anti - $PP2A_C$). Aliquots from the same experiments were examined for protein loading by Coomassie staining of membranes or parallel in-gel silver nitrite staining. (C) HEK293 membranes were solubilized by 2% SDS, pre-cleared with an excess of GST and incubated with a GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads. The affinity precipitates were resolved by SDS-PAGE and were immunoblotted with an antibody raised against the catalytic subunit of protein phosphatase 2A (anti - $PP2A_C$). Aliquots from the same experiments were examined for protein loading by parallel in-gel silver nitrite staining.

Figure 2. The N-terminus of AC8 interact with the PP2A core enzyme. A GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads was incubated with GST pre-cleared brain membranes. The affinity precipitates were resolved by SDS-PAGE and immunoblotted with (A) an antibody raised against the scaffolding subunit of protein phosphatase 2A (anti - $PP2A_A$) or (B) an antibody raised against the catalytic subunit of protein phosphatase 2A (anti - $PP2A_C$). The lower panel shows the Coomassie stained membrane.

Figure 3. The N-terminus of AC8 interacts with catalytically active $PP2A_C$. GST or a GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads were incubated with Triton X-100 solubilized HEK293 cell lysates. The resultant, washed affinity precipitates were incubated with myelin basic protein that had been previously phosphorylated with the catalytic subunit of PKA in buffer containing γ [^{32}P]ATP. (A) Proteins were resolved by SDS-PAGE and the level of

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myelin basic protein dephosphorylation determined by autoradiography. (B) The lower panel shows the Coomassie stained membrane.

Figure 4. A competitive interaction between Ca^{2+}/calmodulin and $PP2A_C$ for association with the N-terminus of AC8. (A) GST or GST fusion proteins containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose were incubated in the absence (-) or presence (+) of 0.5μM calmodulin and 20μM Ca^{2+}. The pull downs were resolved by SDS-PAGE and immunoblotted with an antibody raised against calmodulin (anti - calmodulin). The lower panel shows the Coomassie stained membrane. (B) A GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads was incubated with GST pre-cleared brain membranes containing either 1mM EDTA/EGTA, no exogenous additions, 20μM Ca^{2+}, 0.5μM calmodulin or 20μM Ca^{2+} and 0.5μM calmodulin. The affinity precipitates were resolved by SDS-PAGE and immunoblotted with an antibody raised against the catalytic subunit of protein phosphatase 2A (anti - $PP2A_C$). The lower panel shows the Coomassie stained membrane. (C) A GST fusion protein containing the full length N-terminus of AC8 immobilized on glutathione-Sepharose beads was incubated with GST pre-cleared brain membranes containing either 1mM EDTA/EGTA or 20μM Ca^{2+} and 0.5μM calmodulin. The affinity precipitates were resolved by SDS-PAGE and immunoblotted with an antibody raised against calmodulin (anti - calmodulin). The lower panel shows the Coomassie stained membrane.

Figure 5. Amino acids critical for Ca^{2+}/calmodulin association are not essential for binding $PP2A_C$. (A) GST, a GST fusion proteins containing the full length N-terminus of AC8 or a GST fusion protein containing the full length N-terminus of AC8 with point mutations in the calmodulin binding region (8M34) that were immobilized on glutathione-Sepharose beads were incubated in the presence or absence of 20μM Ca^{2+} and 0.5μM calmodulin. After washing with 1% Triton X-100 the pull downs were resolved by SDS-PAGE and immunoblotted with an antibody raised against calmodulin (anti-calmodulin). The lower panel shows the same blot stripped and re-probed with an antibody raised against GST (anti-GST). The relative amount of the three fusion proteins

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was quantified by densitometric analysis of the GST immunoblot. (B) GST fusion proteins containing the full length N-terminus of AC8 or the full length N-terminus of AC8 with point mutations in the calmodulin binding region (8M34), immobilized on glutathione-Sepharose beads were incubated with equivalent volumes of GST pre-cleared brain membranes. The affinity precipitates were resolved by SDS-PAGE and immunoblotted with an antibody raised against the catalytic subunit of protein phosphatase 2A (anti-$PP2A_C$). The lower panel shows the Coomassie stained membrane. (C) A GST fusion protein containing the full length N-terminus of AC8 with point mutations in the calmodulin binding region (8M34) immobilized on glutathione-Sepharose beads was incubated with GST pre-cleared brain membranes containing either 1mM EDTA/EGTA or 20μM Ca^{2+} and 0.5μM calmodulin. The affinity precipitates were resolved by SDS-PAGE and were immunoblotted with an antibody raised against the catalytic subunit of protein phosphatase 2A (anti-$PP2A_C$). The lower panel shows the Coomassie stained membrane.

