AN ABSTRACT OF THE DISSERTATION OF

Anand Venkataraman for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on November 10, 2009.

Title: *In vitro* and *In vivo* Approaches for Functional Characterization of the Scaffold Protein, GRASP.

Abstract approved:

__________________________________________________________

Mark Leid

Studies using the pluripotent embryonic carcinoma cell line, P19, as a retinoic acid (RA)-responsive model system have been instrumental towards our understanding of the RA-dependent signaling pathways in development and homeostasis. Grp1-associated scaffold protein (GRASP; also known as Tamalin) was first identified by our group, as a gene robustly induced by RA treatment in P19 cells. GRASP was reported to influence the cell-surface expression of interacting membrane receptors in vitro albeit by an unknown mechanism. Furthermore, the in vivo role of GRASP is poorly understood. Mice with a germline deletion of *Grasp* (*GRASP*\textsuperscript{−/−}) do not exhibit any gross morphological, behavioral or sexual deficits and exhibit mild, altered sensitivities to morphine and cocaine administration. The goals of our studies herein were to investigate the mechanistic details of GRASP for the role in trafficking and cell-surface, localization of membrane receptors in vitro and the role of GRASP in vivo.
Previously, our group had identified a strong interaction of GRASP with Grp1, a guanine nucleotide exchange factor for the small G protein, Arf6. In this study we show GRASP localizes in intracellular recycling endosomal compartment(s), recruits Grp1 to these structures, and facilitates activation of Arf6. Activation of Arf6 regulates key aspects of vesicular trafficking, actin reorganization and cellular migration. Furthermore, we show that GRASP preferentially regulates intracellular membrane trafficking by the Arf6-dependent/clathrin-independent pathway.

We have shown that GRASP is expressed in the adult murine skin. Our studies demonstrate a robust induction of GRASP transcripts in murine dermis and epidermis following exposure to ultraviolet (UV) rays. We also report the generation of novel mice with germline deletion of Grasp (GRASP\(^{-/-}\)), that exhibit altered proliferative and apoptotic responses to UV exposure. Ongoing investigations suggest that the observed phenotypes of GRASP\(^{-/-}\) mice after UV exposure maybe due to the dysregulation of the subcellular localization of the tumor suppressor protein, p53.

Taken together, our \textit{in vitro} and \textit{in vivo} approaches have provided new insight in the role of GRASP as scaffold protein that regulates intracellular trafficking pathways.
In vitro and In vivo Approaches for Functional Characterization of the Scaffold Protein, GRASP

by
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APPROVED:

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I understand that my dissertation will become part of the permanent collection of the Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

___________________________________________________
Anand Venkataraman, Author
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CONTRIBUTION OF AUTHORS

Dr. Mark Leid and Anand Venkataraman designed research. Anand Venkataraman performed the research and with Dr. Mark Leid analyzed and wrote the manuscripts.
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Chapter 1

*In vitro* and *in vivo* approaches for functional characterization of the scaffold protein, GRASP.
Introduction

By the late 90's, it was known that vitamin A (retinol) and its active derivatives (collectively referred to as retinoids) exert a wide variety of effects on vertebrate embryonic body shaping and organogenesis, tissue homeostasis, cell proliferation, differentiation, and apoptosis (1-6). Amongst all biologically active retinoids, the all-trans retinoic acid (ATRA) and its 9-cis isomer (9-cis RA) were known to have the most pleiotropic functions. Retinoic acid can substitute for the role of vitamin A during embryogenesis and allow rescue from symptoms of vitamin A deficiency in adults (1). These apparently simple structured retinoid molecules are able to elicit such a broad range of functions by binding to two classes of RA-binding transcriptional regulators belonging to two classes of the nuclear receptors (NRs) superfamily. The retinoic acid receptors (the multiple isoforms of the RARα, β, and γ isotypes), which bind ATRA and 9-cis RA, and the rexinoid receptors (the multiple isoforms of the RXRα, β, and γ isotypes), which bind 9-cis RA only (1). In vitro studies, performed either in cell-free systems or transfected cells in culture, revealed that RAR/RXR heterodimers could control the transcription of RA-target genes through binding to DNA response elements (Retinoic acid response elements; RARE). These target genes encode transcription factors, metabolic enzymes, growth factor receptors and extracellular matrix proteins and secreted proteins postulated to convey positional information in the developing embryo (5,6). Subsequent to these findings an emerging
focus was to understand the precise mechanism involved in the RAR/RXR-mediated cell-signaling pathways which ultimately led to the observed phenotype during development and homeostasis. Towards this purpose, the use of P19 cells, a kind of embryonic stem cell line has proven to be extremely fruitful (7).

Embryonic stem (ES) cells can give rise in-vivo, to the ectodermal, endodermal, and mesodermal germlayers and, in-vitro, can differentiate into multiple cell lineages, offering broad perspectives in regenerative medicine (8,9). A special class of these stem cells called embryonic carcinoma (EC) cells have been derived from malignant teratocarcinomas which have the ability to transform from a malignant to non-malignant state by a process of cellular differentiation. These teratocarcinomas arise spontaneously in testes of mice and humans from defective germ cells or can be induced artificially by implanting early murine embryonic tissue to extrauterine sites (7). Once isolated the EC cells can grow indefinitely in an undifferentiated state and have proved to be invaluable to the scientific community. Examples of such cells include the Tera-2 EC cells from humans and F9 and P19 EC cells from mice.

P19 cells were extensively studied in the early 90's as a retinoic acid (RA)-responsive model system and have helped understand the retinoic acid signaling pathways (7,10). P19 cells can differentiate into either the ectodermal, endodermal or mesodermal germ layers, based on the levels of retinoic acid present in the culturing
medium. Several years before the P19 EC cells were established in culture, it was observed that efficient differentiation of EC cells depends on the prior formation of non-adhering aggregates or embryonic bodies, which resemble the inner cell mass of an embryo. In embryoid bodies, initial differentiation occurs when the outer cells of the aggregate differentiate into endoderm-like cells that surround undifferentiated core (7). In addition it was observed, that hormones like retinoic acid, induced differentiation of some EC cells, like F9, in monolayer culture, without aggregation phase. A combination of embryoid body formation and drug application was then used to induce P19 EC cells to differentiate into derivatives of all the three germ layers. When grown in a monolayer and treated with ATRA, P19 cells undergo differentiation along endodermal and mesodermal pathways (11). In contrast ATRA has a biphasic effect on P19 cells grown in aggregates. When treated with low (1-10 nM) concentrations of ATRA, P19 cells aggregates differentiate primarily via the endodermal pathways, while high concentrations (>300nm) of this retinoid induce differentiation into neurons, glia and fibroblast like cells (12). Additionally, P19 cells aggregates treated with DMSO (0.5-1%) led to the formation of wide variety of endodermal and mesodermal tissues like cardiac and skeletal muscle cells (13).
Discovery of GRASP

To understand the precise signaling mechanism involved in RA-induced P19 differentiation pathway, it was necessary to dissect the molecular players involved in this pathway. In other words, it was necessary to identify the genes induced by retinoic acid treatment in P19 cells. At the time of this these studies, a predecessor of the micro-array technique called subtractive hybridization screen was widely being used to identify genes induced by a given treatment (14). cDNA libraries from cells treated with retinoic acid were created and allowed to hybridize (subtracted) with a reference cDNA library (from untreated cells). Only the unsubtracted cDNA copies of induced genes would in theory remain after subtraction, which were amplified using PCR techniques and the entire process iterated to obtain a very high signal to noise ratio. This technique had already led to the isolation of genes like Ephrin B1 (Stra1) and Hox3a as retinoic acid genes in P19 cells (15).

Our lab adapted the subtractive hybridization screen and identified a 2.1-kilobase pair transcript, referred to hereafter as GRASP (also known as tamalin) as a gene robustly induced by ATRA treatment in P19 cells (16). We demonstrated that GRASP transcripts were induced within 2 hour of initiation of ATRA treatment, reaching a maximum of 18-fold induction at 24 hours, in both monolayer and aggregate cultures of P19 cells (16). Furthermore the use of a protein synthesis
inhibitor, cycloheximide, in the culturing medium, suggested that at least a part of this dose dependent induction of GRASP transcript by ATRA treatment, did not require protein synthesis (16).

The cDNA GRASP fragment isolated from the subtractive hybridisation screening was used to isolate the full-length clone from a randomly primed cDNA library. The full length mouse GRASP transcript spanned 1,978-base pair containing an open reading frame encoding a 392-amino acid protein with a predicted molecular mass of 42,382Da. A database search of GRASP sequence (NCBI Reference Sequence: NM_019518.3) with the mouse genomic sequence reveals that GRASP genomic locus located on Chromosome 15 spans ~9 kilobases and is made of 8 exons. A multi-sequence alignment of GRASP homologs across 44 vertebrate species reveals regions of high evolutionary conservation (both intronic and exonic), indicating a selection pressure for the conservation of this gene structure and sequence (Fig. 1.1). The only known functional paralog of GRASP is CASP (cytohesin associated scaffold protein). Though CASP (also known as Cybr or Cytip) is structurally and functionally similar to GRASP, it is only known to be expressed in tissues devoid of GRASP expression namely the hematopoietic system (17,18).
**GRASP structure and protein-protein interactions**

Based on comparison of predicted amino-acid sequence with protein databases, GRASP is predicted to have four principal protein-protein interacting motifs/domains (see Fig. 1.2). Reading from the N-terminus the principal domains of GRASP are -

(a) alanine/proline (A/P) rich domain - This domain harbors a putative Src homology 3 (SH3) domain binding epitope (PXXP) which binds to the hydrophobic pocket of the SH3 domain (16). SH3 domains are found in proteins of signaling pathways regulating the cytoskeleton, Ras protein, Src kinase and many others. This A/P rich domain also has been shown to harbor an immunoreceptor tyrosine-based activating motif (ITAM) which is doubly phosphorylated by Src family of kinases (19). In immune cells, paired tyrosine phosphorylation of ITAM in antigen receptors and their accessory proteins is essential for activation of intracellular signaling cascades (20,21).

(b) PDZ domain - The 95-kDa postsynaptic density protein (PSD-95)1/discs-large/ZO-1 (PDZ) domain is a key protein-binding domain comprised of 90 amino acid residues and interacts with a PDZ binding motif with the consensus sequences S/TXV/I/L (X is any amino acid) (22). In neurons, postsynaptic PDZ domain-containing scaffold proteins, PSD-95 and S-SCAM, interact with a number of membrane and cytoplasmic proteins, including NMDA and AMPA receptors, and are important in sub-cellular
trafficking of their partner proteins (23). Using full-length GRASP as bait in a yeast-2 hybrid experiment, numerous neuronal proteins were identified as protein-interaction partners of GRASP. Subsequently, using GST-pulldown assays the PDZ domain of GRASP has been shown to interact directly with receptors like group I metabotropic glutamate receptors (mGluR1 and 5), GABA (B2) receptors, kinase deficient neurotrophin receptors (TrKCT1) and with other scaffold proteins like SAP90/PSD-95-associated protein 3 (SAPAP3) and S-SCAM (24-26).

(c) Leucine rich domain - A yeast two-hybrid screen was employed with the leucine-rich region of GRASP (amino acids 180-257) as bait, to screen for potential protein interaction partners. This led to the isolation of a clone that coded for 1-156 amino acids of a nucleotide exchange factor, GRP1 (General receptor for phosphoinositide-1) from a mouse embryo cDNA library (16). The leucine rich region of GRASP was subsequently confirmed to directly interact with the N-terminal coil-region of GRP1 and an associated family member cytohesin-2 (ARNO), using GST-pulldown assays.

(d) PDZ binding motif - The carboxy-terminal PDZ binding motif of GRASP has been suggested to be involved in intra- and inter-molecular interaction PDZ-domain of GRASP (27). By means of such association, GRASP has been suggested to adopt an auto-inhibitory state which is relieved in the presence of competing PDZ-domain containing interaction partners such mGluRs and S-SCAM (27). In other words, the
self-association of GRASP leading to the auto-inhibitory state, has been suggested to function as a molecular sensor for the levels of its corresponding interaction partners.

The presence of these diverse protein-interaction domains and partners, led us to suggest the potential role of GRASP as a scaffold protein (16). Scaffold proteins as the name suggests function as molecular platforms and can play diverse roles, including trafficking, anchoring and clustering of membrane proteins; linking membrane proteins to their downstream signaling proteins; organizing multiple components into large signaling complexes; and interfacing with and regulating the dynamics of cytoskeletal structures (23).

Insights in the physiological role of GRASP from in vitro studies

Several reports over the last decade have suggested the role of GRASP, as a molecular scaffold involved in regulating intracellular trafficking pathways. The following discussion will first provide a brief introduction on the major intracellular trafficking pathways followed by our current understanding of GRASP as a regulator of these pathways.
Intracellular trafficking pathways: Eukaryotic cells differ fundamentally from their prokaryotic counter-parts by their possession of internal, membrane-bound, compartments. Intracellular communication within the compartmentalized eukaryotic cell necessitated the co-evolution of trafficking pathways (28). Three decades of research has identified many entry pathways into cells, which vary in the cargoes they transport and in the protein machinery that facilitates the endocytic process (29-31). Endocytosis is the process by which ligands, nutrients, proteins and lipids from the plasma-membrane are internalized into the cell interior. Another facet of intracellular trafficking is the inter-organellar delivery or exchange of proteins in the eukaryotic cell. Proteins synthesized de novo in the endoplasmic reticulum are either trafficked to the Golgi apparatus for post-translational modifications and/or directed into other organelles (or organellar membranes) such as peroxisomes and mitochondria.

