REGULATION OF SUBCELLULAR LOCALIZATION AND FUNCTIONS OF RGK PROTEINS BY 14-3-3 AND CALMODULIN

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Summary of work

Kir/Gem, Rad, Rem and Rem2 (RGK) are members of a distinct family of Ras GTPases. Two important functions of RGK proteins are the regulation of voltage gated calcium channels (VDCCs) and cell shape remodeling.

In the current study, I did a comprehensive analysis of the interaction of RGK proteins with 14-3-3 and calmodulin (CaM). The two proteins alter the subcellular localization of RGK proteins through regulation of nucleocytoplasmic transport. While 14-3-3 binding sequesters the RGK proteins in the cytosol, abolition of CaM binding allows them to translocate to the nucleus. In addition to the effect on cellular localization, 14-3-3 and CaM also modulate the cell shape changes induced by RGK proteins.

The mechanism of regulation of calcium channel activity by RGK proteins was also studied. Current results show that RGK proteins interact with the β_3 subunit of calcium channel and this association prevents the interaction of the β_3 subunit with the α subunit, thereby affecting cell surface expression of the α subunit, which in turn downregulates calcium channel activity. Further, any possible roles for CaM or 14-3-3 in the regulation of VDCCs by RGK proteins was investigated and found that CaM but not 14-3-3 affects the modulation of calcium channel activity by RGK proteins.

Since nucleocytoplasmic transport was found to play a significant role in regulating the functions of RGK proteins, I analyzed if RGK proteins possess any nuclear localization signals. Indeed, three NLSs were identified in Kir/Gem, which were conserved in the other RGK members. While NLS1 and NLS2 are non-canonical signals,

NLS3 is a typical bi-partite motif consisting of basic amino acid clusters. The study also revealed that RGK proteins associate with specific importins, which are essential for nuclear transport of RGK proteins. Furthermore, phosphorylation regulates the subcellular localization of RGK proteins and 14-3-3 binding to RGK proteins.

Thus our investigations reveal that RGK family of Ras related small GTPases are subjected to multiple regulatory mechanisms, which may be critical for the selective control of their effects on the dynamics of cytoskeleton and calcium channel activity.

List of publications

1. *Béguin, P., *Mahalakshmi, R.N., Nagashima, K., Cher, D.H., Takahashi, A., Yamada, Y., Seino, Y. and Hunziker, W. (2005a). 14-3-3 and calmodulin control subcellular distribution of Kir/Gem and its regulation of cell shape and calcium channel activity. J. Cell Sci. *118*, 1923-1934

2. *Béguin, P., *Mahalakshmi, R.N., Nagashima, K., Cher, D.H., Kuwamura, N., Yamada, Y., Seino, Y. and Hunziker, W. (2005b). Roles of 14-3-3 and calmodulin binding in subcellular localization and function of the small G-protein Rem2. Biochem. J. *390*, 67-75

3. *Béguin, P., *Mahalakshmi, R.N., Nagashima, K., Cher, D.H., Ikeda, H., Yamada, Y., Seino, Y. and Hunziker, W. (2006). Nuclear sequestration of beta-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. J. Mol. Biol. *355*, 34-46.

4. Mahalakshmi, R.N., Nagashima, K., Ng, M.Y., Inagaki, N., Hunziker, W. and Beguin, P. (2007). Nuclear transport of Kir/Gem requires specific signals, importin α5 and is regulated by calmodulin and serine phosphorylation. Traffic.

5. Mahalakshmi, R.N., Ng, M.Y. Beguin, P and Hunziker, W. (2007). Nuclear transport blocks cell shape remodeling and serine phosphorylation regulates 14-3-3 binding and subcellular distribution of RGK proteins. Traffic.

6. Béguin, P., Kruse, C., Ng, A., Mahalakshmi, R.N., Ng, M.Y. and Hunziker, W. (2006). RGK small G protein interaction with the nucleotide kinase domain of Ca^{2+} channel beta-subunit using an uncommon effector binding domain. J. Biol. Chem.

*** First co-authors**

List of figures

Abbreviations

aa or a.a	amino acids
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
Ca^{2+}	Calcium
cAMP	cyclic AMP
CC	coiled coiled
CACN	Calcium channel
CaM	Calmodulin
COOH-terminus	carboxy-terminus
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GK	Guanylate Kinase
GST	Glutathione *S*-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HA	Haemagglutinin
HEPES	Hydroxyethylpiperazine ethanesulfonic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IP	immunoprecipitation
Kb	kilobase(s)
kDa	kilodalton
L	Liter
LB	Luria-Bertani
M	Molar
MAGUK	Membrane Associated Guanylate Kinase

Min	minute
ml	milliliter
miniprep	Small scale plasmid isolation
midiprep	Medium scale plasmid isolation
NLS	Nuclear localization signal
NES	Nuclear export signal
NPC	Nuclear pore complex
mM	millimolar
μg	microgram
μm	micrometer
nm	nanometer
NH_2-terminal	amino terminal
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase Cγ
PM	Plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
sec	second
SDS	Sodium dodecyl sulphate
SH3	Src homology 3
SV40	Simian virus 40
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
WT	wild type

CHAPTER 1

Introduction

1.1 Ras Superfamily of small GTPases: GTPases, along with their regulators and effectors, function as central elements in signal transduction pathways that control virtually every aspect of cell biology. These GTPases fall within a superfamily named RAS. The Ras superfamily of GTPases are a large group of proteins comprising over 150 members with evolutionarily conserved proteins in D.melanogaster, C.elegans, S.cerevisiae, S.pombe, D.discoideum and plants (Colicelli, 2004). GTPases of the Ras superfamily regulate a wide variety of cellular functions, acting as biotimers that initiate and terminate specific cell functions and thus control the duration of different processes. They play key roles not only in the temporal but also in the spatial determination of cellular functions. Generally small GTPases act as molecular switches alternating between an inactive GDP bound "OFF" state and an active GTP bound "ON" state. They exhibit high affinity binding for GDP and GTP and possess low intrinsic GTP hydrolysis rates. The key regulators of the switch process are GTPase activating proteins (GAPs) and Guanine exchange factors (GEFs), which determine the equilibrium between active and inactive states. Following an upstream signal, GEFs exchange the GDP to GTP, thus promoting the formation of active GTP bound protein. This leads to a conformational change in the effector binding region of the GTPases, which allows interaction with downstream effectors, thereby triggering a number of cellular functions. GAPs accelerate the GTP hydrolysis rate in the active protein and help in the conversion to the inactive GDP bound state, leading to the release of the bound effectors. Thus, the GEFs function as positive regulators and GAPs function as negative regulators of Ras signalling

and together, they alternate cycles of activation and inactivation, which transduces an upstream signal to a downstream effector. In addition to the switch process, the spatial and temporal distribution of the small GTPases , as well as of their regulators, are equally important determinants of Ras signaling. These include variations in structure, post-translational modifications that may specify defnite subcellular localizations and the regulators and effectors, which allow the small GTPases to function as sophisticated modulators of cellular processes.

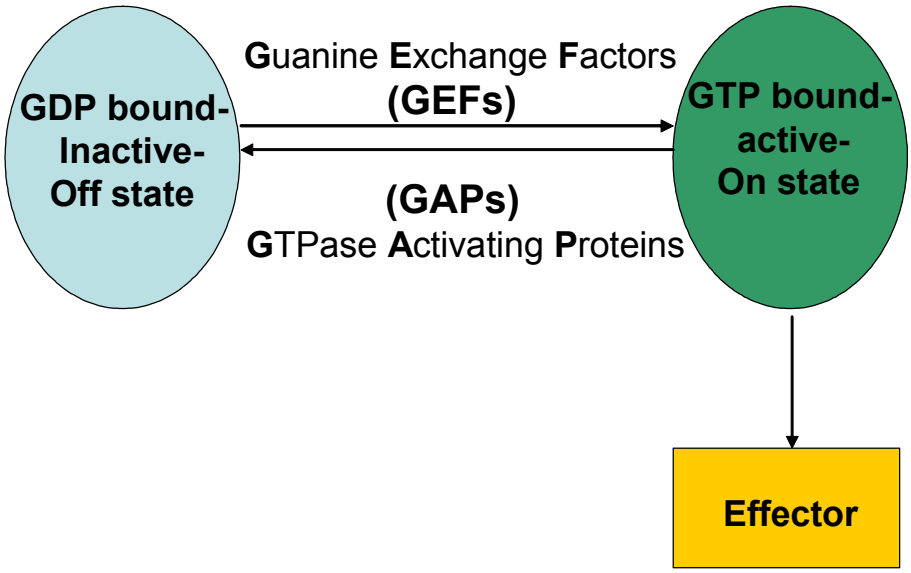


Fig. 1-1 Mechanism of action of small GTPases. Guanine exchange factors or GEFS bind to inactive small GTPases and convert them to the active GTP bound form. The proteins in the active state can interact with effectors and trigger a number of signaling events. The active GTPase is inactivated by GTPase activating proteins or GAPS.

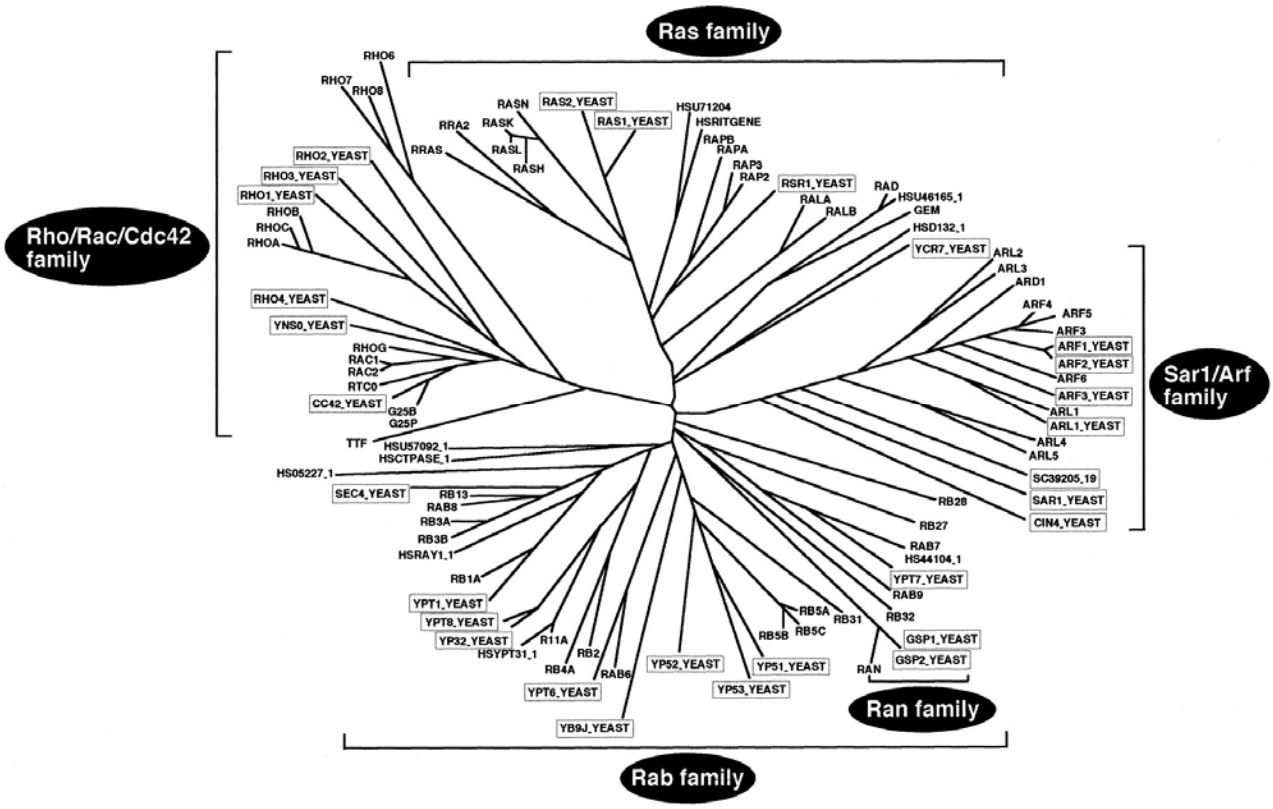
The Ras superfamily can be grouped into five subfamilies based on their sequence and functional similarities: Ras, Rho, Rab, Arf and Ran (Takai et al., 2001). In general, the subfamilies have distinct functions: the Ras subfamily mainly regulates gene expression, the Rho subfamily regulates cytoskeletal reorganization and gene expression, the Rab/Arf members control intracellular vesicle trafficking and the Ran members are involved in nucleocytoplasmic transport.

Members of the Ras superfamily share a conserved core region composed of a set of so called G domains namely G1, G2, G3, G4 and G5. These domains play specific roles in phosphate binding, guanine binding and effector binding. Key amino acids and motifs in the various domains of the core region are conserved among the different members of the Ras family: G1-GXXXXGKS/T; G2-T; G3-DXXGQ/H/T; G4-T/NKXD and G5-C/SAK/L/T (Colicelli, 2004 and Wennerberg et al., 2005). The structural changes during the conversion between inactive and active states are confined to two loop regions called switch I (Ras residues 30-38) and switch II (Ras residues 59-67). These regions exhibit pronounced differences in conformations during the switch process and it is mainly through these conformations that the regulators and effectors sense the nucleotide status of the small GTPases. A second important biochemical feature of Ras proteins is their post translational modification by lipids. Majority of the Ras GTPases contain a C-terminal CAAX (C-Cysteine, A-aliphatic and X-any amino acid) sequence. The CAAX motif is the recognition sequence for farnesyl transferase and geranylgeranyl transferase, which catalyze the covalent addition of a farnesyl or geranylgeranyl group to the cysteine residue. This modification is normally required for the specific subcellular location of the GTPases.

Rho and Rab GTPases are alos regulated by a third class of molecules called Guanine Dissociation Inhibitors (GDIs), which mask the prenyl modification and promote cytosolic sequestration of these GTPases (Seabra et al., 2004). It is interesting to note that some Ras members do not appear to be modified by lipids, but still associate with membranes (e.g. Rit, Miro, sar1) while some others are not lipid modified and are not bound to membrane (e.g. Ran, Rerg) (Wennerberg et al., 2005).

While the branching of the Ras superfamily into 5 subfamilies is largely based on functional and structural similarities, grouping into the various subfamilies is often arbitrary (Wennerberg et al., 2005).

Fig. 1-2 Classification of Ras superfamily



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Subfamilies of the Ras Superfamily

The Ras subfamily: The RAS (**Ra**t **S**arcoma) oncoproteins have been of immense interest due to their critical role as human oncogenes. Mutations in three Ras genes have been detected in ~ 30% of human cancers. Ras proteins were first identified in the Harvey and Kersten strains of acutely transforming reteroviruses. There are about 35 members in this subfamily and they show high conservation in the core domain.

Most Ras proteins are localized in the membrane due to prenylation of the Cys in a C-terminal CAAX motif. While some Ras proteins possess other lipid modifications like geranylgeranylation, farnesylation, acetylation and palmytoylation, others lack lipid modifications. N-terminal lipidation like myristoylation and palmitoylation are also found in some Ras proteins. Well characterized Ras effectors include RAF1, PI3K, RIN1, RAL GEFs etc. The best characterized Ras signaling pathway is the activation of Ras by the epidermal growth factor receptor tyrosine kinase through the Ras GEF SOS (Repasky et al., 2004). Activated Ras binds to Raf and translocates it to the plasma membrane, where it undergoes additional phosphorylation for complete kinase activation. Raf phosphorylates and activates MEK, which further phosphorylates and activates ERK, a MAP kinase. Once activated, ERK translocates into the nucleus, where it triggers activation of downstream promoters. Thus a number of Ras family proteins regulate different signaling networks. They not only regulate cell proliferation, but also control differentiation, cellular morphology and apoptosis. Interestingly, some Ras proteins such as Rerg, Noey2 and D-Ras, seem to act as tumor suppressors, rather than as oncogenes (Colicelli, 2004). This exemplifies the diversity and complexity of the functional roles of the different members of the Ras subfamily.

The Rho subfamily: Rho (**R**as **ho**molog) proteins are key regulators of signaling networks that mediate actin organization, cell cycle progression and gene expression (Etienne-Manneville and Hall, 2002). RhoA, Rac and Cdc42 are the best studied among the 23 members that have been categorized into this subfamily. RhoA is involved in actin stress fibre formation and focal adhesion assembly, Rac1 promotes lamellipodia formation and membrane ruffling and Cdc42 promotes actin micropikes and filopodia formation (Wennerberg et al., 2005). Rho GTPases have been implicated in multiple aspects of cell polarity, cell motility, cell shape remodeling, cell-cell interaction and regulation of endocytosis and exocytosis (Ridley, 2001). Rho GTPases are regulated by a large diversity of GAPs and GEFs and possess a varied range of downstream effectors. Some of the Rho family effectors include ROCK1, mDia, PLCB etc. Like the Ras proteins, this subfamily is also subject to a number of post translational modifications. Although the G boxes are highly conserved between the Rho and Ras family members, the former has an additional insert sequence that is absent in Ras subfamily members. Significantly distinct proteins of the Rho subfamily are the Miro or RHOT proteins which show a notable sequence divergence from the other members, lack the insert sequence as well as lipid modifications. Miro proteins possess two EF-hand domains that may confer calcium binding, a function that is unique to them. They also possess a C-terminal GTPase like domain, the significance of which is not known yet. Miro proteins, being localized to mitochondria, regulate the integrity of the compartment (Krister et al., 2005).

The Rab subfamily: The Rab proteins were originally identified as **Ra**s proteins in **b**rain. They are the largest subfamily of the Ras superfamily with around 61 members (Pereira-Leal and Seabra, 2001). Rab proteins function in protein trafficking pathways

by regulating vesicle formation, budding of vesicles from donor compartments, transport to acceptor compartments and vesicle fusion. Effectors of Rabs are rabphilin, RILP, M6PRBP1 etc. Most Rab proteins undergo C-terminal prenylation, which determines their subcellular localization and function. Rab5, for example, localizes to early endosomes and regulates clathrin coated vesicle mediated transport from plasma membrane to early endosomes.

The Ran subfamily: The **Ra**s like **n**uclear proteins (RAN) are very closely related to Rab proteins by sequence homology and are the most abundant small GTPase in cells. They are involved in the regulation of nucleocytoplasmic transport of RNA and proteins, which is primarily dependent on the spatial gradient of the GTP-bound Ran. The presence of Ran GAP in the cytosol and Ran GEF (RCC1) in the nucleus establishes a gradient of Ran activity across the nuclear membrane and pore complex, determining the directionality of nuclear import and export. Nuclear Ran-GTP binds to importins and transports them to the cytosol where they are released and Ran GTP is converted to Ran GDP by Ran GAP. Ran-GDP binds to exportin and diffuses back to the nucleus where GDP is exchanged to GTP by Ran GEF. Ran is characterized by an acidic C-terminal region and is not known to undergo prenylation.

The ARF subfamily: ADP **R**ibosylation **F**actors or ARFs are regulators of intracellular trafficking and cytoskeletal remodeling. Arf proteins lack C-terminal lipid modifications but are subject to N-terminal myristoylation. Conformational differences between the GDP and GTP bound forms not only occur in the switch I and II regions, but also in the N-terminal region, where the myristate group interacts with membranes in the active state. Effectors mediating Arf functions include Arfaptins and Arfopilins.

There are six known Arf members, Arf 1-6. Arf1 is the best characterized and is involved in the regulation of vesicle formation in endocytic and exocytic pathways. Arf6 is known to function in actin reorganization and endocytosis.

1.2 RGK subfamily of Ras related proteins: The **RGK** (**R**ad, **R**em/**G**em/**K**ir) family belongs to the Ras-related GTPase family and consists of **Kir/Gem** (Cohen et al., 1994; Maguire et al., 1994), **Rad** (Reynet and Kahn, 1993), **Rem or Rem1** (Finlin and Andres, 1997) and **Rem2** (Finlin et al., 2000).

The RGK proteins exhibit several unique structural and functional features that differentiate them from other Ras related proteins. The basic structure of RGK proteins consists of a ras related core domain flanked by distinct C- and N-terminal extensions. The Ras related core domain is divided into G1, G2, G3, G4 and G5 regions which are involved in guanine, phosphate and effector binding. The RGK proteins differ among themselves in the effector (G2) domain, which implies that they might have different interacting partners. The RGK family members differ from other Ras like GTPases in a number of characteristic features. Firstly, the G3 motif, which in Ras participates in binding and hydrolysis of GTP, is unique (DXWE instead of DXXG), implying that the RGK proteins probably share an exclusive molecular mechanism for GTP hydrolysis or do not hydrolyze GTP. Secondly, RGK members contain a notable N- and C-terminal extension flanking the Ras-like core region. The C-terminal extension includes a calmodulin binding region, linking these proteins to calcium signaling events (Fischer et al., 1996; Moyers et al., 1997). Thirdly, they do not have classical lipid modification motifs at the C-terminus, which in other Ras-like proteins undergo lipid modifications that are important for membrane anchorage. Another distinctive characteristic is their

tissue specific expression patterns and regulation at the transcriptional level (Cohen et al., 1994; Finlin and Andres, 1997; Maguire et al., 1994; Reynet and Kahn, 1993). Further key amino acids in Ras like the G12 and Q61 have been substituted for other amino acids in RGK proteins.

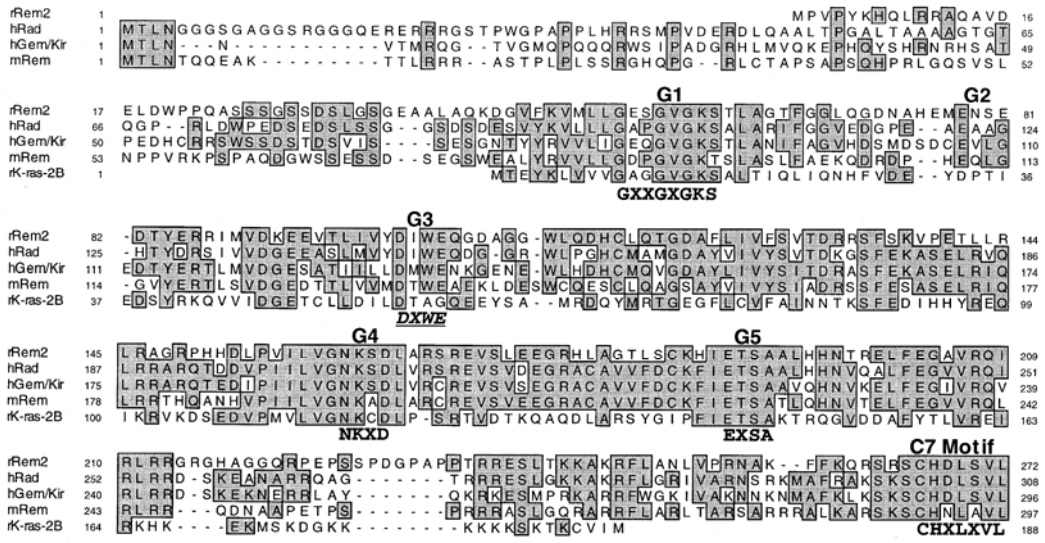

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Fig. 1-3 Clustal alignment between Ras and RGK proteins. Comparison of the amino acid sequences of rat (r) Rem2, mouse (m) Rem, human (h) Gem, hRad, hKir and rK-ras-2B proteins. Hyphens represent gaps introduced for optimal alignment. Numbers are residue numbers. Amino acid residues that are conserved in at least two of the five proteins in the alignment are shaded in grey. Consensus sequences for GTP-binding regions (G1–G5) and the conserved C7 sequence motif are labelled. The G3 consensus is unique to the RGK family and is underlined and in italics.

1.2.1 Kir/Gem: Gem (**G**ene **e**xpressed in **M**itogen stimulated T cells) is the human orthologue of mouse Kir (**K**inase **i**nducible **r**as like). Kir was identified by a differential screen aimed at isolating genes involved in malignant transformation by the BCR/ABL oncogene. BCR/ABL is a fusion gene resulting from the t(9;22) chromosomal translocation, a cytogenic marker of chronic myelogenous leukemia. Kir and Gem are about 35kDa in size and their expression is highly regulated. Gem has been shown to be an immediate early gene in primary lymphocytes, monocytes, endothelial cells and human embryonic fibroblasts (Maguire et al., 1994, Van hove et al., 1997). Gem mRNA is detected in thymus, spleen, kidney, testis and lungs (Maguire et al., 1994). Gem is constitutively expressed in neuroblastoma cell lines and ectopic Gem expression stimulates cell flattening and neurite extensions in human (SH-SY5Y) and mouse (N1E115) neuroblastoma cell lines (Leone et al., 2001). It could be interesting to analyze the metastatic potential of neuroblastoma expressing ectopic Gem. Overexpression of Kir in Saccharomyces cerevisiae induces invasive pseudohyphal growth. Kir induced pseudohyphae formation requires a MAP kinase cascade involving ste20, ste11 and ste7 (Dorin et al., 1995). Calmodulin binds to Kir/Gem and inhibits GTP binding to the protein. The C-terminus of Kir/Gem exhibits hallmarks of a typical calmodulin binding domain. A point mutant W269G was identified to completely abolish calmodulin binding to Gem (Fischer et al., 1996).

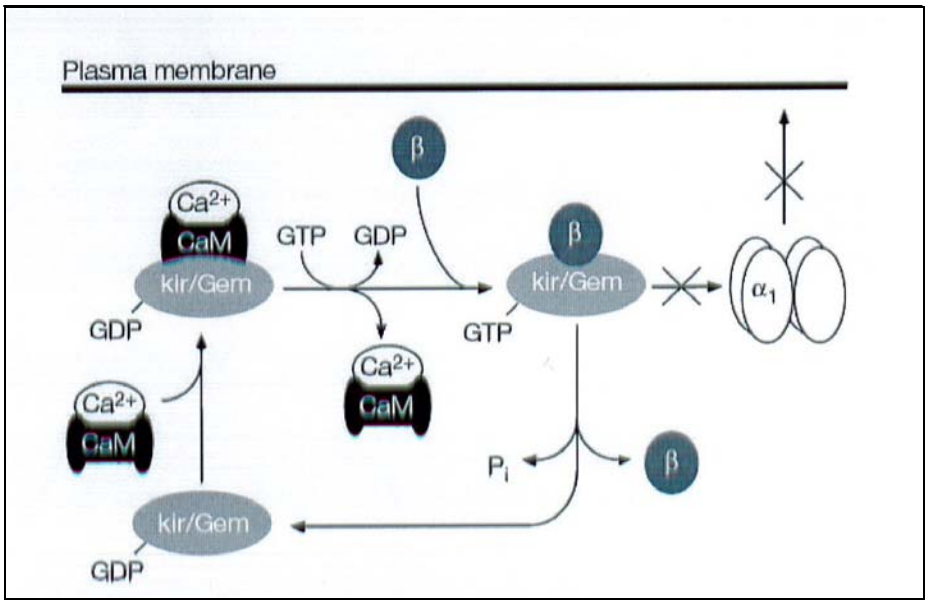
Gem interacts with a kinesin like molecule called KIF9 and has been shown to be associated with microfilaments and microtubules. The dynamics of Gem mediated formation of long cellular extensions were studied by time lapse video microscopy and events such as cell body retraction, increased filopodia formation, increased nuclear

migration and cortical contraction were observed. It was also reported that the Gem induced extensions need intact actin and microtubules. Both Gem and Kif9 display identical expression patterns in different tissues and developmental stages indicating a molecular link between Gem and the cytoskeleton. The group also showed that nucleotide binding was required for the complete elongation activity of Gem since Gem S89N, bearing a single point mutation in the GTP binding site was significantly less active compared to the WT, in terms of cell morphology changes (Piddini et al., 2001). The potential role of Gem-Kif9 interaction could be manifold. Gem might be regulating the motor activity of KIF9, as already proposed for rab6 on rabkinesin6 or the interaction could provide ways of recruiting Gem on microtubules, which would put it in the vicinity of its effectors/regulators. A number of evidences state that microtubules act as signal transduction platforms where key components are recruited through kinesins for downstream signaling.

Kir/Gem is involved in the negative regulation of Rho pathway through its interaction with Rho kinase β. It prevents Rok-β mediated cell rounding and neurite retraction, thereby implying a role in cytoskeletal organization. Gem binds Rokβ independently of RhoA in the Rokβ coiled-coil region. The interaction affects the substrate specificity of Rokβ by inhibiting phosphorylation of myosin light chain and myosin phosphatase, but not LIM kinase (Ward et al., 2002).

Kir/Gem also functions in the regulation of voltage dependent calcium channels. The Ca^{2+} transporting α_1 subunit of voltage-dependent Ca^{2+} channels associates with auxiliary subunits (β, $\alpha_2\delta$ and γ subunits) that have regulatory functions (Catterall, 1998)). Kir/Gem interacts with the Ca^{2+} channel β-subunit in a GTP dependent manner

and causes downregulation of functional Ca^{2+} channels. This is due to the decrease in the expression of α_1 subunit at the plasma membrane. The binding of calmodulin is necessary for the inhibitory effect of Gem on the calcium channels. Overexpression of RIN, another small G protein, which also binds calmodulin did not abolish currents, indicating that the inhibitory effect is Kir/Gem specific. Further, inhibition of the calcium channels by Kir/Gem also prevents calcium triggered exocytosis in hormone secreting cells, thereby affecting Ca^{2+}-dependent GH secretion. It was also reported that the N-type and P/Q type channels were also down regulated by Kir/Gem(Beguin et al., 2001).



(Adapted by permission from Macmillan publishers Ltd: (Nature) Beguin et al., 2001)

Fig. 1-4 Ca^{2+}/CaM binds to GDP bound Kir/Gem and localizes it to cytoplasm. When Kir/Gem is dissociated from CaM, it is converted to active GTP bound form, which then binds to β_3 subunit of Ca channel. Binding of beta to Kir/Gem interferes with the association of β subunit with α subunit, blocking the trafficking of α to the plasma membrane, thereby affecting Ca channel functioning.

The BID (Beta Interacting Domain) of the β subunit, which interacts with the AID (Alpha Interacting Domain) of the α_1 subunit, is crucial for the association of the β subunit with Kir/Gem. This gives further evidence for a possible competition between Kir/Gem and the α_1 subunit for binding to the β subunit. It was also reported that S88N, a Kir/Gem mutant which has reduced guanine nucleotide binding, does not bind β subunit and therefore does not downregulate VDCCs and hence has no effect on GH secretion. This implies that guanine binding to Kir/Gem is required for its association with β subunit (Sasaki.T et al., 2005). Interestingly a possible link between the signaling pathways of RhoA and Gem was suggested by the interaction of Kir/Gem with a Rho-GAP GMIP (Gem interacting protein). GMIP was identified in a two hybrid screen using Gem as the bait and interacts with the core region of Gem. It was reported that GMIP stimulates GTPase activity of RhoA, but not Rac and Cdc42 (Aresta S et al., 2002).

Tau, a microtubule associated protein (MAP) which is predominantly expressed in neurons is a major component of paired helical filaments found in brains of patients with Alzheimer's disease. A comparison study was performed to analyze the brains of wild type and Tau deficient mice to identify any undefined roles of Tau. The study revealed that the expression of Gem GTPase was significantly increased. Though Tau and Kir do not directly bind, Tau antagonizes Kir induced cell elongation in Chinese hamster ovary (CHO) cells. The antagonistic effect of Tau was attributed to the microtubule binding domain of Tau. The analysis indicates that Tau may be involved in Gem mediated signal transduction pathway (Oyama F et al., 2004). The levels of Tau in neurodegenerative diseases like dysphasic disinhibition dementia2 is found to be

profoundly reduced and it could be of interest to examine if Gem levels are upregulated in these cases.

Gem has been reported to be phosphorylated on both tyrosine and serine residues. Phosphorylation of serines 260 and 288, located in the C-terminal extension, is required for Gem mediated cytoskeletal reorganization in N1E115. In addition to the role in cytoskeletal rearrangement, phosphorylation of S288 in conjunction with S22 results in bidentate 14-3-3 binding to Kir/Gem. Further, interaction with 14-3-3 stabilizes Kir/Gem by increasing its half-life (Ward et al., 2004).

Thus Kir/Gem seems to be modulated by several molecules that could attribute to its functions involving cytoskeletal rearrangement and calcium channel regulation.

1.2.2 Rad: Rad (**R**as related protein **A**ssociated with **D**iabetes) was the first member to be identified in the RGK family of proteins and shows about 60% identity to Kir/Gem. Rad is expressed in skeletal muscle, lungs and heart. Screening of cDNA subtraction library showed that Rad is overexpressed in human skeletal muscle of Type II diabetes patients but not in Type I or non-diabetic skeletal muscle (Zhu et al., 1994). Rad has been implicated as a negative regulator of insulin stimulated glucose uptake in cultured muscle (C2C12) and fat (3T3-L1 adipocyte) cells and this effect does not interfere with Glut4 translocation to the plasma membrane (Moyers et al., 1996). Rad interacts with skeletal muscle β-tropomyosin, an actin binding protein suggesting a role in skeletal muscle motor function and cytoskeletal reorganization. The interaction with β-tropomyosin was upregulated with increasing concentration of calcium (Zhu et al., 1996).. Further evidence for Rad's role in the regulation of cytoskeleton is indicated by its interaction with Rho kinase-α, counteracting the ROK-alpha mediated cell rounding

(Ward et al., 2002). Rad binds to CaM through its C-terminus suggesting a role in calcium signaling events. Calmodulin does not affect the GTPase activity of Rad and it was reported that Rad and Gem are invitro substrates of calmodulin kinase II. Furthermore Rad also interacts with calmodulin kinase II, the downstream effector of CaM (Moyers et al., 1997). Apart from calmodulin kinase II, Rad also serves as a substrate for phosphorylation by PKA, PKC and Caesein kinase II (Moyers et al., 1998). NM23, a tumour metastasis suppressor acts as GAP and GEF for Rad, modulating both GTP exchange and hydrolysis. In turn, Rad regulates the NDP kinase activity of NM23 and also NM23's ability to undergo autophosphorylation (Tseng, 2001). Reports showed that Rad was highly expressed in breast cancer cell lines with high tumorigencity and metastatic potential. Transfection of Rad in Rad negative breast cancer cell line displayed an increase in tumor growth, suggesting a role of Rad in tumorigencity and metastasis. Co-expression of NM23 markedly reduced the capacity of Rad to induce tumor growth. Thus NM23 can act as dominant negative regulator of Rad (Tseng et al., 2001). Rad binds to 14-3-3 (Finlin et al., 1999), but the interaction sites had not been identified prior to this study. Rad was also reported to bind the β_3 subunit of calcium channel, with the C-terminus playing an important role in binding, leading to the inhibition of channel currents. A potential link between a nonsynonymous single nucleotide polymorphism within the Rad gene (Rad Q66P) and patients with congestive heart failure has been defined and it could be speculated that the RadQ66P mutation could be causing the disease by virtue of its regulation of calcium channel (Finlin et al., 2003).

1.2.3 Rem: Rem1 or Rem (**R**ad and G**em** related) is about 45% identical to the other members of RGK family and is expressed in cardiac muscle, skeletal muscle, lungs and kidney (Finlin et al., 1997). It was identified as a product of polymerase chain reaction amplification using oligonucleotide primers derived from conserved regions of Rad and Gem. It was the first Ras related GTPase whose mRNA level was repressed by stimulation of lipopolysaccharide, a potent activator of inflammatory and immune responses (Finlin et al., 1997). Like Kir/Gem and Rad, Rem1 also interacts with 14-3-3 and this association is phosphorylation dependant (Finlin et al., 1999). Rem binds directly to the β subunit of calcium channel and inhibits L type calcium channel, but not the T type, which does not need the auxiliary β subunit for channel expression. Deletion analysis showed a critical role for the C-terminus of Rem in regulation of calcium channel and β subunit association (Finlin et al., 2003). Recently a study showed that Rem can modulate calcium channel without decreasing the density of L-type channel at the surface. It was also shown that a complex of Rem-Ca_vβ-alpha can be formed formed without disrupting the alpha and beta association. Thus there is no competition between Rem and alpha subunit for interaction with beta subunit (Finlin et al., 2006).

Rem overexpression does not affect myogenic differentiation of C2C12 cells, which is widely used as an invitro model for skeletal muscle differentiation (Finlin et al., 2002). Ges (GTPase regulating endothelial cell sprouting), the human orthologue of mouse Rem was reported to induce endothelial cell sprouting, thereby functioning as a potent morphogenic switch in endothelial cells. Ges function was shown to be sufficient to substitute for angiogenic growth factor signals in promoting endothelial cell sprouting

since overexpression of GES led to development of long cytoplasmic extensions and reorganization of the cytoskeleton (Julie et al., 2000).

1.2.4 Rem2: Rem2 **(R**ad and G**em** related-**2)** is the latest identified member of the RGK family and hence not much is known. It shows about 50% identity with the other RGK proteins. High levels of Rem2 mRNA were detected in brain and kidney, contrasting with the expression patterns of other RGK family members. Rem2 is the only RGK protein highly expressed in neuronal tissues (Finlin et al., 2000). Recently, Rem2 was shown to be a glucose responsive gene in pancreatic β cells whose expression increases with exposure to high glucose. It also plays a role in regulating calcium triggered exocytosis in hormone secreting cells by preventing glucose stimulated insulin secretion in pancreatic β-cells (Finlin et al., 2005). Rem2 has been reported to reduce N type calcium channel without interfering with the channel density and this effect was likely due to Rem2's activity on preexisting N-type channels rather than alterations in channel synthesis (Chen et al., 2005). These data clearly identify Rem2 as a novel and a potential mediator of Ca^{2+} dependent secretion and signaling.

1.3 Regulators and effector of RGK proteins:

1.3.1 Calmodulin: Calmodulin (CaM), a major transducer of Ca^{2+} signaling, is a small, acidic, calcium binding protein and is involved in controlling many of the biochemical processes in cells. Ca^{2+} plays an important role in the physiology of organisms and is involved in the regulation of many cellular processes ranging from gene transcription and neurotransmitter release to muscle contraction and cell survival. The intracellular concentration of free Ca^{2+} is tightly controlled and usually very low inside the cytosol (0.1μM) whereas the extracellular concentration of Ca^{2+} is roughly 1mM.