Figure 6. The calmodulin binding domains of AC8 are not regulated by phosphorylation. Cobalt affinity gel (control) or a His-fusion protein containing the C-terminus of AC8 immobilized on cobalt affinity gel (C2bAC8) were incubated in the presence or absence of 20μM Ca^{2+} and 0.5μM calmodulin. The affinity precipitates were resolved by SDS-PAGE and immunoblotted with an antibody raised against calmodulin (anti-calmodulin). The lower panel shows the Coomassie stained membrane.

Figure 7. AC8 and $PP2A_C$ are present in lipid rafts. Hippocampal neurons at 10 DIV 48 h after transfection with (A) GFP or (B) GFP-AC8. (C) Hippocampal neurons at 10 DIV 48 h after transfection with GFP-AC8 were extracted with 0.5% Triton X-100 at 4^{o}C. The scale bar in all images represents 10μM. (D) Crude mouse forebrain membranes were incubated in cold Triton X-100 at a 10:1 detergent to protein ratio and prepared for sucrose equilibrium density gradient centrifugation as described in "*Experimental Procedures*". Fractions (3 – 10) were resolved by SDS-PAGE and immunoblotted with antibodies raised against flotillin (anti-flotillin), PKA catalytic subunit (anti-PKA), Gαs-olf (anti-Gαs) or the catalytic subunit of protein phosphatase 2A (anti-$PP2A_C$).

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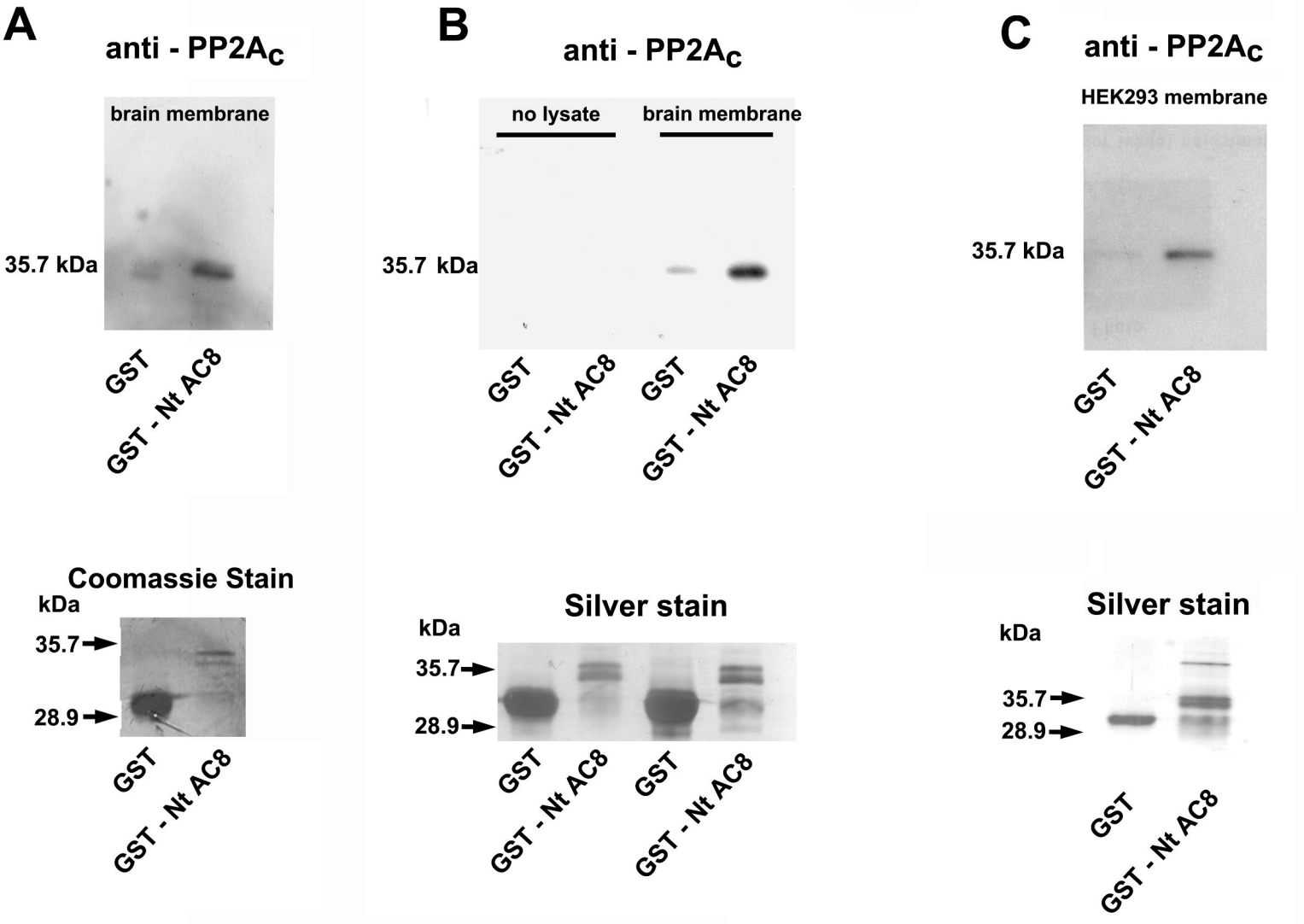