The intracellular trafficking pathways can be broadly classified into clathrin-dependent (CD) and clathrin-independent (CI) pathways (31). Further sub-classification of these pathways has been suggested by Mayor et. al (31), based on dependence on dynamin, a GTPase involved in vesicle scission (see Fig. 1.3). Membrane proteins that are trafficked by the CD pathway harbor cytoplasmic signal peptides which can bind to clathrin and recruit a special class of adaptor proteins (30,32). In addition to these adaptor proteins and clathrin, there are numerous ancillary proteins that are involved in CD internalization including epsin, endophilin,
amphiphysin and dynamin (33). Immediately after endocytosis, the clathrin/AP coat is released and the vesicle then fuses with the “classical” early endosomal compartment that is defined by the presence Rab5, a small GTPase of the Rab family, and phosphatidylinositol 3-phosphate (PI3P). Once in the early endosome, membrane proteins, lipids and the fluid content of the endosome are sorted and transported either to the trans-Golgi network, to Rab7+ late endosomes and lysosomes for degradation, or into Rab11+ membrane carriers that recycle back to the PM (34). The CD pathway is important not only to facilitate the uptake of nutrients, such as iron-loaded transferrin and LDL into the cell, but also for the rapid internalization/recycling of most signaling receptors after ligand binding.

An alternative route for cargo molecules is the clathrin-independent (CI) pathway, which as the name suggests is categorized based on the absence of the clathrin-machinery. A distinguishing feature of the CI pathways is its dependence on lipids and high sensitivity to cholesterol binding drugs like filipin (30,32). The caveolae-mediated trafficking is the best understood dynamin-dependent clathrin-independent pathway (35). Caveolae are 50-80nm flask-shaped invaginations that are marked by the presence of a member of the caveolin protein family. These caveolar membrane fractions have been reported to be enriched in sphingolipids and cholesterol, signaling proteins and clusters of glycosyl phosphatidylinositol-anchored proteins (GPI-APs). A common feature of the dynamin and clathrin-independent
subclass is the involvement of small G-proteins like CDC42 or the ADP-ribosylation factor (Arf) family member, Arf6 (31). This pathway was first identified as a major route for the cellular uptake of cholera toxin B (CtxB), the plant protein ricin and the *Helicobacter pylori* vaculating toxin (VacA;36,37,38). Plasma membrane invaginations stimulated by CDC42 are relatively long and wide in surface, leading to large intake of the fluid phase in a single budding event. Therefore CDC42 has been suggested to be an important regulator of the pathways involved in fluid-uptake of the cell (31). Recent investigations are providing an ever expanding role for the other known small GTPase, Arf6, in the clathrin and dynamin independent trafficking pathways (39). Arf6 has been implicated in the internalization of numerous transmembrane receptors which do not harbor the classic clathrin-binding cytoplasmic motif such as major histocompatability complex-I (MHC-I) and CD59 (32). Elegant work by the Donaldson group (40) reveals that although Arf6-dependent CI pathways are clearly distinct, they also intersect/interact with the CD endocytic pathways (at least in HeLa cells). Arf6-dependent cargo such as MHC-I and CD59 once internalized eventually fuse with the classic clathrin and Rab5-positive early-endosomes, whereas signaling molecules like H-Ras can completely bypass the clathrin-dependent machinery (40,41). Once internalized, the Arf6-dependent cargo can be either routed for degradation or recycled back to the plasma membrane. Arf6-dependent recycling of membrane cargo specifically requires Rab11, Rab22 and Arf6.
GTPases and occurs through tubulo-vesicular membrane compartments which are distinct from compartments used by the CD pathway (32).

In summary, a myriad of intracellular trafficking pathways each specialized for a particular function, have presumably co-evolved by paralogous duplication events (28). The intricate differences and the dynamic cross-talk of the above discussed pathways is probably required for the plasticity observed during physiological events such as cytokinesis, cellular migration, wound healing and synapse formation (42).

Role of GRASP as a regulator of trafficking pathways: Several reports that have explored the physiological role of GRASP have suggested its role in intracellular trafficking pathways. First, ectopic expression of full-length GRASP or mutant GRASP lacking the leucine-zipper domain of GRASP in COS-7 cells has been shown to increase or decrease respectively, the cell-surface expression of mGluR1a (26). We have shown earlier that the leucine-zipper domain of GRASP interacts robustly with Grp1, and co-expression of GRASP and Grp1 led to a distinct increase in membrane association of Grp1 in HEK293 cells (16). Grp1 is a nucleotide exchange factor for the small G-protein, Arf6 (43,44). Second, treating neuronal culture with the growth factor neurotrophin led to an Arf6-dependent redistribution of endogenously expressed GRASP and TrkCT1 into punctate structures along the length of the neuron (25). The post-synaptic density (PSD) of neurons is a highly organized signal-processing
machinery composed of supramolecular protein complexes in neurons (45). Third, using the yeast-two hybrid screens, the PDZ domain of GRASP has been shown to interact with other PSD-resident proteins like Membrane associated guanylate kinase proteins (MAGUK) family of scaffolding proteins namely PSD-95, Mint2 and CASK (24). PSD-95 and CASK are the well known to play a role in trafficking of glutamate receptors and ion channels in both neuronal and non-neuronal cell types (46-50). Munc18-1-interacting protein 2 (Mint2) is an essential component of the machinery involved in protein transport from Golgi apparatus to cell surface in epithelial cells (51-54). Taken together, these studies suggest the potential role of GRASP in regulating intracellular trafficking of its interacting proteins, either directly through the Arf6-dependent pathway or indirectly through its interaction with other molecular-scaffold proteins. Finally, the intra- and inter-molecular interactions of GRASP have been suggested to form weak autoinhibitory states (27). mGluR1 can relieve the autoinhibitory confirmation by competing with the intrinsic PDZ-binding motif of GRASP (27). Thus the autoinhibitory confirmation of GRASP can be viewed as a molecular sensor of the physiological concentration of the interacting membrane receptors, in addition to its role in membrane trafficking pathways. In summary, investigative efforts performed in vitro suggest the potential role of GRASP in regulating cell-signaling and/or trafficking pathways, although the nuances of this regulation needs further investigation.
Insights in the physiological role of GRASP from in vivo studies

Expression profile of GRASP: A semi-quantitative reverse transcriptase PCR (RTPCR) analysis of GRASP transcript from murine tissues reveals that GRASP transcripts are highly expressed in the brain and to a lesser extent in heart and lungs (16). In situ hybridization of adult murine brain shows GRASP expression localized mainly in the telencephalic regions, including the olfactory bulb, cerebral cortex, hippocampus, anterior olfactory nucleus, olfactory tubercle, striatum, and nucleus accumbens (26). Within the hippocampus, intense signals were seen in pyramidal cells throughout CA1–CA3 regions as well as in granule cells of the dentate gyrus (26). In the olfactory bulb, prominent expression was observed in mitral cells and internal granular cells (26). In the cerebral cortex, signals were distributed in all but layer I, with the highest intensity at layers II–III (26). Furthermore, the expression of GRASP transcripts was barely detectable by northern blot analysis in embryonic brain tissue. However, GRASP is expressed post-natally, peaking in expression at 2 weeks after birth (24). This ontogeny in GRASP expression closely resembles the expression pattern seen in other major scaffold proteins, such as PSD95 and the Shank families of scaffold proteins (55).

Mice with germline deletion of GRASP (GRASP−/−): A recent article from the Nakanishi group (56) reports the generation of mice with germline deletion of
GRASP (GRASP\(^{-/-}\)). Using the Cre-Loxp strategy they targeted exons 5-7 of GRASP, which encodes the PDZ and leucine-zipper domains of GRASP. The authors report that under basal conditions GRASP\(^{-/-}\) mice do not exhibit any gross morphological, physical, behavioral or sexual defects. The expression levels of GRASP-interacting proteins, including mGluR1a, mGluR2, mGluR5, GABAB2 receptor, cytohesin-2, PSD-95, Mint2, and CASK, remained unchanged in striatum homogenates from GRASP\(^{-/-}\) mice as compared with their levels in WT mice. GRASP\(^{-/-}\) mice also showed no alteration in physical characteristics (body weight, appearance of fur and whiskers, and rectal temperature), in behavior involving sensory/motor functions (wire hang, grip strength, sensory-motor reflex, prepulse inhibition, and rotarod test), or in emotional behaviors (light/dark transition, elevated plus maze, social interaction, and forced swim tests). Furthermore, the authors report that behavioral tests examining the learning and memory faculties (namely radial maze test, contextual and cued fear conditioning test) revealed no obvious deficits in the GRASP\(^{-/-}\) mice.

However, when the authors challenged the GRASP\(^{-/-}\) mice by chemical stress, such as by administration of morphine or cocaine, GRASP\(^{-/-}\) mice exhibited numerous altered behavioral responses. First, acute subcutaneous morphine-induced hyperlocomotion was found to be attenuated in GRASP\(^{-/-}\) mice. Second, GRASP\(^{-/-}\) mice exhibited reduced analgesia following morphine administration as compared to wild-type mice (56). Finally, GRASP\(^{-/-}\) mice exhibit altered conditional place
preference (CPP) to repeated morphine administration. CPP is a behavioral model employed to study relapse in drug addiction (57). These results indicate that GRASP deficiency in vivo selectively impairs the adaptive neural plasticity involved in reinforcement and addiction to drugs of abuse. However, the role of the abundant expression of GRASP in brain structures not directly implicated in drugs of abuse and/or the role of GRASP in tissues other than brain still remains largely elusive.
**Research Objectives**

The goal of this study was to investigate and understand the physiological role of GRASP by *in vitro* and *in vivo* approaches in a mammalian system.

In, Chapter 2 we investigated the precise role of GRASP as a trafficking molecule. We investigated the role of GRASP as a regulator of the ADP-ribosylation factor 6 (Arf6)-dependent membrane trafficking pathway. Using truncation analysis, we determined the precise regions of GRASP involved in sub-cellular localization of the protein, and using cell-culture based assays we ascertained the role of GRASP in the Arf6-trafficking pathway.

In Chapter 3, we investigated the role of GRASP *in vivo* using the murine skin as a model system. Towards this goal, we generated a mouse with germline deletion of GRASP (GRASP−/−). Under basal conditions GRASP−/− mice exhibit no gross behavioral, morphological or sexual deficits. However, we have detected expression and identified a role of GRASP in the adult murine skin. Using exposure to ultraviolet-B (UVB) rays as a proliferative paradigm, we compared the response of GRASP−/− mice to littermate wild-type mice and found altered response in the GRASP−/− mice. Our results from a combination of histological, immunohistochemical and biochemical analysis suggest the potential role of GRASP as regulator of p53-mediated apoptotic response in the murine skin following exposure to UVB rays.
References


**Figure 1.1. Evolutionary conservation of the GRASP locus**

A schematic representation of the human GRASP genomic locus (purple boxes are exons and arrow are introns) with corresponding depiction of evolutionary conservation across 44 vertebrate species (black vertical lines) as measured by *phastcons* algorithm. PhastCons is a hidden Markov model-based method that estimates the probability that each nucleotide belongs to a conserved element, based on the multiple alignment (obtained from UCSC genome browser (58)). The figure also shows alignment of human GRASP genome locus with commonly used animal model systems (each green vertical bars corresponds to a single conserved base pair at a given loci).
Figure 1.1
Figure 1.2. A schematic representation of the GRASP protein domains and interaction partners.

A schematic of the GRASP protein structure showing the different domains and corresponding amino-acid (top). Green arrows represents interaction of the GRASP domain and indicated proteins.
Figure 1.3. Classification of intracellular trafficking pathways. A schematic representation of the classification model (adapted from Mayor et.al (31)). Examples of cargoes or proteins that are trafficked by the different pathways are italicized in the above schematic.
CHAPTER 2: GRASP regulates the Arf6-dependent membrane trafficking pathway

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Abstract

Grp1-associated scaffold protein (GRASP) is encoded by a retinoic acid-induced gene. GRASP interacts with Grp1 (general receptor for phosphoinositides 1; cytohesin 3), which catalyzes nucleotide exchange on and activation of ADP-ribosylation factor-6 (Arf6), a low molecular weight GTPase that regulates key aspects of endocytic recycling pathways. Over-expressed GRASP was found to accumulate in the juxtanuclear endocytic recycling compartment (ERC) in which it co-localized with a constitutively inactive mutant of Arf6, in a manner that was reversed by expression of wild-type Grp1. Co-expression of GRASP and Grp1 promoted membrane ruffling, a cellular hallmark of Arf6 activation. GRASP accumulation in ERC was found to block recycling of the major histocompatibility complex-I (MHC-I), which is trafficked by the Arf6-dependent pathway. In contrast, expression of GRASP had no effect on the recycling of transferrin receptors, which are trafficked by a clathrin-dependent pathway. Finally, we found that agonist-driven internalization of M3-muscarinic acetylcholine receptors occurred at least partially via the Arf6-dependent pathway in a manner that was influenced by over-expression of GRASP. These findings suggest the GRASP regulates the non-clathrin/Arf6-dependent, plasma membrane recycling and signaling pathways.
**Introduction**

GRASP (also known as Tamalin, Ref.1) was identified in our laboratory as an all-trans retinoic acid-induced gene in P19 cells (2). GRASP has been shown to interact with numerous neuronal proteins (3) and has been suggested to play a role in the intracellular trafficking of receptors, such as group 1 metabotropic glutamate receptors (mGluRs; 1), and a kinase-deficient isoform of neurotropin-3 receptor (TrKCT1; 4). Previous work from this laboratory has also demonstrated that GRASP interacts with cytohesin family members Grp1 and Arf nucleotide-binding site opener (ARNO; 2). Grp1 and ARNO, are guanine nucleotide exchange factors (GEFs) for small G proteins of the Arf family.