Various stimuli such as changes in membrane polarization (voltage gated calcium channel) or ligands (ligand gated calcium channel) can trigger the opening of calcium channels residing in the plasma membrane, resulting in the influx of Ca^{2+} ions into the cytosol. The increase in free Ca^{2+} concentration upon stimulation of a cell allows Ca^{2+} proteins to bind Ca^{2+} ions. Several hundred Ca^{2+} binding proteins have been identified and most of them possess a helix-loop-helix motif of about 30 amino acids which is called EF- hand motif. Calmodulin belongs to the EF hand group of proteins and contains four EF hand motifs, each of which binds a calcium ion. CaM is a major component in the regulation of Ca^{2+} channels and pumps (Vetter and Leclerc, 2003)). Kir/Gem and Rad bind CaM via their C-terminal extensions in a Ca^{2+}-dependent manner. CaM inhibits binding of GTP by Kir/Gem (Fischer et al., 1996) and shows a better affinity for the GDP bound form of Rad (Moyers et al., 1997). The role of CaM binding on RGK protein function is not clear, but in the case of Kir/Gem, it may involve the control of nuclear localization (Beguin et al., 2001). Rin, a small GTPase of Ras family of proteins, binds calmodulin through its C-terminus. Inhibition of calmodulin binding to Rin affects the formation of neurite extensions by Rin in PC12 cells (Hoshino et al., 2003). It is also interesting to speculate that the calmodulin binding motifs may represent yet another important module regulating protein-protein interactions in signal transduction pathways.

1.3.2 14-3-3 proteins: 14-3-3 proteins are a family of ubiquitously expressed, 28-33kDa, highly acidic polypeptides, which are highly conserved from yeast to mammals. They were identified in 1967 during a systematic classification of brain proteins. The

name was derived from the elution position on DEAE (Diethylaminoethyl) cellulose chromatography and subsequent migration position on starch gel electrophoresis.

In mammals, there are seven isoforms denoted by β, γ, ε, ζ, η, σ, τ. Most of the isoforms are expressed in all tissues except σ, which is specific to epithelial cells. Around 15 isoforms are present in plants and two have been identified in yeast, drosophila and xenopus. 14-3-3 proteins form homo- and heterodimers with identical or different isoforms. All 14-3-3 proteins share a similar structure, composed of a N-terminal dimerization region and a target binding region. The target binding region involves amino acids from both the N- and C-termini of the protein.

14-3-3 proteins function by binding to phospho-serine or threonine in the context of a consensus binding motif present in the target proteins. Two known 14-3-3 binding consensus motifs are RSX_pSXP and $RXXX_pSXP$, where $_pS$ denote phosphoserine and X any amino acid. An arginine at position -4 or -3, a serine at position -2 and a proline at position +2 are crucial in binding of 14-3-3 to target proteins. Furthermore, there are several proteins that do not possess the consensus motifs, but can bind 14-3-3 either in a phosphorylation dependent or independent manner (e.g. TERT, exoenzyme S).

14-3-3 dimer is characterized by a highly helical, cup shaped structure. The structure provides grooves, where the phosphorylated residues of the ligand fits in and this causes a conformational change in the ligand in most number of cases. Based on the ligand bound, the functions of 14-3-3 proteins may differ; it can alter the ligand's enzymatic activity, subcellular localization, prevent dephosphorylation of ligand, promote stability or inhibit/mediate interaction of ligand with other proteins. In most cases, 14-3-3 exerts its effect by either inducing a conformational change in the target

protein or through steric hindrance. The ligands that can interact with 14-3-3 are diverse; transcription factors, protein kinases, phosphatases, apoptotic proteins, cell cycle regulators etc. Dimerization is needed for target binding in a number of cases. Presence of two 14-3-3 binding sites in the target increases binding affinity by 30 fold. Further, proteins with low affinity binding sites may bind dimeric 14-3-3, but not monomeric 14-3-3. Similarly, high affinity sites may bind monomeric 14-3-3. Thus dimerization of 14-3-3 also plays a role in target binding either directly or indirectly.

14-3-3 by itself can be regulated by a number of possibilities. 14-3-3 interactions are regulated by kinases and phosphatases. Mostly 14-3-3 motifs in the targets are good substartes for basophilic kinases like AGC kinase family and Ca^{2+}/calmodulin dependent kinases e.g. PKA, PKC, CamKI, ChkI, Akt, SDK1, CKI etc. Some of the kinases that have been reported to phosphorylate non consensus motifs are Cdk5 and Lim kinases. Regulation by phosphatases is observed through PP1 and PP2A, where 14-3-3 interaction with targets are affected by dephosphorylation by the two phosphatases. The localization of 14-3-3 has been controversial with reports indicating various subcellular localization slike cytosol, nucleus, cytoskeleton, centrosome etc. This could be due to the various isoforms of 14-3-3. It is possible that some of the isoforms are specific to certain intracellular locations. 14-3-3 interactions are also regulated by phosphorylation of residues in the very close proximity or within the consensus motifs. In p53 and cdc25C, phosphorylation of residues at -2 positions of 14-3-3 binding pS prevents 14-3-3 association.

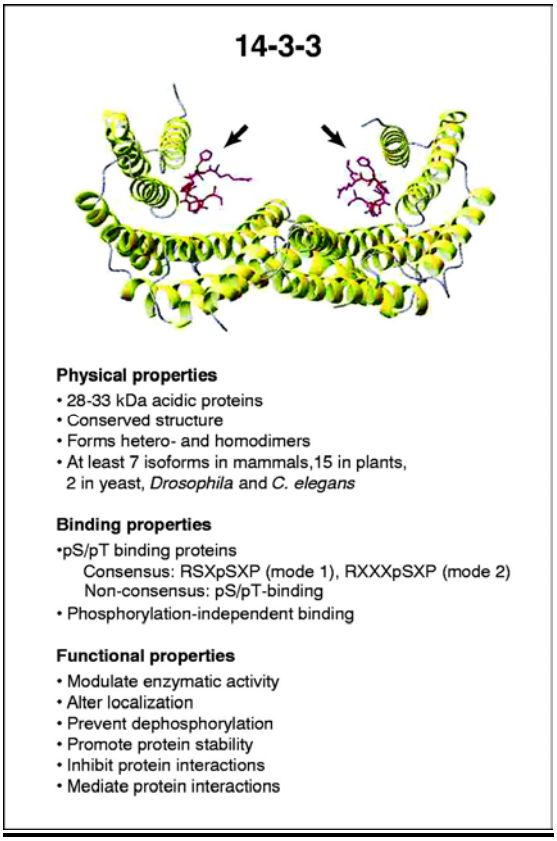
Some of the well known cases where 14-3-3 exerts its effect by affecting either the localization or target binding is discussed below.

1. 14-3-3 and Cdc25c: The association of 14-3-3 with Cdc25c retains Cdc25c in the cytoplasm. This blocks Cdc25c's access to cdc2-cyclin B, thereby preventing mitotic entry. It is hypothesized that 14-3-3 regulates the localization of Cdc25c, by masking the nuclear localization signal in Cdc25c, which is close to the 14-3-3 binding site in Cdc25c. Phosphorylation of serine 214 of Cdc25c abolishes 14-3-3-Cdc25c interaction and Cdc25c translocates to the nucleus.
2. 14-3-3 and TERT (Telomerase Reverse Transcriptase): 14-3-3 interacts with TERT in a phosphorylation independent manner. The binding of 14-3-3 to TERT retains TERT in the nucleus. This is accomplished by masking a nuclear export signal, which in turn prevents the binding of CRM1, thereby affecting the protein export.
3. 14-3-3 and BAD: BAD is a pro-apoptotic gene. Unphosphorylated BAD binds to BCL-X_L and is localized in mitochondria. Interaction of BAD with BCL-X_L interferes with the anti-apoptotic function of BCL-X_L. Phosphorylation of S136 by Akt kinase in BAD activates 14-3-3 binding which translocates BAD from mitochondria to cytoplasm. This inhibits BAD's interaction with BCL-X_L. 14-3-3 obscures the BCL-X_L interaction domain in BAD.

Rad and Rem, but not Rem2, bind 14-3-3 in a phosphorylation dependent manner (Finlin and Andres, 1999). Kir/Gem interacts via two 14-3-3 binding sites (Ward et al., 2004). Although the phosphorylation state of a 14-3-3 binding site in Kir/Gem may regulate cytoskeletal reorganization and stabilize the GTPase (Ward et al., 2004), it is not

clear if this reflects the phosphorylation state per *se* or whether 14-3-3 binding is involved.

Fig. 1-5-Properties of a 14-3-3 dimer



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1.3.3 β_3 subunit of VDCCs: Voltage dependent ion channels exist in plasma membrane of most cells, where they regulate membrane permeability to specific ions. Voltage dependent Ca^{2+} channels (VDDCs) allow Ca^{2+} entry into cells upon membrane depolarization, triggering intracellular events such as gene expression, muscle contraction, hormone secretion and synaptic transmission. The correct trafficking and localization of VDCCs in different cells are of high importance, since many processes are regulated by calcium. Ca^{2+} ions serve as both charge carriers and second messengers. VDCCs are composed of the main, pore-forming α_1 subunit (190kDa) and auxiliary subunits-β, γ and $\alpha_2\delta$, which have regulatory functions. The α_1 subunit is a transmembrane protein composed of four domains, each with six membrane spanning segments (S1-S6). The S4 segment serves as a voltage sensor, and is responsible for transmitting a conformational change that opens the pore. The pore is formed through the S5 and S6 segments and is highly selective for calcium ions. The $\alpha_2\delta$ subunits are linked to each other via a disulfide bond. While the α_2 subunit is extracellular and glycosylated, the δ subunit is a transmembrane protein. The γ subunit, mostly associated with skeletal muscle calcium channels is a glycoprotein composed of four transmembrane helices and does not affect trafficking or regulation of the channel. The intracellular β subunit (55 kDa) is a MAGUK-like protein (Membrane Associated Guanylate Kinase) containing a guanylate kinase (GK) domain and a SH3 (src homology) domain. The GK domain of the β subunit binds to the I – II loop of the α_1 subunit and regulates channel activity. The presence of SH3 and guanylate kinase domains in β increases the possibitly of additional roles for β other than the one in calcium channel function. The β subunit, by its ability to mask the endoplasmic retention signal in the I – II loop of α_1 subunit

plays a crucial role in terms of the trafficking of α_1 from the site of synthesis in the endoplasmic reticulum to the plasma membrane, where it modulates the gating properties of VDCCs. Thus it plays a chaperone like role in transporting the α_1 subunit and regulating calcium channel activity. There are four isoforms of the β subunit with a number of splice variants for each of them. VDCCs are tightly regulated pertaining to their central role in calcium signalling. Recently, the β subunit was shown to interact with Kir/Gem (Beguin et al., 2001), Rad and Rem (Finlin et al., 2003) and Rem2 (Beguin et al., 2005), making it a common effector for all RGK proteins.

1.4 Biological functions of small GTPases

Cell signaling or signal transduction is the study of discovering how a cell responds to extracellular stimuli and analyzing the molecular events leading to cellular responses. Normal cell function depends on the proper response of cells to stimuli or extracellular signals. The first critical component of cell signaling is communication of signal from its origin outside the cell across the cell membrane to evoke a response inside the cell, a function known as signal transduction. Hence, the stimulation of signal transduction pathways requires the receptor to be activated through binding to the specific ligand or hormone. Once the receptor is activated, the signal will be transduced inside the cells and result in the stimulation of different signal transduction pathways.

An important set of proteins involved in signal transduction is the small GTPases which serve as molecular switches to regulate growth, morphogenesis, cell mobility, axonal guidance, cytokinesis and trafficking.

Role of small GTPases in cytoskeletal reorganization

The actin cytoskeleton mediates a variety of essential biological functions in all eukaryotes. In addition to providing a structural framework for cell shape and polarity, it also gives the driving force for cells to move and divide. Understanding the biochemical mechanisms that control the actin organization is thus a major goal of cell biology, with implications for health and disease. Reorganization of actin cytoskeleton plays crucial roles in many cellular functions like cell shape changes, cell motility, cell adhesion and cytokinesis. One of the best-characterized functions of Rho GTPases is the rearrangement of the actin cytoskeleton. They are important regulators of the actin cytoskeleton and consequently influence cell shape and migration. Cell shape changes

underlie important biological processes like movement of immune cells for defense, neuron process extension and connection, wound repair, cell division and cancer progression and metastasis. Several lines of evidence have implicated that small GTPases of the Rho family are the major regulators of signalling pathways that link the external signals to the assembly of focal adhesions and other associated cytoskeletal structures. The first remarkable observation was the capability of serum or lysophosphatidic acid (LPA) to trigger activation of Rho that induces formation of focal adhesions and actin stress fibres (bundles of actin filaments that traverse the cell) in serum-starved Swiss 3T3 fibroblasts (Ridley & Hall 1992). Cdc42 triggers formation of filopodia (long and thin extensions from the cell membrane), whereas Rac1 is responsible for the formation of lamellipodia (broad, web shaped protrusions at the cell periphery) and membrane ruffles. Thus the Rho GTPases play an important role in the regulation of cytoskeletal network.

Small GTPases in cancer

Ras is a well-known oncogene that has been identified in an activated state in various human cancers including epithelial carcinomas of the lung, colon, and pancreas. The Ras proteins were originally identified in retroviruses that trigger sarcoma-type tumours. They are crucial switches of intracellular signalling networks that regulate cell growth and differentiation. Mutations of the *Ras* proto-oncogenes (H-*Ras*, N-*Ras*, K-*Ras*) are found in about 25% of all human tumors. Most of these mutations result in the abrogation of the normal GTPase activity of Ras. Malignant transformation may arise from the unregulated stimulation of Ras signaling pathways, which either stimulate cell growth or inhibit apoptosis.

Activation of Ras proteins proceeds through their activated kinase receptors (most notably receptor tyrosine kinases, RTKs) or G protein-coupled receptors, and when activated they control cellular signalling via multiple downstream effectors. A major intracellular signalling pathway mediated by Ras is initiated by the epidermal growth factor (EGF) receptor (EGFR) leading to cell proliferation. Depending on cell type and environment EGFR signalling can induce mitosis or apoptosis, proliferation, oncogenic transformation, enhanced motility, protein secretion and differentiation. The best understood effector pathway is activation of Ras by the recruitment of son-of-sevenless (SOS) to the cell membrane via the adapter proteins Shc and/or Grb2. This promotes interaction of GTP-bound Ras with downstream effectors such as the kinase Raf, which ultimately leads to the activation of mitogen-activated protein kinase (MAPK).

Rho GTPases and especially their activating GEFs are also involved in the evolution of cancer. Ras-transformed mammary epithelial cells or MDCK cells resemble fibroblasts with increased focal adhesions and stress fibres (Zondag *et al.* 2000). Activated Rho is responsible for some characteristics of Ras-transformed mammary cells. Its inhibition by C3 exoenzyme or the presence of inactive Rho restores partially the normal epithelial phenotype of the cells, but leads to loss of stress fibers and focal adhesions. Thus, high level of activated Rho is responsible for the cytoskeletal alterations in these cells. On the other hand, activation of Rac promotes epithelial phenotype in MDCK cells. However, Ras is able to decrease Rac activity (down-regulation) and increase Rho activity leading to mesenchymal MDCK cells (Zondag *et al.* 2000). Tiam1, a GEF for Rac GTPase is considered an invasion-inducing gene.

Overexpression of activated Tiam-1 or activated Rac is shown to induce invasion and metastasis of T-lymphoma cells.

Regulation of cell-cell adhesion

Small GTPases of the Rho family regulate the cadherin mediated cell-cell adhesions in many ways. Cell adhesion to a large extent is a calcium regulated process. Adhesion to neighbouring cells is achieved by a group of plasma membrane associated proteins called cadherins. Cadherins are Ca^{2+} binding proteins and the calcium binding is critical for their function. Transfection studies of MDCK cells with RhoA, Rac1 and Cdc42 constructs have indicated that Rac strengthens the cadherin-based cell-cell adhesion by increasing the amounts of actin filaments, E-cadherin and β -catenin to the cell-cell adhesion sites. In contrast, Rac disrupts cell-cell adhesion in small preconfluent colonies of keratinocytes (Braga *et al.* 2000). IQGAP1, one of the effectors of Cdc42 and Rac plays a role in decreasing cadhrin mediated cell-cell adhesion. The interaction of IQGAP1 with Cdc42/Rac prevents association of IQGAP1 with β -catenin and results in strong cell-cell adhesion (Aspenstrom, 2004). Inactive Cdc42 and Rac, in turn, are unable to interact with IQGAP1, which then promotes its binding to β -catenin and dissociation of α-catenin from the cadherin-catenin complex, leading to weak adhesion in mouse L fibroblasts expressing E-cadherin.

Rho has multiple functions in epithelial cells, since ROCK, a downstream effector of Rho GTPase disrupts the adhesion of MDCK cells, whereas another Rho downstream regulator, mDia, stabilises it (Sahai & Marshall 2002). Rho and Rac GTPases also regulate the tight junctions. Active GTPases perturb the gate function of junctions as well as change the morphology of junction strands and the localization of tight junctional

components, occludin and ZO-1. Inactive GTPases only perturb the gate function (Jou *et al.* 1998). RhoA-ROCK dependent and independent mechanisms, in turn, control the permeability of tight junction and phosphorylation of occludin (Hirase *et al.* 2001).

Rho GTPases in inflammation

One of the major functions of the Rho GTPases is the reorganisation of actin cytoskeleton in response to various extracellular stimuli. Thus, they especially regulate the cellular processes where filamentous actin plays a central role. Furthermore, they are involved in diverse vital cellular processes such as regulation of transcription via the c-Jun N-terminal kinase (JNK) signalling pathway, induction of apoptosis, cell growth control, inflammation and malignant transformation.

Some bacterial toxins inactivate or activate Rho GTPases and in this way affect the actin cytoskeleton of the host. Clostridium botulinum exoenzyme (C3) inhibits Rho by ADP-ribosylation and Clostridium sordellii lethal toxin inhibits Rac, Ras and Rap GTPases with the same mechanism (Just *et al.*, 2001).

Cytokines are soluble proteins produced by nucleated cells throughout the body and secreted into circulation. They affect cell growth and differentiation but also mediate immune responses and have a role in tissue repair. Cytokines produced by cells of the innate defense system play an essential role in influencing the immune response towards protective anti-tumour immunity. These cytokines might act as first "danger signals" in alerting the immune system. Interleukin-1β (IL-1β) is one of the inflammatory cytokines that exerts its effect through Rho GTPases. For induction of infection, bacterium binds to cell surface and/or enters the cell interior. Helicobacter pylorus is a human-specific pathogen that causes chronic active gastritis and peptic ulcer disease. Its adherence to

surface of the gastric cells promotes activation of Rho GTPases and sequential activation of jun and p38 kinases, responsible for stimulation of transcription. As a result transcription factors, such as NF-κ B are activated, followed by secretion of interleukins 1, 6, 8 and tumour necrosis factor-α, TNF-α (Wessler *et al.* 2000).

Small GTPases and neuronal morphogenesis

Brain function requires the precise connections of millions of nerve cells (neurons), which make distinct neuronal circuits that serve multiple functions, from a simple reflective response to complex operations such as learning and memory. An important challenge in neurobiology is to understand at the molecular level how this intricate pattern of neuronal connections is established.

It is known that during development, extensive migration and maturation of neurons, as well as extension of neurites (axons and dendrites) take place to allow the establishment of proper neuronal connections. Once a neuron has migrated to its final destination and sometimes even before, it begins to extend an axon and several dendrites. Axon will grow towards its target (dendrites of other neurons) to forma a synaptic junction for communication, and the dendrites will undergo extensive growth preparing to receive signals from axons of other neurons. In response to guidance cues, both axon and dendrite extend at their growing tips by means of a highly motile structure called the growth cone, which contains both actin and microtubule. Most actin filaments in a growth cone accumulate in the periphery, forming filopodia and lamellipodia that give the characteristic ruffled and spiky appearance. Microtubules are concentrated in the central core of the growth cone.

Small GTPases of the Rho family involved in actin cytoskeletal reorganization (stress fibres, filopodia and lamellipodia formation) in fibroblasts are also important signaling molecules regulating neurite outgrowth and growth cone remodeling in neurons, linking extracellular guidance cues to actin cytoskeletal reorganization, thereby enabling them to advance, retract or turn. Expression of constitutively active Rac affected the growth of axons but not dendrites in cerebellar Pukinje cells (Luo et al., 1996). Activation of Rac and cdc42 in a neuroblastoma cell line, N1E-115, has been shown to promote the formation of filopdia and lamellipodia, respectively (Kozma et al., 1997). Activation of Rho in neuronal cells has been shown by a number of groups to induce neurite retraction and the expression of dominant negative Rho prevents the neurite retraction.

Two GTPases, RIT (RAS-like Protein in All Tissues) and RIN (RAS-like Protein in Neurons) are positive factors for neuronal cell survival as well as initiation, elongation and branching of neurites in culture (Spencer et al., 2002). The enhanced branching of RIT and RIN in developing and mature neurons supports the biological relevance of these properties. RIN includes a Ca^{2+} / calmodulin binding site, which appears to be required for its neurite outgrowth function (Hoshino et al., 2003).

Regulation of small GTPases by Ca^{2+}

$Ca^{2+}$is a critical second messenger for a number of biological processes like gene expression, muscle contraction, electrical excitability of neurons and secretion. Ca^{2+} signals play a role from fertilization to the execution of cell death and hence defects in Ca^{2+}regulatory mechanisms can contribute to disorders and diseases like cancer. It also plays a role in cell migration in neutrophils, eosinophils, smooth muscle cells and

neurons. Cell migration is a sequential and interrelated multistep process which involves the formation of lamellipodia/membrane protrusion at the front edge, cycles of adhesion and detachment, cell body contraction and tail retraction (Yang et al., 2005). Increase of calcium levels is generally achieved by either calcium influx through the plasma membrane or calcium release from intracellular stores. Among the membrane channels are the voltage dependent calcium channels, which selectively allows the entry of Ca^{2+} ions through an electrochemical gradient, from a high concentration outside the cell to a low concentration inside the cell, to raise the Ca^{2+} levels.

The Ras subfamily has been implicated in cell cycle and proliferation. Calcium is also a critical factor in regulating cell proliferation. The relationships between Ras like GTPases and Ca^{2+} signaling is diverse. In PC12 cells, membrane depolarization and calcium influx results Ras activation (Rosen et al., 1994). The Ca^{2+}induced activation of Ras can be explained by the presence of Ca^{2+} regulated Ras GEFs (Ras GRFs), GAPs and Ras effectors (phospholipase C) which aid in the integration of Ras and Ca^{2+} regulated pathways (Aspenstrom , 2004, Cullen et al., 2002)). Interestingly, RGK proteins have been shown to have a direct influence on Ca^{2+} signaling by regulating the expression and function of voltage dependent calcium channels (Beguin P et al., 2001, Finlin et al., 2005). In addition, the Rho GTPases, as well as some of their regulating proteins have been shown to have roles in Ca^{2+} dependent cell adhesion, cell migration and exocytosis. Fivaz and Meyer (2005) have described a novel mechanism in hippocampal neuronal cultures whereby Ca^{2+} / CaM regulates the levels of K-Ras, and other small GTPases containing polybasic-prenyl targeting motifs such as Rap, at the plasma membrane following NMDA receptor activation and Ca^{2+} entry. Relocation to intracellular

membranes was rapidly activated by Ca^{2+} / CaM in a reversible fashion because the small GTPase tail was sequestered away from the plasma membrane. Since H-Ras and N-Ras do not possess a similar polybasic motif, their distribution was unaffected by Ca^{2+} / CaM. Interestingly, it was also shown that K-Ras was delivered to intracellular organelles in the active GTP-bound form within minutes of cell stimulation and that activation at these sites was prolonged in comparison to that at the plasma membrane. Interpretation of these results was primarily dependent on overexpression data but is consistent with other studies showing an interaction of CaM with RGK, Rab, Ral and K-Ras small GTPases (Cook & Lockyer et al., 2006).

Small GTPases in trafficking

Transmembrane proteins and secreted proteins are transported from one compartment to another by vesicles. Newly synthesized secretory proteins are translocated into the ER and are then transported to the plasma membrane via the Golgi apparatus by vesicles. In parallel, the molecules that are taken up from the plasma membrane are transported inward to endosomes and lysosomes by vesicles. Some proteins including receptors for extracellular ligands transit through recycling endosomes to be recycled back to plasma membrane. Thus, exocytosis, endocytosis and recycling are achieved by vesicular trafficking. Arfs and Rabs are two main families of GTPases that regulate intracellular vesicular transport and trafficking of proteins between different organelles of the endoyctic and secretory pathways. Arf regulates the formation of vesicle coats at different steps in the exocytic and endocytic pathways. GTP-and-donor-membrane-bound Arf associates with and activates coat proteins. The Arf-coat-protein complex then facilitates cargo sorting and vesicle formation and release. In contrast to

Rab proteins, which function at single steps in membrane trafficking, Arf proteins can act at multiple steps. For example, Arf1 controls the formation of coat protein I (COPI)-coated vesicles involved in retrograde transport between the Golgi and ER, of clathrin/adapter protein 1 (AP1)-complex at the TGN and on immature secretory vesicles. Arf6 can regulate actin organization as well as endocytosis. Rabs regulate the budding/targeting/docking/fusion processes of the vesicles. Rab3A plays a key role in Ca^{2+} dependant exocytosis, particularly in neurotransmitter release from nerve terminals (Takai et al., 2001).

Potential roles of RGKS as GTPases

RGK proteins constitute a relatively novel subfamily of Ras related GTPases. The two identified functions so far for these GTPases are cytoskeletal reorganization and regulation of calcium channel. They perform their roles in these functions through their interaction with specific interacting partners as mentioned in previous sections. Cell shape changes are important in several biological processes like tumor metastasis, cell division, wound healing and tissue repair, neuronal outgrowth and a role for RGK proteins in some of these events cannot be ruled out. The mechanism of regulation of RGK proteins and the roles they play in modulation of different interacting proteins are under study and could be manifold. Apart from the two established functions, a putative role for Rad could be speculated in tumor progression and cell growth (Tseng et al., 2001); although a lot more work has to be performed to ascertain the function. Another distinctive function could be in the regulation of hormone secretion like insulin and growth hormone. Indeed, Kir/Gem inhibits growth hormone secretion through its ability

to down regulate calcium channels (Beguin P et al., 2001). Rem2 regulates insulin secretion in pancreatic β cells respectively (Finlin et al., 2005).

Thus, though the current knowledge about the biological implications of RGK proteins is limited, more evidence is evolving towards placing the small GTPases in a wider picture.

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Aims of study

RGK family of small GTPases (Kir/Gem, Rad, Rem and Rem2) have been of significant interest due to their homology to Ras GTPases and their distinct features that distinguish them from other related GTPases. Despite being identified a number of years ago, little is known about how they regulate physiological processes such as cell shape remodelling and VDCC activity, or the mechanisms by which the RGK proteins themselves are regulated.

Among the unique properties of RGK proteins are the association with CaM. It has been observed that Kir/Gem mutant that cannot bind CaM localizes to the nucleus. I was thus interested to determine if re-localization from the cytoplasm to the nucleus in the absence of CaM binding is common to all RGK family members and, if so, how nuclear transport is regulated and what consequences does it have on RGK proteins' functions.

In addition, I was interested in defining the functional role of a second regulatory protein known to bind RGK proteins, 14-3-3. These studies uncovered unexpected roles for CaM and 14-3-3 in the regulation of the subcellular distribution and function of RGK proteins. The nuclear localization of RGK proteins further led to the identification of NLSs and specific importins required for nuclear translocation in RGK proteins and elucidated the role of several phosphorylation events in this process.

CHAPTER 2

Materials and Methods

Basic DNA manipulation techniques were carried out as described in standard laboratory manuals.

2.1 Cloning techniques:

2.1.1 ESTs: The cDNAs for various constructs were obtained from I.M.A.G.E consortium and cloned into a mammalian expression vector pME18S.

List of ESTs

Rad	5149047
Rem	5389715
Rem2	5328555
14-3-3-β	5482228
14-3-3-γ	5532354
14-3-3-ε	5502318
14-3-3-σ	5476961
14-3-3-τ	5478108
14-3-3-η	5531102
14-3-3-ζ	5492512
Importin α1	4825235
Importin α3	4821602
Importin α4	5261465
Importin α5	2822859
Importin α6	5297277
Importin α7	3913864
Importin β	6518750

2.1.2 Polymerase chain reaction: Point mutations were introduced into various constructs using PCR methods. PCR was carried out using Taq polymerase (Roche) and the corresponding buffer in the presence of a forward and reverse primer (1nmol), template DNA (25ng) and 2.5 mM dNTPs (Roche). After PCR, purifications were done

using QIAquick™ PCR purification kits (QIAGEN). The RGK proteins were Myc epitope tagged, β_3 subunit was Flag tagged, 14-3-3 was GST tagged and α_1 was HA epitope tagged.

2.1.3 Restriction digestion and gel electrophoresis*:* The PCR fragments and vectors were digested using restriction enzymes from Roche or New England Biolabs. Restriction digestion was performed using 1-2μg of DNA in the presence of 1-2 units of restriction enzymes and buffers supplied by the manufacturers. The vector was subjected to Alkaline phosphatase (Roche) treatment to prevent self ligation. Digested DNAs were analyzed by agarose gel electrophoresis (1%w/v) using ethidium bromide staining. Gels were run in 1X TAE (40mM Tris-HCL (pH 8.5), 1mM EDTA, 20mM glacial acetic acid) buffer at a constant voltage of 150V and visualized by UV illumination. The selected fragments were cut from the gel and purified using QIAquick™ Gel Extraction kits (QIAGEN).

2.1.4 Ligation: The purified vector and fragment were ligated using T4 DNA ligase (Roche). All reactions were carried out overnight at 16°C.

2.1.5 Preparation of competent cells: DH-5α bacteria was grown overnight at 37°C to an O.D of 0.4 to 0.5. The culture was then transferred to pre-chilled sterile centrifuge tubes and spun at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet re-suspended in ice cold calcium chloride solution (60mM $CaCl_2$, 10mM PIPES (pH 7.0), 15% glycerol) and left on ice for 30 minutes. After an additional centrifugation at 3000 rpm for 10 min, the supernatant was discarded carefully and the pellet re-suspended in fresh calcium chloride solution and aliquoted in pre-chilled

eppendorfs placed in ice. The tubes were transferred to dry ice and once frozen, stored at -80˚C.

2.1.6 Trasformation: The ligated constructs were mixed with competent E.coli cells in 1:5 ratio and left in ice for 30 minutes. Cells were then heat shocked at 42°C for 90 seconds and immediately placed in ice for 90 seconds. LB medium was added in a 10:1 ratio and tubes were agitated for 1 hour at 37°C, followed by centrifugation. The transformed bacteria were then plated on LB agar plates with ampicillin (100μg/ml) and left overnight at 37°C. Colonies were picked and inoculated for overnight growth and DNA extraction was performed the next day.

2.1.7 Miniprep and Midiprep: Plasmid DNA extraction from bacteria was performed using the Qiagen Hispeed plasmid purification mini and midi kits. All steps were followed according to the manufacturer's instructions. The DNA obtained was quantified by spectroscopy at 260nm. (1 O.D. Unit at A260 for dsDNA = 50 μg/ml).

2.1.8 Sequencing: All constructs were subjected to PCR based sequencing reactions using sequencing protocol V3.1. The sequencing reaction consists of 25 cycles (96°C - 30sec., 50°C – 15sec., 60°C – 4min.) preceded by a 1 min. heating step to 96°C and terminated with a cooling step to 4°C. The samples were ethanol precipitated and sent for sequencing by the IMCB sequencing core facilities.

2.2 Cell culture: The cell lines commonly used during the study were Cos-1, 293T and PC-12.

Cos-1 are fibroblastic and adherent cells derived from the kidney of *Cercopithecus aethiops* (African green monkey). They were maintained in Dulbecco's

Modified Essential Medium (DMEM-high glucose) with 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine and 1% 1M Hepes at 37°C in 5% CO_2.

PC12 cells are polygonal in shape and appear as loosely adherent, multicellular aggregates. They originate from adrenal gland pheochromocytoma of *Rattus norvegicus* (Rat) and were maintained in DMEM (high glucose) with 5% FCS, 10% Horse serum (Sigma), 1% Penicillin/Streptomycin, 1% Glutamine and 1% 1M Hepes at 37°C in 5% CO_2. The addition of horse serum is to prevent differentiation in these cells. Collagen coated tissue culture dishes (Iwaki) were used to maintain PC12 cells to ensure good adhesion of these cells.

HEK 293 cells are human (*Homo sapiens)* fetal kidney cells with adherent properties and epithelial morphology. They were propagated in Dulbecco's Modified Essential Medium (DMEM-high glucose) with 10% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine and 1% 1M Hepes at 37°C in 5% CO_2.

Other cell lines used less frequently for various analyses during the study are listed below:

Hela	Human cervix
A549	Human lung
NIH3T3	Mouse embryo
SH-SY5Y	Human neuroblastoma
N1E115	Mouse neuroblastoma
NIT-1	Mouse insulinoma
Rin-5F	Rat insulinoma
AtT20	Mouse pituitary
GH3	Human pituitary
αt3	Mouse pituitary(immature)
LβT2	Mouse pituitary(mature)
CHO	Chinese hamster ovary
C2C12	Mouse muscle
H9C2-(2-1)	Rat myocardium

2.2.2 Freezing of cells: The cells to be frozen for maintenance of stocks were grown to subconfluency in 10 cm dishes, trypsinised and spun at 1000 rpm for 5 min. After the spin, the medium was carefully aspirated without disturbing the pellet and 0.5 ml of fresh medium with 20% FCS was added and the pellet was re-suspended. The tube was then placed in ice for 15 min and 0.5 ml of medium with 20% FCS and 10% DMSO was added with gentle agitation. After 10-15 min on ice, the cells were transferred into a sterile, pre-chilled and labeled cryovial and stored in liquid nitrogen.

2.2.3 Thawing of cells: Frozen cells were quickly thawed by placing the cryovial in a 37°C water bath. Once the cells were thawed, they were transferred to a 15 ml falcon tube containing 9ml of medium and spun at 1000rpm for 5 min. The medium was carefully aspirated and the pellet re-suspended in 5 ml of fresh medium and transferred to a 10 cm tissue culture dish with 5 ml of medium.

2.2.4 Transfection: The plasmid DNA from midiprep was used for transfection of cells. A combination of lipofectamine plus and reagent along with optimem I (Invitrogen) were used, following the manufacturer's protocol. Other reagents such as lipofectamine 2000 and oligofectamine were occasionally used. Transfected cells were analyzed 24 or 48 hours after transfection.

For RNAi experiments, Hela cells were transfected with 100 nM of RNAi (Ambion and Invitrogen) using oligofectamine reagent (Invitrogen) and incubated for 24 hours. Following this, cDNAs encoding the proteins and the RNAi were co-transfected using lipofectamine 2000 and incubated for the next 24 hours.

2.3 Protein analysis

2.3.1 Cell lysis and homogenate preparation: Cells were washed twice with ice cold PBS followed by lysis with homogenization buffer (50mM Tris-HCl, 100mM NaCl, 1mM $MgCl_2$, 1mM DTT, 0.2% Tween-20 supplemented with 5ug/ml each of protease inhibitors leupeptin, pepstatin, antipain, chymostatin and 1mM PMSF). Harvested cells were subjected to two cycles of freeze (liquid nitrogen) and thaw (37°C) and briefly sonicated. After agitating at 4°C for 20 min, the lysate was spun down and the supernatant was carefully transferred to a fresh tube. The protein concentration in the supernatant was determined using a Bradford assay (Bio-Rad).

2.3.2 Preparation of GST fusion proteins

An overnight culture of the PGEX vector based plasmid transformed in BL21 competent cells was grown at 37°C in LB medium containing ampicillin (100µg/ml). The following day, the culture was mixed with 900ml of fusion protein medium (10g/l Tryptone, 5g/l Yeast extract, 10g/l NaCl, 2g/l glucose) along with 100µg/ml ampicillin and left under agitation for an hour at 37°C, before induction with 0.2 mM IPTG for 4 hours. Following induction, the culture was centrifuged at 7000rpm for 15 min and the pellet was re-suspended in ice cold sonication buffer (50mM Tris-HCl, 50mM NaCl, 1mM EDTA, pH 8.0) and stored at -20°C.

The following day, the re-suspended pellet is thawed at room temperature, mixed with lysozyme (Sigma) and left on ice for 15 minutes. Protease inhibitors (1mM PMSF, 5 µg/ml each of leupeptin, pepstatin and antipain were added and subjected to sonication, followed by centrifugation at 10000 rpm for 10 minutes. The centrifugation is repeated after removing the supernatant carefully. The lysed cells were then mixed with 50%

Protein G sepharose beads (Amersham Biosciences) which were prepared by washing with PBS and left on agitation at 4°C until needed. Once the beads were added, the cells were left at 4°C for 1.5 hour on a rotator. This was followed by two washing steps using PBS and centrifugation at 3000 rpm for 3 minutes. The fusion protein was eluted by adding elution buffer (100 mM Tris-HCl, 120 mM NaCl, 6.2mg.ml reduced glutathione, pH 8.0) and left under rotation for 30 minutes at 4°C, followed by centrifugation at 10000 rpm for 20 seconds. The elution step was repeated twice and the concentration of the fusion protein was measured at a wavelength of 280nm, using a spectrophotometer. The protein thus obtained was subjected to overnight dialysis with PBS and stored at -80°C.