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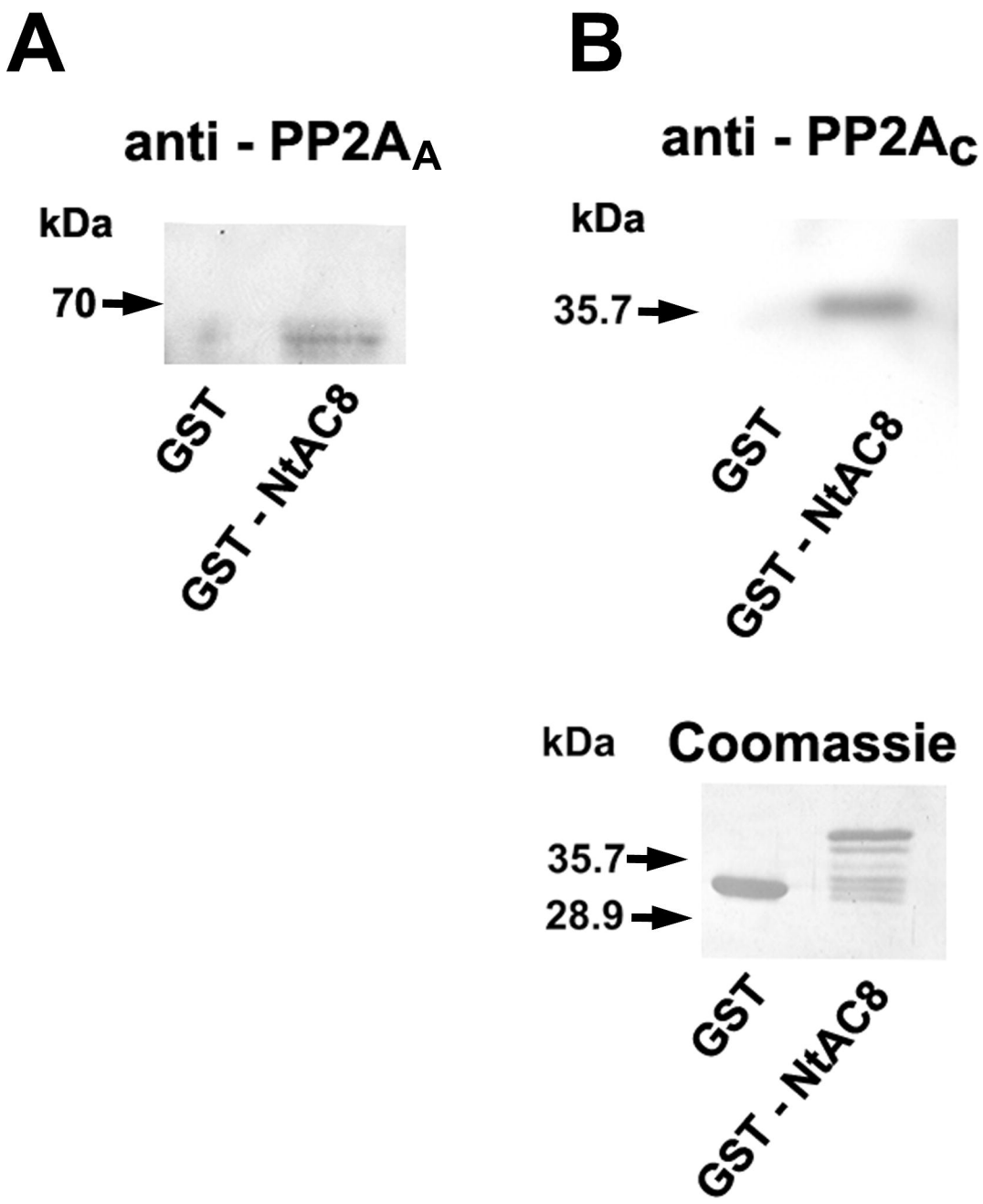