Arf proteins, like other G-proteins, cycle between inactive, GDP-bound, and active, GTP-bound conformations, which interact differentially with various classes of effector proteins (5-7). The majority of Arf effector proteins that have been identified generally interact with more than one Arf protein in *in-vitro* binding assays. However, it seems that Arf proteins have distinct localization characteristics, and this compartmentalization may be an important determinant that imparts unique functions to individual members of the Arf family (8,9). Arf proteins are divided into three classes based on phylogenetic analysis and size (9). Class I Arfs, which include Arf 1-3, function in vesicular trafficking pathways in the Golgi apparatus, while little is known about the function of class II Arfs (Arf 4-5), and the sole class III Arf, Arf6, localizes to the plasma
membrane and endosomal compartments. Arf6 is known to regulate key aspects of vesicular trafficking, actin reorganization and cellular migration (5,9,10).

Arf6 has also been described to regulate a novel trafficking pathway, utilized by membrane proteins that lack cytoplasmic, clathrin-binding motifs, such as major histocompatibility complex I (MHC-I; 11). The list of cargo molecules that traverse through this pathway have now expanded to include β-integrins, cytokine receptors, and G-protein coupled receptors such as M2-muscarinic acetylcholine receptor (11-15).

Arf6 plays a critical role in mouse development as germline disruption of the Arf6 locus results in embryonic lethality accompanied by severe defects of liver development (16). Moreover, Arf6 is required for cytokinesis in Drosophila spermatocytes, possibly by regulating formation of the cleavage furrow (17).

The mechanistic details of the Arf6 pathway have only been recently investigated and its role in cellular trafficking is at least partially mediated by its effects on phospholipid metabolism (9,18-21). However, the nuances of this pathway require further investigation, and the use of constitutively active (Arf6 Q67L; GTP-bound), and constitutively inactive (Arf6 T27N, GDP-bound; Arf6 N122I, nucleotide-free) point
mutants has been helpful in delineating the role of Arf6 in these processes. Arf6 Q67L localizes within invaginations of the plasma membrane, and appears to be responsible for the generation of membrane ruffles, and increased internalization of membrane proteins (19,22-24). In contrast, Arf6 T27N accumulates in large aggregates of tubulovesicular structures and its expression reduces the recycling of membrane proteins (11,15,25,26). Arf6 N122I is a lesser known point mutant of Arf6, which mimics functional and localization characteristics of Arf6 T27N variant (19,27,28).

Small G proteins of the Rab family have been used for subcellular identification of the various endosomal compartments. Canonical markers include Rab5 for early endosomes, Rab7 for late endosomes and Rab4 and Rab11 for labeling recycling endosomes in the cell (29). Arf6 T27N has been demonstrated recently to reside predominantly in Rab22- and Rab11-positive recycling endosomal compartments in HeLa cells (15,30). These results have led to the hypothesis that nucleotide exchange and activation of Arf6 occurs on the juxtanuclear endocytic recycling compartment (ERC; 11,23,31). To complete the cycle, GTP hydrolysis by Arf6 at the plasma membrane appears to be required for internalization and its subsequent localization to the ERC (9,31-33).
Nucleotide exchange on and GTP hydrolysis by Arf6 proteins, are facilitated by numerous GEFs and GTPase activating proteins (GAPs), respectively (7,34,35). The cytohesin family of Arf6-GEFs are insensitive to the fungal toxin brefeldin A, and includes cytohesin 1 (PSCD1), ARNO (PSCD2), Grp1 (ARNO3; PSCD3;cytohesin 3), and cytohesin 4 (PSCD4; 7). The cytohesins share a common, five-domain structure consisting of an amino terminal coiled-coil (CC) domain, followed by a Sec7 domain that is responsible for GEF activity, a pleckstrin homology (PH) domain that binds phosphoinositides, and a polybasic region at the carboxyl terminus (34). We have previously shown that the Grp1 CC-domain directly interacts with GRASP (2), while the PH domain of Grp1 has been shown to have very high selectivity for phophatidyl 3,4,5-triphosphate (PIP3) species of phosphoinositides (36).

In this study we have identified GRASP as a regulator of the Arf6-dependent trafficking pathway. GRASP, when expressed in HeLa cells, localizes in the ERC and along the plasma membrane. Results of the present study suggest that GRASP recruits Grp1 to endosomal structures and likely facilitates the activation of Arf6, leading to cortical cytoskeletal rearrangements. We also show that GRASP regulates Arf6-dependent receptor internalization and recycling in HeLa cells. Thus, GRASP is a component of the carefully orchestrated Arf6-dependent trafficking and signaling pathways.
Materials and Methods

Cell Culture: HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 g/ml streptomycin, and 100 U/ml penicillin. Glass coverslips were coated with 0.02% gelatin as per standard protocol. Cells were seeded in a 12-well plates and transfected after they reached 80-90% confluency with Lipofectamine 2000 reagent (Invitrogen, USA) as per manufactures protocols.

Antibodies, Plasmids and Transient Transfection: Arf6 expression vectors were kindly provided by Dr. K. Nakayama (University of Tsukuba, Ibaraki, Japan; Ref. 37). The Grp1 construct (36) was kindly provided by Drs. Michael Czech and Jes Klarlund (University of Massachusetts Medical Center). The Flag-M3-muscarinic acetylcholine receptor (Flag-M3AchR) construct was kindly provided by Dr. Neil Nathanson (University of Washington; Ref. 38). The GFP tagged Rab4, Rab5, Rab7 and Rab11 constructs (39) were kindly provided by Drs. Volker Vogt (Cornell University) and Mark Johnson (University of Missouri). All constructs were subsequently subcloned into pCDNA3.1(+) (Invitrogen, USA) or into pEGFP-C1 (Clontech, USA) vectors using standard molecular biology protocols. Alexa-546-conjugated transferrin (546-Tfr) and Alexa-350-conjugated phalloidin were purchased from Molecular Probes (Invitrogen, USA). Secondary antibodies conjugated to Cy3 fluorophores was purchased from Jackson
Immunoresearch (USA). Other antibodies were goat polyclonal antibody raised against purified GST-GRASP (Bethyl Laboratories; Ref. 2), mouse anti-HA (12CA5; Roche Applied Sciences, USA), mouse anti-Myc (Ab-1; OP10, Oncogene Research Products), goat anti-Grp1(N-17; sc 9730; Santa Cruz Biotech), rat anti-GFP (kindly provided by Drs. Chrissa Kioussi and Michael Gross, OSU) and mouse anti-human MHC-I (#311402, BioLegend, USA).

Fluorescence Microscopy: Approximately 6-24 hrs. following transfection, HeLa cells were fixed in 4% paraformaldehyde (PFA) for 10 min. and processed for immunofluorescence microscopy. Until otherwise mentioned, all images were captured using a ZEISS LSM 510 confocal microscope with 63x Plan Apo 1.3 NA objective. These images were then processed using Photoshop CS4 (Adobe Systems, Inc., USA). For GRASP endosomal scoring assays, a minimum of 50 cells were counted and scored for the accumulation of GRASP$^+$ endosomes. Only cells that exhibited a large accumulation of GRASP in the endosomal compartment were scored as positive. All experiments were conducted in triplicates, and then verified in 3-5 independent transfections.

Receptor internalization and recycling assays: MHC-I recycling assays were conducted 16 hrs. after transfection. Cells were chilled to 0-4°C for 60 min. to stop all endocytic activity, followed by incubation in a mouse anti-human MHC-I antibody (7.5 µg/ml) for
60 min. at 0-4°C. Surface labeled, MHC-I receptors where allowed to internalize at 37°C in complete medium for 1 hr., followed by stripping of the uninternalized surface receptors by multiple washes in a mild acid-stripping buffer (0.5% acetic acid, 0.5 M NaCl, pH 3.0), followed by three washes of ice-cold 1x PBS. The cells were then chased for indicated time intervals in prewarmed, complete medium at 37°C, followed by a second wash with the stripping buffer, and fixation with 4% PFA for 10 min. Samples were processed for indirect-immunofluorescence to visualize receptors that failed to be recycled (and/or degraded).

The continuous transferrin recycling assay was adapted from previous work (40). Briefly, cells were serum starved in internalization media (DMEM containing 2 mg/ml bovine serum albumin) for 60 min. at 37°C. Subsequently, these cells where incubated with 10 µg/ml Alexa 546-Transferrin (Tfr-546) in prewarmed internalization media for 60 min. These cells where then rapidly washed with prewarmed recycling media (complete medium with 100-fold excess of unlabelled transferrin and 100 µm deferoxamine mesylate to prevent re-internalization of the receptors) and chased in this medium for indicated time intervals. At the end of indicated times, recycled transferrin receptors present on the cell-surface were stripped by multiple washes with a mild acid-stripping buffer (0.5% acetic acid, 0.5 M NaCl, pH 3.0), fixed with 4% PFA for 10 min. and processed for fluorescence microscopy to visualize receptors that failed to be recycled (and/or degraded).
The M3-muscarinic acetylcholine receptor (M3AchR) internalization assay was conducted 16 hours after transfection. Cells were chilled to 0-4°C for 60 min. to stop all endocytic activity followed by a incubation with mouse anti-HA antibody (4 µg/ml of complete medium) for 60 minutes at 0-4°C. Next, the cells were rinsed briefly with ice-cold DMEM containing 10% FBS (complete medium), and incubated at 37°C with prewarmed complete medium in presence of 1mM carbachol for indicated intervals of time. Then the cells were chilled again to 0-4°C to by rinsing three-times with ice-cold 1x PBS followed by fixation with 4% PFA for 10 min. These cells were then processed for indirect-immunofluorescence microscopy in the absence of any permeablizing agents to allow detection of M3AchR present only at the cell-surface.

Quantitation of cellular signal intensity: The fluorescence signal intensity of the receptors at the cell-surface was measured according to published techniques (14,40). Briefly, 30 cells were randomly selected and imaged using a 510 LSM confocal microscope (Zeiss) with an oil 40x plan Apo objective. The pinhole was completely open, and all the images were taken with identical acquisition parameters, those previously optimized for the fluorescent signals to be in the dynamic range. Under these conditions the total fluorescence measured is proportional to the amount of receptors present per cell. The total fluorescence of the individual cell was measured by manually demarcating the edges and using the ‘measurement tool’ in Photoshop CS4 to obtain the integrated pixel density which was expressed in arbitrary units/cell.
Coimmunoprecipitation analyses: These studies were conducted as described previously (2).

Statistical analyses: A standard "t-test" was performed to determine if the difference between means of two groups were statistically significant. For comparison of three or more groups, values from control and experimental groups were compared using a two-way, repeated measures ANOVA followed by Bonferroni post hoc analyses. All statistical analyzes was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., USA)
Results

GRASP co-localizes with a constitutively inactive point mutant of Arf6 in a Rab11$^+$ recycling endosomal compartment.

We have previously reported that GRASP localized predominantly to the plasma membrane of transfected HEK293 cells (2). In addition, we have consistently observed endogenous GRASP staining in perinuclear, endosomal-like structures, in HeLa cells (Fig. 2.S1). For the advantages of a strong fluorescent signal and enhanced resolution, we decided to use ectopic expression of either Myc-GRASP or GFP-GRASP to visualize GRASP in all further experiments. We also observed that HeLa cells transfected with expression vectors expressing either GFP- or Myc-GRASP localized similarly to endogenous GRASP, i.e., in juxtanuclear, tubular, endosomal like structures and along the plasma membrane (Figs. 2.1A and 2.S2). Additionally, over 20% of these transfected cells exhibited enrichment of GRASP in punctate, endosomal-like aggregates (Fig. 2.1B), and this proportion appeared to be a function of GRASP expression levels (Fig. 2.1C). Arf6 T27N, a constitutively inactive point mutant of Arf6 is known to localize within a sub-population of the Rab11$^+$ ERC (ERC; 6,15,23,30,41). Arf6 N122I was also found to localize predominantly in Rab4- and Rab11-positive recycling endosomes, and did not appear to co-localize with either Rab5 (early endosomal marker) or Rab7 (late endosomal marker; Fig. 2.2A-D). The punctate aggregation of over-expressed GRASP resembled
that of the constitutively inactive Arf6 point mutants, Arf6 T27N and Arf6 N122I, and localized predominantly in Rab11-positive recycling endosomes (Fig. 2.2 E-H).

We conducted studies to determine if the localization of GRASP was similar to and/or affected by the co-expression of wild-type Arf6, Arf6 Q67L or Arf6 N122I. The punctate localization of GRASP was not coincident with that of either wild-type Arf6 (Fig. 2.2I) or Arf6 Q67L (Fig. 2.2J), but completely co-localized with Arf6 N122I (Fig. 2.2K). Furthermore, co-expression of Arf6 N122I with GRASP dramatically increased the proportion of cells exhibiting punctate localization of GRASP, from 22% to 79%, while no such effect was observed with either wild-type Arf6 or Arf6 Q67L (Fig. 2.2L). Furthermore, we observed a partial overlap of endogenous GRASP with wild-type Arf6-positive compartments and the enrichment of endogenous GRASP in Arf6 N122I-positive endosomal structures, when expressed in HeLa cells (Fig. 2.S3). Collectively, these results suggest that over-expression of GRASP mimics the localization pattern of the constitutively inactive Arf6 point mutants, both of which appear to localize predominantly in Rab11+ recycling endosomes.

Arf6 is known to regulate a distinct, clathrin-independent pathway of plasma membrane recycling, which is blocked by the expression of constitutively inactive Arf6 point-mutants. The data above suggest that the propensity for GRASP to localize in ERC
is increased by the expression of Arf6 N122I, indicating that GRASP might be involved in the Arf6-dependent pathway of plasma membrane recycling under physiological conditions.