2.3.3 Immunofluorescence: Cells were washed with ice cold PBS and fixed with 3.7% paraformaldehyde for 30 min. After fixation, cells were washed with PBS, PFA was quenched with 50mM Ammonium chloride for 10 min and cells permeabilized with 0.2% Triton X-100 for 10 min. Cells were then incubated in blocking solution (PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin (BSA) and 250mM NaCl) for 30-45 minutes, followed by incubation with primary antibodies {rabbit anti-Myc (Upstate biotechnology) for myc tagged constructs, mouse anti-GST (Cell Signaling Technology) for GST tagged constructs, mouse anti-Flag (Sigma) for Flag tagged constructs and rat anti-HA (Roche diagnostics) for HA tagged constructs}.

Following a 1 hour incubation at RT with the primary antibody, the cells were washed thrice with the blocking solution and incubated with the appropriate secondary antibodies {Cy3-labeled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories), Alexafluor 488 goat anti mouse IgG (Molecular Probes), Alexafluor 488 goat anti rat IgG(Molecular probes)}. The cells were then washed thrice with the

blocking solution and finally with PBS. The coverslips were mounted on glass slides using crystal mount from Biomeda and stored in the dark until visualization of the specimens with an Axiocam microscope (Carl Zeiss) at 100x magnification.

2.3.4 Co-Immunoprecipitation: Flag agarose or myc agarose beads (Sigma) were washed with washing buffer (50mM Tris HCl, 100mM NaCl, 1mM $MgCl_2$, 1mM DTT) and then incubated with cell lysates (400 μg protein) in interaction/binding buffer (washing buffer with 0.2% Tween or 0.2 % Triton X-100, 1mM PMSF and a cocktail of protease inhibitors (5ug/ml of leupeptin, papstatin and antipain)) at 4°C for either 4 hours or over night under constant agitation. Following incubation, beads were extensively washed and the bound protein complexes eluted by heating at 95°C for 5 min. Following centrifugation, the eluted protein complexes were subjected to a SDS PAGE and Western blot analysis.

2.3.5 GST pull down: In order to verify the interaction between two proteins, pull down experiments were performed using cell lysates expressing one protein and GST fusion protein for the second one. GST-agarose beads (Sigma) were extensively washed with the washing buffer (50mM Tris HCl, 100mM NaCl, 1mM $MgCl_2$, 0.1mM DTT, pH 7.5) and incubated with 2 ug of the GST fusion protein in interaction/binding buffer (washing buffer supplemented with a cocktail of protease inhibitors) for 1.5 hours at 4˚C. The beads were then extensively washed and incubated with 400 ug of cell lysates for 3 hours at 4°C. Upon subsequent washes after incubation period, protein complexes were eluted and subjected to SDS PAGE and Western blot analysis.

2.3.6 Western blot:

SDS PAGE: The resolving gel (0.125 M Tris Glycine (pH 8.9), 7-9.5% (depending on the proteins to be analyzed) Acrylamide-Bis, 0.1% SDS, 0.05% TEMED and 0.05% APS) was cast and overlayed on the stacking gel (0.125 M Tris Glycine (pH 6.9), 3% Acrylamide-Bis, 0.1% SDS, 0.05% TEMED and 0.1% APS). The electrophoresis buffer consisted of 0.125M Tris, 0.1M glycine and 0.5% SDS. To verify protein expression, 20ug of total protein was dissolved in sample buffer (6% SDS, 85% sucrose, 0.125% bromophenol blue and β-mercaptoethanol) and heated at 95°C for 3 min, prior to loading on to the gel. Pre-stained kaleidoscope protein molecular weight markers (Bio Rad) were used to estimate the molecular weight of the proteins under study.

Wet transfer and Immunoblotting: Following the SDS PAGE, the gel was briefly washed in transfer buffer (25Mm Tris-HCl, 0.19M glycine, 20% methanol and 0.05% SDS, pH 7.5). The PVDF membranes (Millipore) to be used were first rinsed in 100% methanol and then in transfer buffer. A sandwich consisting of Whatmann 3mm filters, the polyacrylamide gel and the PVDF membrane stacked in the correct orientation was assembled. Proteins transfer from the gel to the membrane was performed overnight at 4°C at a constant 40V in transfer buffer.

Following the transfer, the PVDF membrane was washed using TBS-Tween buffer (0.1M NaCl, 0.1% Tween, 0.01M Tris-HCl, pH 7.5) and blocked for an hour with 5% milk powder in TBS tween buffer. The membrane was then washed twice for 5 min each time, followed by 1 hour incubation with primary antibody (monoclonal anti flag (Sigma) diluted 1: 16000 or monoclonal anti-c-myc (Roche) diluted 1:3000) in TBS-Tween buffer with 2% BSA (96%, fraction V, Sigma). After subsequent washes (3

times, 15 min), the membrane was incubated with the secondary antibody (sheep anti-mouse-conjugated to horse radish peroxidase (Amersham biosciences) diluted 1: 4000) in TBS-Tween buffer with 5% milk buffer. The membrane was washed again for three times and subjected to chemiluminescence detection (Amersham biosciences) following manufacturer's instructions. The membrane was then exposed to hyperfilm (Amersham biosciences).

List of antibodies used during the study

Mouse anti flag	Sigma
Rabbit anti flag	Sigma
Chicken anti DDDDK	Abcam
Rabbit anti myc	Upstate, Sigma
Mouse anti myc	Roche
Chicken anti myc	Abcam
Rat anti HA	Roche
Mouse anti HA	Roche
Rabbit anti HA	Upstate
Mouse anti GST	Cell signaling technology
Rabbit anti GST	Zymed

2.4 Electrophysiology: Electrophysiological recordings were done by our collaborator (Dr.Kazuaki Nagashima, Graduate school of Medicine, Kyoto University, Japan). Recordings for calcium currents were made 48h after transfection using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The whole-cell VDCC currents in PC12 cells were recorded as described (Beguin et al., 2001). Briefly, Ba^{2+} was used as a charged carrier for measurement of VDCC currents. The extracellular solution contained 40 mmol/l $Ba(OH)_2$, 20 mmol/l 4-aminopyridine, 110 mmol/l tetraethylammonium hydroxide, 10 mmol/l tetraethylammonium chloride, 140 mmol/l

methanesulfonate and 10 mmol/l 3-(N-morpholino)propanesulfonic acid (pH 7.4). The pipette solution contained 10 mmol/l CsCl, 130 mmol/l Cesium aspartate, 10 mmol/l EGTA, 10 mmol/l 3-(N-morpholino)propanesulfonic acid and 5 mmol/l Mg-ATP (pH 7.2). Cells were maintained at a holding potential of -60 mV. For recording VDCC currents, square pulses of 400 ms duration at potentials between -40 and +60 mV in steps of 10 mV were applied every 4 s. For normalization, the currents were divided by the membrane capacitance measured for each cell (Kawaki et al., 1999). Statistical significances were determined using unpaired Student's t-tests and results are expressed as mean +/- SE.

CHAPTER 3

Regulation of RGK proteins by CaM and 14-3-3

3.1 Identification of 14-3-3 binding sites in RGK proteins

Ward et al (2004) showed that Kir/Gem binds to 14-3-3 via an N- and C-terminal site, S22 and S288 (S23 and S289 in human Kir) respectively. As an approach to characterize the interaction further, the sequences of RGK proteins were compared to look for any consensus 14-3-3 binding sites conserved with those in Kir/Gem. Sequence analysis revealed that the C-terminal 14-3-3 binding serine residue (S300 in Rad and S290 in Rem) is conserved. Further analysis indicated S25/S38 in Rad and S18/S38 in Rem has two putative N-terminal 14-3-3 binding serine residues.

A previous study reported that 14-3-3 does not bind to Rem2 (Finlin et al ., 1999). Sequence analysis of EST clones and genome BLAST of the rat, mouse and human sequences of Rem2 (Genebank accession number: rat, CB802662; mouse, BY794767 and human, BM546743) was done using the NCBI online resources. The analysis revealed an in frame methionine (M1) present upstream of the initiation methionine (M70) previously assigned to the rat cDNA (AF084464). An in frame stop codon upstream of methionine 1 (M1) (Fig. 3-1) was present in mouse EST clones, but not in EST clones from human and rat. Blasting of the mouse EST sequence against the human and rat genome subsequently confirmed the presence of an in frame stop codon upstream of M1 in the human and rat cDNAs. More detailed analysis indicated that a likely sequencing error in AF084464 (a C missing in a GC rich region), probably led to the erroneous assignment of an in frame termination codon upstream of M70. In contrast, M1 is conserved across species and is preceded by an in frame stop codon in all three species.

Thus, AF084464 used in previous studies encodes an N-terminally truncated form of Rem2 that lacks the first 69 amino acids.

S334 in Rem2 is analogous to the C terminal 14-3-3 binding site in other RGK proteins. S55 and S69 were identified as putative 14-3-3 binding sites in the N-terminus. The lack of S55 and S69 in the truncated form used by Finlin et al (1999) probably accounts for the lack of an interaction between Rem2 and 14-3-3, as observed in their study.

Fig. 3-1 A Sequence analysis of Rem2 and critical binding sites in RGK proteins. The previously reported sequence for Rem2 (AF084464) is a truncated form that starts at M70 and lacks S69, the N-terminal 14-3-3 binding site in Rem2. **B.** 14-3-3 and CaM binding sites in RGK proteins. The residues in brackets are non-functional putative 14-3-3 binding sites.

3.2 Characterization of 14-3-3 binding to RGK proteins

To study the interaction between 14-3-3 zeta and RGK proteins, N-GST-tagged 14-3-3 zeta and N-Myc tagged RGK proteins were co-expressed in Cos1 cells and subjected to co-immunoprecipitation. Since 14-3-3 and RGK proteins are ~ 35 kDa in size, 14-3-3 was GST tagged in order to differentiate the bands clearly. This would also enable to distinguish the endogenous (~ 35 kDa) and over expressed (~ 60 kDa) 14-3-3. In addition to WT, two mutants of 14-3-3 were used as controls. One, 14-3-3 K49E, is incapable of target binding and the other is a dimerization defective mutant that can bind to target but cannot dimerize.

The following page lists the various constructs used for analysis.

KIR/GEM

1. **Kir/GemWT**
2. **S22A-mutant in which N-terminal 14-3-3 binding is mutated to Alanine**
3. **S288A-mutant in which C-termnal 14-3-3 binding site is mutated to Alanine**
4. **S22A-S288A-mutant in which both N- and C- terminal 14-3-3 binding sites are mutated.**
5. **W269G-mutant deficient in calmodulin binding**
6. **W269G-S22A-mutant deficient in calmodulin binding and lacks N-terminal 14-3-3 binding site.**
7. **W269G-S288A- mutant deficient in calmodulin binding and lacks C-terminal 14-3-3 binding site.**
8. **W269G-S22A-S288A-triple mutant deficient in calmodulin and 14-3-3 binding.**
9. **R18-a chimera in which the C-terminal 14-3-3 binding region is replaced by the R18 peptide.**

RAD

1. **RAD WT**
2. **S38A-mutant in which N-terminal 14-3-3 binding is mutated to Alanine**
3. **S300A-mutant in which C-termnal 14-3-3 binding site is mutated to Alanine**
4. **S38A-S300A-mutant in which both N- and C- terminal 14-3-3 binding sites are mutated**
5. **L281G-mutant deficient in calmodulin binding**
6. **L281G-S38A-mutant deficient in calmodulin binding and lacks N-terminal 14-3-3 binding site.**
7. **L281G-S300A- mutant deficient in calmodulin binding and lacks C-terminal 14-3-3 binding site.**
8. **L281G-S38A-S300A-triple mutant deficient in calmodulin and 14-3-3 binding**

REM or REM1

1. **REM WT**
2. **S18A-mutant in which N-terminal 14-3-3 binding is mutated to Alanine**
3. **S290A-mutant in which C-terminal 14-3-3 binding site is mutated to Alanine**
4. **S18A-S290A-mutant in which both N- and C- terminal 14-3-3 binding sites are mutated**
5. **L271G-mutant deficient in calmodulin binding**
6. **L271G-S18A-mutant deficient in calmodulin binding and lacks N-terminal 14-3-3 binding site.**
7. **L271G-S290A- mutant deficient in calmodulin binding and lacks C-terminal 14-3-3 binding site.**
8. **L271G-S18A-S290A-triple mutant deficient in calmodulin and 14-3-3 binding.**

REM2

1. **REM2 WT**
2. **S69A-mutant in which N-terminal 14-3-3 binding is mutated to Alanine**
3. **S334A-mutant in which C-termnal 14-3-3 binding site is mutated to Alanine**
4. **S69A-S334A-mutant in which both N- and C- terminal 14-3-3 binding sites are mutated**
5. **L317G-mutant deficient in calmodulin binding**
6. **L317G-S69A-mutant deficient in calmodulin binding and lacks N-terminal 14-3-3 binding site.**
7. **L317G-S334A- mutant deficient in calmodulin binding and lacks C-terminal 14-3-3 binding site.**
8. **L317G-S69A-S334A-triple mutant deficient in calmodulin and 14-3-3 binding**

Binding experiments showed that all RGK proteins associated with GST-14-3-3 (Fig. 3-2A, lanes 2, 8, 13, 18) but not with GST-14-3-3 K49E (lanes 3, 9, 14, 19), confirming a specific interaction. 14-3-3 did not bind to the serine mutants where the putative binding sites were mutated to alanine, S22A or S288A of Kir/Gem (lanes 4, 5), S38A or S300A of Rad (lanes 10, 11) and S69A or S334A of Rem2 (lanes 15, 16). In the case of Rem, while single mutations (S18A or S290A) did not affect the interaction (lanes 20, 21), simultaneous mutation of both binding sites, S18 and S290, completely abolished the interaction with 14-3-3 (lane 22). Mutation of other putative sites (S25 in Rad, S38 in Rem and S55 in Rem2) did not have any effect on the interaction (data not shown) showing that these sites are non-functional in terms of 14-3-3 binding. A chimera (Kir/Gem-R-18) was also generated in which the domain from K283 to H290 of Kir/Gem was substituted by the R18 peptide-PHCVPRDLSWLDLEANMCLP. R18 peptide was identified in a phage display and can mediate constitutive binding to 14-3-3 (Wang et al., 1999). This chimera showed binding to endogenous and overexpressed 14-3-3 (lane 6, panels B and C). Western blot of the cell lysates confirmed that the transfected cells expressed similar levels of the WT or mutated RGK proteins and GST-14-3-3 WT or mutants (panels D and E).

As previously shown (Leone et al., 2001), Kir/Gem migrated as a doublet in SDS-PAGE (panel D, lane 1), indicative of a posttranslational modification. The ratio between the slower and faster migrating band was affected following mutation of the 14-3-3 binding sites or overexpression of GST-14-3-3 (panel D; compare lanes 1 with 2-5), suggesting that 14-3-3 either induces or stabilizes this modification. Intriguingly, 14-3-3 associated mainly with the slower migrating form of Kir/Gem (panel B, lane 2).

The presence of two 14-3-3 binding sites in RGK proteins suggests an association with 14-3-3 dimers. Consistent with this hypothesis, the dimerization-defective 14-3-3 mutant failed to associate with Kir/Gem, Rad and Rem2 or Kir/Gem-R-18 (Fig 3-2B, lanes 2, 6, 8 and 4).

Interestingly, as mentioned before, Rem S18A and Rem S290A still associated with GST-14-3-3 and only simultaneous mutation of both serine residues (Rem S18A/S290A) prevented the interaction. Furthermore, Rem1 also bound to the dimerization defective mutant of 14-3-3 (lane 10), which implied that 14-3-3 dimerization is not essential for the association and therefore suggesting that Rem1 can interact with 14-3-3 monomers. The ability of Rem1 to interact with 14-3-3 monomers thus correlates with the requirement of only one of its two 14-3-3 binding sites for the interaction.

In conclusion, while Kir/Gem, Rad and Rem2 can interact with 14-3-3 through the N- and C-terminal binding sites and associate with 14-3-3 dimers, Rem can associate with 14-3-3 monomers with either one of its two binding sites.

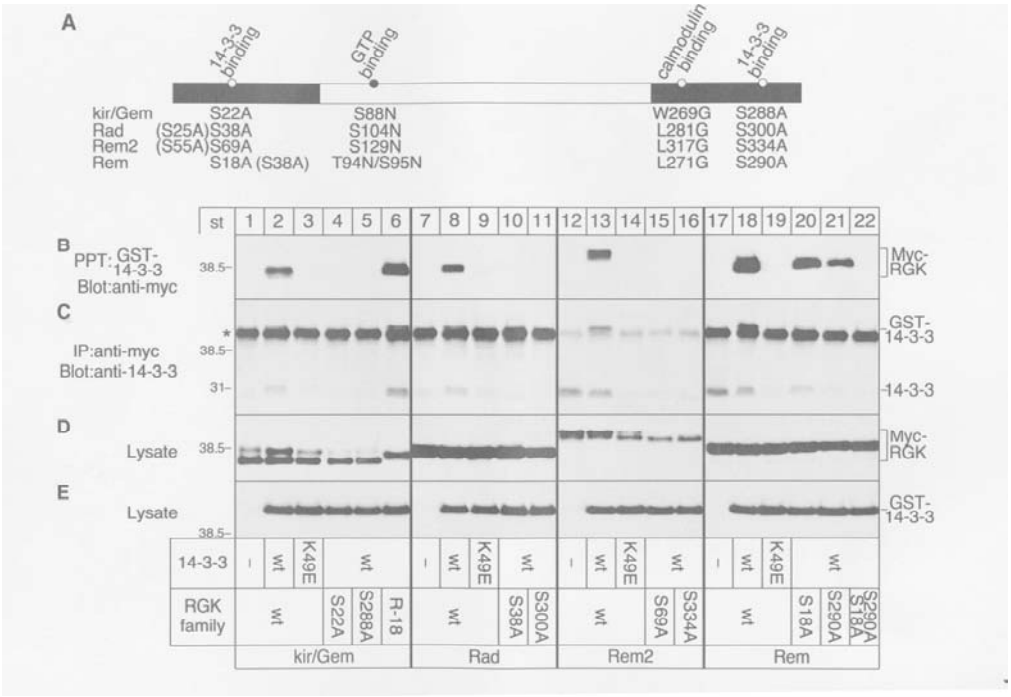


Fig. 3-2A Binding of 14-3-3 to RGK proteins.

A. The Ras like core domain (white bar), N- and C-terminal extensions (black bar) and the location of 14-3-3, CaM and GTP binding sites are indicated with putative, non functional 14-3-3 sites in the brackets.

B and C. Co-immunoprecipitation. Cells were co-expressed with WT or mutant N-myc-RGK proteins and GST-14-3-3 WT or mutants. GST-14-3-3 proteins were precipitated and bound RGK proteins detected by western blot using Myc antibodies.

B. RGK proteins were immunoprecipitated and associated overexpressed GST- and endogenous 14-3-3 were detected by western blot using 14-3-3 antibodies

C. Cell lysates were blotted with Myc (D) or GST (E) antibodies to monitor the expression levels of RGK proteins or GST-14-3-3. Kir/Gem R-18 is a chimera in which the C-terminus (including S288A) was substituted by a stretch of 18 amino acids (R-18) that mediate constitutive 14-3-3 binding

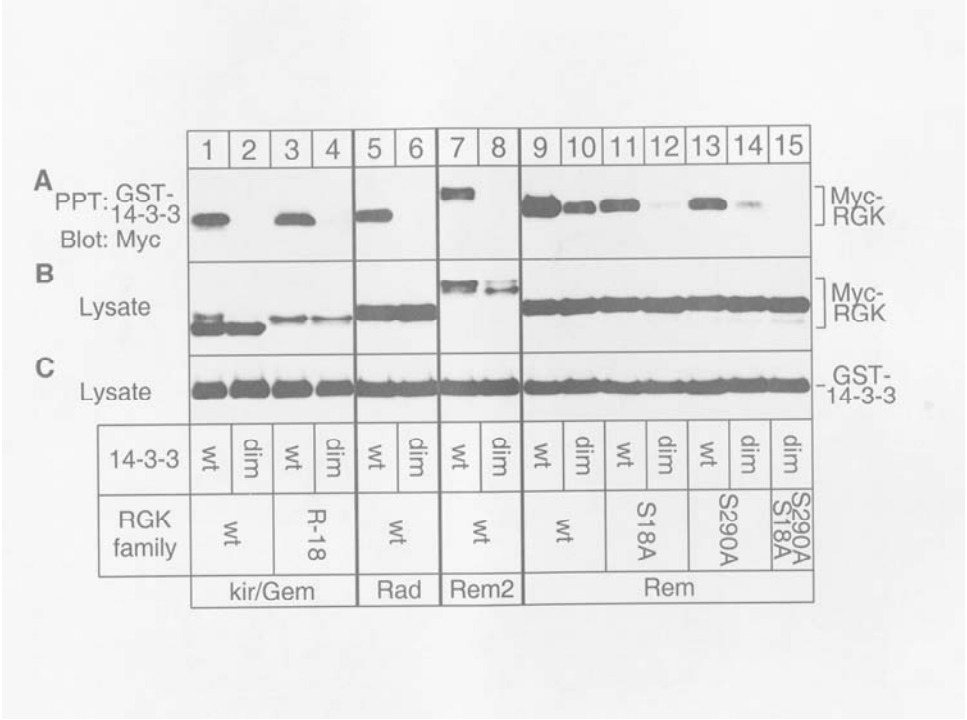


Fig. 3-2B Association of RGK proteins with 14-3-3 dimers

A. Cos1 cells were co-transfected with cDNAs for WT or mutant N-Myc-RGK proteins and GST-14-3-3 WT and dimerization deficient mutant (dim). GST-14-3-3 is precipitated and associated RGK proteins were detected by western blot using Myc antibody.

B and C. Cell lysates were blotted with Myc(B) and GST(C) antibodies to monitor protein expression levels.

3.3 14-3-3 regulates the subcellular distribution of RGK proteins

14-3-3 is known to regulate the subcellular localization of many of its interacting partners e.g. BAD, Tesk etc. It can sequester the target proteins in various cellular compartments like plasma membrane (Raf1), mitochondria (BAD) or nucleus (Tert) (Dougherty et al., 2004). To explore the physiological role of 14-3-3 binding with RGK proteins, I analyzed if the interaction with 14-3-3 had any effect on the intracellular distribution of the small G proteins. Cos-1 cells were transfected with cDNAs for RGK WT or mutants lacking one or both 14-3-3 binding sites, either in the absence or presence of WT or mutant GST-14-3-3 zeta. The subcellular localization of the RGK proteins was then analyzed by immunofluorescence microscopy. RGK proteins expressed in Cos-1 cells were distributed throughout the cell and slightly enriched at submembranous and perinuclear regions and in the nucleus (Fig. 3-3A, panel a). Mutants in which 14-3-3 binding sites were mutated also showed a similar distribution to that of WT. Kir/Gem-R18 chimera, which binds to 14-3-3 constitutively, showed a more prominent nuclear localization (panel l).

Interestingly, co-expression of GST-14-3-3 and RGK WT led to the efficient nuclear exclusion/cytoplasmic sequestration of the small G proteins. Using Kir/Gem as an example (Fig 3-3A), co-expression of Kir/Gem WT and 14-3-3 led to an efficient nuclear exclusion (c-c"). This effect was not observed if 14-3-3 K49E, which is devoid of binding to target, was co-expressed with Kir/Gem (d-d"). Also, the dimerization defective mutant of 14-3-3 was unable to cause an effect following co-expression with Kir/Gem, implying that 14-3-3 dimerization is essential for association with Kir/Gem (e-e"). Furthermore, Kir/Gem mutants in which either the N-or C- terminal or both 14-3-3

binding sites were mutated, failed to show any effect when co-transfected with 14-3-3 (g-k"). Remarkably, Kir/Gem R-18 chimera, which predominantly localized to the nucleus, was also efficiently redistributed to the cytoplasm by exogenous 14-3-3 (m-m"). Thus the binding of 14-3-3 with Kir/Gem causes nuclear exclusion and/or cytoplasmic sequestration of the small GTPase.

Similar experiments with the other RGK proteins were also performed (Fig 3-3B-D). Rad wt was efficiently excluded from the nucleus when co-expressed with 14-3-3 wt Fig 3-3B, b-b"). This was not observed when the 14-3-3 binding sites were mutated (e-e", h-h", k-k"). In the case of Rem (Fig 3-3C), WT Rem showed nuclear exclusion when co-expressed with 14-3-3 wt (b-b"). While mutation in the C-terminal 14-3-3 site failed to show any effect (e-e"), mutation of the N-terminal 14-3-3 site (S18A) resulted in an intermediate nuclear exclusion, when co-expressed with 14-3-3 (d-d"). No effect was observed when both the sites were mutated (h-h").

For Rem2 (Fig 3-3D), when the WT protein was co-expressed with 14-3-3, it was observed that the cell population showing nuclear exclusion was significantly smaller as compared to other RGK proteins, where the majority of the cells co-expressing RGK and 14-3-3 wt proteins displayed efficient nuclear exclusion. Rem2 mutants lacking single or both 14-3-3 binding sites did not display an effect.

Data from 3 to 5 independent experiments were subjected to quantification analysis, where the population of cells that showed complete, partial or no nuclear exclusion was counted under the microscope. Quantification of this data confirmed that over expression of 14-3-3 leads to nuclear exclusion of RGK proteins (Fig. 3-6A, C, D and E).

Fig. 3-3-Cytoplasmic relocalization of RGK proteins by 14-3-3

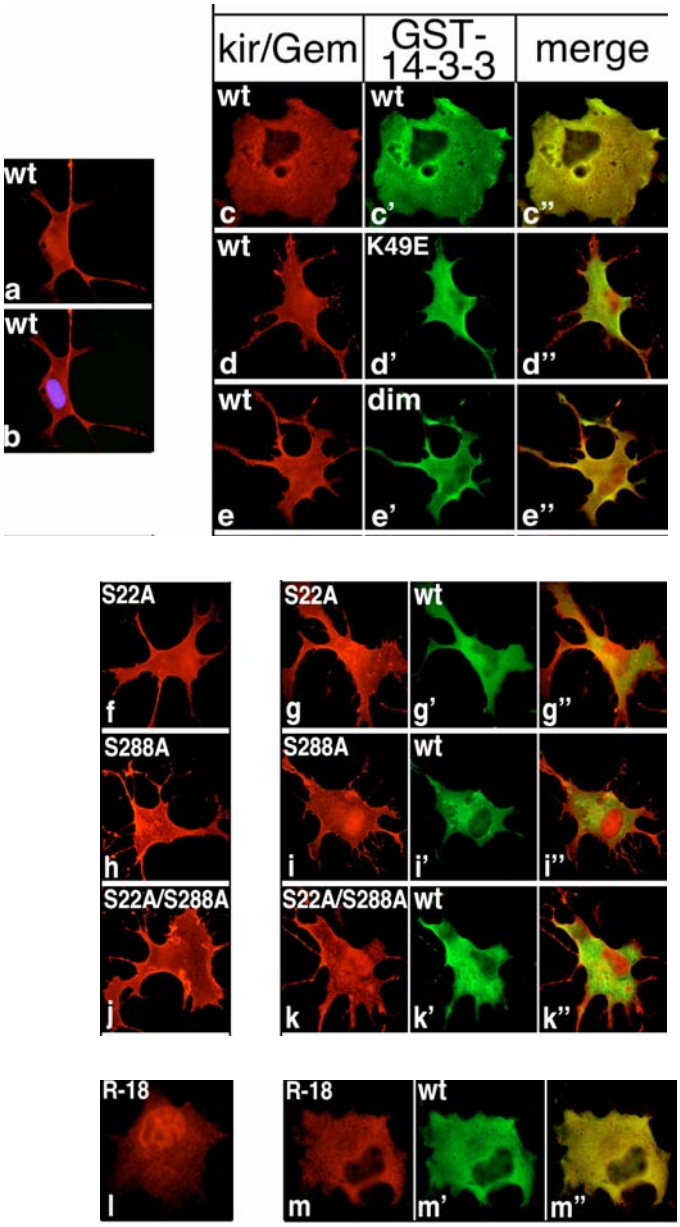


Fig. 3-3A Regulation of localization of Kir/Gem by 14-3-3
Cos1 cells were transfected with cDNAs for Kir/Gem WT and mutants either alone or with 14-3-3 WT or K49E mutant and processed for immunoflurescence using rabbit anti Myc (red) antibody for Kir/Gem and Mouse anti GST (green) antibody for GST-14-3-3. Areas of co-localization are in yellow in the merged image. Nuclei were stained with Hoescht in panel b.

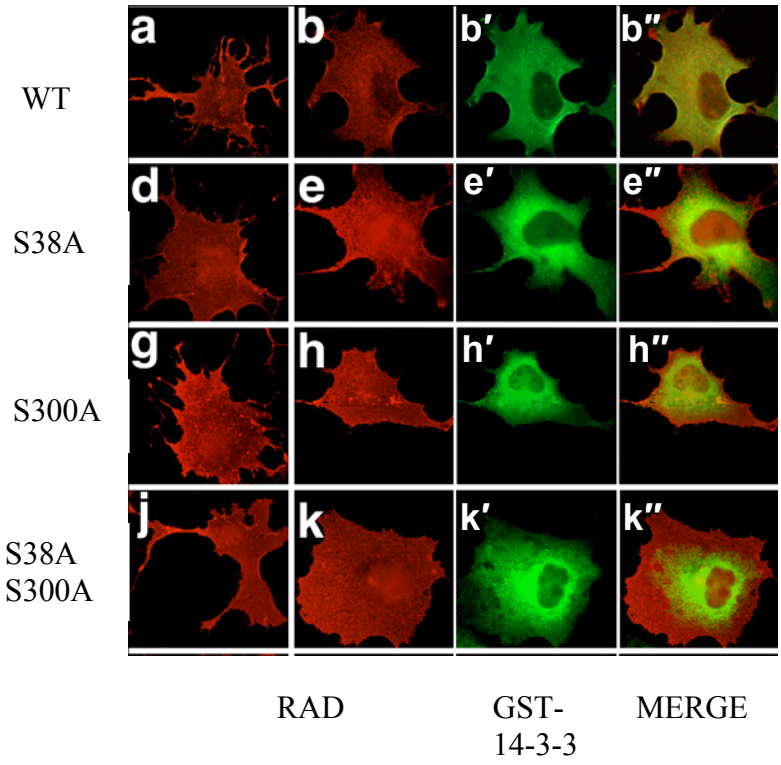


Fig. 3-3B Regulation of localization of Rad by 14-3-3
a, d, g, j show Rad WT, S38A, S300A and S38A-S300A mutants expressed alone in Cos1 cells. b-b", e-e", h-h", k-k" are the respective proteins co-expressed with 14-3-3.

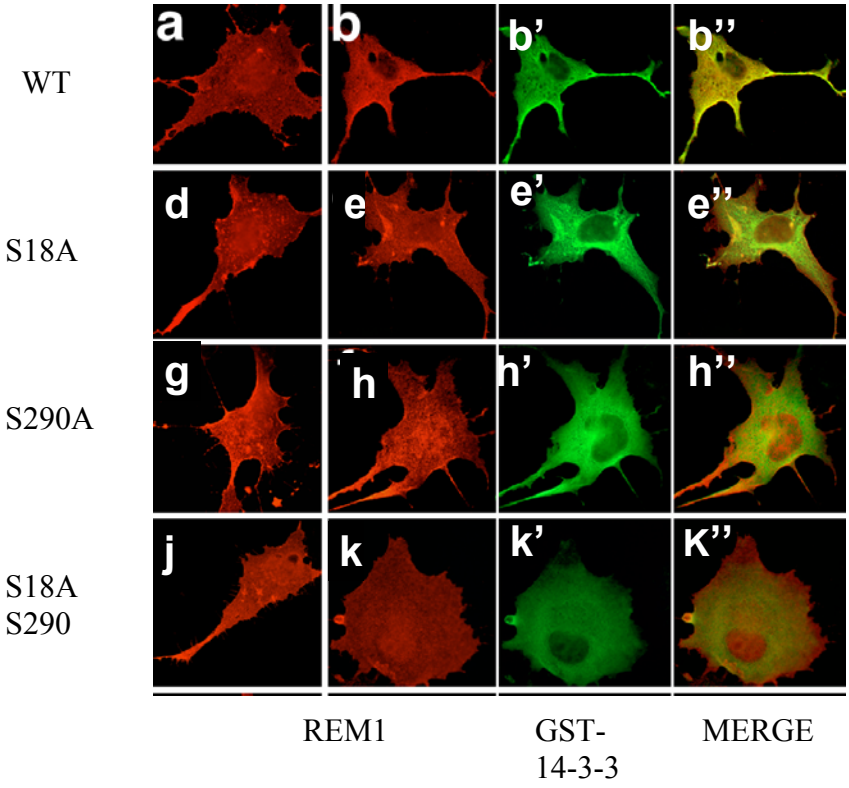


Fig. 3-3C Regulation of localization of Rem1 by 14-3-3
a, d, g, j are Rem1 WT, S18A, S290A and S18A-S290A mutants expressed alone in Cos1 cells. b-b", e-e", h-h", k-k" are the respective proteins co-expressed with 14-3-3.

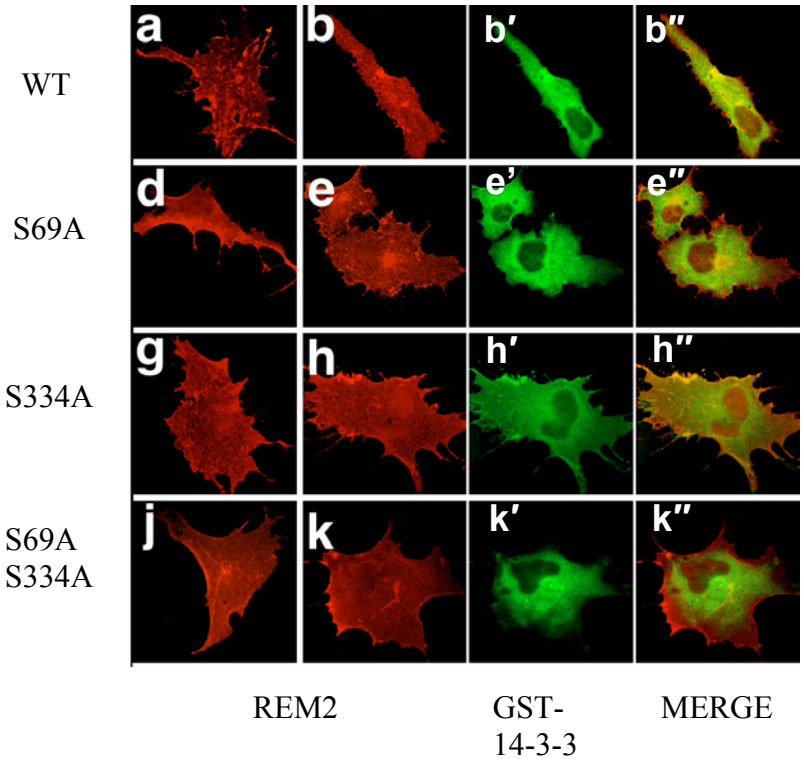


Fig. 3-3D Regulation of localization of Rem1 by 14-3-3
a, d, g, j are Rem2 WT, S18A, S290A and S18A-S290A mutants expressed alone in Cos1 cells. b-b", e-e", h-h", k-k" are the respective proteins co-expressed with 14-3-3.

3.4 Modulation of subcellular localization of RGK proteins by CaM

Earlier studies showed that Kir/Gem binds to CaM through the C-terminal CaM binding domain and a point mutation in Kir/Gem (W269G), which prevents the binding to CaM (Fischer et al., 1996), translocates the protein to the nucleus. Similar to what has been reported in HEK 293 cells, (Beguin et al., 2001) it was found that W269G showed a predominant nuclear localization in cos1 cells following transient transfection. Though reports showed that Rad bound CaM, the residues crucial for the binding had not been identified. Data from Dr. P. Beguin showed that Rem and Rem2 also bind CaM. The amino acid critical for CaM binding, in analogy to Tryptophan of Kir/Gem, in other RGK family members is Leucine. Subsequently, Rad L281G, Rem1 L271G and Rem2 L317G were generated. In order to understand the functional significance of CaM binding to RGK proteins, RGK WT and CaM binding deficient mutants were expressed in Cos-1 cells and the localization was analyzed. The WT proteins showed a diffused and submembranous localization with induction of dendrite-like extensions. Dendrite-like extensions are described as the cell morphology changes caused due to the development of neurite like processes from the cell body. In contrast, the CaM binding deficient mutants translocated to the nucleus and also showed an effect on the induction of extensions. In the case of Kir/Gem, the extensions were lost and for the other members, it was an obvious reduction in extensions. Further, it was observed that the nuclear translocation of Rem2 L317G was consistently less pronounced compared to the other RGK proteins.

From the immunofluorescence data (Fig. 3-4) it is evident that CaM plays a crucial role in the localization of RGK proteins. A comparison of WT and mutants lacking CaM binding is depicted in Fig. 3-4.