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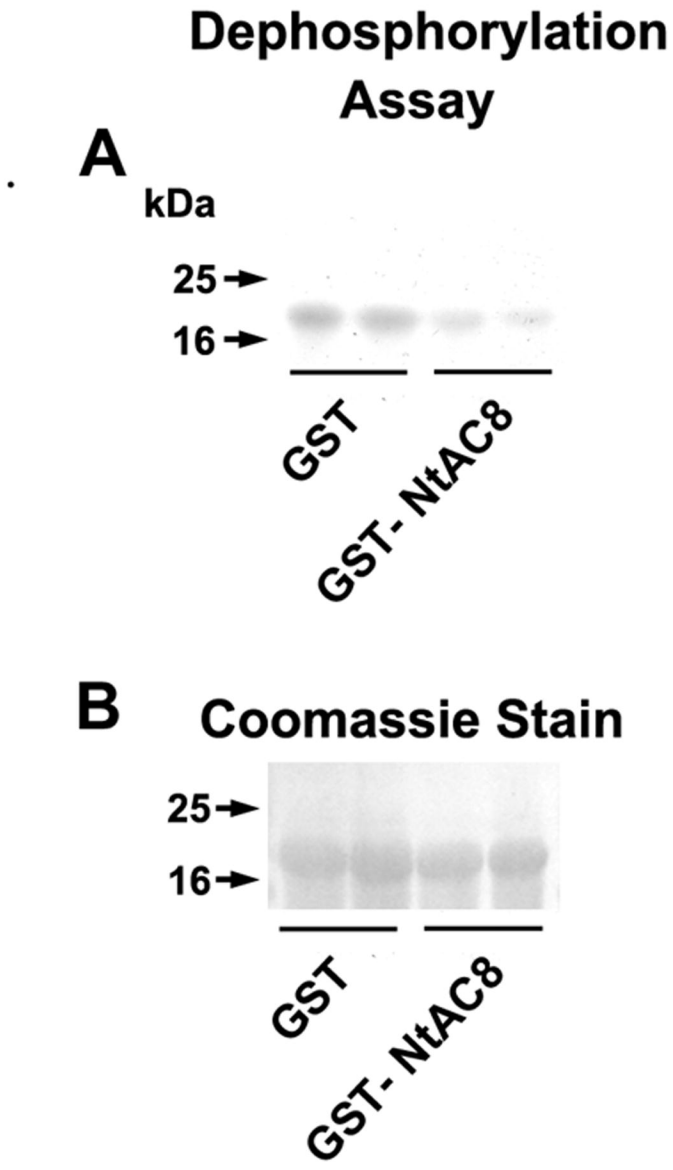