**Co-expression of GRP1 prevents accumulation of GRASP in the endosomal compartment.**

We hypothesized that over-expression of GRASP may titrate cellular levels of Arf6-GEF(s), leading to a relative scarcity of free Arf6-GEF(s), and compromised Arf6 function, which we would observe as intense localization of GRASP in ERC. If true, co-expression of an Arf6-GEF should reduce GRASP localization in the endosomal compartment. To test this hypothesis, HeLa cells were transfected with expression vectors encoding GFP-GRASP in the presence and absence of wild-type Grp1-HA, an Arf6-GEF, which interacts with GRASP (2). All transfections were normalized with empty vector to minimize artifactual interpretations. We observed that the co-expression of Grp1 and GRASP resulted in a striking increase in membrane ruffling, in which both GRASP and Grp1 localized (Fig. 2.3A-E). In contrast, membrane ruffles, which are associated with activated Arf6, were not observed in HeLa cells individually transfected with GRASP or Grp1 expression vectors (Fig. 2.S4). Moreover, co-transfection of Grp1 resulted in a dramatic reduction of transfected cells exhibiting punctate localization of GRASP (55% to 25%; Fig. 2.3E). The ability of Grp1 to reduce GRASP aggregation
in ERC required both the coiled-coil (CC) domain of Grp1, which interacts directly with GRASP (2), and the catalytic Sec7 domain (Grp1 Coil-Sec7; Fig. 2.4). The isolated CC domain of Grp1 (Grp1 Coil) did not affect localization of GRASP in ERC in any detectable manner, and the combined Sec7 and PH domains nearly doubled the fraction of transfected cells exhibiting punctate localization of GRASP (Fig. 2.4). However, none of the Grp1 truncation mutants, including Grp1 Coil-Sec7, promoted membrane ruffling with GRASP in a manner observed for wild-type Grp1 (data not shown). Thus, the coiled-coil region and catalytic activity of Grp1 were found to be necessary to reduce GRASP accumulation in ERC, while the generation of membrane ruffles required all the known functional domains of Grp1. To confirm these findings, we generated catalytically inactive (Grp1 E161K) and PIP3 binding-deficient (Grp1 R284D) point mutants of Grp1 based on homology with the corresponding mutants of ARNO, as described elsewhere (42-45). These point mutants interacted with GRASP in a manner that was indistinguishable from that of wild-type Grp1 (data not shown) but neither reduced the aggregation of GRASP in ERC (Fig. 2.4). All Grp1 mutants co-transfected with GRASP were expressed at levels equivalent to that of wild-type Grp1 and GRASP (Fig. 2.S5).

Considered all together, these data suggest that GRASP either recruits or is recruited by Grp1 to recycling endosomes, within which Grp1 may stimulate guanine nucleotide exchange and activation of Arf6. However, over-expression of GRASP leads to accumulation of GRASP in ERC, perhaps by sequestering soluble pools of Grp1. This
blockade was reversed by co-transfecting catalytically active forms of Grp1 that are competent to interact with GRASP.

**GRASP localizes in ERC independent of Grp1.**

In order to define the region(s) of GRASP responsible for endosomal targeting, we examined the subcellular distribution of several GRASP mutants by fluorescence microscopy and found that endosomal localization required the leucine-rich domain (amino acids 180-257) of GRASP (data not shown), which also interacts with the Arf6 GEFs, Grp1 and ARNO (2). These findings suggest that interaction with cytohesins may be necessary for endosomal targeting of GRASP. Alternatively, endosomal targeting and interaction with GEFs of the cytohesin family may be separable functions of the leucine-rich region of GRASP. A series of deletion mutants was used to distinguish between these two possibilities. The expression levels of all GRASP mutants were similar as verified by immunoblotting (see Fig. 2.5).

First, we sought to identify the regions within the leucine-rich domain of GRASP that specified interaction with Grp1 in HeLa cells. Grp1 co-immunoprecipitated with GFP-GRASP, GFP-GRASP 180-257 and 180-230 (Fig. 2.5A, lanes 8-10). In contrast, Grp1 did not co-immunoprecipitate with GFP-GRASP 231-392 or 208-392 (Fig. 2.5A,
lanes 11 and 12 respectively). These results indicate that amino acids spanning 180-207 of GRASP are critical for interacting with GRP1. However, a construct encoding GFP-GRASP 191-207 did not co-immunoprecipitate with GRP1 (not shown). Thus, the GRP1 interaction domain of GRASP appears extensive and likely spans amino acids 180-230 residues of the leucine-rich domain.

We next determined the region of the leucine-rich domain of GRASP that was responsible for targeting GRASP to endosomes. We transfected HeLa cells with expression constructs encoding GRASP truncation mutants, GFP-GRASP 180-257, 180-230, 231-392 or 208-392. We observed that the propensity with which GFP-GRASP 180-257 accumulated in ERC was similar to that of wild-type GRASP (~55%; Figs. 2.5B and 2.S7A), while the GFP-GRASP 180-230, exhibited reduced ability to form punctate endosomal aggregates (~30%; Figs. 2.5B and 2.S7B). We next analyzed truncation mutants that failed to interact with GRP1 (see Fig. 2.5B). GFP-GRASP 231-392, localized in ERC but lost the ability to aggregate in these ERC structures efficiently (Figs. 2.5B and 2.S7C), while GFP-GRASP 208-392, exhibited localization in Arf6 N122I-positive ERC in a manner indistinguishable from wild-type GRASP (54%; Figs. 2.5B,C). Intrestingly, co-expression of wild-type Grp1 leads to redistribution of GRASP mutants GFP-GRASP 180-257 and 180-231 to the plama-membrane and into the nucleus(Fig. 2.S8). However, co-expression of wild-type Grp1 did not prevent accumulation of GFP-GRASP 208-392 in the ERC (Fig. 2.5D). These results define two
separable regions within the leucine-rich domain of GRASP, one conferring interaction with Grp1, and the other responsible for endosomal targeting of GRASP. GRASP residues 180-230 were sufficient to interact with Grp1, while GRASP truncation mutants GFP-GRASP 208-392 and 231-392 were unable to interact with Grp1 and yet successfully localized in endosomal structures. This revealed that endosomal localization of GRASP was independent of its binding with Grp1 while the release of GRASP from ERC required its interaction with Grp1 (see Fig. 2.5D). Further, GRASP residues between amino acids 208 and 230 of GRASP seem to harbor a minimal endosomal localization signal, without which efficient targeting of GRASP to endosomes was lost (see Fig. 2.5B,C and Fig. 2.57C). Amino acids flanking this minimal endosomal targeting sequence, i.e., amino acids 231-392, also affected the frequency with which GRASP was targeted to the endosomes. Addition of residues 231-257 to GRASP 180-230 resulted in a two-fold increase in endosomal targeting (see Fig. 2.5B). Therefore, in addition to the minimal endosomal localization signal of GRASP (residues 208-230), and the Grp1-interacting residues of leucine-rich domain, other domains of GRASP and/or unknown factors interacting with those domains may collectively fine tune the subcellular localization characteristics of GRASP in HeLa cells. In summary, these results demonstrate the presence of an endogenous endosomal targeting signal within GRASP that is separable from the Grp1-interaction residues in the leucine-rich domain of GRASP.
GRASP regulates the Arf6-dependent recycling of membrane proteins

The GTP-binding deficient mutant of Arf6, Arf6 T27N, has been reported to block plasma membrane recycling of Arf6-dependent cargo, such as interleukin-2α receptor (IL-2αR) and major histocompatibility complex I (MHC-I), but not the recycling of clathrin-dependent cargo, such as transferrin receptors (TfR; 11,24,26,31). Based on the similarities between localization characteristics of constitutively inactive Arf6 point mutants (Arf6 T27N and N122I; see above), and the punctuate aggregates formed in cells expressing high levels of GRASP, we hypothesized that over-expression of GRASP would phenocopy Arf6 T27N, with respect to the recycling of MHC-I receptors. To determine this we carried out a MHC-I recycling assay and a continuous TfR recycling assay in cells transfected with vectors encoding either GFP or GFP-GRASP. A fluorescent signal corresponding to internalized MHC-I receptors was lost rapidly in the absence of transfected GRASP (not shown) or in cells expressing low levels of GRASP (compare Figs. 2.7A-C and D-F), most likely indicating that these receptors had been recycled back to the cell surface (and/or degraded). In contrast, internalized MHC-I receptors co-localized with the punctate aggregates of GRASP (Fig. 2.7G-I) in cells expressing high levels of GRASP, and these MHC-I receptors were unable to recycle out of the cell efficiently (compare Figs. 2.7G-I and J-L). Internalized MHC-I receptors are known to recycle back to the cell-surface rapidly in HeLa cells (26). In agreement, we found that about 50% of the internalized receptor population was either recycled back to
the plasma membrane and/or degraded after a 10 min. chase in cells expressing either GFP or low levels of GRASP. In contrast, appreciable amounts of internalized MHC-I receptors remained in ERC-like structures even after a 60 min. chase in cells expressing high levels of GRASP (~70%; Fig. 2.7M).

High levels of GRASP did not block recycling of clathrin-dependent cargo, such as TfrR. We observed that internalized TfrR partially co-localized with GRASP-positive endosomal structures (Fig. 2.7A-C and G-I). However, internalized TfrRs were recycled back to the plasma membrane (and/or degraded) equivalently and efficiently in cells expressing both low and high levels of GRASP (compare Fig. 2.7A-C and D-F; and Fig. 2.7G-I and J-L respectively; Fig. 2.7M). These findings suggest that GRASP, like Arf6 N122I and T27N, does not likely play a role in TfrR recycling.

Collectively, our data suggest that high levels of GRASP recapitulate the activity of Arf6 T27N in blocking the release of MHC-I receptor, but not that of TfrR, from ERC in HeLa cells, suggesting a potential role of GRASP in the Arf6-dependent pathway.
Activated Arf6 triggers rapid internalization of M3-muscarinic acetylcholine receptor in Hela cells.

The third intracellular loop (i3-loop) of M3-muscarinic acetylcholine receptor (M3AchR) has been thought to be critical for its agonist-mediated, clathrin-dependent, endocytosis (46). However, a recent study in HeLa cells suggests that mutant M3AchR lacking the i3-loop exhibits agonist-dependent internalization by a clathrin-independent pathway (14). Furthermore, several reports have linked Arf6 and M3AchR downstream signaling events in HeLa cells (47,48). These results indicate that agonist-dependent internalization of M3AchR could potentially utilize both the clathrin-dependent and -independent mechanisms of endocytosis, the latter of which may involve Arf6. We investigated this possibility by performing a M3AchR internalization assay in cells co-transfected with expression vectors encoding HA-M3AchR and either wild-type GFP-Arf6, GFP-Arf6 point mutants, or GFP-GRASP, and treated with 1mM carbachol, a muscarinic receptor agonist. HA-M3AchR did not appear to undergo rapid (within 30 min.), carbachol-induced internalization (Fig. 2.8). However, cells co-expressing HA-M3AchR and Arf6 Q67L exhibited a ~50% loss of cell-surface of M3AchR, within 30 min. of carbachol treatment (Fig. 2.8). As GRASP over-expression mimicked the effects of constitutively inactive point mutant of Arf6 (Arf6 T27N), we were surprised to find that HA-M3AchR was internalized rapidly in cells expressing both low and high levels of GRASP (40-50% loss in cell surface of M3AchR within 30 min. of carbachol treatment; Fig. 2.8). In this respect, over-expressed GRASP appeared to phenocopy the effects of
Arf6 Q67L, a constitutively active point mutant of Arf6. After 60 and 90 min. of carbachol treatment, substantial M3AchR receptor internalization was observed and this appeared unaffected by any of the co-transfected GRASP or Arf6 constructs (Fig. 2.8).

These results suggest that M3AchR exhibits an initial delay in agonist-dependent receptor endocytosis, and this delay was shortened by increasing the pool of activated Arf6 (Arf6 Q67L). In this regard, it is conceivable that over-expression of GRASP similarly increased the active pool of Arf6 in HeLa cells, thus, facilitating the burst phase of agonist-induced M3AchR internalization. Taken together, our results indicate that internalization of M3AchR in HeLa cells is influenced by the active pool of Arf6, which might be in concert with or parallel to the clathrin-dependent internalization of M3AchR.
Discussion

GRASP has been shown to facilitate the cell-surface expression of group 1 metabotropic glutamate receptors (mGluRs; 1) and kinase-deficient neurotrophin receptor (TrKCT1; 4); however, in both cases the precise mechanisms for these effects remain unknown. In the present study we established GRASP as a novel regulator of the Arf6-dependent, receptor trafficking pathway. GRASP was shown to localize in juxtanuclear the ERC and GRASP•Grp1-mediated regulation of the Arf6 pathway was crucial for Arf6-dependent recycling of receptors from the this compartment.

In HeLa cells, both endogenously and exogenously expressed GRASP (at low levels) resulted localization in endosomal-like compartments and along the plasma membrane (see Figs. 2.1, 2.2, and 2.S1). However, when expressed exogenously at higher levels, GRASP aggregated selectively in the endosomal compartment and its localization characteristics were reminiscent of the constitutively inactive Arf6 T27N mutant (27,41). In contrast to a previous report (37), we found little or no co-localization of Arf6 N122I with late-endosomal marker Rab7. Rather, we consistently found that the subcellular localization of Arf6 N122I mimicked that of Arf6 T27N, predominantly in Rab11- and Rab4-positive, juxtanuclear endosomal recycling compartments (ERC; see Fig. 2.2A-D). Furthermore, co-expression of Arf6 N122I with low levels of GRASP resulted in
enhanced accumulation of GRASP in ERC, indicating that GRASP and Arf6 likely function in similar plasma membrane trafficking pathway(s).