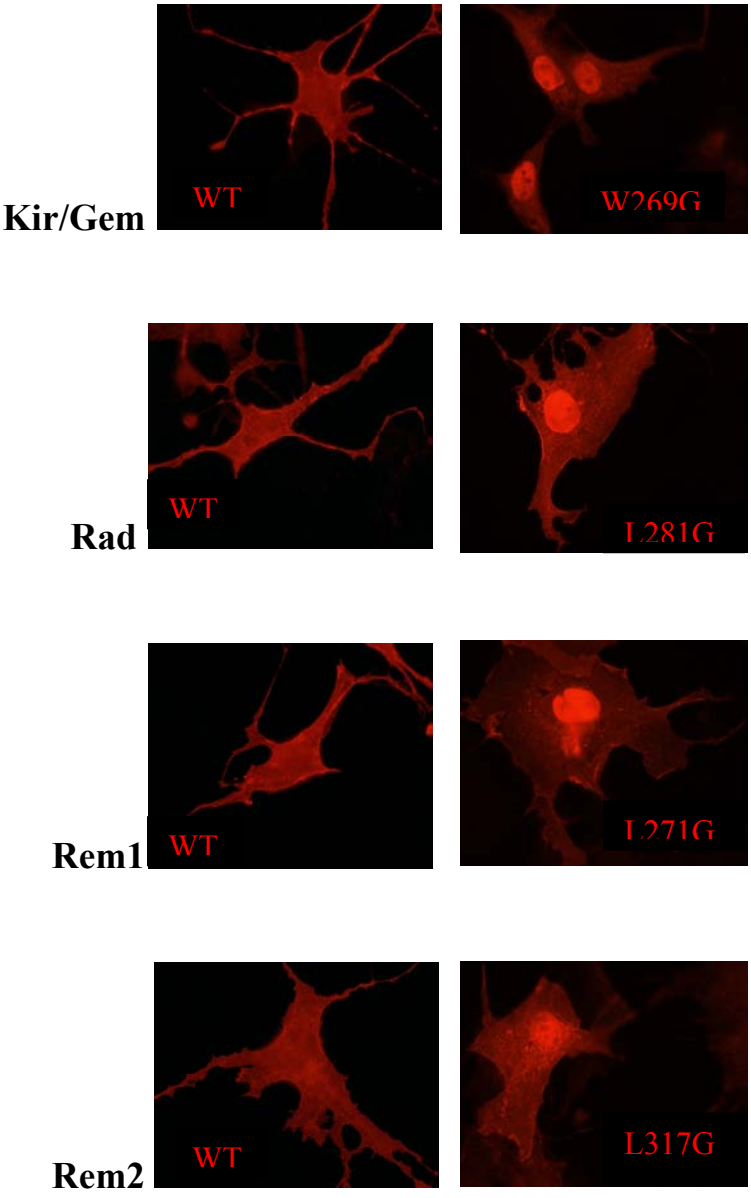


Fig. 3-4 RGK proteins deficient in CaM binding localize to nucleus and show an effect on RGK induced dendrite-like extensions.

3.5 Modulation of localization of RGK proteins by 14-3-3 in the absence of CaM binding

The 14-3-3 and CaM binding sites are in close proximity at the C-terminus of RGK proteins. CaM had already been shown to affect the subcellular localization of Kir/Gem. To explore a possible interplay between 14-3-3 and CaM binding on the localization of RGK proteins, the study was first performed in Kir/Gem. As shown in Fig. 3-4, Kir/Gem W269G, which does not bind CaM, showed increased nuclear localization. Mutants lacking 14-3-3 binding sites in Kir/Gem W269G (W269G-S22A or W269G-S288A or W269G-S22A-S288A) were generated and these mutants also showed predominant nuclear localization (Fig. 3-5A, f, i, l).

Co-transfection of W269G with 14-3-3 WT, but not 14-3-3 K49E, led to the nuclear exclusion of W269G (Fig. 3-5A, c-c", d-d"). While there was no nuclear exclusion observed for W269G-S288A or W269G-S22A-S288A when co expressed with 14-3-3 WT (j-j", m-m"), W269G-S22A was efficiently excluded, indicating the importance of the C-terminal 14-3-3 binding site in the absence of CaM binding (g-g"). Since 14-3-3 can efficiently clear W269G-S22A from the nucleus, it was hypothesized that, in the absence of CaM binding, 14-3-3 monomer can interact with the C terminal 14-3-3 binding site of Kir/Gem. To test this, the dimerization defective 14-3-3 mutant was co-expressed with W269G-S22A. This resulted in an intermediate state of nuclear clearance of W269G-S22A (h-h"). This indicated that 14-3-3 monomer can associate with S288 in the absence of CaM binding and cause a partial nuclear clearance, although efficient nuclear exclusion can result only through binding to 14-3-3 dimers. Co-immunoprecipitation experiments also showed that 14-3-3 bound to W269G and W269G-S22A, but not to W269G-S288A or W269G-S22A-S288A (Fig. 3-5B).

To ascertain if the effects of 14-3-3 binding in the absence of bound CaM on the subcellular localization of Kir/Gem were of general validity for the entire RGK family, the analysis was extended to the other members. As described earlier, Rad L281G, Rem L271G and Rem2 L317G are analogous to Kir/Gem W269G. Though these mutants were localized in the nucleus similar to Kir/Gem W269G (Fig. 3-4), it was consistently observed that Rem2 L317G was less efficiently translocated when compared to the other RGK proteins. Mutants lacking both 14-3-3 and CaM binding were also generated and were found to localize in the nucleus {(Rad L281G-S38A, Rad L281G-S300A, Rad L281G-S38A-S300A), (Rem1 L271G-S18A, Rem1 L271G-S290A, Rem1 L271G-S18A-S290A), (Rem2 L317G-S69A, Rem2 L317G-S334A, Rem2-S69A-S334A)} (Fig. 3-5C-E, a, c, e and g respectively). Binding of CaM to various mutants used in the study is shown in Fig. 3-5G (*data provided by Dr.P.Beguin*).

The localization of RGK proteins either with or without 14-3-3 co-expression in Cos1 cells was studied by immunofluorescence microscopy. As observed for Kir/Gem W269G, co-expression with 14-3-3 led to efficient nuclear exclusion of Rad L281G, Rem1 L271G and Rem2 L317G (Fig. 3-5C-E, b-b"). No re-distribution was observed if both the 14-3-3 binding sites were mutated (h-h"). In case of Rad, mutation of either N- or C-terminal sites did not cause any redistribution (L281G-S38A or L281G-S300A) (Fig. 3-5C, d-d", f-f"). While no nuclear exclusion was seen if only the C-terminal site was mutated in Rem1 and Rem2 (Rem1 L271G S290A and Rem2 L317G S334A) (Fig. 3-5D and E, f-f"), mutation in N-terminal site showed a partial redistribution (Fig. 3-5D and E, d-d").

Binding experiments (Fig. 3-5F) showed results that were consistent with the immunofluorescence data for all RGK proteins except Rem2 L317G S69A, which showed a partial nuclear exclusion. No binding could be detected, possibly due to a weak or transient interaction that is lost during co-precipitation.

Further, to substantiate the effect of cytosolic sequestration of RGK proteins by 14-3-3 binding, quantification of 3 to 5 independent experiments was done and the results shown in Fig. 3-6(A, C, D and E). Quantification analysis was also done for the loss of dendritic extensions for Kir/Gem by 14-3-3 binding (Fig. 3-6B).

Thus, despite subtle differences, the localization of all RGK proteins, is regulated by 14-3-3 and CaM binding.

Fig. 3-5 Cytoplasmic relocalization of RGK mutants lacking CaM binding

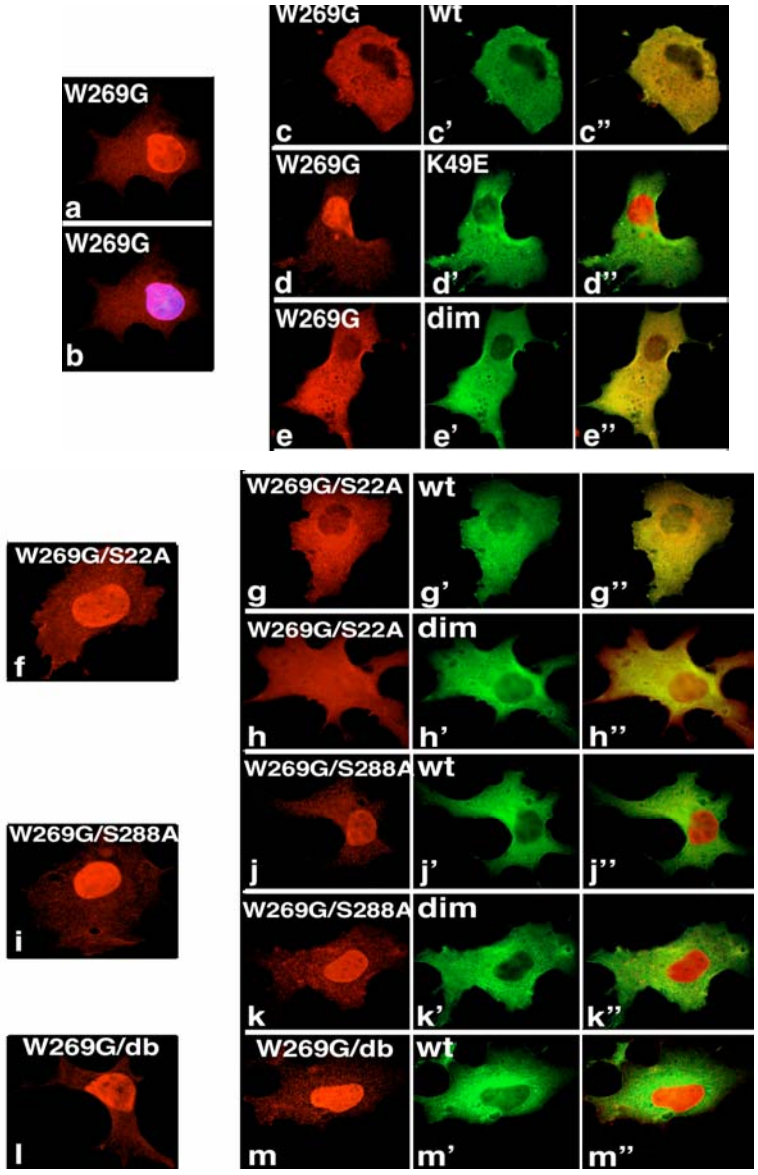


Fig. 3-5A Regulation of localization of Kir/Gem W269G and mutants by 14-3-3
Cos-1 cells were transfected with cDNAs for Kir/Gem W269G and corresponding 14-3-3 binding mutants either alone or with 14-3-3 WT or K49E mutant and processed for immunoflurescence using rabbit anti Myc (red) antibody for Kir/Gem and mouse anti GST (green) antibody for GST-14-3-3. Areas of colocalization are in yellow in the merged image. Nuclei were stained with Hoechst in panel b

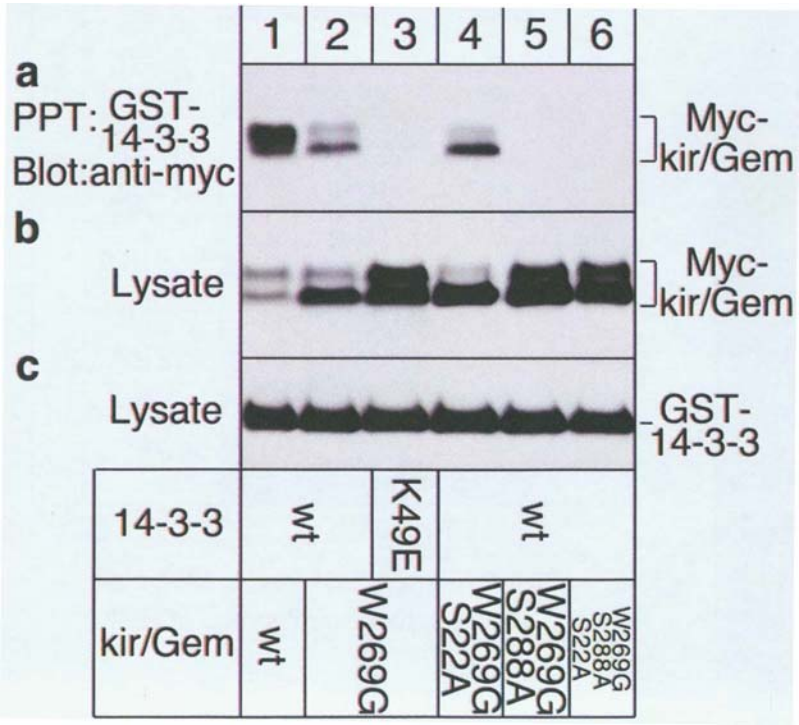


Fig. 3-5B Binding of 14-3-3 to Kir/Gem mutants lacking CaM binding
Cells were cotransfected with cDNAs for WT or mutated Myc-kir/Gem and GST-14-3-3 WT, GST-14-3-3 K49E.
a. GST-14-3-3 proteins were precipitated and associated Myc-kir/Gem was detected by Western blot using Myc antibody.
b and **c**. Cell lysates were blotted with Myc (**b**) or GST (**c**) antibodies to monitor kir/Gem and GST-14-3-3 expression levels.

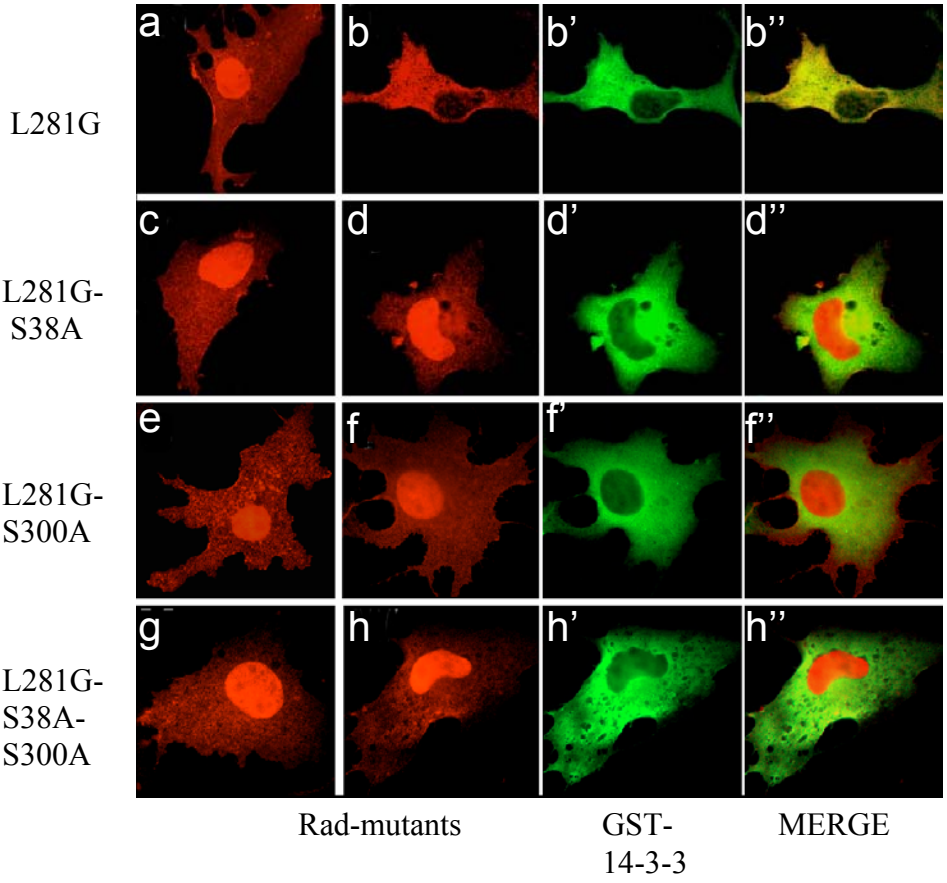


Fig. 3-5C Regulation of localization of Rad L281G and mutants by 14-3-3
a, c, e, g show Rad L281G, L281G-S38A, L281G-S300A and L281G-S38A-S300A mutants respectively expressed alone in Cos-1 cells respectively. b-b", d-d", f-f", h-h" show the respective proteins co-expressed with 14-3-3.

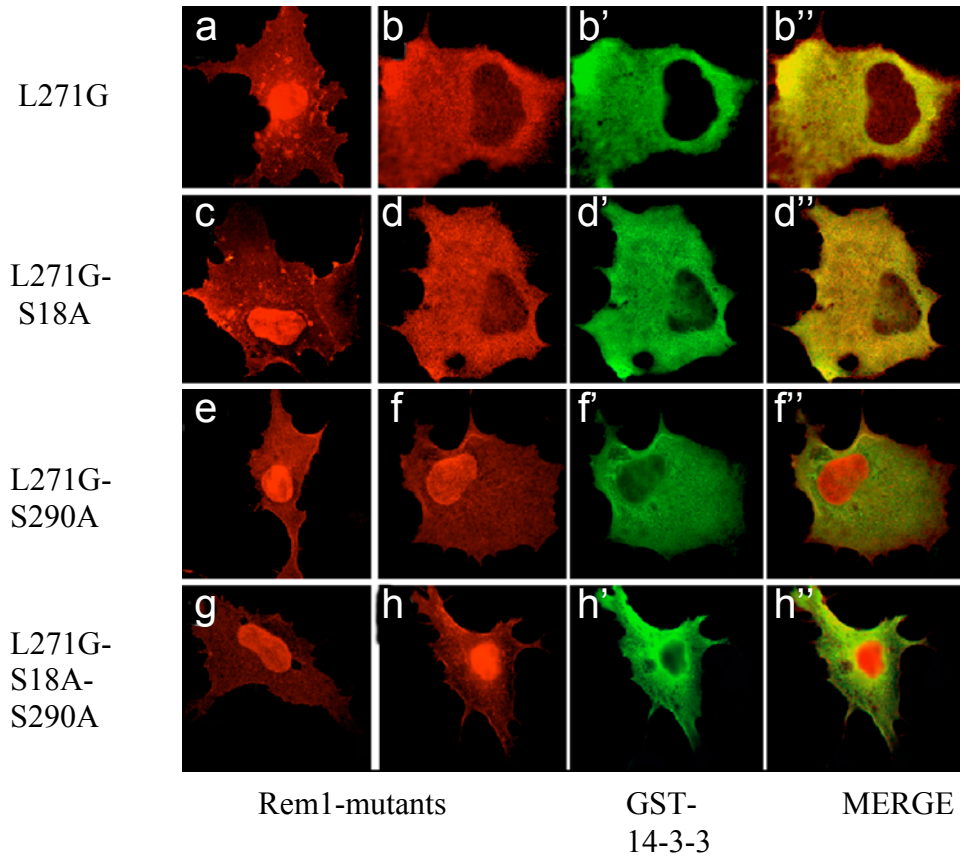


Fig. 3-5D Regulation of localization of Rem1 L271G and mutants by 14-3-3
a, c, e, g show Rem1 L271G, L271G-S18A, L271G-S290A and L271G-S18A-S290A mutants respectively, expressed alone in Cos-1 cells. b-b", d-d", f-f", h-h" are the respective proteins co-expressed with 14-3-3.

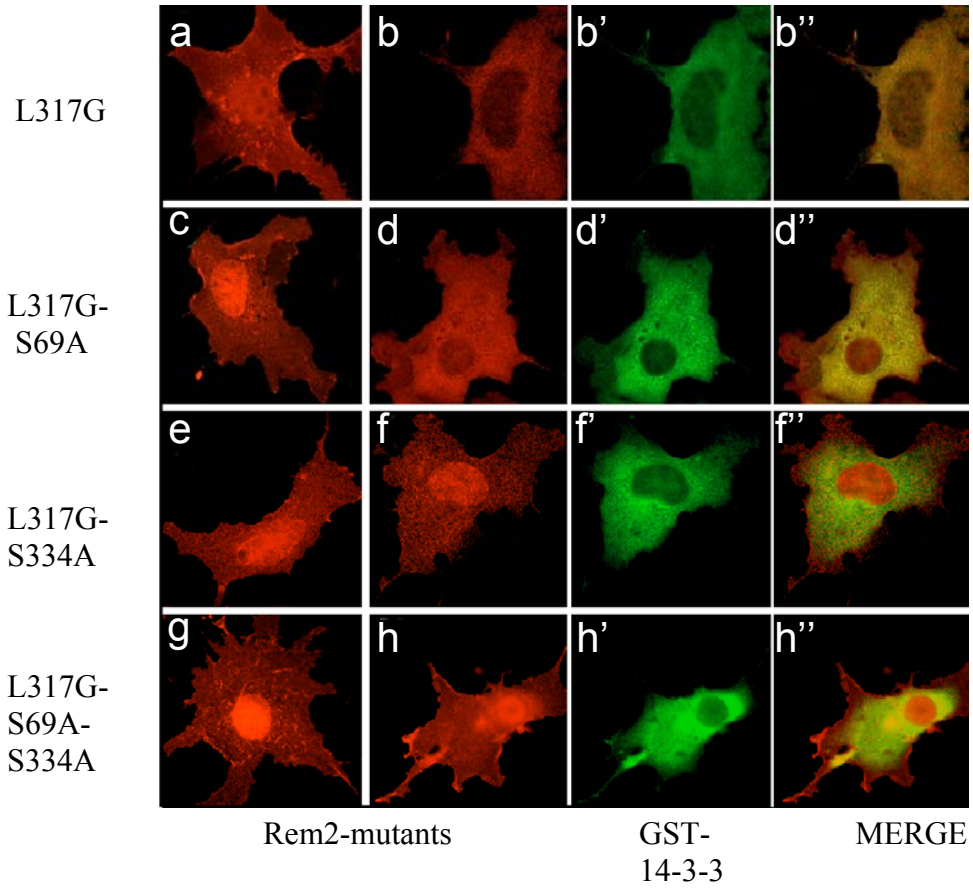


Fig. 3-5E Regulation of localization of Rem2 L317G and mutants by 14-3-3.
a, c, e, g show Rem2 L317G, L317G-S69A, L317G-S334A and L317G-S69A-S334A mutants respectively, expressed alone in Cos-1 cells respectively. b-b", d-d", f-f", h-h" show the respective proteins co-expressed with 14-3-3.

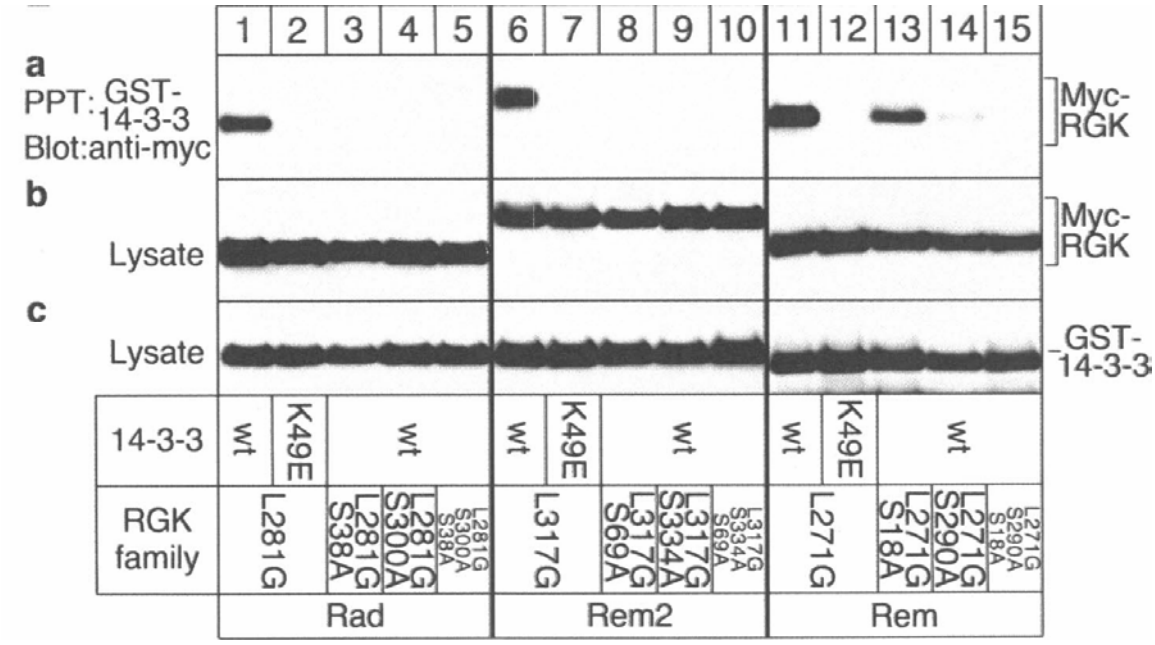
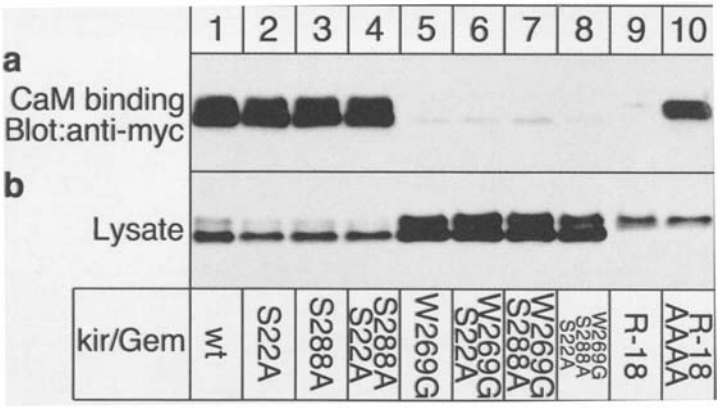


Fig. 3-5F Binding of 14-3-3 to RGK proteins lacking calmodulin binding.
Cells were co-transfected with cDNAs for WT or mutated Myc-RGK protein and GST-14-3-3 WT or mutant.
a. GST-14-3-3 proteins were precipitated and the associated Myc-RGK proteins detected by western blot using Myc antibody.
b and **c**. Cell lysates were blotted with Myc (b) or GST (c) antibodies to monitor expression levels of RGK proteins and 14-3-3 respectively.



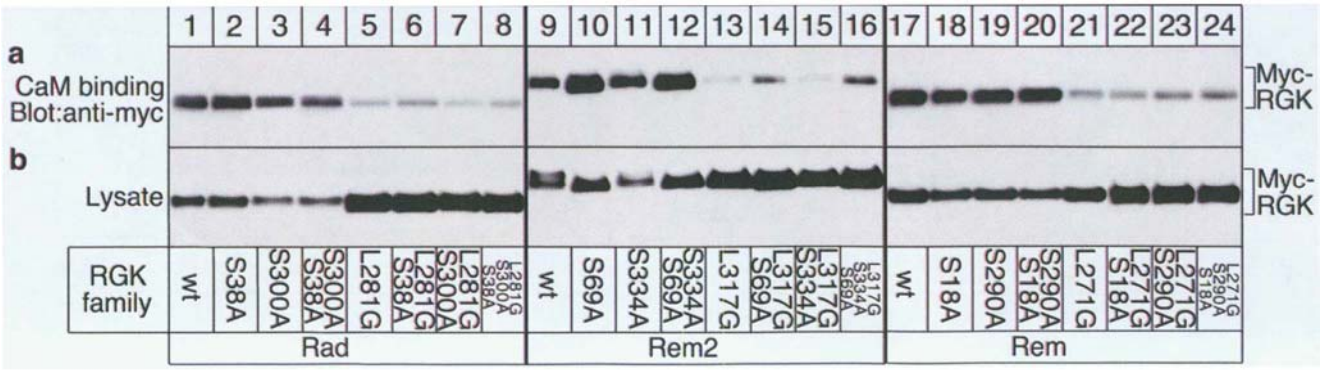
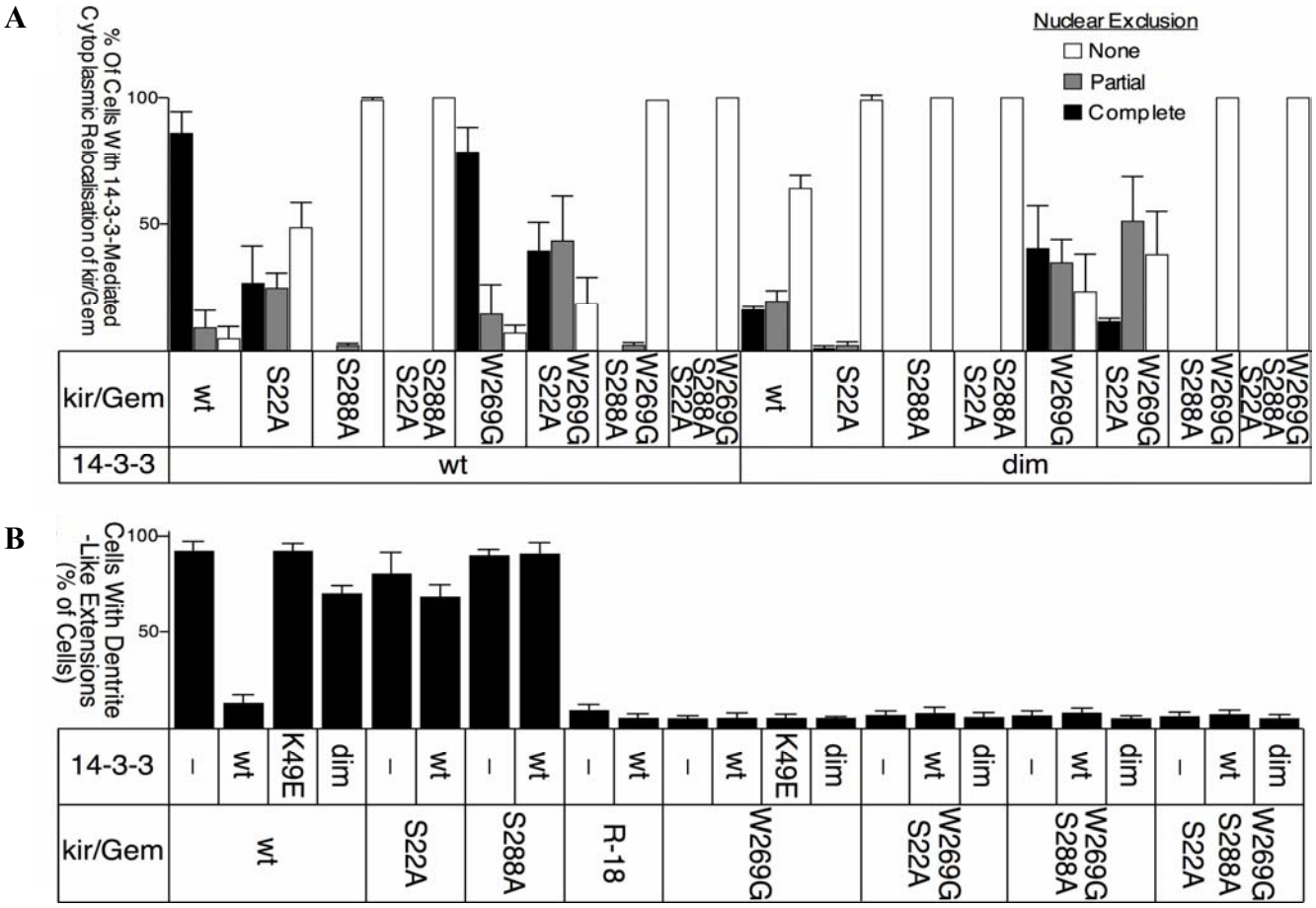


Fig. 3-5G CaM binding to RGK proteins.

a. Cells were transfected with cDNAs for WT or mutant Myc-RGK proteins. Cell homogenates were incubated with CaM beads and bound Myc-RGK proteins were detected by western blot using Myc antibody.

b. Cell lysates were blotted with Myc antibodies to monitor RGK protein expression levels.

Quantification data



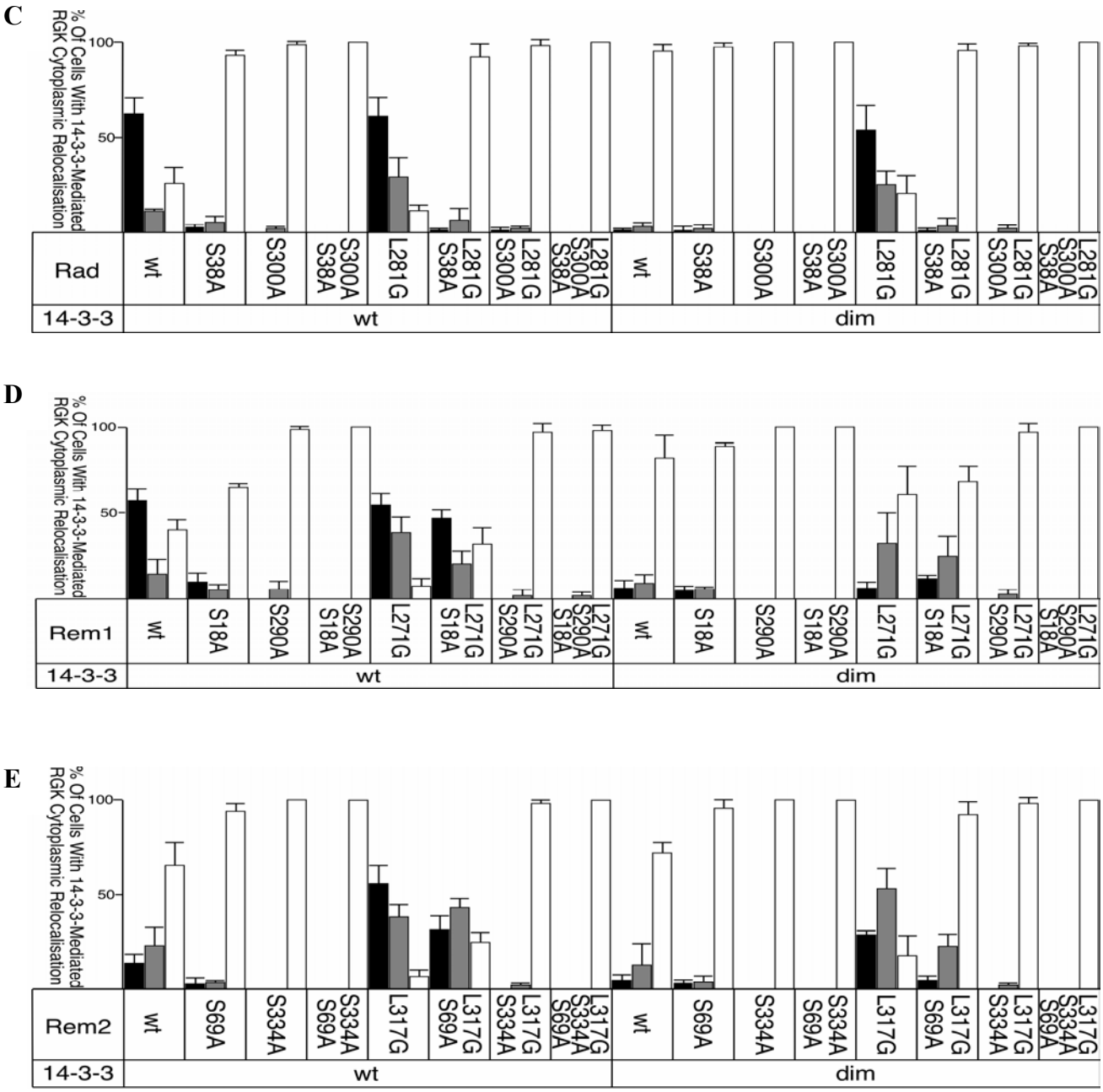


Fig. 3-6A-E Quantification of cytoplasmic redistribution and dendritic extensions in RGK proteins 100-200 transfected cells were randomly selected from 3-5 independent experiments and analyzed visually. Quantification of the 14-3-3 mediated cytosolic relocalization of Kir/Gem (A), Rad (C), Rem (D) and Rem2 (E). The fraction of cells showing efficient (black bar), partial (grey bar) or no (white bar) nuclear clearance is plotted. Quantification of Kir/Gem induced morphological changes (B). The fraction of cells showing dendritic extensions is plotted.

CHAPTER 4

Roles of CaM and 14-3-3 in cell shape remodeling and downregulation of calcium channel activity by RGK proteins

4.1 14-3-3 and CaM modulate RGK mediated cell shape changes

Since Kir/Gem (Leone et al., 2001; Ward et al., 2002), Rad (Ward et al., 2002) and Rem (Pan et al., 2000) mediate an effect on cell shape (induction of dendrite-like extensions), I examined if 14-3-3 or CaM play a role in RGK induced morphological changes in cell shape.

As shown in chapter 3, expression of RGK proteins induced dendrite-like extensions in Cos-1 cells and this was not affected by mutating the 14-3-3 binding residues (Fig. 3-3A). Overexpression of 14-3-3 abolished the induction of dendrite-like extensions, which was more obvious in the case of Kir/Gem when compared to the other members of the RGK family. This shows that the inhibitory effect of 14-3-3 on the morphological changes needs 14-3-3 binding, since it was neither observed for mutants lacking 14-3-3 binding nor for RGK proteins co-expressed with 14-3-3 K49E.