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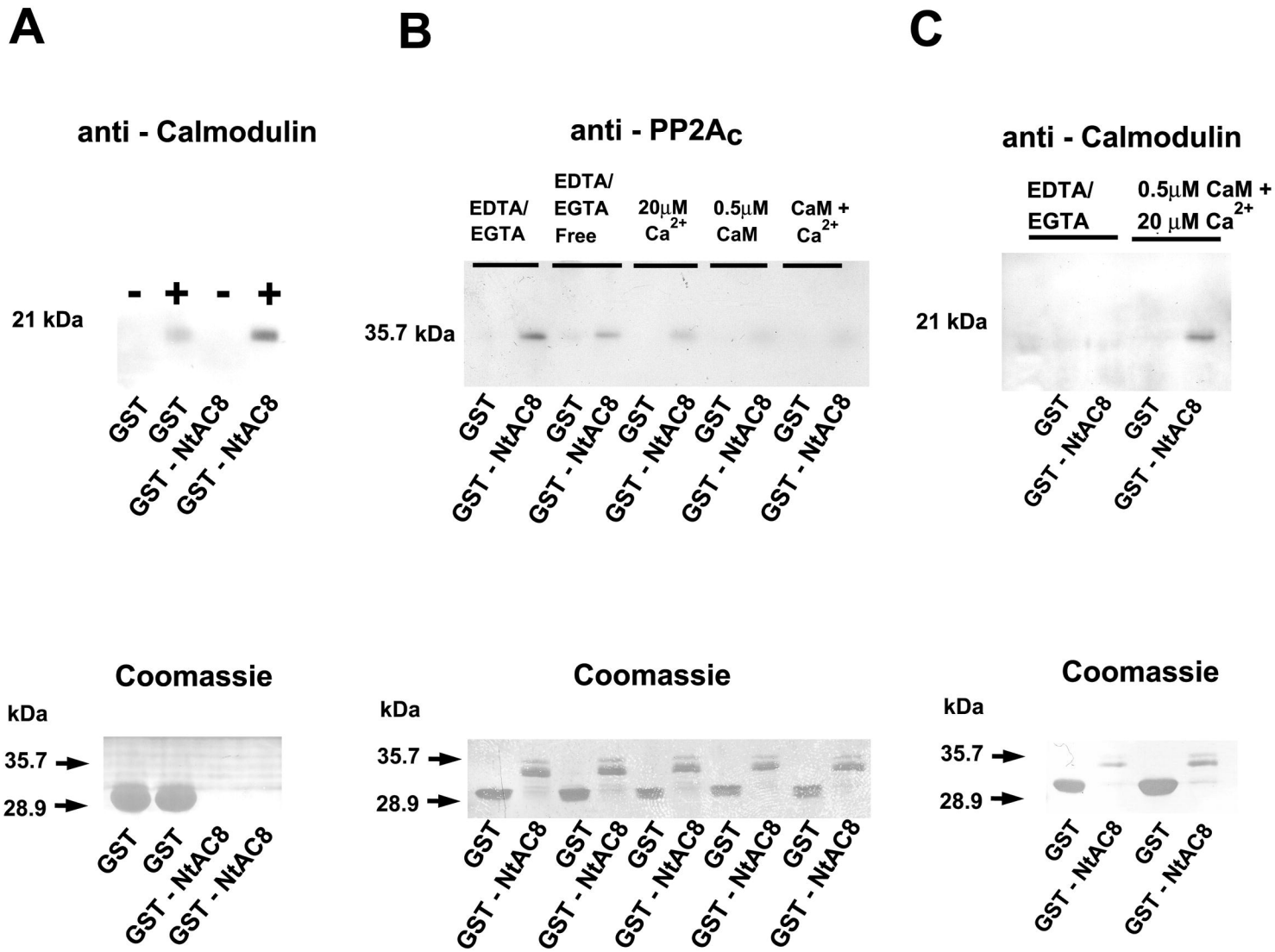