Our studies with GRASP truncation mutants suggested that GRASP may harbor an endogenous endosomal localization signal(s) and likely recruits Grp1 to ERC. Furthermore, we hypothesized that GRASP aggregation in these ERC structures is due to sequestration of freely soluble Grp1 in the cytosolic fractions, thereby preventing the entry of Grp1 in the endosomal trafficking pathway. This hypothesis was supported by our finding that co-expression of Grp1 dramatically reduced the accumulation of GRASP in ERC (see Fig. 2.3E). However, our results cannot rule out an alternative hypothesis in which a third unknown protein competes with Grp1, to bind to the coil region of GRASP. Regardless of the nuances of this mechanism, GRASP definitively can localize to the ERC independent of Grp1 and requires Grp1 to prevent its accumulation in the ERC (see Figs. 2.3, 2.4, 2.5, 2.8, and 2.9). Co-expression of Grp1 with GRASP also led to a large increase in cortical membrane ruffling and GRASP and Grp1 co-localized in these structures along the plasma membrane. However, neither the catalytically inactive Grp1 nor a Grp1 mutant lacking the GRASP-interaction domain affected the aggregation of GRASP in ERC and/or resulted in the generation of membrane ruffles (see Figs. 2.4, 2.8, and data not shown). Membrane ruffling is a well-documented, downstream phenotype of Arf6 activation (5,9,10,49). These findings therefore suggest the existence of a GRASP-Grp1-Arf6 pathway which minimally involves: (1) GRASP recruitment of
Grp1 to ERC (2) Grp1-catalyzed nucleotide exchange on Arf6, leading to activation of the latter, and (3) translocation of the GRASP•Grp1 complex into membrane ruffles.

Two confounding results arose from our studies involving the Grp1 mutants. First, co-expression of Grp1-mutant, Grp1 Sec7-PH, lacking the GRASP-interaction domain increased the frequency of cells in which GRASP localized as aggregates in ERC (see Fig. 2.4). This finding may indicate that Grp1, independent of its interaction with GRASP, may influence the occurrence of GRASP+ endosomes. Second, the Grp1 mutant lacking a PH domain, Grp1 Coil-Sec7, prevented aggregation of GRASP in ERC, while the point mutant that is defective for PIP3 binding, Grp1 R284D, did not (see Fig. 2.4). Based on the studies from Cohen and coworkers (33), it is clear that the interaction of the Grp1 PH domain with Arf6 facilitates efficient targeting of activated Arf6 to cortical structures. We observed that the Grp1 Sec7-PH fragment localized along the plasma membrane (data not shown), and it is conceivable that this mutant may facilitate cortical localization of basally activated Arf6, leading to increased internalization of GRASP-positive endosomes, and an increased proportion of cells exhibiting GRASP-positive ERC. Furthermore, in addition to the PIP3 sensing abilities, the PH domain of Grp1 was recently shown to inhibit cell spreading when overexpressed in COS-7 cells (44). In contrast, other mutants of the Grp1 PH domain have been shown to bind PIP3 in vitro but were unable to inhibit cell spreading in COS-7 cells (44), suggesting the necessity of a putative protein partner that is necessary for the dominant negative activity of the Grp1
PH domain. Such putative protein partners might also provide the dominant negative effect of R284D, which prevents release of GRASP from ERC.

Under the transfection conditions used in our study, cells individually expressing Grp1 or GRASP did not generate membrane ruffles (Figs. 2.2D, 2.S4 and data not shown). However, cells co-expressing of GRASP and wild-type Grp1 exhibited a dramatic increase in the incidence of membrane ruffling and both GRASP and Grp1 co-localized in these protrusive structures along the plasma membrane (see Fig. 2.3A-D). The physical interaction of GRASP and Grp1, along with the catalytic and PIP3-sensing activities of the latter were found to be collectively required for generation of membrane ruffles (data not shown). Acute activation of Arf6 by treatment with AlF₄⁻ (23) or by expression of Arf6-GEFs (50-52) results in generation of ruffling protrusions along the plasma membrane. Our studies now show the presence of GRASP in these cortical actin structures, suggesting a potential role of GRASP in facilitating the Arf6-mediated cytoskeletal rearrangements. Arf6-mediated cytoskeletal remodeling is thought to be required for the formation of pseudopods and membrane ruffles, neurite outgrowth, cell spreading, cell migration and phagocytosis (9). These results also suggest the potential role of GRASP in the repositioning of receptors, such as mGluRs and TrKCT1, to specific regions of the plasma membrane during neurite outgrowth and cellular migration.
Arf6-dependent internalization of G protein-coupled receptors has been extensively described (14,53). For example, agonist-mediated internalization of the M2-muscarinic acetylcholine receptor (M2AcchR) has been shown to occur initially by an Arf6-dependent pathway in HeLa cells (13), while, M1-, M3-, and M4-muscarinic acetylcholine receptor subtypes are thought to be endocytosed via a clathrin-dependent pathway (54). However, several studies over the last decade have suggested the involvement of the Arf6 pathway in M3AcchR downstream signaling events in HeLa cells (47,48). Indeed, at the time of writing this manuscript, work from the Donaldson group (14) demonstrated that M3AcchRs are constitutively internalized by a clathrin-independent pathway in HeLa cells. Data presented herein suggest that the rate of internalization of M3AcchR depends on the levels of activated Arf6 (see Fig. 2.8). M3AcchR exhibited an initial delay in agonist-dependent receptor endocytosis and this delay was shortened by increasing the pool of activated Arf6. This trend seems to support other studies, which have shown a similar, rapid phase of Arf6-dependent endocytosis, such as that of MHC-I and IL-2αR in cells overexpressing Arf6 Q67L (22). Collectively, these studies indicate that the agonist-dependent endocytosis of M3AcchR occurs by an Arf6-dependent pathway in HeLa cells. However, there exist two caveats to this interpretation. First, our studies do not account for the effects of activated Arf6 on the rate of constitutive M3AcchR internalization. Second, the above mentioned study (14) also reported inhibition of agonist-mediated endocytosis of M3AcchR in cells depleted of clathrin, which may be explained by either of the following: (1) M3AcchR may switch
between Arf6- and clathrin-dependent pathways either stochastically or regulated by an unknown mechanism, or (2) activated Arf6 may recruit the clathrin-dependent machinery to facilitate rapid receptor endocytosis. Future efforts will need to be directed at resolving this paradox, perhaps by studying with the effects of elevated levels of Arf6 on constitutive internalization of M3AchR, agonist-mediated internalization of M3AchR with depleted clathrin machinery, and agonist-mediated internalization of M3AchR in cells depleted of Arf6. Nonetheless, our study indicates that M3AchR internalization in HeLa cells can be influenced by the clathrin-independent and Arf6-dependent receptor trafficking pathways, consistent with recent results from the Donaldson laboratory.

As GRASP over-expression mimicked the effects of constitutively inactive forms of Arf6 with respect to Arf6-dependent receptor recycling, we were surprised to note that over-expressed GRASP appeared to phenocopy the effects of Arf6 Q67L with respect to M3AchR internalization. This apparent paradox in GRASP behavior may be explained by closely inspecting the role of GRASP in the Arf6-dependent trafficking pathway. Under normal growth conditions Arf6 constitutively undergoes a cycle of activation and inactivation, allowing membrane to recycle in and out of the cell (9, 11,23,31-33). Analysis of Arf6 point mutants suggested that activated Arf6 (Arf6 Q67L) promoted rapid internalization, while constitutively inactivated Arf6 (Arf6 N122I) inhibited recycling (11,22,24). Our data suggest that expression of GRASP most likely facilitated Grp1-mediated activation of Arf6 (see above), and thus low levels of GRASP expression
may have increased basal levels of Arf6-GTP in cells. However, in cells expressing high levels of GRASP it seems conceivable that the Arf6-dependent pathway was driven into an extreme position in which the cell mimicked both Arf6-GTP and Arf6-GDP enriched states. Cells expressing high levels of GRASP, at early time points of their transfection, may exhibit elevated levels of Arf6-GTP, mimicking an Arf6 Q67L-like state. However, with increasing levels of GRASP expression in time, GRASP would titrate available endogenous Arf6 GEFs, mimicking the Arf6 N122I mutant. Therefore, cells expressing high levels of GRASP may phenocopy Arf6 Q67L with regard to M3AchR internalization and Arf6 N122I with regards to MHC-I receptor recycling. Such time-dependent effect has also been observed in cells expressing Arf6 Q67L (22).

In summary, our studies provides insight into the role of GRASP in intracellular trafficking of receptors and its potential role in vivo. Our results provide direct evidence for predominant involvement of GRASP in the regulation of Arf6-dependent cargo. Hence, it may be productive to focus future efforts on the study of Arf6-dependent receptors in Grasp−/− mice (55). One such study would be to elucidate the role of GRASP in MHC-I trafficking in vivo.
References

Figure 2.1. GRASP localizes in tubular perinuclear endosomal compartments.

HeLa cells were transfected with increasing concentrations of an expression vector encoding GFP-GRASP, were visualized by fluorescence microscopy. (A and inset) Cells transfected with low levels of GRASP, exhibits GRASP localization in tubular, perinuclear endosomal-like compartments and along the plasma membrane. (B) High expression levels of GRASP exhibits predominantly a punctate aggregated endosomal-like localization. (C) Quantitation of cells expressing punctuate aggregates of GRASP represented as a percentage of the total number of transfected cells. The values represent the mean +/- S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. Statistical significance is indicated by * and ** symbols for (p < 0.05) and (p < 0.01), respectively. Scale bars = 10 μM.
Figure 2.2 A constitutively inactive mutant of Arf6 (Arf6 N122I) co-localizes with and enhances the punctate localization of GRASP in Rab11-positive endosomes.

HeLa cells were transiently co-transfected with GFP-Rab4, -Rab5, -Rab7 or -Rab11 and either HA-Arf6 N122I (A-D) or Myc-GRASP (E-H) as indicated; the punctate localization of both Arf6 N122I and GRASP strongly co-localized with Rab11 recycling endosomal structures. (I-K) HeLa cells were transiently transfected with GFP-GRASP and either HA-Arf6 wild-type (WT), HA-Arf6 Q67L, or HA-Arf6 N122I as indicated; the punctate localization of GRASP again strongly and specifically co-localized with the constitutively inactive Arf6 point mutant, Arf6 N122I. All images were captured using the 63x plan Apo objective with optical slice < 0.1 μm. (L) Quantitation of cells exhibiting punctate aggregated endosomal-like localization of GRASP, represented as a percentage of the total number of transfected
Figure 2.2
Figure 2.3. Co-expression of wild-type Grp1 prevents accumulation of GRASP in ERC.

HeLa cells were transiently transfected with an expression vector encoding GFP-GRASP with or without Grp1 WT-HA. F-actin stained cells were scored for percentage of transfected cells exhibiting accumulation of GRASP as aggregates and for membrane ruffles. (A-D) Loss of punctate localization of GRASP and its translocation into membrane ruffles (see arrows and dense accumulation of F-actin in cortical protrusive structures) when co-transfected with Grp1 WT. Images were captured using Zeiss Imager.Z1 microscope with a 40x oil Plan-Neofluar objective (D) Quantitation and comparison of percentage of transfected cells exhibiting punctate localization of GRASP (solid-black bars) or localization in membrane ruffles (unfilled-bars) in presence or absence of co-transfected Grp1. All values represent the mean +/- S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. The double asterisk denotes a statistically significant (p < 0.01) difference in punctate localization of GFP-GRASP (black bars) or localization in membrane ruffles (white bars) in the indicated comparisons. Scale bars = 10 μM.
Figure 2.4. All known functional domains of Grp1 are collectively required to prevent GRASP accumulation in ERC.

HeLa cells were transiently co-transfected with an expression vector encoding GFP-GRASP with either WT Grp1 or Grp1 mutants as indicated. Cells were scored for percentage transfected cells exhibiting accumulation of GRASP in ERC aggregates. Values represent the mean +/- S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. The double asterisk denotes a statistically significant (p < 0.01) difference in ERC localization of Myc-GRASP between cells singly transfected with Myc-GRASP vs. cells co-transfected with Myc-GRASP and WT Grp1 or Grp1 mutants.
Figure 2.5. GRASP localizes in ERC independent of Grp1.

(A) Co-immunoprecipitation analyses of Grp1 and GFP-GRASP and indicated truncation mutants. (B) Schematic representation of the GRASP truncation mutants and their corresponding propensity/efficiency to aggregate in ERC. Values represent the mean +/- S.E.M. of three independent experiments in which a minimum of 50 transfected cells were scored. Statistical significance at p < 0.05 and p < 0.01 is indicated by * and ** symbols, respectively. (C) HeLa cells expressing GFP-GRASP 208-392 and HA-Arf6 N122I (D) HeLa cells co-expressing GFP-GRASP 208-392 and Grp1-WT. Scale bars = 10 μM.
Figure 2.5

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Anti-GRP1 Immunoblot

43 kDa

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GRP1

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C

GRASP 208-392

Arf6-N1221

C' GRASP 208-392

C'' Arf6-N1221

D

GRASP 208-392

Grp1

D' GRASP 208-392

D'' Grp1
Figure 2.6. Overexpression of GRASP blocked the release of MHC-I receptors from ERC.

(A-F) MHC-I receptor recycling assays in HeLa cells expressing low (A-F) or high (G-L) levels of GRASP. (M) Quantitation and comparison of the MHC-I receptors that remained within the cell at varying chase intervals, in cells expressing either GFP (filled squares), low levels of GRASP (filled circles) or high levels of GRASP (open circles). Values in (M) represent the mean +/- S.E.M. of three independent experiments, and statistical significance at p < 0.01 and p < 0.001 is indicated by double and triple asterisks, respectively. Scale bars in (A-L) = 10 μM.
Figure 2.6

A-C: GFP-GRASP (Low) MHC-I OVERLAY
D-F: GFP-GRASP (High) MHC-I OVERLAY

M: Recycled MHC-I receptors (% of control) vs. Chase (minutes)
Figure 2.7. Overexpression of GRASP did not block the release of transferrin receptors from ERC.