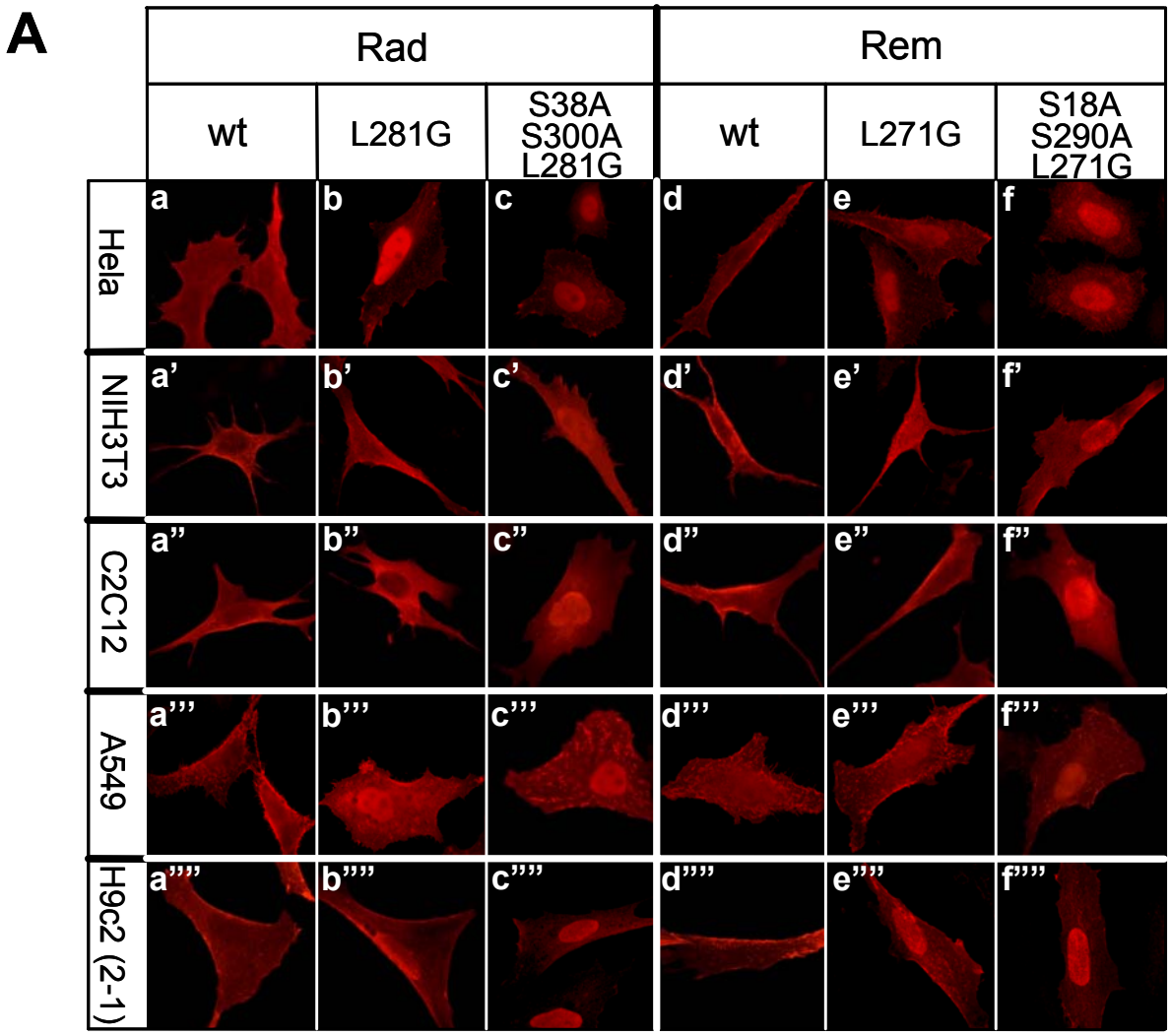
Furthermore, it was observed that CaM binding also plays a role in the RGK mediated cell shape changes. While WT RGK proteins that can bind calmodulin showed dendritic extensions, mutants deficient in CaM binding localized to the nucleus and showed loss/reduction of cell shape changes (Fig. 3-4, compare WT with mutants). This shows that delocalizing RGK proteins from the cytoplasm to the nucleus by mutating the CaM binding site abolishes/decreases the induction of extensions. This effect was very obvious in the case of Kir/Gem W269G and W269G S22A S288A. Since the same effect was not very clear for the other members of the family, I analyzed the nuclear

localization and the changes in cell shape in different cells lines overexpressing WT Rad and Rem, or mutants defective in CaM (i.e. Rad L281G, Rem L281G) or CaM and 14-3-3 (i.e. RadS38A/S300A/L281G, Rem S18A/S290A/L271G) binding. In PC-12 cells, these mutants show a partial or complete nuclear accumulation, respectively (shown later in this chapter). Since, the extent of nuclear localization of RGK proteins may vary among different cell types probably due to differences in phosphorylation state and/or variations in levels of free CaM, 14-3-3 or other regulatory factors, common cell lines like Hela and NIH3T3 as well as cell lines expected to endogenously express RGK mRNA such a C2C12 (muscle), A549 (lungs) and H9c2 (2-1) (heart myocardium) were used.

Similar to Cos1 cells, Rad and Rem showed a diffused and membrane localization in Hela, A549 and H9c2(2-1) cells (Fig. 4-1A, panels a, a”’ and a””, and d, d”’ and d””, respectively) and mutation of either the CaM (Fig. 4-1A, panels b’, b”’, b”” ,and e, e”’, and e””, respectively), or both the CaM and 14-3-3 (Fig. 4-1A, panels c”, c”’ and c””, and f, f”’ and f””, respectively) binding sites resulted in nuclear localization of the mutant RGK proteins. In contrast, in NIH3T3 and C2C12 cells, Rad and Rem were not obviously localized in the nucleus (Fig. 4-1A, panels a’ and a” and d’ and d”, respectively). While mutation of the CaM-binding site (Fig. 4-1A, panels b’ and b”, and e’ and he”, respectively) did not produce clear relocalization, efficient nuclear accumulation of Rad and Rem in these two cell lines required mutation of both the CaM and 14-3-3 binding sites (Fig. 4-1A, panels c” and c”, and f’ and f”, respectively).

Importantly, the capacity of WT Rad or Rem and the corresponding CaM or CaM and 14-3-3 binding deficient mutants to induce dendritic extensions in NIH3T3, C2C12

and Cos1 cells showed a good correlation with their subcellular distribution (Fig. 4-1B). An increase in nuclear relocation of RGK proteins in general correlated with a reduction of long dendrite-like extensions. In NIH3T3 cells, the nuclear localization of Rad S38A/S300A/L281G and Rem S18A/L271G/S290A was less pronounced compared to C2C12 or Cos1 cells and, correspondingly, long dendrite-like extensions were observed in some cells. Thus the nuclear localization of Rad and Rem due to abolition of CaM or CaM/14-3-3 binding correlates with the downregulation of their ability to induce changes in cell shape.


A
Rad
Rem
wt
L281G
S38A
S300A
L281G
wt
L271G
S18A
S290A
L271G
Hela
NIH3T3
C2C12
A549
H9c2 (2-1)
a
b
c
d
e
f
a'
b'
c'
d'
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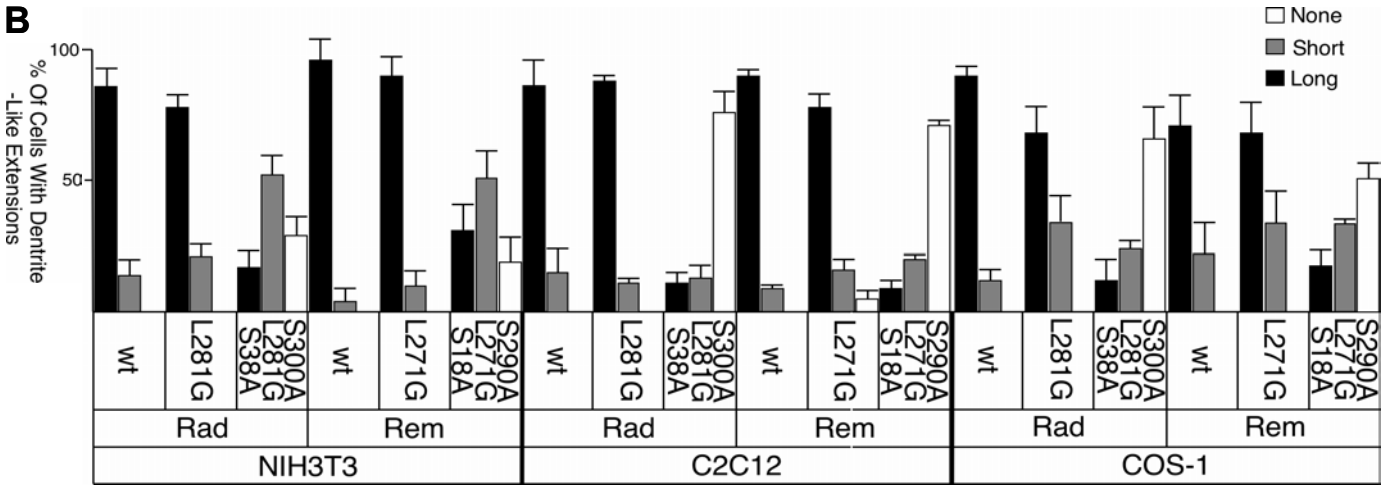


Fig. 4-1 Nuclear localization of Rad and Rem dramatically reduced RGK protein induced changes in cell shape. (**A**).The indicated cell lines were transfected with cDNAs for Myc-WT Rad (panels a-a""), Rad L281G (panels b-b""), Rad S38A/L281G/S300A (panels c-c""), WT Rem (panels d-d""), Rem L217G (panels e-e"") and Rem S18A/L271G/S290A (panels f-f"") and processed for immunofluorescence.
(**B**) Induction of dendrite-like extensions. Since only NHI3T3, C2C12 and COS-1 revealed RGK-induced cell shape remodeling, these cell lines were selected and analyzed. The ability to induce short (< cell diameter) or long (> cell diameter) extensions was monitored in 100 randomly chosen cells expressing the indicated WT or mutated RGK proteins.

Since the nuclear translocation in Rem2 was consistently less efficient than that of the other RGK proteins and to ascertain that the modulation of subcellular distribution by CaM and 14-3-3 is a general feature of RGK proteins, I expressed Rem2 WT, L317G and L317G/S69A/S334A in different cell lines and analyzed their subcellular localization. While the extent of nuclear localization of WT Rem2 differed among different cell lines, a consistent change in the nuclear localization was observed for the mutants defective in CaM and/or 14-3-3 binding, indicating that CaM and 14-3-3 regulate Rem2 localization in all cell lines in a similar fashion (Fig. 4-1C). Compared to WT, CaM deficient mutants displayed a more pronounced nuclear localization, which was further enhanced when the 14-3-3 sites were also mutated. WT Rem2 induced dentrite-like structures in all cell lines analyzed except for the neuroendocrine cell lines GH3, PC-12 and MIN6. In Neuro2a cells, Rem2 did not induce dendrite-like extensions but short protrusions. Consistent with the observation for other cells, the ability to induce morphological changes was reduced for Rem2 mutants that localized to the nucleus. The analysis in different cell lines underscored that the subcellular localization of Rem2 is indeed modulated by 14-3-3 and CaM binding.

In conclusion, RGK mediated cellular extensions are inhibited either if 14-3-3 binds to RGK proteins, or if CaM or CaM/14-3-3 do not bind to the small G proteins. Thus both 14-3-3 and CaM regulate RGK induced cell shape changes by modulation of subcellular distribution.

	morphological changes	cellular localization	Rem2
C2C12 (mouse myoblast)	++++	C/N	wt
	+++	C/N	L317G
	++	C/N	L317G/S69A/S334A
GH3 (rat pituitary)	−	C	wt
	−	C	L317G
	−	C/N	L317G/S69A/S334A
Hela (human cervix)	++++	C/N	wt
	+++	C/**N**	L317G
	−	N	L317G/S69A/S334A
Neuro2a (mouse neuroblastoma)	++(**)	m	wt
	++	m/C	L317G
	++	C/N	L317G/S69A/S334A
MIN6 (mouse pancreatic β-cell)	−	C	wt
	−	**C**/N	L317G
	−	**C**/N	L317G/S69A/S334A
NIH3T3 (mouse embryonic)	++++	C	wt
	++++	C/N	L317G
	++	C/N	L317G/S69A/S334A
PC-12 (rat pheochromocytoma)	−	C	wt
	−	C	L317G
	−	C/N	L317G/S69A/S334A
293T (mouse embryonic kidney)	++	C	wt
	−	C/N	L317G
	−	C/N	L317G/S69A/S334A
COS1 (monkey kidney)	++++	C/N	wt
	++	C/**N**	L317G
	−	N	L317G/S69A/S334A

C- cytoplasm, N- Nucleus

Fig. 4-1C Comparison of localization of Rem2 WT and mutants in different cell types. Rem2 WT, L317G and L317G/S69A/S334A were expressed in the cell lines indicated and the subcellular localization and induction of morphological changes in cells expressing moderate to high levels of Rem2 were monitored. Cells were classified into those showing only cytoplasmic (C), a diffused cytoplasmic and nuclear (C/N) or a predominant nuclear (N) distribution of Rem2. A shift towards a more nuclear or cytosolic localization is indicated in bold (**N** or **C** respectively).

In the case of Neuro2a (**), Rem2 was found at the membrane (m) and induced short protrusions. In Hela cells, Rem2 overexpression produced filopodia like structures that were independent of the subcellular localization of Rem2. C, cytosol; N, nucleus; m, membrane. Morphological changes were scored by determining the fraction of cells with extensions of twice the cell diameter or longer: >75% (++++), 50-75% (+++), 5-50% (++) and <5% (-). Three independent experiments were carried out and 80-100 cells with medium to high Rem2 expression scored in each experiment.

4.2 Introduction to Voltage dependent calcium channels

Voltage dependent calcium channels (VDCCs) are involved in several cellular functions and are controlled by many intracellular signals. VDCCs regulate Ca^{2+} entry into excitable cells, coupling membrane potential changes to various biological activities like muscle contraction, hormone and neurotransmitter release, neuronal migration and gene expression.

VDCCs have been characterized as multisubunit complexes of a pore forming α_1 subunit; a transmembrane, disulfide-linked complex of α_2 and δ subunits; an intracellular β subunit and in some cases, a transmembrane γ subunit (Fig. 4-2). There are ten α_1 subunits, four α_2 δ complexes, four β subunits and two γ subunits known to date (Catterall., 2000). The different types of Ca^{2+} currents are primarily defined by different α_1 subunits. The α_1 subunit forms the pore of the channel and is the voltage sensor and binding site of drugs and toxins that alter the channel activity. Although the biological diversity in native VDCCs is due to the α_1 subunits, the auxiliary β, γ, $\alpha_2\delta$ subunits, present as different isoforms, are important in modulating channel properties such as amplitude, voltage dependence and other kinetics.

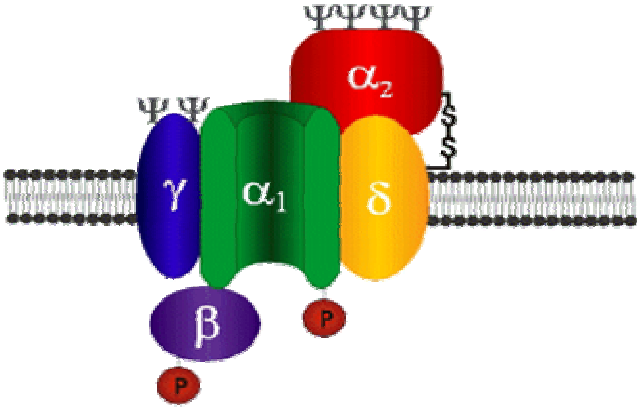


Fig. 4-2 Schematic diagram of the subunits of VDCCs
The channel is made up of five subunits with the α_1 forming the channel pore and the α_2 and δ subunits joined by a disulphide bridge. Sites marked ψ show sites of N-linked glycolsylation. The α_1 and β subunits also have sites for cAMP-dependent protein kinase phosphorylation marked by Ps.

Among the auxiliary subunits, the β subunits are of high importance as they substantially enhance the structure and function of the Ca^{2+} channels. Four β subunit genes have been identified and each of them is subjected to alternative splicing to generate additional isoforms. The association with different β subunits can significantly alter the physiological function of the α_1 subunit (Catterall., 2000). In the absence of beta subunit, the α_1 subunit is retained in the endoplasmic reticulum by ER retention signals. Binding of the β subunit will mask the ER retention signals and allow plasma membrane transport of the α_1 subunit. Thus, the β_3 subunit plays an important role in the functioning of VDCCs by controlling the cell surface expression of α and thereby VDCC activity.

4.3 CaM, but not 14-3-3 plays a role in RGK- mediated downregulation of calcium channel activity

The $Ca_v\beta 3$ subunit is an effector of Kir/Gem (Beguin et al., 2001). Rad, Rem (Finlin et al., 2003) and Rem2 (Beguin et al., 2005, Finlin et al., 2005)) also interact with β_3 subunit. The interaction between β_3 and RGK proteins results in the down regulation of Ca^{2+} currents. While Kir/Gem WT down regulates Ca^{2+} currents, the CaM binding deficient mutant (W269G) did not show any effect on the Ca^{2+} currents (Beguin et al., 2001, Ward et al., 2004)). Since CaM and 14-3-3 modulate the subcellular localization of RGK proteins, we explored a possible role of these regulatory proteins on the down regulation activity of all RGK proteins. The respective WT and CaM binding deficient mutants were expressed in PC12 cells, either alone or together with 14-3-3, and endogenous calcium currents were measured. This study was done in a collaborative work with Dr. Nagashima, Kyoto University, Japan.

Electrophysiology results show that while Rad L281G does not cause any down regulation of currents (Fig. 4-3, panel A, lane 4), Rem L271G and Rem2 L317G did not interfere with the down regulation activity (panel A, lane 12 and panel B, lane 4). Furthermore, 14-3-3 did not have any effect on the RGK mediated regulation of calcium channel functioning, given that the mutants lacking 14-3-3 binding (panel A, lanes 6, 7, 14, 15 and panel B, lanes 6, 7) or 14-3-3 co-expressed with RGK proteins (panel A, lanes 3,5,11,13 and panel B, lanes 3, 5) did not interfere with the downregulation of calcium channel activity mediated by RGK proteins. However, in the absence of bound CaM, 14-3-3 might play a role in the inhibition of currents by Rad (panel A, lanes 8, 9).

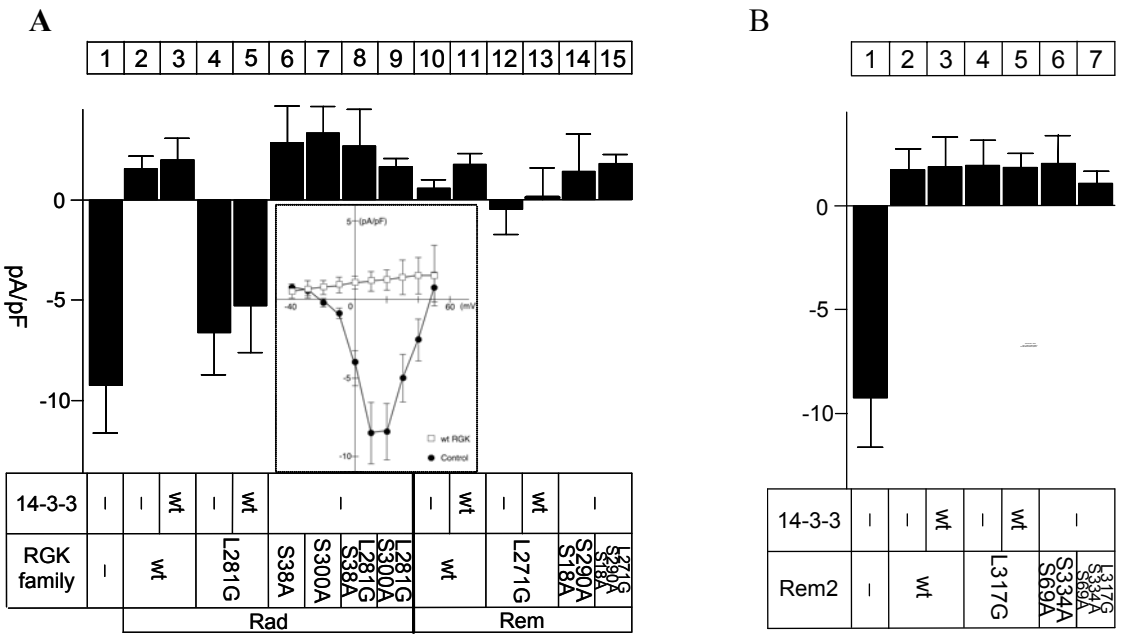


Fig. 4-3 Electrophysiology to study the regulation of calcium channel activity by RGK proteins. PC12 cells were cotransfected with a GFP plasmid and cDNAs for WT or mutant RGK proteins, either with or without 14-3-3. GFP positive cells were selected for electrophysiology and the average of the maximal current detected at +20 mV for endogenous Ca^{2+} channels was measured. Between 9 and 16 independent experiments were carried out for each condition. An example of the I-V relationship of Ca^{2+} channels in PC12 cells is shown in the inset. (A, Ampere; F, Farad). Cells transfected only with the GFP cDNA served as a control.

4.4 RGK proteins block cell surface expression of α_1 subunit of VDCCs

Earlier reports show that co-expression of α_1 and β subunits resulted in the expression of α subunits in the plasma membrane (Beguin et al., 2001). Co-expression of α and β subunits with Kir/Gem WT blocked the cell surface expression of α_1. These results indicated that Kir/Gem, through its association with β_3 subunit interferes with the cell surface expression of α_1 subunit at the plasma membrane.

To determine the mechanism by which Rad, Rem and Rem2 regulate calcium channel activity, and to understand if CaM and 14-3-3 binding modulate RGK protein function, I analyzed the subcellular distribution of the Ca^{2+} channel α_1- and β-subunits in PC12 (Fig. 4-4) cells expressing either WT or mutated RGK proteins. Cells were cotransfected with cDNAs encoding a Cav1.2 α_1-subunit in which the HA-tag was inserted into an external loop (Altier et al., 2002), a Flag-tagged Cavβ3 and Myc-tagged wt or mutated RGK proteins. To ensure homogenous expression of the Ca^{2+} channel subunits, the α- and β-subunit were expressed from an IRES containing vector. The pIRES vector allows to translate two genes of interest from the same mRNA. It contains the internal ribosomal entry site (IRES) from the encephalomyocarditis virus, which permits ribosome binding at an internal location along the mRNA transcript. The expression of the RGK proteins and the α_1- and β-subunit was verified by immunofluorescence in permeabilized cells. Alternatively, live cells were incubated with rat anti HA antibody prior to permeabilization to selectively detect the Ca^{2+} channels present at the cell surface (surface labeling).

As shown in Fig. 4-4A, the α_1-subunit expressed alone showed a cytoplasmic localization (panel c) and could not be detected at the cell surface (panel a). Only in the presence of the β-subunit, the α_1-subunit was detected at the surface of unpermeabilized cells as a diffused staining (Fig. 4-4A, panel b'), confirming the importance of the β-subunit in facilitating surface transport of the α_1-subunit. The β-subunit was diffused in the cytoplasm (panels b and d).

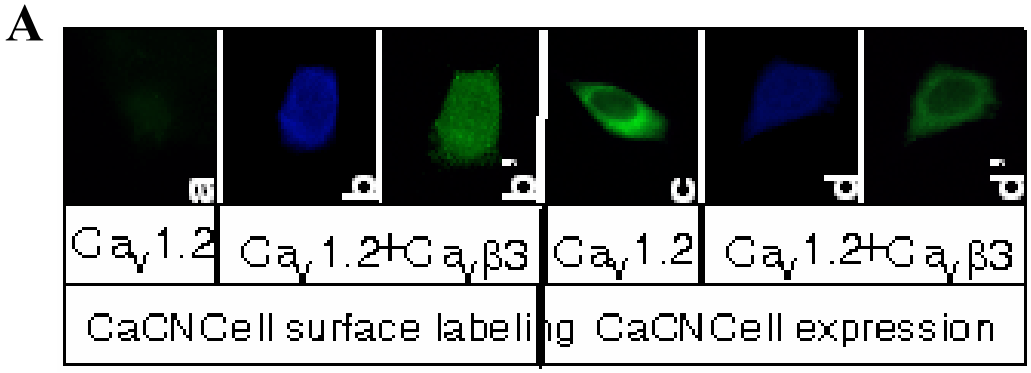
Rad, Rem and Rem2 mutants in which both N- and C-terminal 14-3-3 binding sites were mutated showed a subcellular distribution similar to that of WT proteins. This was consistent with what has been observed in Cos1 cells. The nuclear localization of RGK proteins in PC12 cells was less pronounced when compared to Cos1 cells. In PC12 cells, although Rad L281G and Rem L271G showed a diffused localization, the triple mutants translocated efficiently. For Rem2, while L317G showed a cytosolic distribution, L317G-S69A-S334A showed a diffused localization, indicating an increase in its nuclear localization. Thus, a consistenly enhanced nuclear localization of the mutants was observed.

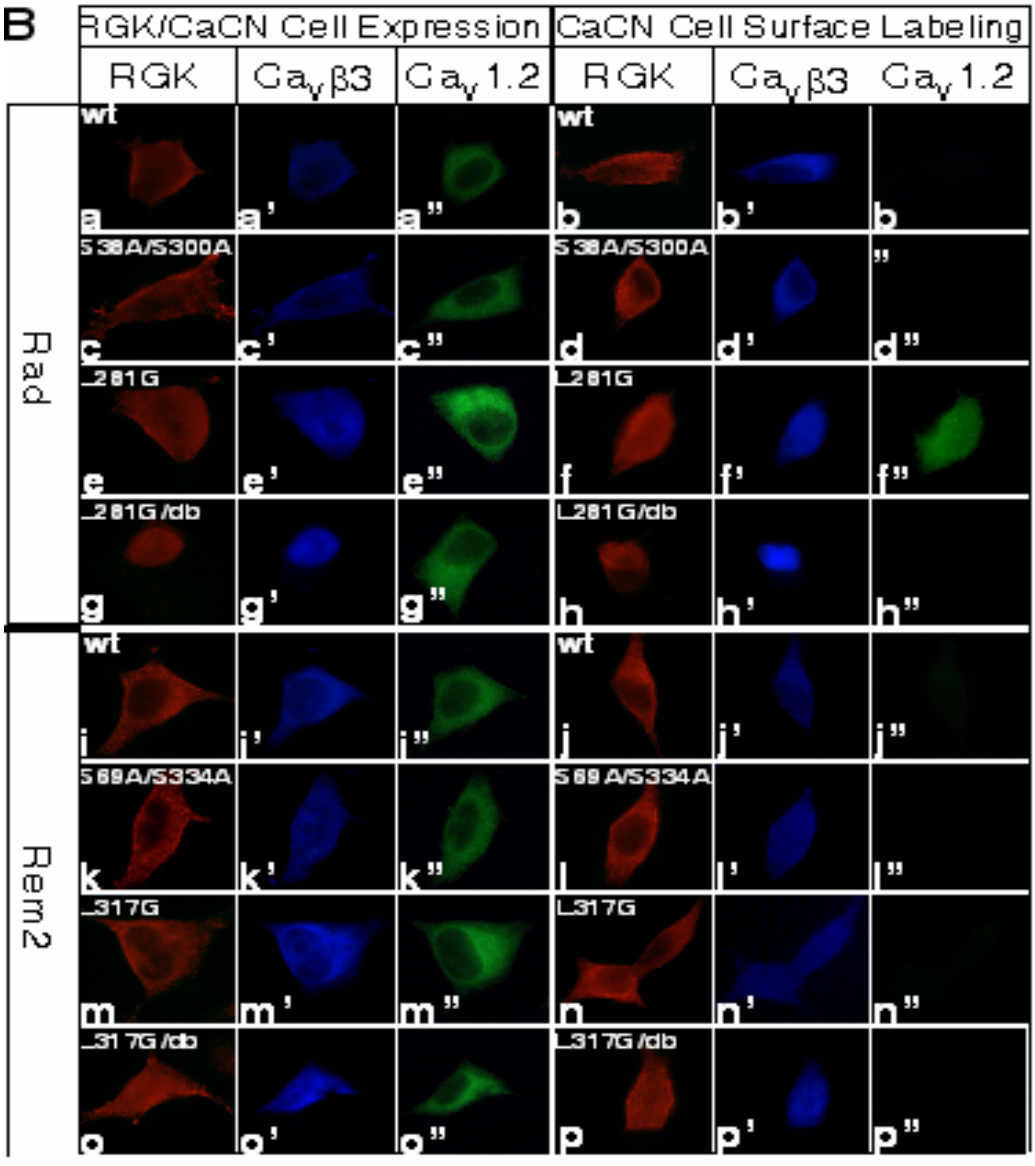
The β-subunit predominantly co-localized with the RGK proteins. Where the RGK proteins were in the cytoplasm, the β-subunit was also cytoplasmic (Fig. 4-4B, panels a'-d', i'-l', q'-t'). If the RGK proteins were in the nucleus, the β-subunit also translocated into the nucleus (panels e'-h', m'-p', u'-x'). The co-localization of the Cavβ3 subunit with the RGK proteins was also observed in Cos1 cells (data not shown).

Cell surface transport of the α_1-subunit in response to the expression of the β-subunit (Fig. 4-4A, compare panel a with b') was blocked upon the expression of either

the WT RGK proteins or mutants (Fig. 4-4B, panels b"-x"). The only exception were the cells expressing Rad L281G, where the α_1-subunit was readily detected at the cell surface (Fig. 4-4B, panel f"). Thus, surface transport of the α_1-subunit in cells expressing different RGK proteins and their mutants correlated with their functional effect on Ca^{2+} channel activity (Fig. 4-3), based on electrophysiology experiments. Similar results were also obtained for Hek 293 cells (data not shown).

In summary, like Kir W269G (Beguin et al., 2001), the CaM binding defective Rad L281G allows cell surface expression of α_1 and therefore cannot inhibit calcium channel activity. However, when both 14-3-3 and calmodulin binding are prevented, Rad can downregulate channel function. This proves that the bound CaM is important for the repression of channel function in the case of Rad and Kir, but not Rem and Rem2. At least in HEK 293 and PC12 cells, 14-3-3 and CaM are apparently not involved in regulating the function of Rem and Rem2 on Ca^{2+} channel surface expression.


A
a
b
c
d
e
f
Ca$_V$1.2
Ca$_V$1.2+Ca$_V$β3
Ca$_V$1.2
Ca$_V$1.2+Ca$_V$β3
CaCN Cell surface labeling
CaCN Cell expression


B
RGK/CaCN Cell Expression
CaCN Cell Surface Labeling
RGK
Ca$_V$β3
Ca$_V$1.2
RGK
Ca$_V$β3
Ca$_V$1.2
Rad
Rem2
wt
S38A/S300A
L281G
L281G/db
wt
S69A/S334A
L317G
L317G/db

(PC12 cells)

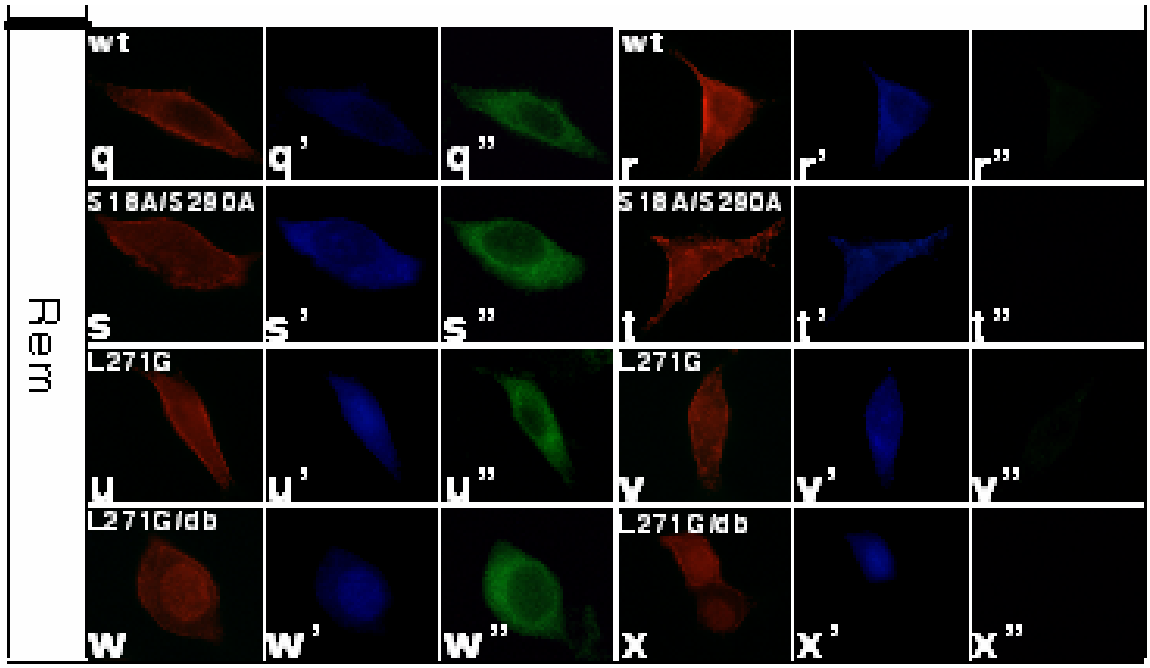


Fig. 4-4 RGK proteins block cell surface expression of α-subunits in PC12 cells
Cells were transfected with cDNAs for WT or mutated Myc-RGK together with an IRES based vector carrying the cDNAs for HA-$Ca_v1.2$ and Flag-$Ca_v\beta3$. Cells were then fixed, permeabililzed and processed for immunofluorescence microscopy using Myc, HA and Flag antibodies to detect RGK (red), $Ca_v1.2$ (green) and $Ca_v\beta3$ (blue), respectively (CaCN cell expression). Alternatively, live cells were first incubated with HA antibodies to selectively label surface exposed $Ca_v1.2$ prior to the fixation, permeabiliziation and labeling with Myc and Flag antibodies (CaCN cell surface labeling). db: double mutant with both 14-3-3 binding sites mutated.

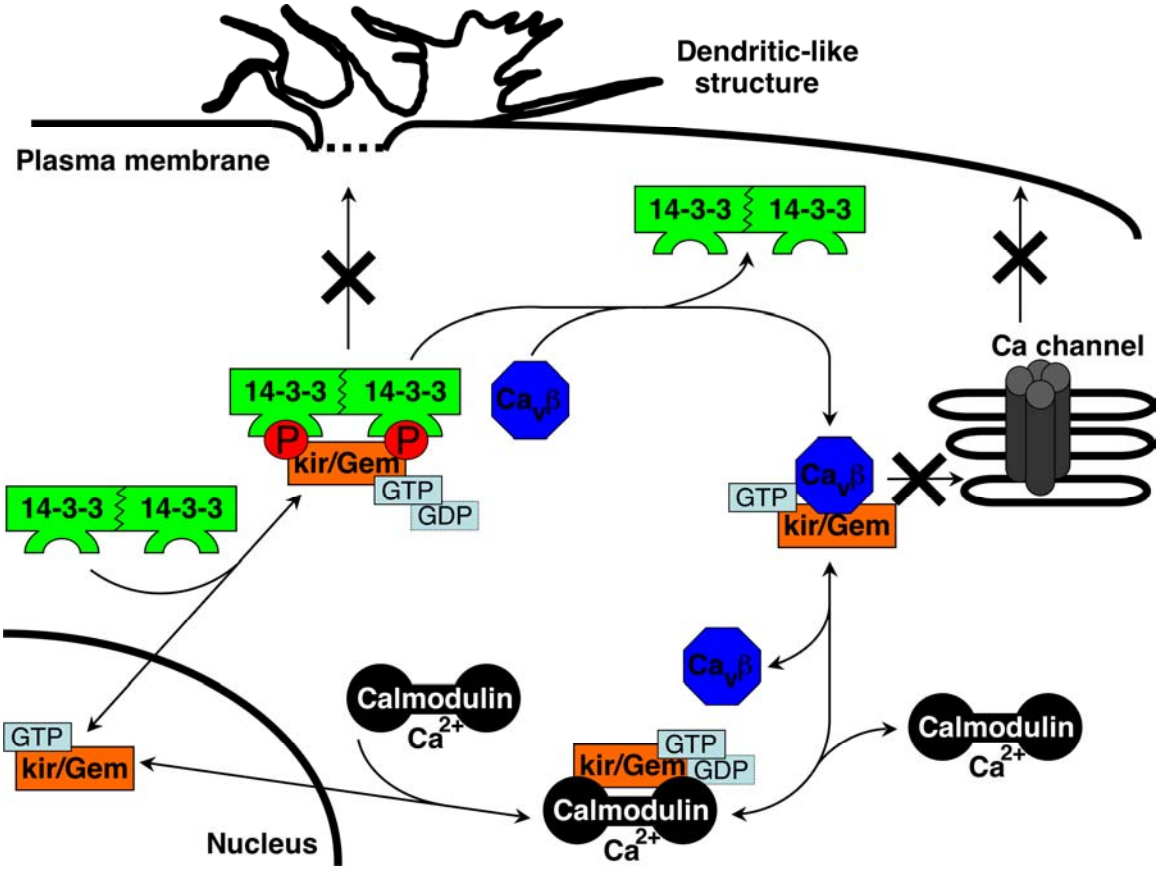


Fig. 4-5 Working model for the regulatory role of 14-3-3 and CaM on Kir/Gem localization and function

Competition assays indicate that binding of CaM and 14-3-3 to Kir/Gem is mutually exclusive. Furthermore, CaM and 14-3-3 compete with β subunit for binding to Kir/Gem and CaM can displace the β subunit from Kir/Gem (*Beguin et al., 2005a*). Therefore, two regulatory pathways can be considered. Activation of CaM by intracellular Ca^{2+} results in association of CaM with Kir/Gem and retains it in the cytoplasm. Dissociation of CaM allows Kir/Gem to bind the Ca^{2+} channel β-subunit, interfering with plasma membrane expression of the α_1 subunit. Alternatively, in the absence of CaM activation, 14-3-3 can re-localize Kir/Gem to the cytoplasm and also block the formation of dendrite-like extensions. 14-3-3 bound to Kir/Gem may be exchanged for the β-subunit. Upon activation of CaM by Ca^{2+}, Kir/Gem dissociates from the β-subunit. Kir/Gem is in its active, GTP-bound form when located in the nucleus or bound to the β-subunit, however, the activation state when associated with CaM or 14-3-3 remains to be determined.

CHAPTER 5

Identification and characterization of nuclear localization signals in the RGK family of proteins

5.1 Introduction

Calmodulin and 14-3-3 are key modulators of the functions of RGK proteins, regulation of calcium channels and cytoskeleton dynamics. Interestingly, abolition of CaM binding results in nuclear accumulation of RGK proteins, whereas 14-3-3 binding causes nuclear exclusion. The mechanism, by which nuclear localization of RGK proteins is regulated, however, is unknown. The highly regulated nuclear localization of RGK proteins indicates that this process is of physiological relevance, possibly as a mechanism to rapidly and reversibly inactivate RGK proteins or to modulate calcium channel activity. Compartmentalization of proteins provides an excellent opportunity for regulation of different interacting partners and our data is consistent with the notion that nucleocytoplasmic shuttling may represent a novel mechanism for the regulation of RGK protein function.

Transport of proteins between the nucleus and cytoplasm is a highly regulated process (Xu and Massague, 2004). The nuclear pore complex (NPC) constitutes a passive diffusion channel of about 9nm in diameter. Proteins above the size limit for passive diffusion can enter the nucleus only by active transport. Nuclear transport requires nuclear localization signals (NLSs), which predominantly are short stretches of basic amino acids. Classical NLSs are characterized by the presence of mono or bipartite clusters of basic amino acid. The NLS of SV40 T-antigen (PKKKRKV) and nucleoplasmin (KRPAATKKAGQAKKKK) are the prototypes of mono- and bi-partite signals.