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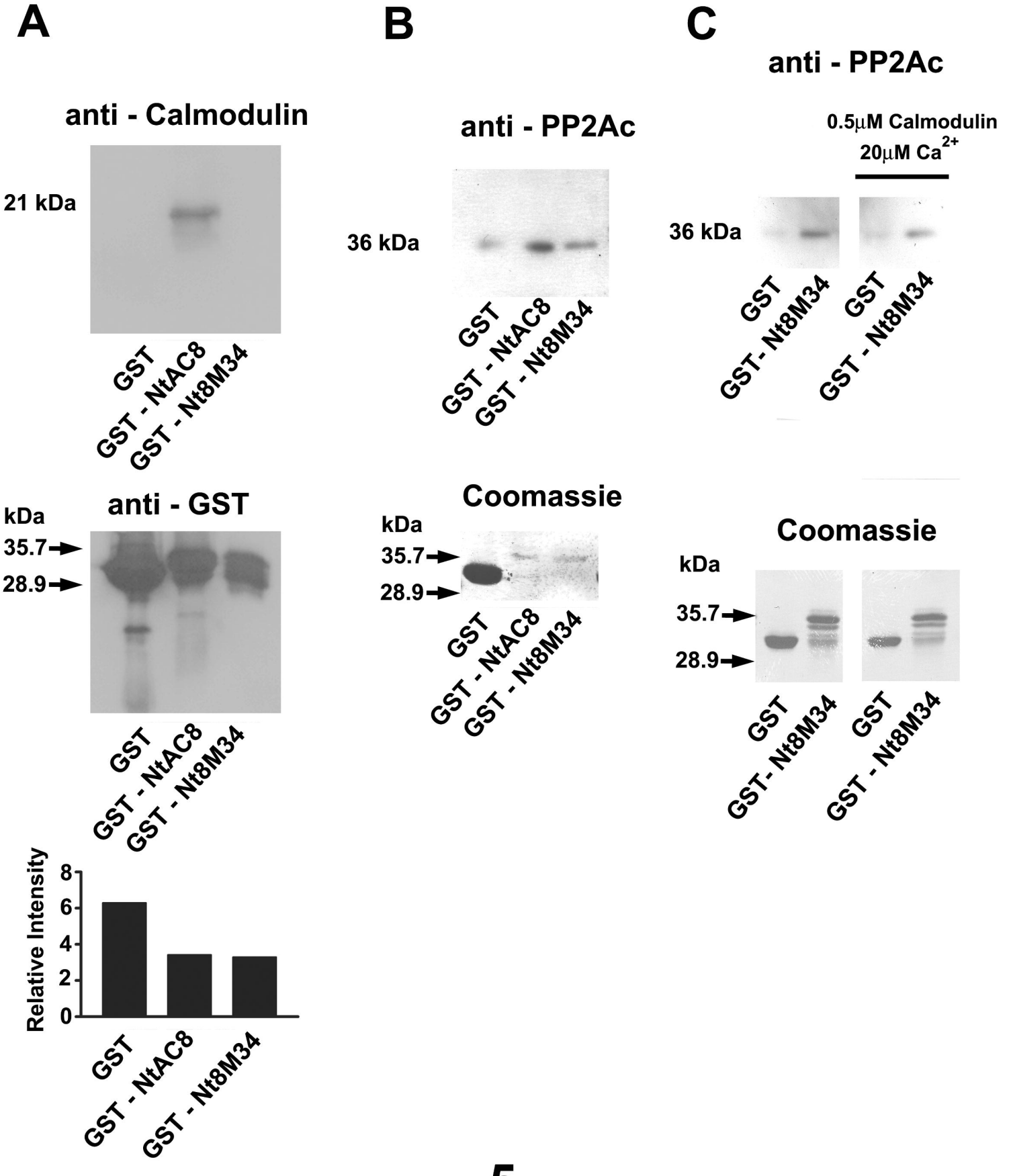

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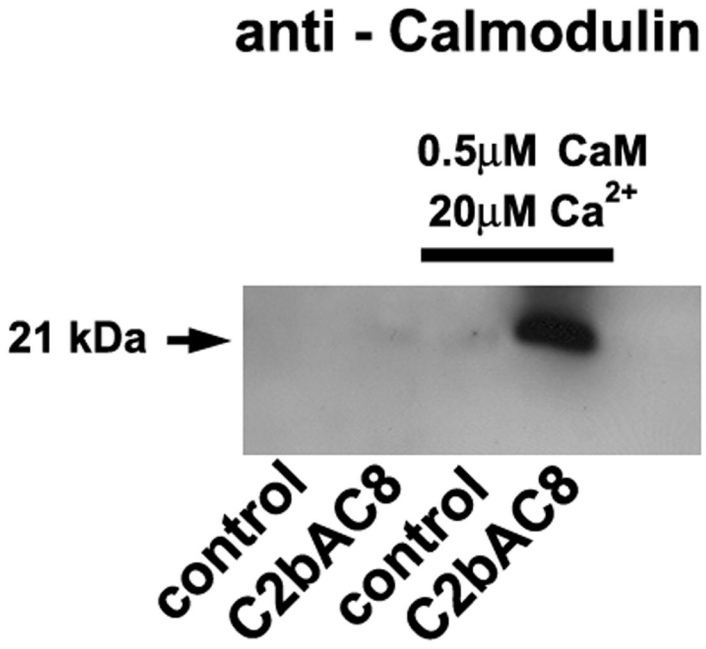

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