(A-F) HeLa cells expressing low levels of GRASP lost fluroscence signal from internalized transferrin receptors (TfrR) after a 60 min. chase. (G-I) The internalized transferrin receptors co-localized with the punctuate aggregates of GRASP in HeLa cells. (J-L) Internalized TfrR recycled out of cells expressing high levels of GRASP after a 60 min. chase; (M) Quantitation and comparison of TfrR that remained within the cell at varying chase intervals in cells expressing either GFP (filled squares), or low (filled circles) or high levels (unfilled circles) of GRASP. Values in (M) represent the mean +/- S.E.M. of three independent experiments. Scale bars = 10 μM.
Figure 2.7

[Images and graphs showing cellular processes and data analysis related to transferrin receptors' recycling.]
Figure 2.8. Activated Arf6 triggers rapid internalization of M3-muscarinic acetylcholine receptors (M3AchR) in HeLa cells.

HeLa cells where transiently co-transfected with expression vectors encoding HA-M3AchR and GFP or the indicated GFP-Arf6 or GRASP expression vectors. Internalization was initiated by treatment of cells with 1 mM carbachol for 30 min. Statistical significance (p < 0.001) between indicated samples and control (GFP cotransfection) is indicated by the triple asterisks.
Figure 9. Proposed model for GRASP mediated regulation of the Arf6-dependent trafficking pathway.

Following GAP-catalyzed hydrolysis of Arf6-GTP, GRASP along with Arf6-GDP is internalized and localizes in the ERC compartment, in which GRASP is predicted to recruit Grp1 (and/or other cytohesins) to ERC, facilitating GRP1-mediated nucleotide exchange on and activation of Arf6. Following activation of Arf6, GRASP ●Grp1●Arf6 translocate to cortical structures, in which the complex initiates Arf6-dependent signaling pathways that lead to membrane ruffling. Elevated levels of Arf6-GTP in cortical structures can then initiate a fresh round of Arf6-dependent internalization to complete the trafficking circuit. GRASP accumulates in ERC when levels of GRP1 are low and/or GRP1 is sequestered in the cytosol by overexpressed GRASP. These GRASP aggregates in ERC mimic constitutively inactive Arf6-GDP point mutants and block Arf6-dependent receptor recycling.
Figure 2.S1. Endogenous GRASP is localized in perinuclear, endosomal-like compartments in HeLa cells.

HeLa cells were fixed with 10% PFA and permeabilized with 0.1% TritonX-100 for 5 min. These cells were blocked with 10% donkey serum for one hour, followed by incubation with either goat anti-GRASP (1:100) or preimmune sera at 37°C for 1 hr. GRASP signal (red) was subsequently visualized using a biotin-streptavidin amplification method using biotin-conjugated to donkey anti-goat secondary antibody and Cy3-conjugated to streptavidin. DAPI was used to counterstain the nucleus (blue). Images were captured using Zeiss Imager.Z1 microscope with a 40x oil Plan-Neofluar objective under identical exposure settings. (A-B) Endogenous GRASP appears to localize in distinct perinuclear, endosomal-like compartments. (C-D) No signal was detected in cells treated with preimmune-sera. Scale bars = 10 μM.
Figure 2.S2. The fusion of GFP to GRASP does not alter its localization characteristics.

HeLa cells were co-transfected with an expression vectors encoding indicated GFP-GRASP and Myc-GRASP. These cells were subsequently processed to visualize Myc-tag using respective antibodies for indirect-fluorescence microscopy. DAPI was used to counterstain the nucleus (blue). Localization of GFP-GRASP is identical with the Myc-GRASP in cells expressing low levels (A-C) and high levels of GRASP (D-F). Scale bars = 10 μM.
Figure 2.S3. Levels of transfected GRASP relative to the endogenous levels of GRASP in HeLa cells.

HeLa cells were transfected with an expression vector encoding GFP-Arf6 WT or GFP-Arf6 N122I. These cells were subsequently processed to visualize endogenous GRASP (as per Fig. S1) by indirect-fluorescence microscopy. DAPI was used to counterstain the nucleus (blue). (A-C) Endogenous GRASP partially co-localizes with Arf6-WT in tubular, perinuclear endosomal-like compartments and along the plasma membrane. (B) Endogenous GRASP distinctly co-localizes with the Arf6-N122I in tubular, perinuclear endosomal-like compartments. Scale bars = 10 μM.
Figure 2.S4. HeLa cells individually transfected with expression vectors encoding GRASP or wild-type Grp1 do not exhibit significant membrane ruffling.

HeLa cells were transfected with an expression vector encoding GFP-GRASP or HA-Grp1 WT and were processed for immunofluorescence microscopy. Indirect immunofluorescence labeling of HA-Grp1 was performed with anti-HA antibody (1:1000) and subsequently visualized using a Cy3-conjugated secondary antibody. F-Actin was stained using Alexa-350 Phalloidin and GRASP was visualized using GFP fluorescence. Images were captured using Zeiss Imager.Z1 microscope with a 40x oil Plan-Neofluar objective. (A-H) Under the mentioned experimental conditions cells transfected with GFP-GRASP or HA-GRP1 WT exhibit F-actin predominantly in stress fibers with only minimal cortical F-actin seen during membrane ruffling. Scale bars = 10 μM.
Figure 2.S5. Grp1 and its mutants are expressed at equivalent levels when co-transfected with GRASP.

HeLa cells were co-transfected with expression vectors encoding GRASP and either HA-Grp1 WT, Grp1 coil-Sec7, HA-Grp1 E161K, HA-GRP1 R284D, HA-Grp1 Coil or HA-Grp1 Sec7 PH. (A) Lysates from cells expressing GRASP and HA-Grp1 WT, Grp1 Coil-Sec7, HA-Grp1 E161K, HA-GRP1 R284D were analyzed by immunoblotting using an anti-Grp1 antibody (1:1000) and subsequently stripped and reprobed with GRASP antibody as indicated. (B) Lysates from cells expressing GRASP and HA-Grp1 WT and HA-Grp1 Sec7 PH were analyzed by immunoblotting with an antip-HA antibody (1:1000) and subsequently stripped and reprobed with the GRASP antibody (1:3000) as indicated. HeLa cells expressing HA-Grp1 WT (C-E) and HA-Grp1 Coil mutant (F-H) were processed for indirect immunofluorescence microscopy using the HA-antibody (1:1000). Images were captured using Zeiss Imager.Z1 microscope with a 40x oil Plan-Neofluar objective under identical exposure settings. Scale bars = 10 μM.
Figure 2.S5

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Grp1-Blot

GRASP-Blot

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HA-Blot

GRASP-Blot

C

HA-Grp1 WT

D

GFP-GRASP WT

E

OVERLAY

F

HA-Grp1 Coil

G

GFP-GRASP WT

H

OVERLAY
HeLa cells were co-transfected with 1.0 μg each of expression vectors encoding HA-GRP1 WT and either GFP-GRASP WT, 180-257, 180-230, 231-392 or 208-392. Whole cell extracts were prepared and analyzed by immunoblotting using the rat anti-GFP antibody (1:5000). This blot was subsequently stripped and re-probed with for GRP1 using goat anti-GRP1 antibody (1:1000). These blots were visualized using respective HRP-conjugated antibodies (1:1000; Southern Biotech Inc., USA) and developed using Amersham ECL™ chemiluminescent western blotting detection reagents (#RPN2109).
Figure 2.S7. Truncated forms of GRASP localize in the same Arf6 N122I+ endosomal recycling compartment as wild-type GRASP.

HeLa cells were co-transfected with 1.0 μg each of expression vectors encoding HA-Arf6 N122I and either GFP-GRASP 180-257 (A), 180-230 (B) or 231-392 (C) as indicated. Indirect immunofluorescence labeling of HA-Arf6 N122I was performed with anti-HA antibody (1:1000) and subsequently visualized using a Cy3-conjugated secondary antibody, while and GRASP mutants were visualized using GFP fluorescence. Scale bars = 10 μM.
Figure 2.S8. Co-expression of Grp1 WT leads to membrane localization of the GFP-GRASP 180-230 and 180-257 mutants.

HeLa cells were transfected with an expression vector encoding HA-Grp1 WT and either GFP-GRASP 180-230 or GFP-GRASP 180-257. These cells were subsequently processed to visualize HA-GRP1 using anti-HA (1:1000) antibody by indirect-fluorescence microscopy. DAPI was used to counterstain the nucleus (blue). (A-C) Coexpression of Grp1 WT leads to membrane localization of GFP-GRASP 180-230. (D-F) Coexpression of Grp1 WT leads to membrane localization of GFP-GRASP 180-257.
CHAPTER 3: Grp1-associated scaffold protein is a regulator of the p53-mediated apoptosis following UVB exposure in the murine skin.

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Abstract:

Grp1-associated scaffold protein (GRASP) is expressed by a retinoic acid induced gene in the post-natal murine brain, heart and lungs. In this study we have identified expression of GRASP transcripts in the epidermal layers of the adult murine skin. GRASP expression in the murine skin is robustly induced in both the epidermal and dermal layers following an acute dose of ultraviolet-B (UVB) exposure. We also report the generation of a novel mice with germline deletion in exon1 of GRASP (GRASP<sup>-/-</sup>). In comparison to wild-type mice, GRASP<sup>-/-</sup> mice exhibit a delayed proliferative response and a transient apoptotic response following an acute UVB exposure. The basal and UVB induced transcript and protein levels respectively, of the tumor suppressor p53, was observed to be similar in wild-type and GRASP<sup>-/-</sup> mice. However, immunohistochemical analysis reveals that the nuclear sub-localization of p53 is significantly attenuated in GRASP<sup>-/-</sup> mice following UVB exposure. Taken together, we report an in vivo physiological role of GRASP as a regulator of the sub-cellular localization of p53 and p53-mediated apoptotic response following UVB exposure in the adult murine skin.
Introduction

Grp1-associated scaffold protein (GRASP; also known as tamalin (1)) was first identified by our group as a gene induced by treatment of all-trans retinoic acid (ATRA) in P19 cells (2). GRASP harbors four principal protein-protein interaction regions namely, the N-terminal alanine/proline rich (A/P) domain, followed by the PDZ-domain, the leucine-zipper domain and the C-terminal PDZ-binding motif (2,3). Each of the four domains of GRASP interacts with numerous proteins including kinases {syk; Kitano, 2003 #632}, other molecular scaffolds {Mint2, CASK; Kitano, 2003 #632}, membrane receptors {GABA, mGluRI and TrkCT1; Kitano, 2003 #632; Esteban, 2006 #629} and guanine nucleotide exchange factors {Grp1, ARNO; Nevrlvy, 2000 #648}. Several reports over the last decade suggest the physiological role of GRASP as a molecular scaffold in regulating the trafficking of interacting membrane proteins like mGluRI and TrkCT1 receptors (1,3,5). In this regard, we have recently demonstrated that GRASP through its interactions with Grp1, uniquely regulates the clathrin-independent/Arf6-dependent membrane trafficking pathway (submitted to JBC).

However, the role of GRASP in vivo has remained largely elusive. GRASP is highly expressed in post-natal murine brain, liver and lungs (1,2,4). According to recent reports, mice generated with germline deletion of GRASP (GRASP−/−) exhibit
no gross morphological, behavioral or sexual deficits and exhibit mild altered sensitivities to morphine and cocaine (6). As GRASP was initially identified as an ATRA induced gene in vitro, an alternative approach towards understanding the in vivo role of GRASP would be to investigate the response of adult GRASP−/− mice to ATRA treatment. Skin is an excellent system to study proliferative paradigms by either exposure to chemical stressors like ATRA (7,8), TPA/DMBA (9) or by treatment with physical stressors such as ultraviolet (UV) radiation (10-14).

The murine skin is comprised of three principle layers – the outermost epidermis, followed by the inner dermis and the innermost layer called the hypodermis. The outer epidermis is further stratified into four layers named inside out as basal, spinous, granular and cornified layers (15). A balance of proliferation, differentiation, and cell death preserves epidermal homeostasis (15). In the basal layer of epidermis, stem cells and transiently amplifying cells give rise to keratinocytes that undergo multiple rounds of proliferation before terminal differentiation into spinous, granular and cornified layers (15).

Two apoptotic pathways of physiologic importance have been identified in skin. The first pathway occurs during the keratinization process and during the basal maintenance of normal hair growth in skin (16). This pathway can be triggered after ligation of the cell surface death receptor Fas, which results in activation of caspase-8
(16), and dysregulation of this pathway has been implicated in skin diseases including cutaneous graft versus host disease (17), toxic epidermal necrolysis (18), and eczematous dermatitis (19). Apoptosis in the skin can also be triggered by the tumor supressor p53 after exposure to ultraviolet (UV) rays (16). The UV region of the electromagnetic spectrum is subdivided into three wavebands: (a) UVA, 320-400 nm, (b) UVB, 290-320 nm and (c) UVC, 200-290 nm. Of these UVC is of least biological importance, as it is efficiently absorbed by the earth's atmosphere (20). UVB on the other hand is highly carcinogenic compared with UVA, and both UVA and UVB are significant etiological factor for skin cancer and photodamage (11-14). Acute UVB exposure has been shown to induce cellular damage, most of which will disappear within about 2 weeks, whereas chronic and repeated exposures causes epidermal cell damage leading to skin cancer (11).