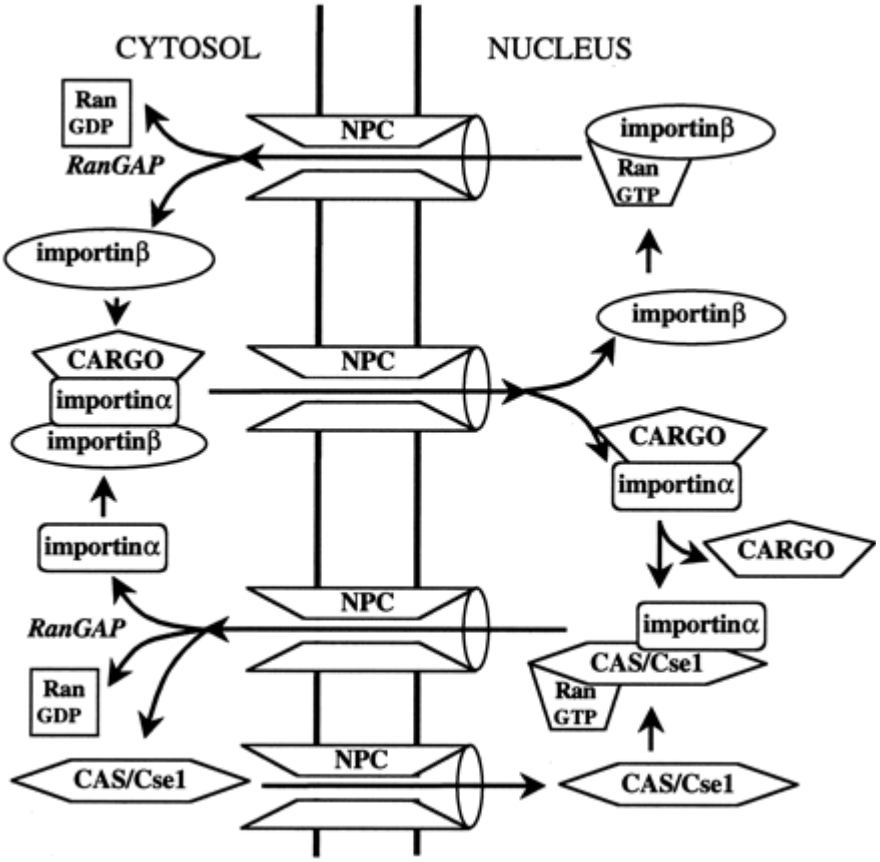
Key players in nucleocytoplasmic shuttling of proteins are the karyopherins, which include importins and exportins. As the name suggests, importins mediate nuclear import, whereas exportins mediate nuclear export. There are two types of importins, importin α and β. Importin α recognizes proteins with NLSs (cargo) and serves as an adapter, linking importin β and the cargo. The importin α/β heterodimer then targets the cargo to the NPC and translocates it into the nucleus.

There are six mammalian importin α and one importin β. Importin α is composed of a flexible N-terminal importin-β-binding (IBB) domain and a highly structured domain comprised of ten tandem armadillo (ARM) repeats. The helical ARM repeats assemble into a twisted slug-like structure with a binding groove for the classical NLS (cNLS) of the cargo. The flexible N terminal IBB domain of importin α interacts either *in trans* with importin β or *in cis* with the cNLS-binding groove. It binds the importin β to target the complex to the NPC for translocation and also contains an autoinhibitory region which mimics a cNLS and regulates binding of cargo to the ARM domain (Goldfarb et al., 2004). Thus the best known function of importin α is to serve as an adaptor which links the NLS containing proteins to importin β, which in turn docks the ternary complex containing importin α/β and the cargo at the NPC and facilitates the translocation into the nucleus.

Based on sequence homology, the different members of the importin α family can be divided into three groups containing α1, α3 and α4 and α5, α6 and α7 (Xu and Massague., 2004). In addition, close to 20 importin-β isoforms are known. Once imported into the nucleus, cargo release occurs through a conformational change in importin-β induced by its interaction with active Ran GTPase. The binding of Ran-GTP

to importin β in the nucleus is a crucial step in the delivery of the cargo as it triggers the dissociation of importin α/β complex and release of the cargo protein. A concentration gradient of Ran GTPase (i.e. preferential localization of Ran-GTP to the nucleus and Ran-GDP to the cytoplasm) drives directional nuclear transport. This gradient is maintained by molecules such as the Ran GAP and Ran GEF. A typical schematic representation of the nuclear import-export pathway is shown in Fig. 5-1.

While nuclear import via the canonical mechanism is most common, a few proteins are known to enter the nucleus independent of importins, probably due to their ability to interact directly with components of the NPC (Xu and Massague, 2004).



(Reproduced with permission from Microbiol. Mol. Biol. Rev. Macara.G. 2001)

Fig. 5-1 Mechanism of cargo import by the importin α - importin β pathway
The cargo binds to importin α, which in turn binds to importin β. The cargo-importin α-importin β complex is transported across the NPC and disassembled in the nucleus by RanGTP. Importin β returns to the cytosol. The importin α requires a carrier for export, called CAS. Assembly of the export complex requires RanGTP. The ternary importinα CAS-RanGTP complex is disassembled in the cytoplasm by GTP hydrolysis.

5.2 Identification of NLSs in Kir/Gem

To identify putative nuclear localization signals in Kir/Gem, a series of Myc-tagged C- terminal truncation constructs (Fig. 5-2A) were generated and their subcellular distribution in Cos1 cells was analyzed by immunofluorescence microscopy. As previously shown, WT Kir/Gem expressed in Cos-1 cells induced dendrite-like extensions and distributed throughout the cell (Fig. 5-2B, panel a). Abolition of CaM binding (W269G) resulted in a predominant nuclear accumulation of Kir/Gem W269G and the induction of dendrite-like extensions was lost (Fig. 5-2B, panel b).

Progressive C-terminal truncations up to amino acid 144 retained a predominant nuclear accumulation (Fig. 5-2B, panels c-e). Deletion up to amino acid 126 resulted in a diffused localization (C-trun 126; Fig. 5-2B, panel f), suggesting the presence of a NLS between amino acids 126-144. Nuclear accumulation was restored if four amino acids were added back (C-trun 130; Fig. 5-2C, panel a), indicating that the IILL motif between amino acids 126-130 acts as a NLS. Indeed, substitution of these four amino acids with alanine (I126A, I127A, L128A or L129A), either individually or together, abolished the nuclear accumulation of the C-trun 144 mutant (compare Fig. 5-2B, panel e with Fig. 5-2C, panels b-f), confirming the role of the IILL motif as a NLS. However, mutation of the IILL motif did not affect the nuclear localization of shorter deletions (i.e. C-trun 166 or C-trun 235; Fig. 5-2C, panels g and h), suggesting the presence of additional NLS in the C-terminus of Kir/Gem between amino acids 144 and 166.

To identify the additional NLS, additional C-trun constructs carrying a mutated IILL NLS were generated. Truncations up to amino acid 159 were still found in the nucleus (Fig. 5-2D, panel c), but removing an additional eight amino acids abolished the

nuclear accumulation (Fig. 5-2D, panel b), indicating the presence of an additional NLS between amino acids 151 and 159. Mutation of either one or both tyrosine residues (Y152 and Y156) abolished nuclear accumulation (Fig. 5-2D, panels d and f), whereas mutating the other residues within this region only had a minor effect (Fig. 5-2D, panel e), indicating that Y152/Y156 can act as a NLS. Indeed, simultaneous mutation of both the IILL motif and the two tyrosines in the C-trun 235 mutant completely abolished its nuclear localization (compare Fig. 5-2C, panel h and Fig. 5-2D, panel g).

Nevertheless, mutation of the two identified NLSs (IILL and Y/Y) did not interfere with the nuclear accumulation of Kir/Gem W269G (Fig. 5-2D, panel h), indicating the presence of yet another NLS, located C-terminal of amino acid 235. Sequence analysis of the C-terminus of Kir/Gem revealed the presence of a putative bipartite NLS. While mutation of the first half of this motif (K256A/R257A/K258A) did not affect nuclear accumulation of Kir/Gem W269G lacking the IILL and YY motifs (Fig. 5-2E, panel b), mutations in the second half (R263A/K264A/R266A/K267A) resulted in a diffused localization (Fig. 5-2-E, panel c). Mutation of both clusters of positive charges led to the efficient exclusion of the protein from the nucleus (Fig. 5-2E, panel d), indicating that the two clusters of positively charged amino acids indeed function as a bipartite NLS. This C-terminal bipartite NLS was dominant over the IILL and tyrosine based motifs since its mutation was sufficient to redistribute Kir/Gem W269G from a predominant nuclear (Fig. 5-2B, panel b) to a diffused (Fig. 5-2E, panel e) localization. A summary of the localization of the different mutants used in the identification of NLSs in Kir/Gem is given in Fig. 5-2F.

Thus, three motifs were identified in Kir/Gem that play a role in localizing the small GTP-binding protein to the nucleus. These three NLSs will subsequently be referred to as the IILL, YY and KRK-RKRR motifs, or NLS1, NLS2 and NLS3 respectively.

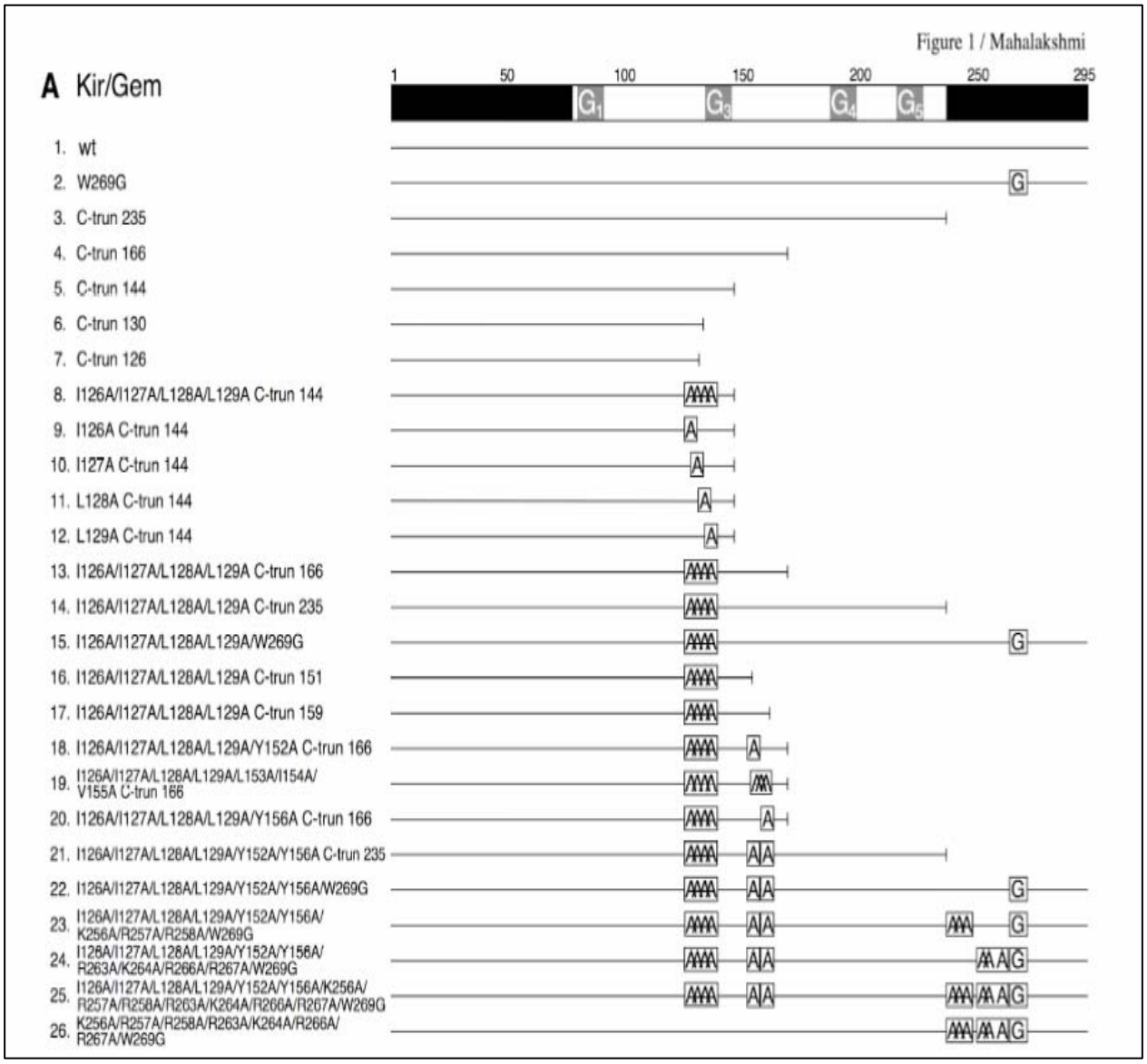


Fig. 5-2A List of mutants used for the identification of NLSs in Kir/Gem
The Ras-like core domain (white), N- and C-terminal extensions (black) and the location of the G1-G5 motifs involved in GTP binding (grey) in WT Kir/Gem are indicated. Corresponding C-terminal truncations (C-trun) and substitutions are depicted. Amino acids are numbered and shown in the one letter code.

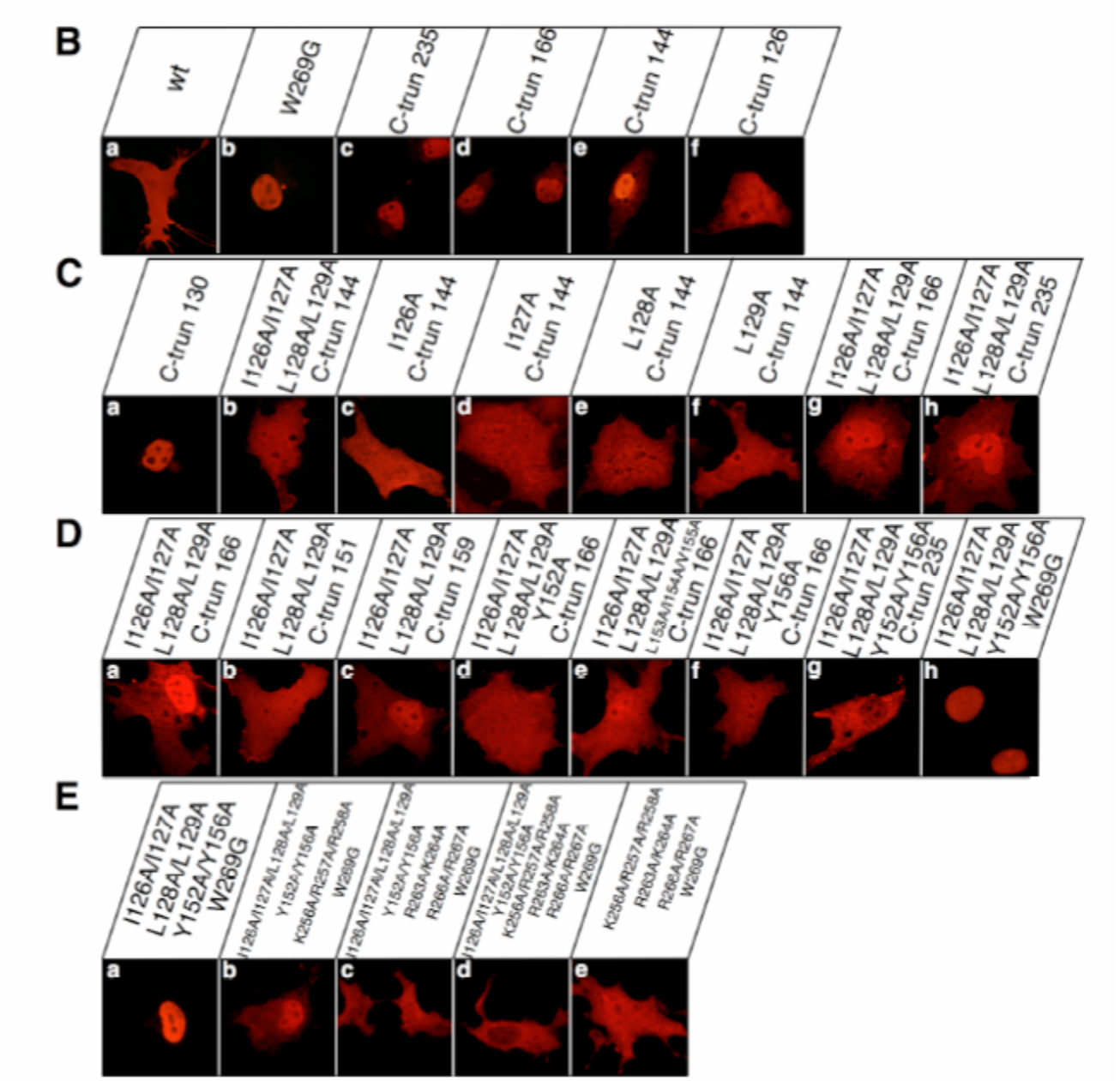


Fig. 5-2B-E Identification of NLSs in Kir/Gem

Cos1 cells were transfected with cDNAs for WT or the indicated mutated Kir/Gem and processed for immunofluorescence microscopy using Myc antibodies to label Kir/Gem (red).

B C-terminal truncation to amino acid 126 abolishes nuclear accumulation

C Identification of an IILL based NLS (NLS1) and the presence of additional C-terminal NLS.

D Uncovering of an YY based NLS (NLS2) and evidence for a third NLS in the C-terminus of Kir/Gem.

E Identification of the third signal as a bipartite NLS (KRK-RKRR; NLS3).

Localization of mutants used in the study of NLS in Kir/Gem		
	Construct	**Localization**
1	WT	Diffused
2	W269G	Nuclear
3	C-trun 235	Nuclear
4	C-trun 166	Nuclear
5	C-trun 144	Nuclear
6	C-trun 130	Nuclear
7	C-trun 126	Diffused
8	I126A/I127A/L128A/L129A-c-trun 144	Diffused
9	I126A-c-trun 144	Diffused
10	I127A-c-trun 144	Diffused
11	L128A-c-trun 144	Diffused
12	L129A-c-trun-144	Diffused
13	I126A/I127A/L128A/L129A-c-trun 166	Nuclear
14	I126A/I127A/L128A/L129A-c-trun 235	Nuclear
15	I126A/I127A/L128A/L129A-W269G	Nuclear
16	I126A/I127A/L128A/L129A-c-trun 151	Nuclear
17	I126A/I127A/L128A/L129A-c-trun 159	Nuclear
18	I126A/I127A/L128A/L129A/Y152A-trun 166	Diffused
19	I126A/I127A/L128A/L129A/L153A/I154A/V155A-C-trun 166	Nuclear
20	I126A/I127A/L128A/L129A/Y156A-C-trun 166	Diffused
21	I126A/I127A/L128A/L129A/Y152A/Y156A-C-trun 235	Diffused
22	I126A/I127A/L128A/L129A/Y152A/Y156A-W269G	Nuclear
23	I126A/I127A/L128A/L129A/Y152A/Y156A/K256A/R257A/R258A-W269G	Nuclear
24	I126A/I127A/L128A/L129A/Y152A/Y156A/R263A/R264A/R266A/K267A-W269G	Diffused
25	I126A/I127A/L128A/L129A/Y152A/Y156A/K256A/R257A/R258A/R263A/R264A/R266A/K267A-W269G	Cytosolic
26	K256A/R257A/R258A/R263A/R264A/R266A/K267A-W269G	Diffused

Fig. 5-2F Localization of Kir/Gem mutants used for identification of NLSs

5.3 Importin α5 interacts with Kir/Gem and is required for its nuclear localization

Since nuclear transport of proteins commonly requires their interaction with importins (Xu and Massague, 2004), it was important to determine if Kir/Gem associates with any importins. Pull down experiments with GST-importin fusion proteins and lysate of Cos1 cells expressing Myc-Kir/Gem W269G, which efficiently localizes to the nucleus, showed that W269G specifically interacted with importin α5 (Fig. 5-3A, panel a, lane 5). No binding was observed with importin β, α1, α3, α6 or α7 (Fig. 5-3A, panel a, lanes 1-4, 6 and 7). Furthermore, neither WT Kir/Gem, which only poorly localizes to the nucleus nor Kir/Gem W269G with all three NLSs mutated, were able to bind importin α5. Similar results were obtained for co-immunoprecipitation experiments (Fig. 5-3B).

To determine whether importin α5 only binds to Kir/Gem or it is also required for nuclear localization of Kir/Gem, importin α5 was depleted in Hela cells using RNAi (Fig. 5-3C, panel a). In RNAi treated cells, Kir/Gem W269G no longer localized to the nucleus and remained cytosolic/diffused (Fig. 5-3C, panel a).

In summary, these experiments establish a specific interaction between Kir/Gem and importin α5. Importin α5 binding is dependent on a functional NLS in Kir/Gem and is selective for Kir/Gem W269G but not WT Kir/Gem, suggesting that bound CaM may interfere with importin α5 binding (see below). Furthermore, nuclear localization of Kir/Gem requires importin α5.

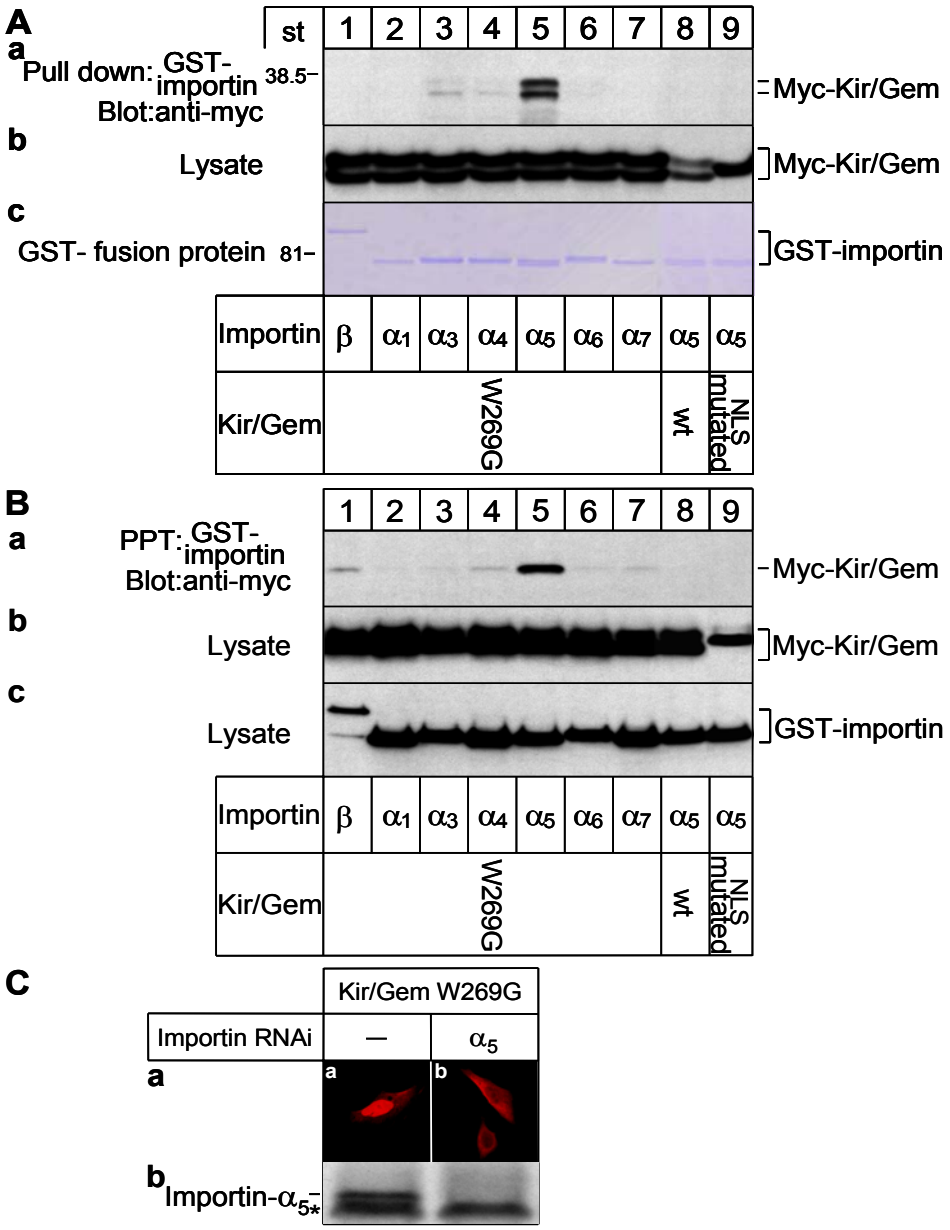


Fig. 5-3 Association of importins with Kir/Gem

A Pull down experiment. (**a**) Immobilized recombinant GST-importins were tested for interaction with WT or mutant devoid of CaM binding (W269G) or in all three nuclear localization signals (NLS mutated) present in homogenates of transfected cells. Kir/Gem proteins were revealed by Western blot using Myc antibody. (**b**) Cell lysates blotted with a Myc antibody to monitor Kir/Gem expression levels. (**c**) Coomassie staining to verify GST-fusion importins. **B** Coprecipitation. (**a**) Cells were cotransfected with WT or mutated Myc-Kir/Gem and GST-importins. GST-importins were precipitated and associated Kir/Gem was detected by Western blot using a Myc antibody. Cell lysates were blotted with Myc (**b**) or GST (**c**) antibodies to monitor Kir/Gem and GST-importin expression levels (*data provided by Dr.P.Beguin*). (**C**) Depletion of importin α5 using RNAi inhibits nuclear localization.

5.4 The NLSs in Kir/Gem can mediate nuclear localization independently

Since two of the three identified NLSs in Kir/Gem were not reminiscent of classical NLSs, I determined if they were able to independently mediate nuclear accumulation of a reporter protein. For this purpose, the regions of Kir/Gem containing the IILL and YY motifs (amino acids 124-166) or the bipartite KRK-RKRR signal (amino acids 253-end) were fused to a β-galactosidase reporter. As controls, the IILL, YY and the positively charged amino acids in NLS3 were substituted to alanine to inactivate the signals. The different β-galactosidase fusion proteins were expressed in Cos1 cells and their subcellular localization analyzed by immunofluorescence microscopy.

As shown in Fig. 5-4A, fusion of a small stretch from amino acids 124-166 that include the N-terminal IILL-YY based NLS of Kir/Gem led to an increase in the nuclear localization of the fusion protein (Fig. 5-4A, panel b) when compared to the diffused pattern observed for β-galactosidase itself (Fig. 5-4A, panel a). Mutation of either the IILL or the YY independently did not significantly affect the nuclear accumulation of the fusion proteins (Fig. 5-4A, panels c and d) and only mutation of both motifs resulted in a diffused distribution (Fig. 5-4A, panel e), similar to that of β-galactosidase itself (Fig. 5-4A, panel a). The β-galactosidase fusion protein carrying the bipartite NLS3 showed an almost exclusive cytosolic localization (Fig. 5-4B, panel c) and only when CaM binding was abolished by introducing the W269G mutation, did the fusion protein show a more obvious nuclear accumulation (Fig. 5-4B, panel a). This was consistently observed in 50-60% of the transfected cells, whereas the remaining cell population showed a diffused pattern. This cellular heterogeneity may reflect differences in the basal phosphorylation

state of the fusion protein. As expected, substitution of the positively charged amino acids in the NLS prevented the nuclear accumulation of the fusion protein (Fig. 5-4B, panel b), confirming the function of the NLS in nuclear import.

In summary, the three NLSs in Kir/Gem can independently mediate nuclear translocation if fused to a reporter protein.

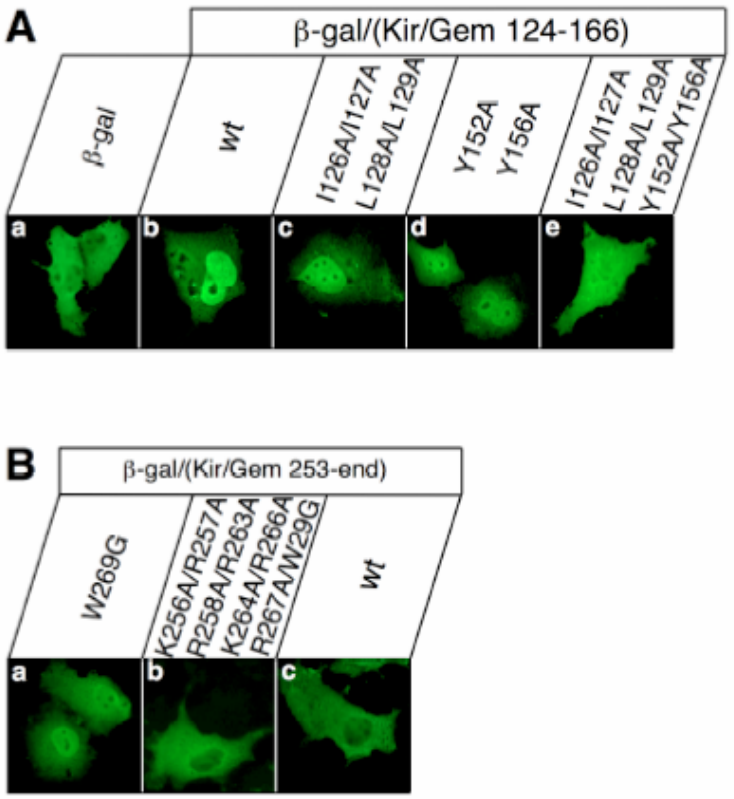


Fig. 5-4 Nuclear translocation of isolated NLSs
A Cos1 cells were transfected with cDNAs for β-galactosidase fusion proteins carrying a region of Kir/Gem containing NLS1 and NLS2 (amino acids 124-166), either in its WT form or with the indicated mutations. Cells were then processed for immunofluorescence microscopy using antibodies to HA to label the fusion protein (green).
B Cos1 cells were transfected with cDNAs for β-galactosidase fusion proteins encompassing WT or the indicated mutant forms of the C-terminal 42 amino acids of Kir/Gem, which includes NLS3, and analyzed as described above.

5.5 CaM associated to Kir/Gem interferes with importin α5 binding

Efficient nuclear localization of Kir/Gem is only observed in mutants defective for CaM binding. Amino acid W269, which is critical for CaM binding, is located in close proximity to the C-terminal KRK-RKRR bipartite NLS. These observations, together with the dominant role of the KRK-RKRR motif in nuclear localization (see Fig. 5-2E), raised the possibility that the association of CaM and importin α5 with Kir/Gem may be mutually exclusive. CaM binding assays showed that WT Kir/Gem bound to importin α5 in the absence but not in the presence of CaM. In contrast, Kir/Gem W269G, which does not bind CaM, was pulled-down by GST-importin α5 irrespective of whether CaM was present or not (Fig. 5-5). These data thus confirms that binding of importin α5 and CaM to Kir/Gem is mutually exclusive.

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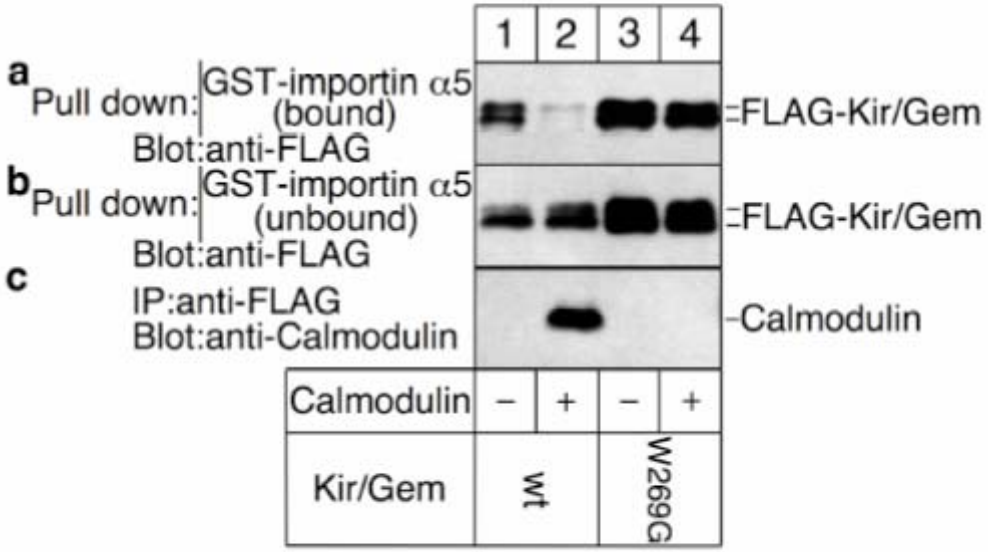


Fig. 5-5 Binding of importin α5 and CaM to Kir/Gem is mutually exclusive
a Cells were transfected with cDNAs for WT or mutated Flag-Kir/Gem. Flag-Kir/Gem was then isolated using Flag antibody (M2) beads and eluted from the beads using excess Flag peptide. The eluate was subjected to a pull-down using immobilized recombinant GST-importin α5 in absence (-) or presence (+) of exogenous CaM. Bound Kir/Gem was revealed by Western blot by probing with Flag antibodies.
(**b**) The supernatant after the GST-importin α5 pull down was blotted with FLAG antibodies to confirm that equivalent amounts of Kir/Gem were used.
(**c**) An aliquot of the cell lysates from (a) were supplemented (+) or not (-) with exogenous CaM, immunoprecipiated with Flag antibodies to isolate Kir/Gem, and bound CaM detected by Western blot using an antibody to CaM. (*data provided by Dr.P.Beguin*)

5.6 Rad, Rem and Rem2 share conserved NLSs

In addition to Kir/Gem, the RGK small G protein family comprises Rad, Rem and Rem2. The four family members share similarities with respect to the regulation of their subcellular distribution and function by CaM and 14-3-3 binding. Therefore, investigations were carried out to check if Rad, Rem and Rem2 also encode NLSs and if so, which importins they bind to.

Alignment of the amino acid sequences of the four RGK proteins revealed that the overall features of the three NLSs identified in Kir/Gem are conserved in Rad, Rem and Rem2 (Fig. 5-6). Reminiscent of the IILL motif in Kir/Gem, a LLVY, LVVM and LIVY motif is present in Rad, Rem and Rem2, respectively. The two tyrosine residues in NLS2 of Kir/Gem are conserved in Rad and Rem, whereas in Rem2, both are substituted by phenylalanine. In addition, two positively charged amino acid clusters similar to the bipartite C-terminal NLS in Kir/Gem (KRK-RKRR) are found in Rad (RRR-KKKR), Rem (RRR-RRR) and Rem2 (RR-KKKR).

To test if these motifs are required for nuclear import of Rad, Rem and Rem2, I took advantage of point mutations that, in analogy to Kir/Gem W269G, fail to bind CaM and hence allow efficient nuclear translocation of the RGK proteins. As shown earlier, while Rad L281G and Rem L271G mutants showed a more predominant nuclear localization, the nuclear accumulation of Rem2 L317G was less apparent as compared to the other family members. N-terminal Myc tagged deletion mutants were generated in Rad, Rem and Rem2 and their localization was analyzed, either with or without the putative NLSs in the corresponding regions. A list of all the constructs used for the study of NLSs in Rad, Rem and Rem2 is given in Fig. 5-7(A-C). The nuclear accumulation of

Rad C-trun 246, Rem C-trun 237, Rem2 C-trun 274 (Fig. 5-7D, E, F-panel b) were affected when their NLS1 and NLS2 were substituted for alanine (Fig. 5-7D, E, F-panel d), indicating the functional importance of these NLSs. Mutating the positive clusters in NLS3 affects the nuclear localization of Rad L281G, Rem L271G and Rem2 L317G showing the significance of NLS3 in these proteins (Fig. 5-7D, E, F-panel i). The nuclear localization of the mutant RGK proteins was abolished if all three putative NLSs were mutated (Fig. 5-7D, E, F- panel h), suggesting that they are required for nuclear transport of Rad, Rem and Rem2.

Pull down assays to sudy the interaction of these proteins with importins showed that Rad bound to importin β, α3 and α5 (Fig. 5-8, panel a) and Rem associated with importin α5 (Fig. 5-8, panel b). Consistent with the low levels of nuclear accumulation observed for Rem L317G, no binding to the importins could be detected (Fig. 5-8, panel c). These associations were greatly reduced or abolished by mutating the NLSs of Rad or Rem (Fig. 5-8, panel d, lanes 3, 6, 9, 12).

RNAi experiments were carried out to determine if the association of Rad and Rem with importins was required for the nuclear localization. Hela cells were transfected with RNAi and the respective RGK mutants. In RNAi treated cells, Rad and Rem mutants showed diffused distribution, indicating that the nuclear localization required the importins.

Taken together, the above findings confirm that the three NLSs initially identified in Kir/Gem are conserved and functional in Rad, Rem and Rem2. Rad can associate with importins β, α3 and α5, whereas Rem only binds importin α5 and the importins mediate nuclear localization of the RGK proteins.