Pilling and coworkers (21) first reported that following UVB treatment, the transcript levels of p53 are unaltered while the half-life of the protein is known to increase significantly. Increased levels of active p53 can inhibit progression of cell cycle for DNA repair processes, or trigger cell death through the highly orchestrated apoptotic pathway (11,13,16,22). Subsequently, numerous reports over the last decade suggest that the fate of the cell is determined by the cumulative effects of a multileveled control at either translation (23,24), post-translation modification (25,26) and/or sub-cellular localization (27-30) of the p53 protein. A mutation in the p53
sequence (31) and/or misregulation at one of the above mentioned controls in the cellular levels of p53 has been documented to be single largest etiological factor for most of the known skin cancers (22).

Another tumor suppressor protein, Phosphatase and tensin homolog detected on chromosome ten (PTEN), is emerging as a novel guardian of the genome (32). Recent reports define one of the roles of PTEN as a molecular interaction partner of p53, leading to the stabilization, transport and activation of p53 in the nucleus (33-36). Another facet of PTEN’s function is the negative regulation of the Akt/PI3K-Mdm2 axis (37), which otherwise would lead to the nuclear export, ubiquitination and degradation of p53 (36,38,39). In summary, PTEN plays a crucial role in the tightly controlled and highly complex regulatory pathways crucial for p53 nuclear stabilization and p53-dependent downstream signaling pathways.

In this study we demonstrate the in vivo role of GRASP in the adult murine skin. We have identified expression of GRASP in epidermal layers and a robust induction of the GRASP gene in the epidermal and dermal layers following an acute dose of UVB exposure, in the murine skin. We report the generation of a novel mice with germline deletion of GRASP (GRASP−/−) that exhibits altered proliferative and apoptotic response to acute dose of UVB. In contrast to sustained nuclear localization of p53 in wild-type littermate controls, the GRASP−/− mice exhibit only transient
stabilization of the nuclear p53 levels following UVB exposure. Our studies suggest that GRASP potentially can regulate the sub-cellular localization of p53 by regulating PTEN mediated processes.
Material and Methods

Antibodies: The antibodies used were rabbit anti-p53 (1:500; #NCL-p53-CM5p, Novacastra), mouse anti-p53 (1:50; #OP29, calcibiochem), rat anti-CTIP2, (1:300; #ab18465 Abcam), mouse anti-PTEN (1:1000; #sc7974, Santacruz Inc.), goat anti-GRASP (1:3000; previously described in (2), Bethyl laboratories Inc.), rabbit anti-K14 (1:100; #PRB-155P,Covance.), rat anti-BrdU(1:100, #1370030, AbD Serotec) and rabbit anti-B-actin (1:5000, #A5441, Sigma-Aldrich).

UVB treatment and sample collection: 2-3 month old male GRASP+/− (n=15) and littermate control wild-type (n=15) mice were housed in cages in a room with controlled temperature, humidity and alternative 12 hour light and dark cycles. The mice were fed with commercial diet and water ad libitum. The dorsal hair of mice were shaved 24 hours prior to UVB exposure to ensure synchronization of the hair-growth cycle. A group of mice from each genotype was not exposed to UV and was used as control (n=3). The other 12 mice from each genotype were placed in on a shelf 20cm below the light source in standard cages (<5 mice/cage), and irradiated with a single acute dose of 750kJ/cm² from a bank of four UVB sun-lamps (#TL-40W,Philips). The irradiance of the sunlamps was measured with a model IL1400A radiometer/photometer with a SL021/FQI detector (International Light, Inc., Newburyport, Massachusetts). A group of 3 mice from each genotype were euthanized.
at 12 hours, 24 hours and 48 hours after UV radiation. Biopsies (5mm²) from the dorsal skin from each group of mice were collected and either immediately fixed in 4% buffered paraformaldehyde or stored in -80°C for future analyzes.

**Immunoblot analysis:** To preserve phosphorylation and SUMOylation of proteins, the skin samples were homogenized and lysed in a denaturing buffer containing 250mM NaCl, 2mM EDTA, 50mM NaF, 5mM Sodium pyrophosphate, 5mM NEM, 0.1mM H.C. (hemin chloride), protease inhibitor cocktail (leupeptin, aprotinin, bestatin) and 1% SDS, boiled for 5 mins, sonicated and centrifuged to remove DNA.

**Coimmunoprecipitation analysis:** Coimmunoprecipitation analysis was carried out as mentioned before (2).

**Histological and Immunohistochemical analysis:** 4% PFA fixed skin samples were cryopreserved in 30% sucrose and frozen down in OCT. The 10 μm cross sections were rinsed with PBS three times, and processed for histological analysis by hematoxylin and eosin (H&E) staining as mentioned earlier (40). For immunohistochemical analysis, the sections were permeabilized with ice-cold methanol for 2 minutes and blocked with blocking buffer (0.3% Boehringer Mannheim blocking reagent, 5% horse serum, 5% fetal calf serum, and 0.1% triton X-100 in PBS). Sections were then incubated with primary antibodies overnight, followed by three washes with PBST.
(PBS+0.01% Tween) and incubation with fluorescently labeled secondary antibody (Cy2 (1:250) or Cy3 (1:500); Jackson ImmunoResearch) for 2 h at room temperature. Nuclei were counterstained using DAPI. After final washes with PBST, sections were dehydrated through a series of ethanol washes, cleared in xylene and mounted with DPX mounting media. All fluorescence images were captured using a ZEISS LSM 510 confocal microscope using a 40x Plan Apo objective and bright field images were capture using the Zeiss Imager.Z1 microscope with a 20x objective. These images where then processed using the Zeiss LSM image browser and Photoshop CS4 (Adobe Systems, Inc., USA).

**TUNEL assay:** The Promega Dead-end colorimetric kit (#TB199) was used to visualize apoptotic cells in the tissue samples. The assay was performed with minor modifications to the manufacturer's protocol: a fluroscent Cy3 flurophore conjugated to streptavidin (SA) was used instead of the colorimetric SA-HRP-DAB visualization procedure, to increase ease and sensitivity of the assay.

**Quantitative real-time PCR (qRT-PCR) analysis:** RNA extraction and cDNA preparation were performed as described (40). qRT-PCR was performed on an ABI 7500 Real-Time PCR system using SYBR green methodology and analyzed as described (40,41). RT-PCR conditions and primers sequences for most of the genes have been previous described (40). Other primer sequences are: GRASP: (F) 5'
AGCAGCTGGAGGACTATCAC; (R) 5'-CGAGATCCAGACATATGGC; p53: (F) 5'-
AGTTCATTGGGACCATCCTG, (R) 5'-AATGTCTCTGGCTCAGAGG.
Results

Generation of the GRASP⁻/⁻ mice

The GRASP targeting vectors was constructed using ~9 kb of the mouse GRASP genomic locus (Fig. 3.1A,B). After electroporation, two ES cell lines (out of 113) were verified to have undergone homologous recombination (HR) as judged by Southern analysis using 5' and 3' probes outside the targeting vector (Fig. 3.1C,D). Both of these clones were injected into blastocysts, and gave rise to three chimeric founder animals that were bred with C57BL/6 mice to allow germ line transmission (GLT) of the targeted L3 allele. We found no phenotypic difference between the three lines and only one of the lines was maintained for further studies. Transgenic mice expressing the Flp recombinase under the control of the CMV promoter (42), were crossed with the GRASP L3 mice, to excise the neo marker and generate the L2 (floxed) GRASP allele. Subsequently, the mice harboring the L2 allele (LoxP-Exon1-LoxP) were crossed with transgenic mice expressing Cre recombinase under the control of the Prm promoter (43), to excise the floxed exon 1 of GRASP. These mice were bred to homozygosity (GRASP L⁻/⁻, referred to as GRASP⁻/⁻ hereafter). Furthermore, immunoblot analysis from brain extracts confirmed the loss of GRASP expression in the GRASP⁻/⁻ mice. Under basal conditions GRASP⁻/⁻ mice showed no gross morphological, behavioral or sexual defects (data not shown), recapitulating previously reported observations of GRASP-null mice (6).
Acute dose of UVB leads to induction of GRASP in skin

We wished to determine if GRASP is induced in vivo by topical application of retinoic acid, recapitulating its induction in vitro in P19 cells (2). Although we found no induction of the GRASP transcript by ATRA treatment in adult murine wild-type skin (unpublished results), we investigated the effects of alternative proliferative paradigms in the adult murine skin. Quantitative real-time PCR (qPCR) analysis of the GRASP transcript revealed a 20 fold upregulation of GRASP transcript in the murine skin after 48 hours of a single acute dose of UVB (Fig. 3.2A). Using in situ hybridization with full-length GRASP probe, we observed that GRASP expression under basal conditions is predominantly restricted to the epidermal layers of the adult murine skin (Fig. 3.2B). Following UVB exposure, a significant proportion of GRASP transcript is induced in dermal layers and epidermal layers of the murine skin (compare Fig. 3.2B and 3.2C ). No appreciable signal was detected by in situ hybridization carried out using the same probe in tissues from GRASP<sup>−/−</sup> mice (Fig. 3.2C,D). Taken together, our results identify the expression of GRASP transcripts in the epidermal layers of murine skin. Furthermore, GRASP is induced robustly following UVB exposure of the dermal and epidermal layers of the murine skin.
Acute dose of UVB leads to a delayed epidermal thickening in GRASP\textsuperscript{−/−} mice

UVB rays are well characterized to induce a epidermal hyperplastic response after 24 hours of the exposure (11). We performed histological analysis by hematoxylin and eosin (H&E) staining and observed a significant increase in the epidermal thickness of wild-type mice after 24 hours (compare Fig. 3.2A and 3.2B) and the epidermal thickness returned to basal state after 48 hours of the acute dose of UVB (compare Fig. 3.2A and 3.2C). In contrast, the GRASP\textsuperscript{−/−} mice show no obvious change in epidermal thickness 24 hours post-UV (compare 3.2D and 3.2E) and exhibit a more delayed epidermal hyperplastic response after 48 hours of the UVB treatment (compare Fig. 3.2D and 3.2F). Interestingly, even at basal state GRASP\textsuperscript{−/−} mice exhibit a subtle but consistently thicker epidermis as compared to the wild-type litter mate controls (~30% thicker; Fig. 3G). Furthermore, the epidermal response to UVB exposure though delayed in GRASP\textsuperscript{−/−} mice is more pronounced after 48 hours of UVB exposure (Fig. 3G). Taken together, in comparison to wild-type mice, the GRASP\textsuperscript{−/−} mice exhibit an altered response in epidermal thickening after an acute dose of UVB.

GRASP\textsuperscript{−/−} mice have altered proliferative response to UVB treatment

We hypothesized that the delay in epidermal response after UVB treatment can be attributed to a delayed proliferative response in the GRASP\textsuperscript{−/−} mice. To determine this
we compared the proliferative index of GRASP<sup>−/−</sup> and wild-type mice skin, by measuring the levels of BrdU<sup>+</sup> nuclei in the basal layer of the epidermis after UVB exposure. Under basal conditions no significant difference in the %BrdU<sup>+</sup> epidermal cells was observed in the wild-type and GRASP<sup>−/−</sup> littermate mice (compare Fig. 4A and B; Fig. 4G). Following UVB treatment the basal cells of the inter-follicular epithelium are known to rapidly enter the S-phase and proliferate (44). As our BrdU administration was 2 hours prior to euthanizing the mice, the majority of the detectable BrdU<sup>+</sup> cells in skin samples obtained 24 hours post-UV from both the wild-type and GRASP<sup>−/−</sup> littermate mice, were restricted to the slow cycling stem-cells of follicular-epidermis (Fig. 3.4C,D and G). After 48 hours of UV exposure, significant increase in the detectable levels of BrdU<sup>+</sup>-cells are observed in the inter- and intra-follicular epidermis of both wild-type and GRASP<sup>−/−</sup> littermate mice (compare Fig. 3.4A to 3.4E and Fig 3.4B to 3.4F respectively). However, after 48 hours %BrdU<sup>+</sup> epidermal observed in GRASP<sup>−/−</sup> was lower (~35%) as compared to the wild-type littermate mice (Fig. 4G). Taken together, our results suggest that the GRASP<sup>−/−</sup> mice exhibits altered proliferative response to UVB exposure.
GRASP\(^{-/-}\) mice have misregulated apoptotic response to UVB treatment

The delay in epidermal response after UVB treatment can also be due to misregulated apoptotic response in the epidermal layers of the GRASP\(^{-/-}\) mice. To determine this, we visualized apoptotic epidermal cells by the TUNEL assay. After 12 hours of the UVB treatment, TUNEL positive cells were detected in the epidermal layers of wild-type mice and GRASP\(^{-/-}\) mice (Fig. 3.5A and 3.5B respectively). This robust apoptotic signal persisted in skin samples collected after 24 and 48 hours of the UVB treatment in wild-type mice (Figs. 3.5C and 3.5E respectively). However, in GRASP\(^{-/-}\) mice the apoptotic signal was attenuated at 24 hours and was almost completely absent in skin samples obtained 48 hours post-UV treatment (Figs. 3.5B and 3.5D, respectively). Taken together our results suggest that in comparison to wild-type littermates, the GRASP\(^{-/-}\) mice exhibit a transient apoptotic response to UVB exposure.