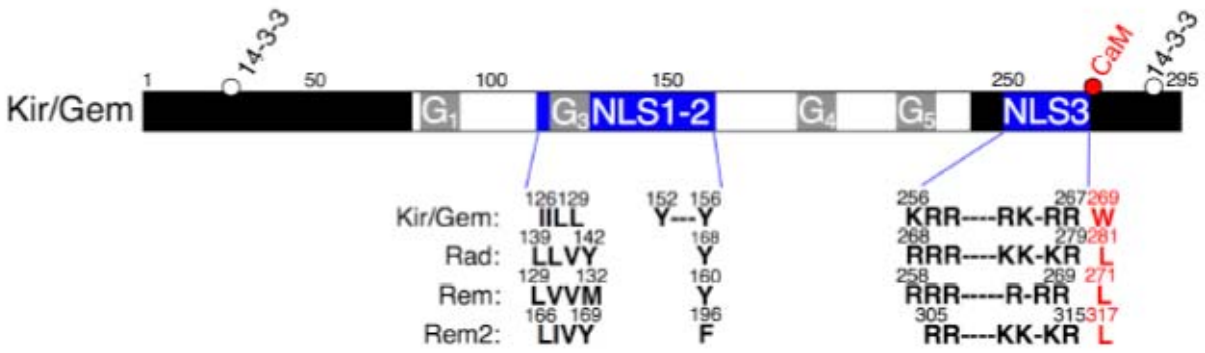
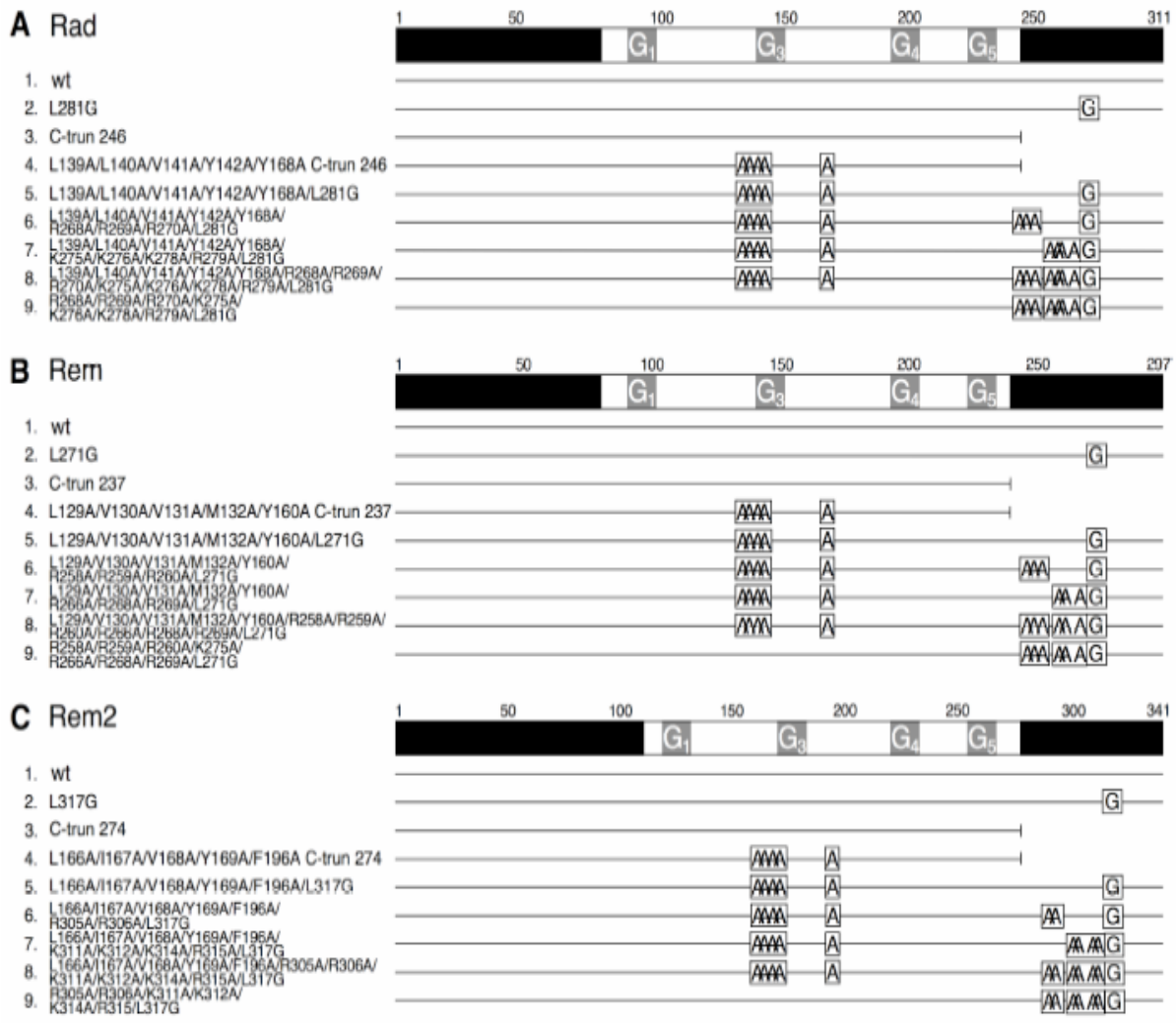


Fig. 5-6 NLSs in RGK proteins are conserved
The Ras-like core domain (white), N- and C-terminal extensions (black), the location of the G1-G5 motifs involved in GTP binding (grey), NLS1-3 (blue) and the 14-3-3 (white circles) and CaM (red circle) binding sites in WT Kir/Gem are indicated. Conserved amino acids within NLS1, NLS2 and NLS3 are shown in the one letter code for the different RGK proteins as well as the amino acid critical for CaM binding (red).


A Rad
1 50 100 150 200 250 311
G1 G3 G4 G5
1. wt
2. L281G
3. C-trun 246
4. L139A/L140A/V141A/Y142A/Y168A C-trun 246
5. L139A/L140A/V141A/Y142A/Y168A/L281G
6. L139A/L140A/V141A/Y142A/Y168A/ R268A/R269A/R270A/L281G
7. L139A/L140A/V141A/Y142A/Y168A/ K275A/K276A/K278A/R279A/L281G
8. L139A/L140A/V141A/Y142A/Y168A/R268A/R269A/ R270A/K275A/K276A/K278A/R279A/L281G
9. R268A/R269A/R270A/K275A/ K276A/K278A/R279A/L281G
B Rem
1 50 100 150 200 250 297
G1 G3 G4 G5
1. wt
2. L271G
3. C-trun 237
4. L129A/V130A/V131A/M132A/Y160A C-trun 237
5. L129A/V130A/V131A/M132A/Y160A/L271G
6. L129A/V130A/V131A/M132A/Y160A/ R258A/R259A/R260A/L271G
7. L129A/V130A/V131A/M132A/Y160A/ R260A/R268A/R269A/L271G
8. L129A/V130A/V131A/M132A/Y160A/R258A/R259A/ R260A/R268A/R269A/L271G
9. R258A/R259A/R260A/K275A/ R265A/R268A/R269A/L271G
C Rem2
1 50 100 150 200 250 300 341
G1 G3 G4 G5
1. wt
2. L317G
3. C-trun 274
4. L166A/I167A/V168A/Y169A/F196A C-trun 274
5. L166A/I167A/V168A/Y169A/F196A/L317G
6. L166A/I167A/V168A/Y169A/F196A/ R305A/R306A/L317G
7. L166A/I167A/V168A/Y169A/F196A/ K311A/K312A/K314A/R315A/L317G
8. L166A/I167A/V168A/Y169A/F196A/R305A/R306A/ K311A/K312A/K314A/R315A/L317G
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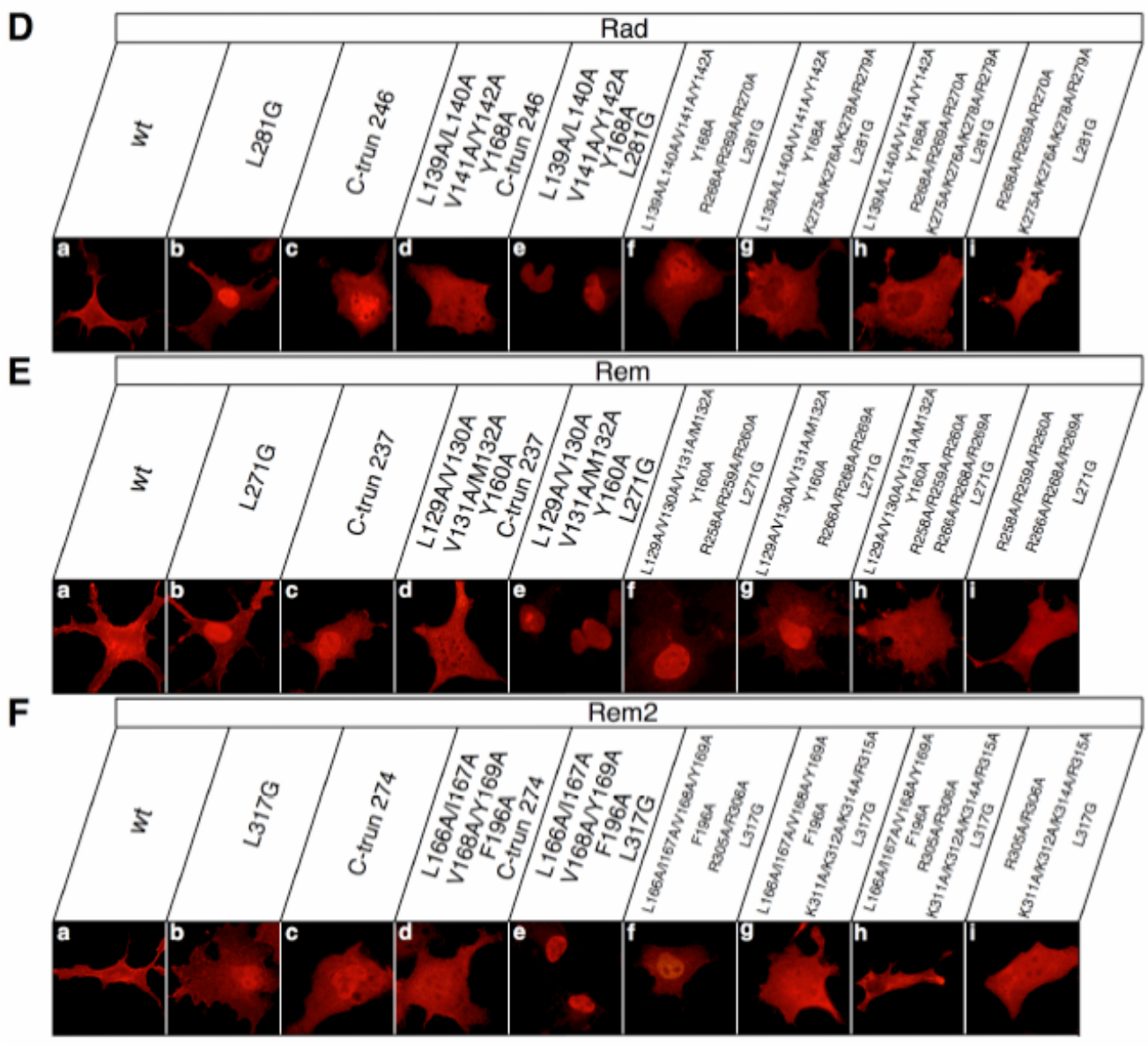


Fig. 5-7 Mutants used in the identification of NLSs in Rad, Rem and Rem2

A-C Schematic representation of RGK protein mutants used in the study. Corresponding C-terminal truncations (C-trun) and substitutions are depicted. Amino acids are numbered and shown in the one letter code.

D-F Subcellular distribution of WT and mutated RGK proteins. Cos1 cells were transfected with cDNAs for WT or the indicated mutated Rad (D), Rem (E) and Rem2 (F) and processed for immunofluorescence microscopy using Myc antibodies to label RGK proteins (red).

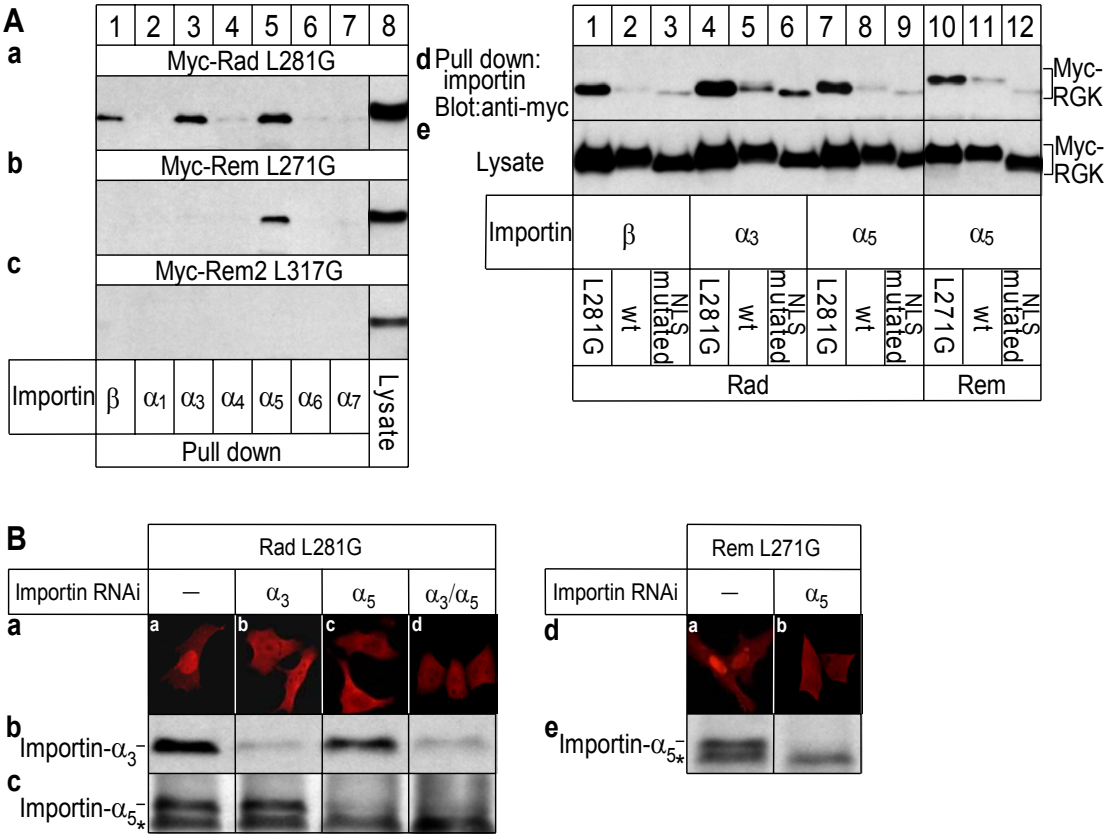


Fig. 5-8 Association of importins with Rad and Rem.

A. (**a-c**) Pull down experiment. Immobilized recombinant GST-importins were tested for their interaction with Myc-Rad L281G (**a**), Myc-Rem L271G (**b**) and Myc-Rem2 L317G (**c**) present in homogenates of transfected cells. RGK proteins were revealed by Western blot using Myc antibody. Cell lysates were blotted with a Myc antibody to monitor Myc-Kir/Gem protein expression levels (lane 8). (**d**) Pull down experiment. Immobilized recombinant GST-importins were tested for interaction with WT or mutated Myc-RGK proteins defective in their CaM binding or nuclear localization signal (NLS mutated) present in homogenates of transfected cells. RGK proteins were revealed by Western blot using Myc antibody. (*Data provided by Dr.P.Beguin)* (**e**) Cell lysates were blotted with a Myc antibody to monitor Myc-Kir/Gem protein expression levels.

B. Depletion of importins using RNAi inhibits nuclear localization of Rad and Rem.

CHAPTER 6

C-terminal phosphorylation modulates the subcellular localization of RGK proteins

6.1 Nuclear accumulation of Kir/Gem is regulated by C-terminal phosphorylation

Kir/Gem has been reported to be phosphorylated on several residues (i.e. Ser260, Ser286 and Ser288; Fig. 6-1A) (Ward et al., 2004) in the C-terminal region containing the bipartite NLS. Phosphorylation of these serine residues may alter the positively charged landscape provided with the KRK-RKRR bipartite signal and thus regulate the function of this crucial NLS. To test this hypothesis, the serine residues were substituted, individually or in different combinations, to either alanine or aspartate to prevent or mimic phosphorylation, respectively. The subcellular distribution of the mutant proteins expressed in Cos1 cells was determined by immunofluorescence microscopy.

As shown in Fig. 6-1B, panel b, Kir/Gem S260A displayed an enhanced nuclear localization as compared to the diffused distribution of the WT protein (Fig. 6-1B, panel a), suggesting that phosphorylation of S260 prevents nuclear accumulation. In contrast, mutation of S286 or S288 to alanine, either individually or combined, had little effect on nuclear localization of Kir/Gem (panels c, d and e). Interestingly, introducing negative charges to mimic phosphorylation at positions 286 and 288 (i.e. S286D/S288D) compensated for the lack of phosphorylation on S260A by showing a diffused distribution (panel h), while individual substitutions (i.e. S286D or S288D) failed to do so (panel f and g).

To explore the role of serine phosphorylation in the absence of CaM binding, S260, S286 and S288 were substituted to alanine or aspartate in Kir/Gem W269G, which displays a predominant nuclear localization. Mimicking phosphorylation on one of these

three serine residues individually did not affect nuclear accumulation of Kir/Gem W269G (Fig. 6-1C, panels a-d and f-h), but introducing two negative charges resulted in an increased presence in the cytosol (panel e and i-l). Consistent with the predominant role of S260 phosphorylation in preventing nuclear accumulation of Kir/Gem (Fig. 6-1B, panel b), cytosolic re-localization was less pronounced for the mutant carrying the S260A substitution (Fig. 6-1C, panel k).

Corresponding mutations were also introduced into the β-galactosidase fusion protein carrying the C-terminal region of Kir/Gem (amino acids 253-end) and their effect on the subcellular localization analyzed. While the fusion protein carrying the WT C-terminal fragment was mostly cytosolic (Fig. 6-1D, panel a), substituting S260, S286 and S288 with alanine resulted in complete nuclear accumulation (panel b). The introduction of two negative charges led to a diffused distribution (panels, c-e) and substituting all three serine residues for aspartate resulted in complete nuclear exclusion (panel f). These results are consistent with a constitutive phosphorylation of the C-terminus of WT Kir/Gem.

Nuclear localization of the β-galactosidase fusion protein carrying the W269G mutation was also analyzed (Fig. 6-1D, panel g). Substitution of all three serine residues to alanine resulted in nuclear accumulation (panel h), while an efficient clearance from the nucleus was observed if they were changed to aspartate (panel l). An intermediate, diffused distribution was obtained where two negative charged residues were introduced (panels i-k) and, as seen for Kir/Gem itself, the cytosolic relocalization was less efficient for the mutant carrying the S260A replacement (panel k). The results for all the possible combinations of serine mutations in the β-galactosidase fusion protein carrying the

W269G substitution are presented in Fig. 6-1F. Taken together, this data indicate that phosphorylation on S260, in conjunction with that of one or both downstream phosphorylation sites, is required for cytosolic localization of Kir/Gem.

The above observations indicated that the introduction of negative charges into the C-terminal region of Kir/Gem, as would be the case for phosphorylation on serine residues, might interfere with the function of the positively charged bipartite NLS. Hence the ability of the different mutants to interact with importin α5 was checked using pull down assays. Compared to WT Kir/Gem, preventing phosphorylation on S260 resulted in an increased association of the S260A mutant with importin α5 (Fig. 6-1E, panel a, lanes 1 and 2), whereas substituting S260, S286 and S288 by aspartate completely abolished the interaction (Fig. 6-1E, panel a, lane 3). Similarly, the association of Kir/Gem W269G with importin α5 was decreased if single serine residues were substituted by aspartate (panel a, lanes 5-7) and completely abolished by the introduction of two (panel a, lanes 8 and 9) or three (panel a, lane 11) negative charges. Consistent with the immunofluorescence data showing a less efficient cytosolic relocalization for the S260A replacement (see Fig 6-1C and D, panel k), this mutant retained residual importin α5 binding (Fig 6-1E, panel a, lane 10). Similar results were obtained in co-precipitation experiments from lysates of Cos1 cells co-expressing the different importins with either WT or mutated Myc-Kir/Gem (data not shown).

Since abolishing binding of CaM to Kir/Gem leads to nuclear accumulation and enhanced importin α5 binding (see chapter 5), it was important to establish that the serine substitutions did not affect the interaction between Kir/Gem and CaM. CaM binding experiments confirmed that Kir/Gem S260A, which like Kir/Gem W269G localizes to the

nucleus and binds importin α5, bound CaM to a similar extent as WT Kir/Gem. Likewise, substituting S260, S286 and S288 with aspartate did not affect CaM binding in WT Kir/Gem, nor did it restore the lack of CaM binding in Kir/Gem W269G (*Dr.P.Beguin, pers. Comm.*). Similar results were also obtained for β-galactosidase fusion proteins carrying the C-terminal part of Kir/Gem with the corresponding mutations. A summary of all β-galactosidase constructs and their localization is shown in Fig. 6-1F.

In conclusion, the above results indicate that the introduction of negative charges in the C-terminus of Kir/Gem, as would be the case for the phosphorylation on serine residues S260, S286 and S288 in the C-terminus of Kir/Gem, favors a cytosolic localization of the RGK protein. In particular, the phosphorylation status of S260, which is located within the KRK-RKRR NLS, appears to be critical in regulating the subcellular distribution of Kir/Gem, most likely by interfering with the recognition of the positively charged NLS by importin α5.

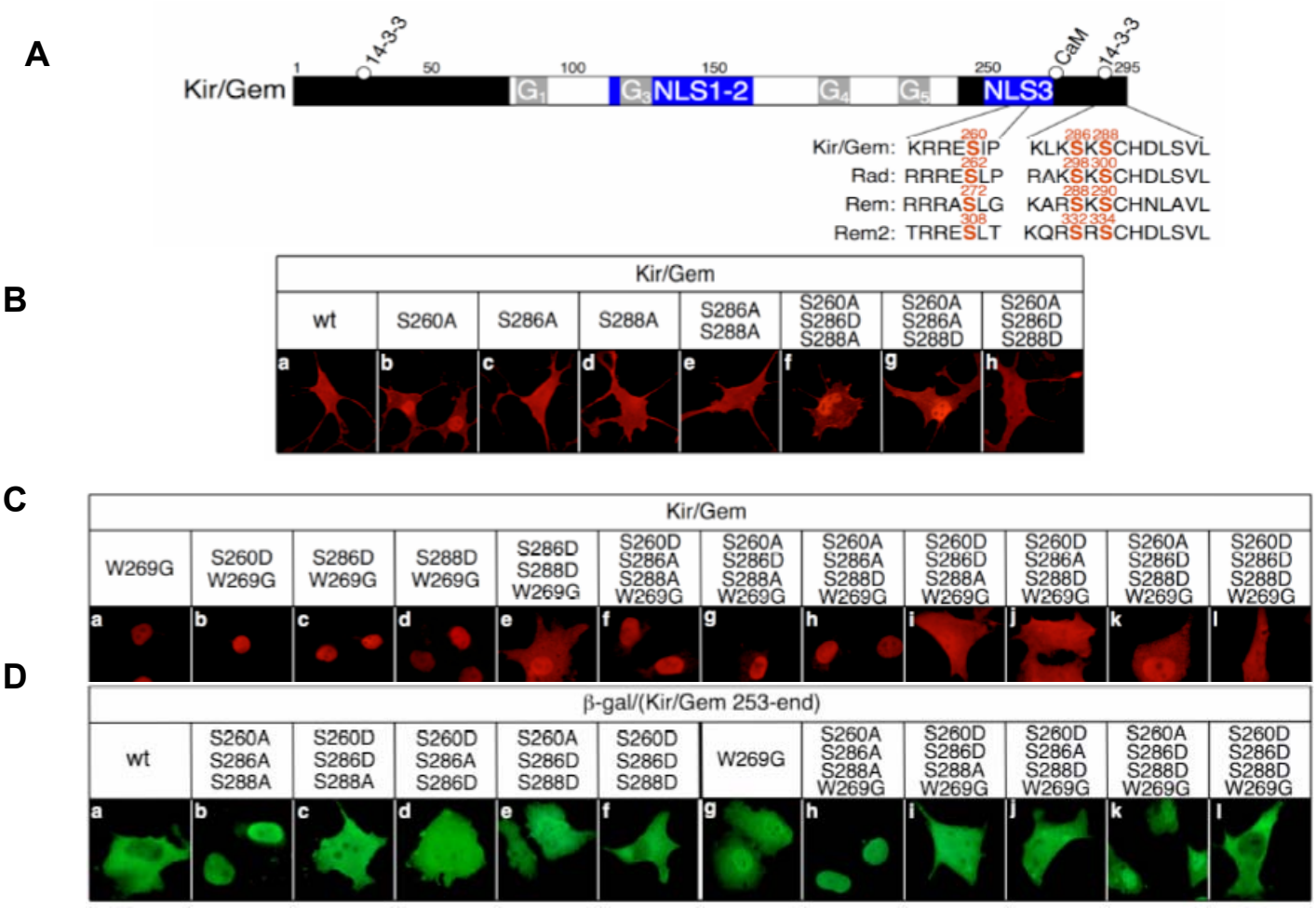
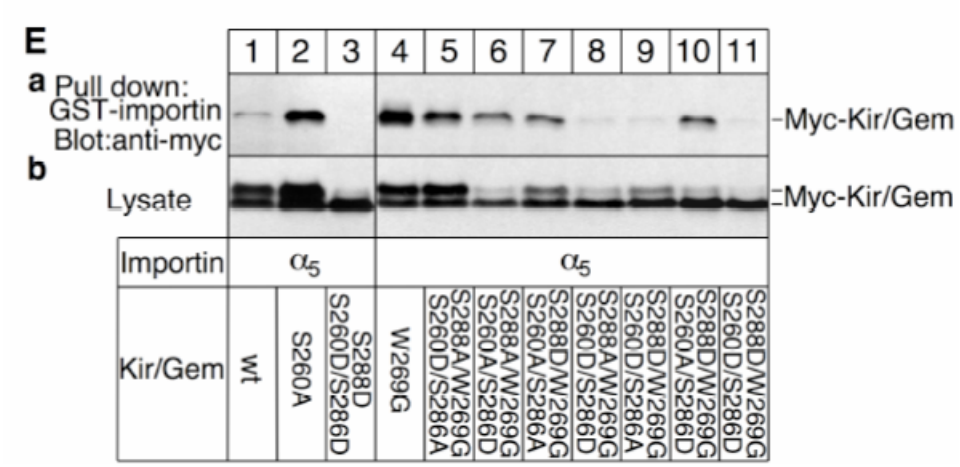


Fig. 6-1 Serine phosphorylation regulates subcellular distribution of Kir/Gem.

A Location of the Ras-like core domain (white), N- and C-terminal extensions (black), G1-G5 motifs involved in GTP binding (grey) and the NLSs (blue), 14-3-3 binding motif and the CaM binding site in Kir/Gem. The amino acid sequence of the region within the NLS3 and the 14-3-3 binding site containing the conserved serine residues (in red) involved in the regulation of the subcellular distribution and 14-3-3 binding in Kir/Gem is shown.

B-D Preventing or mimicking phosphorylation of critical serine residues affects the subcellular distribution of Kir/Gem or β-galactosidase fusion proteins. Cos1 cells were transfected with cDNAs for either WT or mutated Myc-Kir/Gem **(B and C)** or N-HA-β-galactosidase fusion proteins containing WT or mutated forms of the C-terminal 42 amino acids of Kir/Gem (**D**). Cells were then processed for immunofluorescence microscopy using anti-myc or anti-HA to label Kir/Gem (**B** and **C**, red) or the fusion protein (**D**, green).



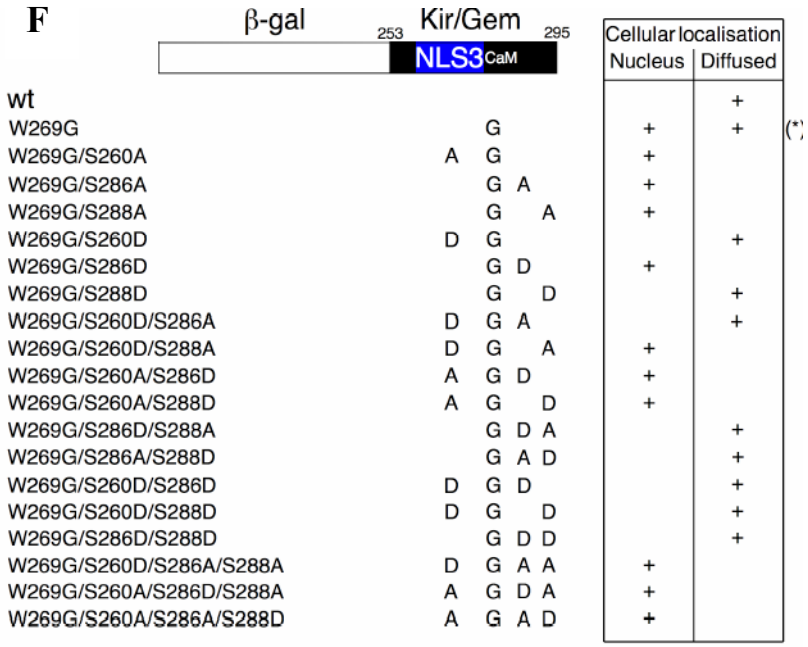


Fig. 6-1E Binding of importin α5 to Kir/Gem mutants carrying critical serine substitutions. (**a**) Pull down experiment. Immobilized recombinant GST-importin α5 was tested for interaction with WT or mutated Myc-Kir/Gem present in homogenates of transfected cells. Kir/Gem proteins were revealed by Western blot using Myc antibody. (**b**) Cell lysates were blotted with a Myc antibody to monitor Myc-Kir/Gem protein expression levels.

F Summary of subcellular distribution of β-gal fusion proteins carrying the C-terminal NLS3 of Kir/Gem. (*) As mentioned in the text, the fusion protein with the W269G mutation displayed a nuclear staining for 50-60% of the transfected cells, whereas the remaining cell population showed a diffused pattern.

6.2 Regulation of subcellular localization of Rad, Rem and Rem2 by serine phosphorylation differs as compared to Kir/Gem

Prevention of phosphorylation on Ser260 leads to nuclear localization of Kir/Gem and this serine is conserved in Rad (S272), Rem (S262) and Rem2 (S308) (Fig. 6-1A). To determine if serine phosphorylation also regulates the subcellular distribution of these RGK proteins, Rad S272A, Rem S262A and Rem2 S308A were expressed in Cos1 cells and their subcellular distribution was analyzed by immunofluorescence microscopy. In contrast to Kir/Gem, the Rad and Rem2 mutants did not accumulate in the nucleus (Fig. 6-2, panels a and b, e and f). Rem S262A, however, showed a nuclear redistribution (Fig. 6-2, panels c and d), similar to that observed for Kir/Gem S260A.

This shows that, similar to Kir/Gem, nuclear translocation of Rem, but not Rad and Rem2, is regulated by phosphorylation of a serine residue located within the C-terminal bipartite NLS.

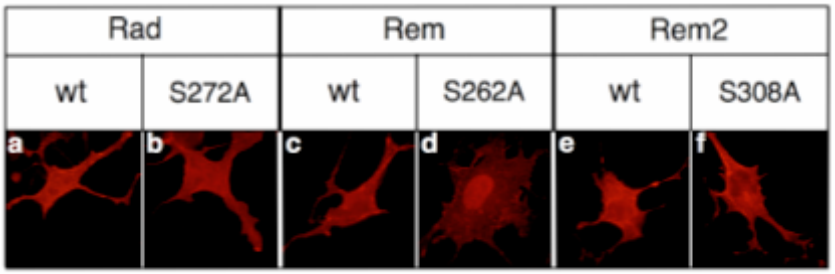


Fig. 6-2 Phosphorylation state of the serine residue located within the NLS3 determines subcellular distribution of Rem but not Rad and Rem2

Cos1 cells were transfected with cDNAs for WT or the indicated mutated RGK proteins and processed for immunofluorescence microscopy using Myc antibodies to label the RGK protein (red).

6.3 Serine (S286) phosphorylation modulates 14-3-3 mediated nuclear exclusion of Kir/Gem

Dimeric 14-3-3 proteins associate with Kir/Gem by binding to phosphorylated S22 and S288 and retain the protein in the cytosol (see chapter 3). Given the role of phosphorylation on serine residues, including S288, in regulating the subcellular distribution of Kir/Gem, the effect of key substitutions of S260, S286 and S288 to alanine or aspartate on 14-3-3 function and its interaction with Kir/Gem was examined.

As shown earlier, expression of 14-3-3 in Cos-1 cells led to the nuclear clearance of Kir/Gem and prevented the changes in cell shape normally induced by the GTP binding protein (Fig. 6-3A, panels a-a"). These effects were not observed for the S288A substitution (Fig. 6-3A, panels d-d"). While Kir/Gem S286A was relocated to the cytosol by overexpressed 14-3-3, no nuclear clearance was observed for the S286D substitution (panels b-b" and c-c"). Similarly, Kir/Gem S260A or Kir/Gem S260A/W269G, which showed a more predominant (panel e) or exclusive (panels i) nuclear localization, respectively, were efficiently re-localized to the cytosol by 14-3-3 (panels i', i" and e', e"). While nuclear clearance of Kir/Gem S260A or W269G by 14-3-3 was not affected if S286 was replaced by an alanine (panels f-f" and j'-j"), it was abolished if substituted with an aspartate (panels g-g" and k-k") or, as expected, if S288 was changed to alanine (panels h-h" and l'-l"). The results obtained were quantified using 3 to 5 independent experiments to establish the consistency of localization and translocation of these mutants.

Similar results were obtained in co-immunoprecipitation experiments where S286A showed only a slight reduction in binding whereas S286D failed to bind 14-3-3.

This was also true in the case of alanine and aspartic acid substitutions in the context of a S260A or W269G mutation.

In summary, these data indicate that phosphorylation of S286 prevents binding of 14-3-3 to Kir/Gem and thereby affects the 14-3-3 mediated cytosolic relocalization of the GTP binding protein.

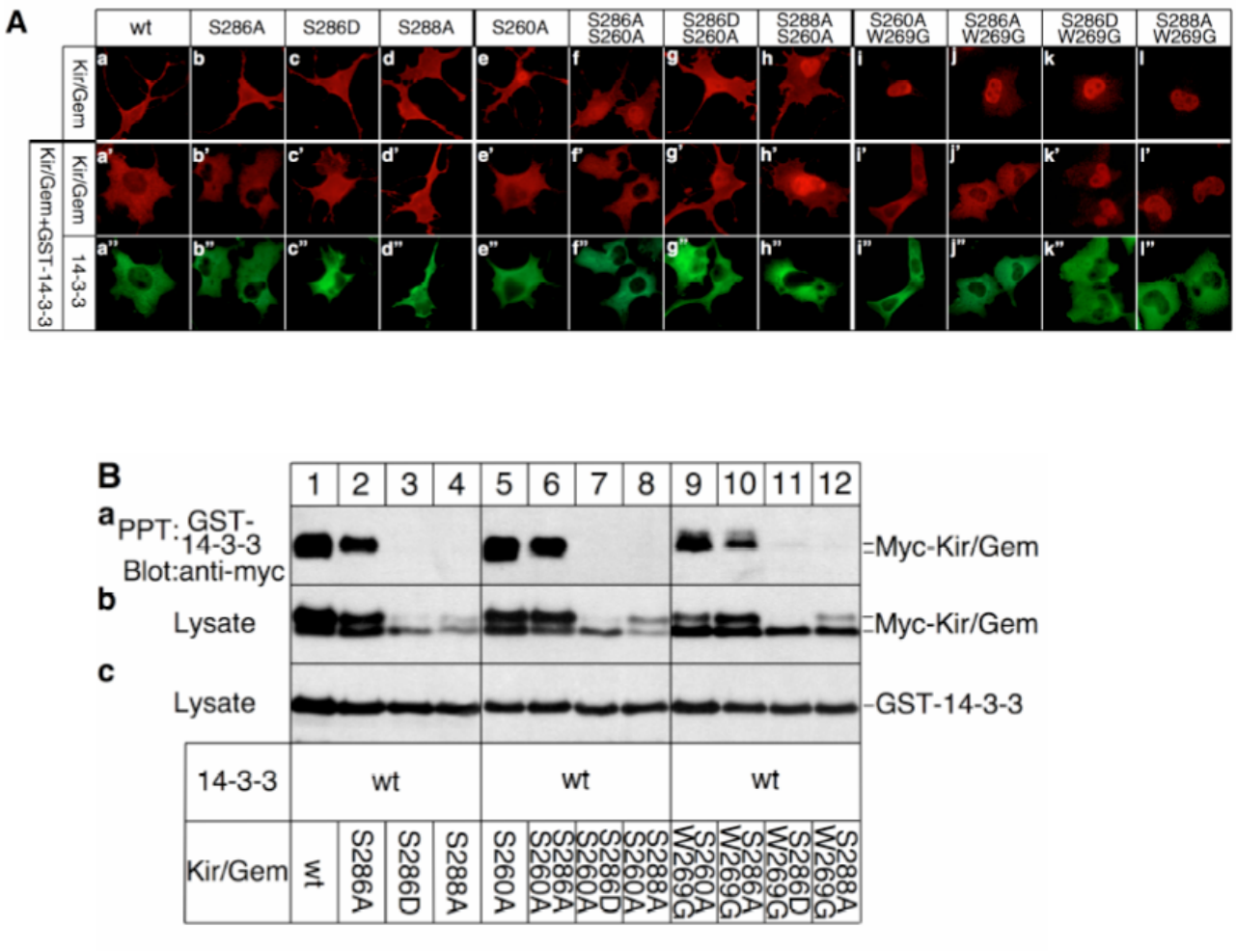


Fig. 6-3 Regulation of 14-3-3 binding by C-terminal phosphorylation in Kir/Gem

A Preventing or mimicking phosphorylation of critical serine residues affects the subcellular distribution of Kir/Gem and the 14-3-3 induced nuclear clearance of Kir/Gem. Cos1 cells were transfected with cDNAs for myc-Kir/Gem in their WT form or with the indicated mutations, either alone (**a-l**) or with GST-14-3-3 (**a'-l'** and **a"-l"**). Cells were then processed for immunofluorescence microscopy using Myc- or GST antibodies to label Kir/Gem (**a-l** and **a'-l'**, red) or 14-3-3 (**a"-l"**, green).

B Binding of Kir/Gem mutants carrying critical serine substitutions to 14-3-3 **(a)** Precipitation experiment. (*Data provided by Dr.P.Beguin*) GST-14-3-3 was precipitated and tested for interaction with WT or mutated Myc-Kir/Gem present in homogenates of cotransfected cells. Bound Kir/Gem was revealed by Western blot using Myc antibody. (**b**) Cell lysates were blotted with a Myc antibody to monitor Kir/Gem protein expression levels. As observed previously (Ward *et al.*, 2004; Béguin *et al.*, 2005b), mutants lacking the 14-3-3 binding site displayed reduced cellular expression levels. (**c**) GST-14-3-3 expression levels were determined by blotting lysates with a GST antibody.

6.4 Serine phosphorylation of Rad, Rem and Rem2 regulates 14-3-3 binding and function

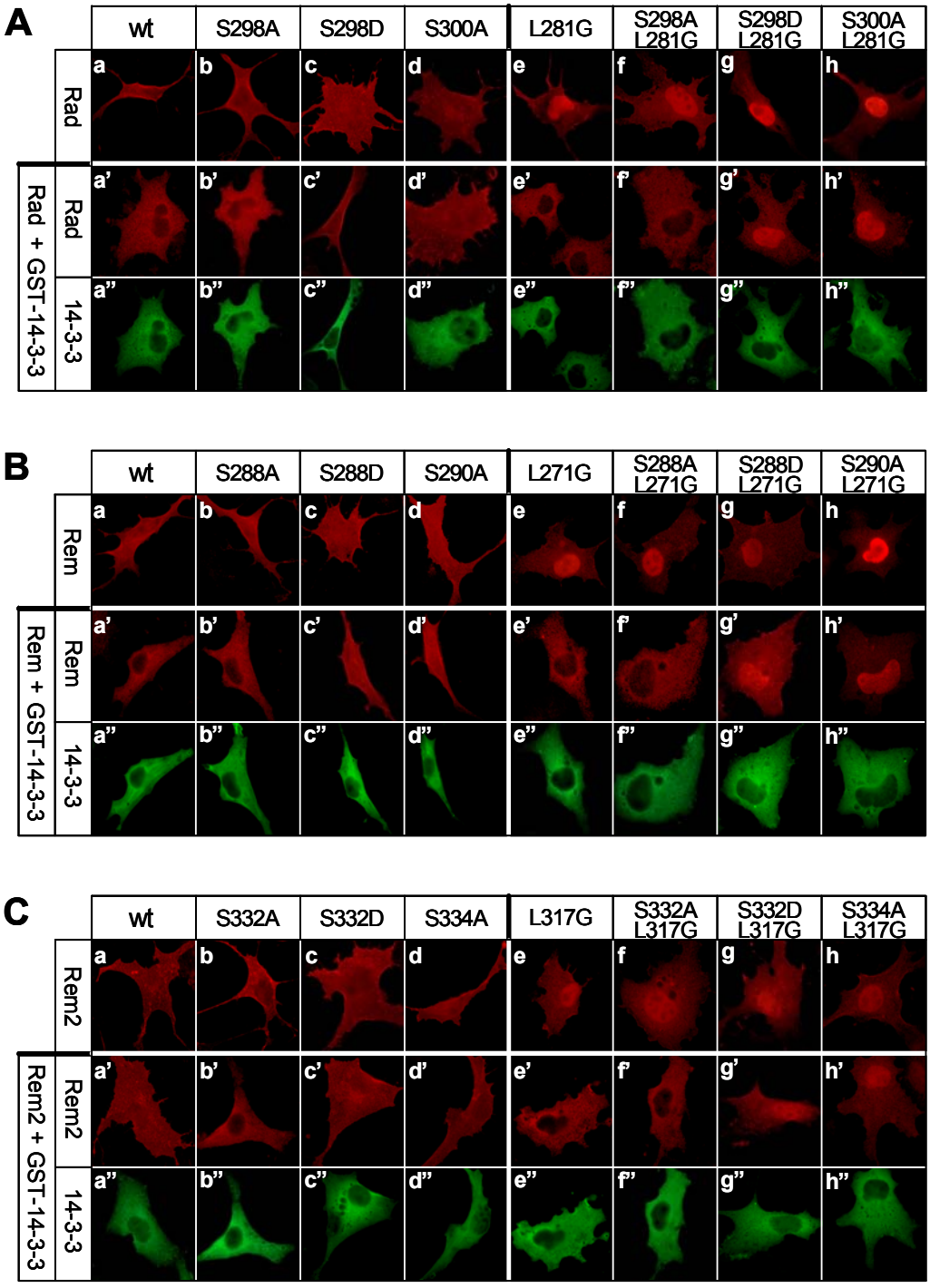
Ser286 is in close proximity to the C-terminal 14-3-3 binding site (S288 of Kir/Gem) and conserved in other RGK family members (see Fig. 6-1A). It was therefore crucial to determine if phosphorylation of the corresponding serine affects the subcellular distribution of Rad, Rem and Rem2 and their ability to bind 14-3-3. Alanine or, to mimic phosphorlyation, aspartate substitutions of this serine residue were generated in the WT RGK proteins (i.e. Rad S298, Rem S288 and Rem2 S332) and the corresponding CaM-binding defective mutants (i.e. Rad L281G, Rem L271G and Rem2 L317G) and analyzed by immunofluorescence and co-immunoprecipitation experiments.

As previously shown (chapter 3), WT and CaM binding defective mutants of Rad and Rem were efficiently excluded from the nucleus, when co-expressed with 14-3-3 (Fig. 6-4A and B, panels a-a", e-e"). For Rem2, only Rem2 L317G underwent cytoplasmic relocalization (Fig. 6-4C a-a", e-e"). Mutation of S298 in Rad or S288 in Rem to Ala did not interfere with the 14-3-3-mediated nuclear exclusion, either in the context of the WT (Fig. 6-4A and B, panels b-b") or the CaM-binding defective RGK protein (Fig. 6-4A and B, panels f-f"). Interestingly, in contrast to WT Rem2, the S332A mutant relocalized to the cytosol after 14-3-3 co-expression (Fig. 6-4C, panels b-b"). Importantly, the 14-3-3-mediated cytoplasmic relocalization was impaired if the corresponding serine residues were changed to aspartate in Rad (i.e. Rad S298D), Rem (i.e Rem S288D) or Rem2 (i.e. Rem2 S332D), either in the context of the WT (Fig. 6-4A, B and C, panels c-c", respectively) or the CaM binding defective mutants (Fig. 6-4A, B and C, panels g-g", respectively). The effect of the aspartate substitution in Rem was much less pronounced (see quantification Fig. 4E). This most likely reflects the fact that,

in contrast to the other RGK proteins that require both the N- and C-terminal sites for 14-3-3 binding, the N-terminal S18 is already sufficient for binding of 14-3-3 by Rem (chapter 3). A quantification of the effects of 14-3-3 on the subcellular distribution of the different RGK protein mutants is provided in Fig. 4D, E and F.

To corroborate the role of serine phosphorylation on the 14-3-3-mediated subcellular redistribution of Rad, Rem and Rem2, the binding of 14-3-3 to different RGK mutants was checked. As previously shown (chapter 3), Rad, Rem and Rem2, as well as the corresponding mutants defective in CaM binding, associated with 14-3-3 (Fig. 6-5, panel a, lanes 1, 5, 9, 13, 17 and 21, respectively). This interaction was not affected if the serine residue upstream of the C-terminal 14-3-3 binding site (i.e. Rad S298, Rem S288 and Rem2 S334) was substituted with alanine (Fig. 6-5, panel a, lanes 2, 6, 10, 14, 18 and 22). As previously observed for Kir/Gem, the slower migrating form of Rem2 S332A was stabilized by 14-3-3. Substitution with aspartate, or mutation of the C-terminal 14-3-3 binding site (i.e. S300 in Rad, S290 in Rem and S334 in Rem2), abolished (Rad and Rem2) or reduced (Rem) 14-3-3 binding (Fig. 6-5, panel a, lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23 and 24). Thus, the 14-3-3-mediated redistribution of the different RGK protein mutants correlated well with their ability to bind 14-3-3.

In summary, the above data thus confirm that the binding of 14-3-3 to RGK proteins and 14-3-3 mediated nuclear exclusion of RGK proteins is regulated by phosphorylation on a conserved serine residue present upstream to the C-terminal 14-3-3 binding site.


A
wt
S298A
S298D
S300A
L281G
S298A L281G
S298D L281G
S300A L281G
Rad
Rad + GST-14-3-3
Rad
14-3-3
a
b
c
d
e
f
g
h
a'
b'
c'
d'
e'
f'
g'
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h''
B
wt
S288A
S288D
S290A
L271G
S288A L271G
S288D L271G
S290A L271G
Rem
Rem + GST-14-3-3
Rem
14-3-3
C
wt
S332A
S332D
S334A
L317G
S332A L317G
S332D L317G
S334A L317G
Rem2
Rem2 + GST-14-3-3
Rem2
14-3-3

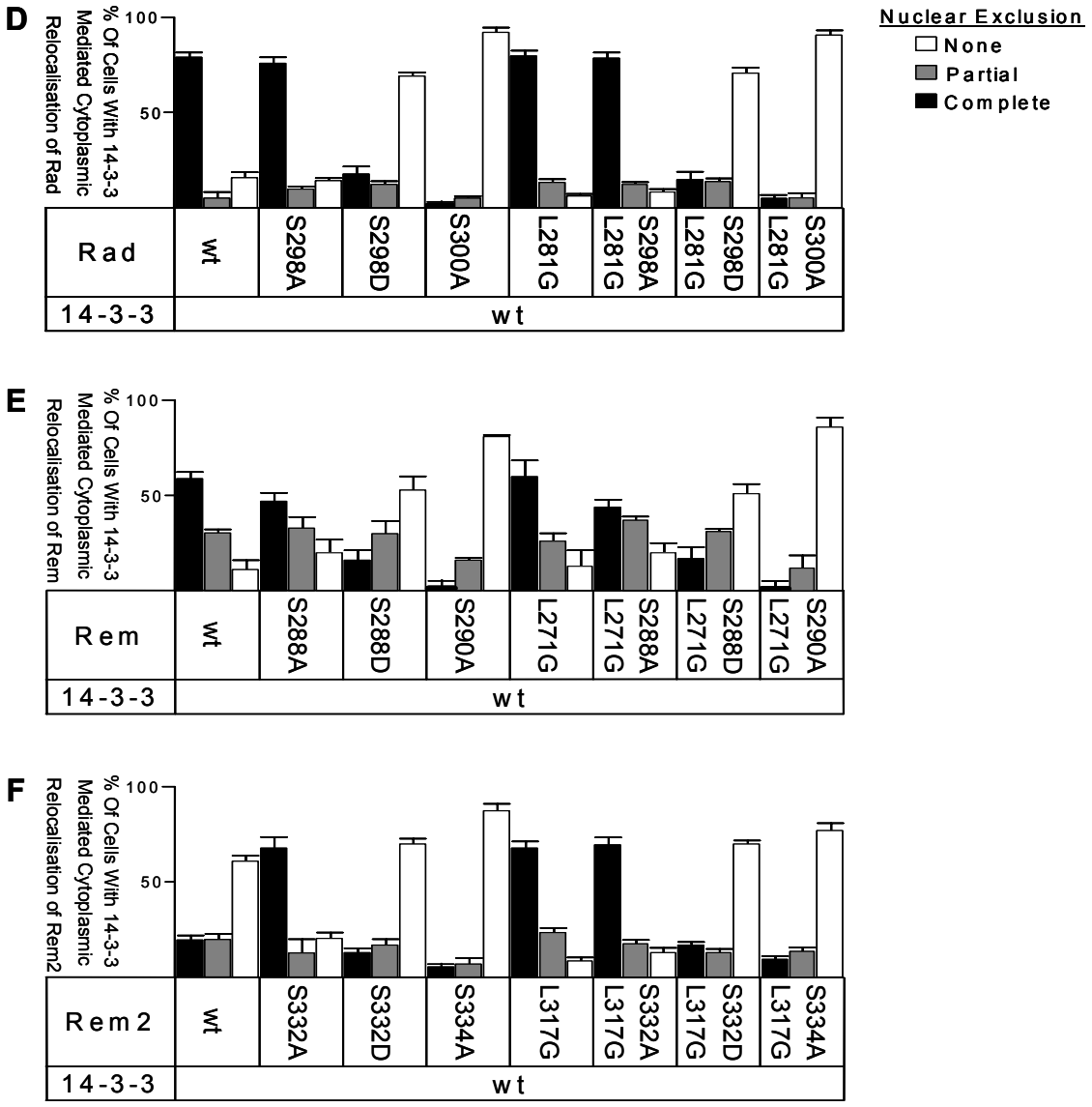

Fig. 6-4 Serine phosphorylation modulates 14-3-3 mediated subcellular redistribution of Rad, Rem and Rem2.

(**A-C**) Preventing or mimicking phosphorylation of critical serine residues (see Fig. 3A) affects the subcellular distribution of RGK proteins and the 14-3-3 induced nuclear clearance. COS-1 cells were transfected with cDNAs for WT or mutated Myc-tagged Rad (**A**), Rem (**B**) or Rem2 (**C**), either alone (**a-h**) or with GST-14-3-3 (**a'-h'** and **a"-h"**). Cells were then processed for immunofluorescence microscopy using anti-Myc or anti-GST antibodies to label the RGK protein (**a-h** and **a'-h'**, red) or GST-14-3-3 (**a"-h"**, green).

(**D-F**) Quantification of the subcellular distribution of WT or mutated RGK proteins. The subcellular distribution (no, partial or complete exclusion from the nucleus) for Myc-Rad (**D**), Rem (**E**) or Rem2 (**F**) in their WT or mutated forms was determined in 100-150 randomly chosen cells coexpressing the RGK protein and 14-3-3 in 3-5 independents experiments

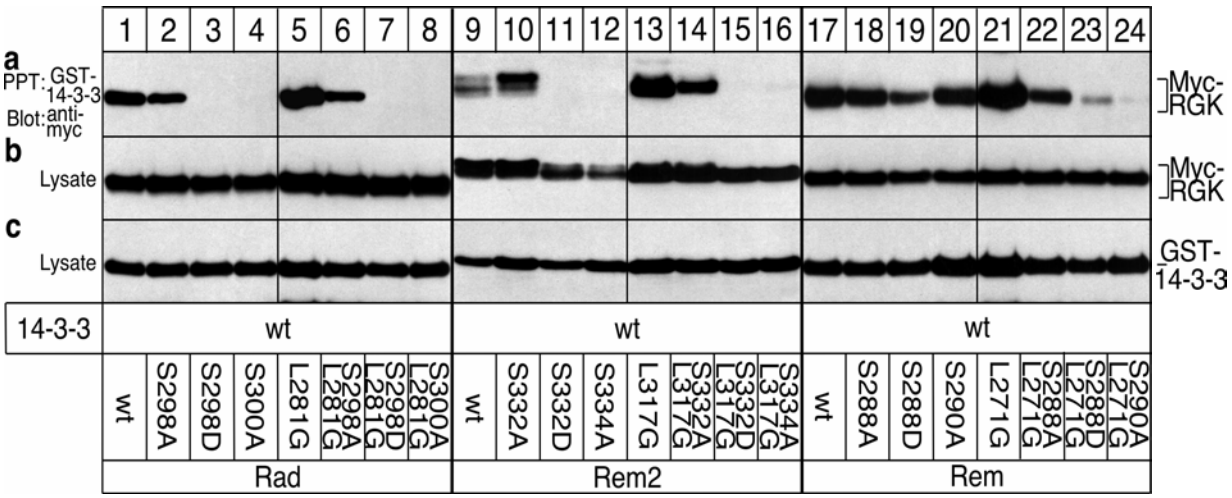

Fig. 6-5 Phosphorylation of a serine upstream from the 14-3-3 binding site regulates 14-3-3 binding to RGK proteins.

Precipitation experiment. GST-14-3-3 was precipitated and tested for interaction with WT or mutated Myc-Rad, Rem and Rem2 present in homogenates of cotransfected cells. Bound RGK protein was revealed by Western blot using Myc antibody. (b) Cell lysates were blotted with a Myc antibody to monitor Kir/Gem protein expression levels. (c) GST-14-3-3 expression levels were determined by blotting lysates with anti-GST antibody. Since Rem can independently bind monomeric 14-3-3 via either the N-terminal or C-terminal binding site, the role of S288 phosphorylation on 14-3-3 binding to the C-terminal site can be only detected in the context of either the CaM deficient Rem mutant or a Rem mutant in which the N-terminal 14-3-3 binding site had been mutated (i.e. Rem S18A, (data not shown)).

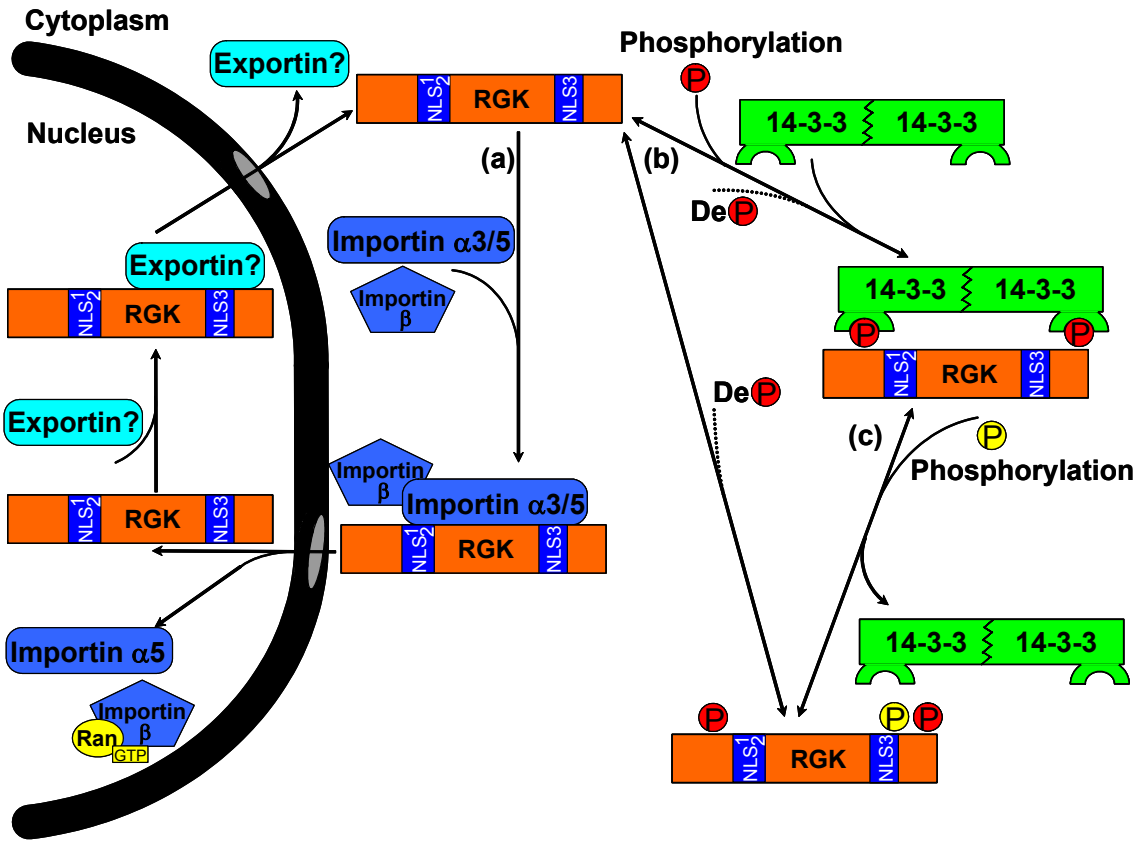

Fig. 6-6 Working model for the regulation of the nucleocytoplasmic shuttling of RGK proteins.

Nuclear import of RGK proteins involves binding of importins α3, α5 and β (Rad) or only importin α5 (Rem and Kir/Gem) to NLSs in the RGK protein (**a**). Export is likely mediated by yet to be identified exportins. Binding of 14-3-3 to Kir/Gem retains the RGK protein in the cytosol, possibly by preventing importin association ((**b**), our unpublished data). Phosphorylation on an N- and C-terminal serine is required for binding of 14-3-3 (**b**), whereas phosphorylation on a serine adjacent to the C-terminal 14-3-3 binding site prevents the association or favors the dissociation of 14-3-3 via an unknown mechanism (**c**). The subcellular localization of RGK proteins can also be modulated by CaM binding or, in the case of Rem and Kir/Gem, phosphorylations on serine residues located within NLS3, both of which interfere with importin binding and hence prevent nuclear accumulation.

CHAPTER 7

Discussion

RGK proteins are multifunctional small GTP-binding proteins that are involved in the modulation of calcium channel activity and cytoskeletal dynamics. They possess unique structural properties such as the presence of N- and C-terminal extensions and lack residues known to be crucial for GTP hydrolysis in other Ras like GTPases. A characteristic feature of RGK proteins in comparison to other small G proteins is their interaction with the regulatory proteins 14-3-3 and CaM. In our study, I have shown that the interaction of 14-3-3 and CaM with the RGK proteins regulate the subcellular localization and the functions of the small GTPases.

CaM and 14-3-3 regulate the subcellular localization of RGK proteins

The distinct C-terminal extension in RGK proteins is characterized by a CaM binding domain. Mutation of a critical residue (W269 in Kir/Gem, L281 in Rad, L271 in Rem and L317 in Rem2) to Gly prevents CaM binding and results in nuclear localization of RGK proteins. This observation indicates that CaM plays a role in the modulation of the subcellular localization of RGK proteins. Under steady state conditions, RGK proteins show cytoplasmic, submembranous and nuclear localizations and our results demonstrate that nuclear accumulation in the absence of functional CaM binding, as in the case of the Kir/Gem W269G, Rad L281G, Rem1 L271G and Rem2 L317G mutants, is a common feature of RGK proteins.

All RGK proteins carry an N- and C- terminal 14-3-3 binding site. Interestingly, the identified 14-3-3 binding sites are distinct from canonical motif for 14-3-3 binding. Although S260 in Kir/Gem is located within a consensus 14-3-3 binding motif (RXX_PSXP), it is not involved in 14-3-3 binding (Ward et al., 2004). RGK proteins can associate with all the isoforms of 14-3-3 (β, γ, ε, ζ, η, σ, τ) with the exception of Kir/Gem, which does not bind 14-3-3-σ (Beguin et al., 2005). A previous study failed to detect binding of 14-3-3 to Rem2 (Finlin et al., 1999) and this was due to the use of a truncated form of Rem2 (starting at M70) that lacked the N-terminal 14-3-3 binding site. Kir/Gem, Rad and Rem2 require both 14-3-3 binding sites and only bind 14-3-3 dimers, whereas, each 14-3-3 binding site in Rem can individually associate with 14-3-3 monomers and hence dimerization is not required. In the absence of bound CaM, the C terminal 14-3-3 binding site in Kir/Gem and Rem1 is sufficient for 14-3-3 binding, whereas, Rad and Rem2 still require both 14-3-3 binding sites. Both monomeric and dimeric forms of 14-3-3 are found in cells and dimerization is regulated by phosphorylation (Woodcook et al., 2003) and thought to be required for high affinity, stable binding (Yaffe et al., 2002). Thus, while 14-3-3 monomers are able to bind to RGK proteins in the absence of CaM, high affinity interaction of dimers may be required to displace CaM from the small GTP binding protein.

The association of 14-3-3 with RGK proteins may be tightly regulated by kinases or phosphatases that act on the 14-3-3 binding sites. While the C-terminal 14-3-3 binding site is common to all RGK members and does not show an obvious motif for a particular kinase, the N-terminal binding site appears to be specific for each of the four RGK proteins. For example S69A in Rem2, but not S22A in Kir/Gem, is within a consensus

sequence for PKA phosphorylation. Thus, depending on the extracellular stimulus, 14-3-3 may associate with a specific RGK protein.

As previously observed for Kir/Gem (Leone et al., 2001), RGK proteins migrate as a doublet on SDS-PAGE, with the slower migrating form likely corresponding to a posttranslational modification. Interestingly, the fraction of the higher molecular weight form of the protein is increased in response to either 14-3-3 over-expression or inactivation of CaM binding, but decreased if the 14-3-3 binding sites are mutated, suggesting that 14-3-3 and CaM affect the posttranslational modifications.

14-3-3 also regulates the subcellular localization of RGK proteins. Interestingly, only 14-3-3 overexpression but not mutation of the 14-3-3 binding site in RGK proteins led to their nuclear exclusion. This, together with the observation that little if any endogenous 14-3-3 was bound to RGK proteins, suggests that, at least in Cos1 cells, most of the endogenous 14-3-3 is sequestered and that nuclear exclusion of RGK proteins requires over-expressions of 14-3-3. In PC12 cells, however, the subcellular localization of RGK proteins was shifted towards a more cytoplasmic localization and the abolition of the 14-3-3 and CaM association was required for efficient nuclear translocation. This is consistent with a more prominent role of endogenous 14-3-3 in regulating the subcellular localization of the RGK proteins in PC12 cells, possibly reflecting a larger pool of available free 14-3-3. The variations in different cell types probably reflect differences in the pools of free 14-3-3 and CaM in these cell lines available to interact with the overexpressed RGK proteins. Alternatively, since binding of 14-3-3 to RGK proteins is likely to be regulated by phosphorylation and/or dephosphorylation, the expression level of kinases and phosphatases that are involved in the posttranslational modification of

RGK proteins may differ. Nuclear exclusion is particularly pronounced for the CaM binding mutants because they are localized in the nucleus, and when co-expressed with 14-3-3, efficiently sequestered to the cytoplasm.

14-3-3 and CaM not only compete for binding to the RGK proteins (*Dr.P.Beguin., pers. comm.*), but their association is likely to be regulated for example by phosphorylation and/or dephosphorylation, implicating that also nuclear localization of RGK proteins is tightly controlled. Whether RGK members exert additional functions in the nucleus remains to be analyzed but is an intriguing possibility given than they are present in the nucleus in their active form (Beguin et al., 2005). Several members of the Rho family of GTPases, including Rnd 1, 2 and 3, RhoH, RhoBTB1 and 2, are also locked into a GTP-bound conformation and unable to hydrolyze GTP (Foster et al., 1996; Nobes et al., 1998; Li et al., 2002). Several amino acids that are critical for GTP hydrolysis are absent in Rnd3 (Fiegen et al., 2002) and the corresponding residues are likewise not present in RGK proteins. In contrast to most other small GTPases, RGK proteins (Reynet and Kahn, 1993; Cohen et al., 1994; Maguire et al., 1994; Finlin and Andres, 1997) and RhoH (Li et al., 2002) expression can be regulated at the transcriptional level. Coupled to a short half-life in cells as observed for Kir/Gem (Ward et al., 2004), this may provide a mechanism to regulate the activity of these small G proteins. Alternatively, post-translational modifications and/or nuclear sequestration could represent mechanisms to rapidly inactivate RGK proteins.

CaM and 14-3-3 interfere with the RGK induced cell shape remodeling

Kir/Gem (Leone et al., 2001; Piddini et al., 2001; Ward et al., 2002), Rad (Ward et al., 2002) and Rem (Pan et al., 2000) have been shown to regulate cell morphology. Similar to the other RGK proteins, Rem2 expression results in the formation of dendrite like extensions. 14-3-3 overexpression led to the redistribution of the RGK proteins form a submembranous to a more cytoplasmic localization and affected the formation of dendrite-like extensions. In a recent study (Ward et al., 2004), disruption of the C-terminal, but not the N-terminal, 14-3-3 binding the site abolished the ability to induce neurite extensions in neuroblastoma cells. At least in Cos-1 cells, little if any endogenous 14-3-3 associates with Kir/Gem, explaining why mutation of the N- or C-terminal does not interfere with the ability of Kir/Gem to induce extensions. In contrast, the presence of exogenous 14-3-3, but not a 14-3-3 mutant defective in target protein binding, abrogates Kir/Gem mediated induction of extensions. Rad, Rem and Rem2 showed a similar behavior but the effect on cell shape was less pronounced compared to Kir/Gem. Thus, at least in Cos-1 cells, 14-3-3 binding to RGK correlates with its inhibitory effect on RGK induced morphological changes. Mutation of the CaM or CaM and 14-3-3 binding sites abrogated the ability of RGK to induce dendtite-like extensions, due to the nuclear localization of the proteins. Thus nuclear transport also affects cell shape remodeling by RGK proteins. Since Kir/Gem and Rad associate with ROK kinases to regulate actin dynamics (Ward et al., 2002), it will be of interest to determine whether 14-3-3 and/or CaM affect this interaction.

CaM, but not 14-3-3 affects RGK mediated down regulation of calcium channel activity

The RGK proteins down regulate calcium channel activity by interacting in their GTP bound form with the β-subunit, thereby preventing association of the latter with the ion transporting α_1-subunit (Béguin *et al.*, 2001; Sasaki *et al.*, 2005). Plasma membrane expression of functional calcium channels requires the association of the α_1 and β subunits (Catterall, 1998) and I propose that RGK proteins downregulate Ca^{2+} channel activity by interfering with cell surface expression of α_1 through binding and sequestering the β-subunit. Overexpression of 14-3-3 neither interfered with the down regulation of calcium channel activity by RGK proteins, nor with their association with the β-subunit. These results, together with the lack of a functional effect by mutating the 14-3-3 binding site in Kir/Gem (Ward et al., 2004), argue against a role of 14-3-3 in the RGK mediated regulation of Ca channel activity. While the expression of all RGK proteins led to an inhibition of endogenous Ca currents in PC12 cells, abolishing CaM binding only affected the functions of Kir/Gem and Rad, but not Rem and Rem2**.**

Co-expression studies of RGK proteins with β_3 revealed that both proteins co-localized. Indeed, a close co-distribution of the β-subunit with RGK proteins was observed. Thus, cell surface expression of VDCCs may be regulated by two distinct mechanisms, one involving the association of the RGK proteins with the β-subunit in the cytosol, the other due to nuclear sequestration of the β-subunit by the RGK protein. The extent to which one or the other mechanism is utilized may in turn be determined by signaling events that control the association of CaM and 14-3-3 with the RGK proteins.

Identification of NLSs in RGK proteins

RGK proteins may exert their function in the cytoplasm, on membranes of the ER and the plasma membrane, or on the cytoskeleton, hence raising the question about the functional relevance of their nuclear transport. Nuclear sequestration may provide an attractive mechanism to rapidly and reversibly inactivate RGK proteins by removing them from their site of action. If nuclear transport indeed serves as a mechanism to spatially control RGK protein activity in cells, the mechanism itself needs to be regulated. Our study revealed that RGK proteins have three conserved NLSs. While two of these signals, NLS1 and NLS2, are non-canonical, the C-terminal NLS3 exhibits the features of a classical bipartite signal. In the context of the full length RGK protein, the bipartite NLS3 appears to be dominant over the two non-canonical NLSs. NLS1 and NLS2 are located within the core GTP-binding domain. NLS1 and NLS2, when isolated together, can mediate nuclear import of a reporter protein. If NLS3 in Kir/Gem is isolated, the specificity of the interaction with importin α5 is lost and it is thus conceivable that NLS1 and NLS2 are secondary sites of interaction that specify the interaction with a particular importin. While Kir/Gem, Rad and Rem associate with importin α5, Rad also binds importins β and α3. Importin binding was only detected for RGK protein mutants defective in CaM binding, which efficiently localize to the nucleus, but not for the WT proteins, which show a diffused distribution. The requirement for efficient nuclear localization to detect importin binding probably also explains why no binding could be detected for Rem2 L317G, which only poorly translocates to the nucleus.

Furthermore, to check if importins only bind to RGK proteins or if they mediate nuclear transport, RNAi experiments were performed, where the specific importins (α5 for Kir/Gem, α3 and α5 for Rad and α5 for Rem) were depleted in hela cells and the localization of the CaM binding deficient mutants of the respective proteins was analyzed. Our analysis revealed that the CaM binding mutants displayed a diffused or cytosolic localization after the depletion of the importins, thus confirming that the importins are needed for the efficient nuclear transport of RGK proteins.

Since only the CaM binding deficient mutant and not the WT RGK proteins associate with importins, it was hypothesized that CaM may interfere with importin binding. Indeed, purification of WT Kir/Gem restores binding to importin α5 and this is blocked by the addition of exogenous CaM *(Dr.P.Beguin, pers. commun*.). These observations indicate that binding of CaM and importins to RGK proteins is mutually exclusive and provides a rationale for why mutants defective in CaM binding show a more predominant nuclear localization when compared to the WT RGK proteins.

Serine phosphorylation regulates nuclear transport and 14-3-3 binding to RGK proteins

Interestingly, nuclear localization of RGK proteins is not only regulated by CaM binding, but also by at least two serine phosphorylation events. The C-terminus of RGK proteins contain at least three serine residues that can be phosphorylated (Ward *et al.*, 2004). One of these serine residues (i.e. S260, S262, S272 and S308 in Kir/Gem, Rad, Rem and Rem2, respectively) is located within NLS3 (Fig. 6-1A), the others in close proximity. Since NLS3 is a typical bipartite signal that depends on the presence of positively charged amino acids, phosphorylation is likely to alter the charged landscape

within this domain (Xu and Massague, 2004). Indeed, mutations that prevent (i.e. alanine substitutions) or mimic (i.e. aspartate substitutions) phosphorylation on these serine residues favored a more nuclear or cytosolic distribution, respectively. In particular, preventing phosphorylation of the serine residue (S260) located within NLS3 resulted in efficient nuclear localization. The effects of the serine substitutions on the subcellular distribution of the mutant RGK proteins correlated with changes in importin binding. Thus, alanine substitution of the serine residue, which localized to the nucleus, dramatically enhanced importin binding, whereas replacement with aspartate, which shoed a diffused localization, completely abolished the association. An inhibitory role of phosphorylation in importin binding and nuclear import has been observed for other proteins (Carvalho et al., 2001; Harreman et al., 2004; Schwindling et al., 2004; Shin et al., 2005). Importantly, this regulation by phosphorylation/dephosphorylation seems to differ among the RGK family members. Despite similar consensus phosphorylation sites, alanine substitution of this serine residue induced nuclear relocalization of Kir/Gem and Rem, but not Rad and Rem2. Since the C-terminus is the site of association for CaM and 14-3-3, the different behavior of the RGK family members may probably be explained by differences in binding affinity of these regulatory proteins. Phosphorylation on S260 and S288 in Kir/Gem is crucial for neurite extensions in neuroblastoma cells and Kir/Gem expressed in T cells in response to mitogen activation is also phosphorylated on both serine residues (Ward *et al.*, 2004). S260 is a consensus site for several kinases, including PKC-ζ (Ward et al., 2004), PKA, and Akt, and it will be of interest to determine if RGK proteins are constitutively phosphorylated on this serine residue. In such a case, dephosphorylation, possibly by a calcium dependent phosphatase, rather than

phosphorylation, may be key to the regulation of RGK protein function and subcellular localization. In contrast, the phosphorylation state of this serine residue did not affect 14-3-3 binding.

Phosphorylation state of a second serine residue (i.e. S286, S298, S288 and S332 in Kir/Gem, Rad, Rem and Rem2, respectively), which is located just upstream from the C-terminal 14-3-3 binding site, influenced 14-3-3 binding to RGK proteins. Mimicking phosphorylation by substituting the serine residue upstream from the C-terminal 14-3-3 binding site completely abolished 14-3-3 binding, thereby preventing cytosolic sequestration of RGK proteins by 14-3-3. Whether this phosphorylation regulates the phosphorylation of the serine residue in the 14-3-3 binding site, interferes with 14-3-3 binding, or induces the release of 14-3-3 from the RGK proteins, remains to be established.

In conclusion, RGK proteins shuttle between nucleus and cytoplasm by a mechanism that is tightly regulated by CaM and 14-3-3. While the functions of RGK proteins overlap, the expression of individual family members is cell-type specific and regulated at the transcriptional level (Kelly, 2005). RGK proteins regulate diverse cellular functions including cell shape and calcium signaling, which require different cellular localizations of the RGK proteins. RGK proteins, for example, can regulate VDCC activity at the level of the ER by preventing cell surface transport of the α-subunit or at the plasma membrane by modulating channel activity (Chen et al., 2005; Finlin et al., 2005). Given their multifunctional nature, the multilayered and complex regulation of the nuclear transport of RGK proteins may be required to selectively regulate individual cellular functions of these small GTP binding proteins.

CHAPTER 8

Conclusion

In this study, I have investigated various regulatory mechanisms that are involved in determining the localization and function of the members of RGK family of proteins, Kir/Gem, Rad, Rem and Rem2.

The calcium binding protein, CaM plays a significant role in retaining the RGK proteins in cytosol. Therefore, abolition of CaM binding results in nuclear translocation of RGK proteins. In addition, the ubiquitous phospho-serine/threonine binding protein 14-3-3 interacts with RGK proteins and causes cytosolic re-localization and/or nuclear exclusion. The association with 14-3-3 affects RGK mediated changes in cell shape, a function that is more prominent for Kir/Gem compared to Rad, Rem and Rem2. Our study also shows that the nuclear transport of RGK proteins affects their ability to induce dendritic extensions.

Members of the RGK family modulate voltage gated calcium channels by interfering with the cell surface expression of α_1 subunit through their association and co-localization with the β_3 subunit, thereby causing a down regulation of channel activity. While binding of CaM is probably required for inactivation of VDCCs by Rad, down regulation of calcium channel activity by rem and rem2 is not modulated by CaM. Though 14-3-3 does not alter the down regulation of calcium channel function by RGK proteins, a role for 14-3-3 in the absence of bound CaM may not be excluded.

The nuclear transport mechanism of RGK proteins and its regulation was elucidated in some detail. Three conserved nuclear localization signals (NLSs) were identified and the specific importins involved in nuclear transport of RGK proteins were

identified. While nuclear translocation of Kir/Gem and Rem is linked to importin α5, Rad associated with importins β, α3 and α5. Binding of importins to Rem2 was not detectable, consistent with the inefficient nuclear translocation of Rem2.

Further, phosphorylation of three serine residues in the C-terminus of Kir/Gem interferes with the nuclear localization, most likely by introducing negative charges into the positively charged landscape required for importin binding. In addition, phosphorylation of a serine just adjacent to the C-terminal 14-3-3 binding site interferes with 14-3-3 binding and the 14-3-3 mediated effects on RGK protein localization and function.

In summary, the analysis uncovers a complex network of interacting partners that are involved in the regulation of RGK proteins.

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