Nuclear accumulation of p53 after UVB treatment is significantly reduced in the epidermal cells of GRASP\(^{-/-}\) mice

We hypothesized that the misregulation of the proliferative and apoptotic responses in GRASP\(^{-/-}\) mice are due to altered regulation at one or more levels in the expression, stability and/or localization of the p53 protein. We carried out quantitative PCR analysis (qPCR) analysis of the p53 transcript in wild-type and GRASP\(^{-/-}\) mice skin. In
accordance to previous published reports (21), we found no difference in the relative levels of the p53 transcripts in samples taken before and after UVB treatment of both GRASP^{−/−} and wild-type littermate controls (Figs. S1A). We next quasi-quantitated the total protein levels of p53 by immunoblot analysis and found that both GRASP^{−/−} and wild-type littermate mice exhibited a robust and sustained increase in the levels of p53 protein at 24 and 48 hours post-UV (Fig. S1B).

We next determined if the sub-cellular localization of p53 in response to UV treatment is altered in GRASP^{−/−} mice. We performed standard immunohistochemical analysis on the skin samples using the CM5-p53 antibody (which recognizes both the wild-type and mutant confirmations of the p53 protein). Using confocal microscopy we observed that the sensitivity of this technique allowed the detection of only the nuclear fraction p53 protein. The nuclear identity of the p53 signal was confirmed by its co-localization with nuclear counterstain DAPI and the transcription factor CTIP2, which is highly expressed in the basal layers of the skin (45; Fig. 7). In wild-type mice, we observed a robust increase in the nuclear localization of p53 (~40% of the epidermal cells) within 12 hours of UVB treatment and the proportion of nuclear p53^{+} cells was maintained till 48 hours post-UV (~30% of the epidermal cells; Fig. 7A-D and 7I). GRASP^{−/−} mice exhibited comparable proportion of nuclear p53^{+} to its wild-type littermate controls at 12 hours post-UV (~40% of the epidermal cells; Fig. 7E,F and I). However, GRASP^{−/−} mice exhibited loss of p53^{+} cells at 24 and 48 hour post-UV (~28% and ~11% respectively of the epidermal cells; Fig. 7G-I). Taken together, our
results suggest that in comparison to wild-type mice the GRASP⁻/⁻ mice exhibit a transient increase in nuclear localization of the p53 protein, in response to UVB treatment. We have also shown that this difference in p53 localization is not due to loss of control at the level of transcription of the p53 transcript or at the level of translation of the p53 protein. This suggests the potential role of GRASP in the post-translational modification and/or sub-cellular localization of the p53 protein.

**GRASP interacts with p53 and PTEN**

Recent reports suggest PTEN to function as a guardian of p53 against mdm2 mediated ubiquitination/degradation and to facilitate nuclear import of p53, enhancing the sensitization of cancerous cells to apoptosis (33-38). We hypothesized that GRASP, induced after cellular stress like UVB exposure, could potentially interact with PTEN and influence the nuclear import/stabilization of p53. To test this hypothesis, we transfected HEK 293T cell lines with expression vectors encoding Myc-GRASP and either GFP-PTEN WT, GFP-PTEN C124S (a catalytically inactive point mutant of PTEN) or GFP-PTEN 399STOP (PTEN lacking the C-terminal PDZ-motif ). We observed that GRASP co-immunoprecipitates (co-IP) with PTEN WT >PTEN C124S> PTEN 399 STOP (Fig. 8 compare lanes 9,10 and 12 of the PTEN blot). In summary, our results suggest that the PDZ-binding motif and/or the catalytic activity of PTEN influence GRASP interaction with PTEN.
Discussion

In this study we report the generation of a mice with germline deletion of GRASP (GRASP\textsuperscript{−/−}). We have observed the expression of GRASP transcripts in the epidermis of murine skin and have demonstrated the induction of the GRASP transcript in the epidermal and dermal layers following UVB exposure. Our data indicates the potential role of GRASP \textit{in vivo} as a regulator of p53-mediated cell-cycle arrest and/or apoptotic response after UVB exposure.

GRASP is well characterized as a molecular scaffold highly expressed in neuronal tissue (1,2,4). However, understanding the role of GRASP \textit{in vivo} was hindered by the relative inaccessibility and complexity of the nervous tissue as a model system. Skin provides an excellent alternative with its accessibility, simplicity and well characterized robust phenotype to external physical or chemical insults (7-14). We found that unlike cells from neuronal origin, topical application of ATRA did not induce GRASP transcripts in murine skin (unpublished data). This indicates a strong tissue-specific regulation of signaling cascades which potentially regulate GRASP expression. Additionally, we observed robust induction of GRASP by quantitative real-time PCR analysis after an acute dose of UVB in skin (see Fig. 3.2A). Using \textit{in situ} analysis we demonstrate that this induction of GRASP transcript occurs predominantly in the dermal compartment of skin (see Fig. 3.2B,C). Our current
understanding of the dermal response to UVB exposure has been largely been limited to one of the following two categories: (i) a hyper-vascularization response accompanied with a large increase in infiltrating macrophage, neutrophils, langerhan cells and a cascade of event that leads to suppression of the innate immunity of the skin (46-52), (ii) a poorly understood signaling cascade which causes major alteration in the dermal extracellular matrix triggered largely by activation of metalloproteinases (53-55). As GRASP has known to be poorly expressed in the immune system, the large induction of dermal GRASP could potentially be in reticular fibroblasts, involved in the signaling cascade leading to the activation of metalloproteinase. Future efforts should also be focused on investigating the status of the dermal elastin and collagen contents following UVB exposure in GRASP−/− mice, which could provide new insights relevant to photo-damage and aging.

A well established outcome of exposure to an acute dose of UVB is a robust increase in the epidermal thickness i.e. epidermal hyperplasia, as observed by histological analysis (10-14). Concurrently, we observed a robust epidermal hyperplastic response after 24 hours of the UVB exposure in our wild-type control mice (see Fig. 3.3A,B). Interestingly, after 48 hours of the UVB exposure the epidermal thickness of our wild-type control mice returned to basal levels (Fig. 3.3C), indicative of a high UVB resistance background of this strain. Furthermore, a robust apoptotic response was observed from 12-48 hours post-UVB exposure in the wild-
type mice. In contrast, apoptotic response in GRASP−/− mice is attenuated and virtually absent at 48 hours post-UVB (see Fig. 3.5B,D,F and H), suggesting a potential mechanism for the increased epidermal thickness seen at this time point. A confounding observation was the absence of increased epidermal thickness in GRASP−/− mice after 24 hours of UVB exposure (Fig. 3.3E). This suggests a potential proliferation defect in addition to the misregulated apoptotic response in the GRASP−/− mice. Indeed 48 hours post-UVB exposure, we observe a subtly reduced %BrdU+ epidermal cell count in GRASP−/− mice as compared to wild-type littermate control (Fig. 3.4G). Future efforts should be focused on using alternative proliferative markers like PCNA, Ki-67 and/or a bivariate BrdU/DNA flow-cytometric analyses to determine the percentage of cells in each cell cycle phase of the GRASP−/− epidermis. In summary, our results clearly indicate an imbalance in the proliferative and apoptotic response of GRASP−/− mice to UVB exposure.

One of the classic molecular hallmarks of UVB induced photodamage is the formation of cyclobutane pyrimidine dimer and (6-4) photoproducts, which triggers the activation of DNA repair machinery (13). The tumor suppressor p53, is known to be robustly induced by DNA damage following UVB exposure, triggering cell-cycle arrest and/or apoptosis to prevent the accumulation of photodamaged cells in the skin. Misregulated apoptosis or the uncontrolled proliferation of cells i.e. cancerous cells, observed following acute UVB damage, is often attributed to a disruption of the
extremely complex multi-leveled regulatory process of p53 induction, stabilization and/or sub-cellular localization (11,13,16,22-30). We investigated if the misregulated apoptotic response in GRASP−/− following damage is due to similar disruption in the regulation of p53 induction/stabilization. Our data suggest that the steady-state levels of the p53 transcript and the induction of p53 protein levels following UVB exposure in wild-type mice are comparable to the GRASP−/− littermate mice (see Figs. 3.S1A,B). However, GRASP−/− mice exhibit significant attenuation in the nuclear accumulation of p53 following UVB treatment, indicative of misregulated nuclear import/export of the p53 protein (see Fig. 3.6A-I). Interestingly, the loss of nuclear p53+ cells perfectly coincided with the loss of TUNEL+ cells in the in the epidermis. This strongly indicates that the transient apoptosis following UVB exposure in GRASP−/− mice is due to misregulation of the p53-mediated apoptotic pathway.

A recent review exhaustively summarizes known regulatory pathways influencing the sub-cellular distribution of p53(29). Amongst these pathways the direct role of Phosphatase and tensin homolog detected in chromosome ten (PTEN) and the indirect effect of PTEN through the Akt/PI3K-mdm2 axis, in sub-cellular localization of p53 has received the most attention. We have now identified a strong interaction between the c-terminal PDZ-binding motif of PTEN and GRASP (see Fig. 3.7). Furthermore, our results also show that the GRASP-PTEN interaction is influenced by the phosphatase activity of PTEN (see Fig. 3.7). Taken together,
GRASP can potentially regulate p53 sub-cellular localization either by interacting and modulating the PTEN-p53 complex and/or the PTEN-Akt/PI3K-mdm2 axis.

In summary, our results indicate a potential role of GRASP in UV induced p53-mediated response of the skin. The underlying importance of this study is highlighted by the finding that over 50% of all known cancers have been linked to misregulation in any one of the many p53-dependent response pathways (22,31). Future, research should be directed by further dissecting the mechanism of GRASP-mediated nuclear transport/stabilization of p53. Furthermore, our study and that published elsewhere, indicates that GRASP likely plays minimal role in embryological development or post-natal basal homeostasis. However, under conditions of induced stress such as UVB exposure or morphine administration (6), the in vivo role of GRASP becomes self-evident. This suggests the potential role of GRASP as 'late gene' which tends to upregulated following an imbalance in the dynamic equilibrium of a given system. It will therefore be prudent to test other proliferative paradigms in the skin (and/or other model systems) to further our understanding of the in vivo role of GRASP.
References

Figure 3.1. Generation of mice with germline deletion of GRASP (GRASP\textsuperscript{−/−}).

(A) Schematic representation of the genomic organization of the mouse GRASP locus with exons 1-8 (B) Schematic representation of the GRASP targeting vector indicating the upstream and downstream LoxP site and the PGK-Neo cassette. PGK-Neo cassette was flanked by Frt sites for excision by Flp recombinase. (C-D) Southern analysis of homologous recombination at the GRASP locus using long template PCR amplified fragments. (C) At the 5’ end PCR products from A1/A2 primers was probed with P1 and P2 (inside and outside respectively of the targeting vector) to identify the transgenic clones. (D) At the 3’end PCR products from A3/A4 primers was probed with P3 and P4 (inside and outside respectively, of the targeting vector) to identify the transgenic clones. (E) Immunoblot analysis of whole brain extracts prepared from wildtype and GRASP\textsuperscript{−/−} mice using anti-GRASP and anti-CTIP2 (as loading control) antibody.
Figure 3.1
Figure 3. 2. GRASP transcripts are induced in the epidermal and dermal layers of the murine skin following an acute UVB exposure

(A) Quantitative real-time PCR (qRT-PCR) analysis of the GRASP transcript in skin samples prepared from wild-type and GRASP−/− mice at the indicated time after UVB exposure. (B) Section *in situ* hybridization of skin samples using full length GRASP transcript as a probe. Skin samples were collected at indicated time after UVB exposure from either wild-type or GRASP−/− mice.
Figure 3.3. GRASP\(^{-/-}\) mice exhibit delayed epidermal thickening to UVB exposure.

(A-F) Hematoxylin and Eosin (H&E) stained histological sections obtained at indicated time intervals after UVB exposure from wild type (A-C) and GRASP\(^{-/-}\) mice (D-F). Scale bars = 50 \(\mu\)M. (G) Quantitation and comparison of the epidermal thickness at indicated times intervals following UVB exposure in wild-type and GRASP\(^{-/-}\) mice respectively. Values in (G) represent the mean +/- S.E.M. of three littermate controlled mice for each time point and genotype. Statistical significance is indicated by */#/ and **/## symbols for (p < 0.05) and (p < 0.01), respectively. Scale bars = 50 \(\mu\)M.
Figure 3.4. GRASP<sup>−/−</sup> mice exhibit a reduced proliferative response to UVB exposure.

(A-F) BrdU (Red) and K14 (Green) immunohistochemical staining in skin samples. Samples are obtained at indicated time intervals after UVB exposure, from wild type and GRASP<sup>−/−</sup> mice. Scale bars = 50 μM. (G) Quantitation and comparison of the BrdU<sup>+</sup>/K14<sup>+</sup> cells of the epidermis at indicated times intervals following UVB exposure in wild-type and GRASP<sup>−/−</sup> mice respectively. Values in (G) represent the mean +/- S.E.M. of three littermate controlled mice for each time point and genotype. Statistical significance is indicated by *** symbol for (p < 0.001). Scale bars = 50 μM.
Figure 3.4

The images depict the effects of UV exposure on the skin, comparing WILD-TYPE and GRASP<sup>−/−</sup> mice.

**A** and **B** show the skin without UV exposure.

**C** and **D** show the skin after 24 hours of UV exposure.

**E** and **F** show the skin after 48 hours of UV exposure.

**G** presents a graph showing the percentage of BrdU positive epidermal cells over time (0, 24, and 48 hours).
Figure 3.5: GRASP<sup>−/−</sup> mice exhibit attenuated apoptotic response to UVB exposure.

(A-H) TUNEL assay was performed in skin are obtained at indicated time intervals after UVB exposure, from wild type and GRASP<sup>−/−</sup> mice. The skin samples were counterstained with nuclear marker DAPI. Scale bars = 50 μM.
Figure 3.5
Figure 3.6: The nuclear localization of p53 following UVB exposure is transient in the GRASP<sup>−/−</sup> mice.

(A-F) Immunohistochemical staining for p53 (Red) and CTIP2 (Green) in skin samples obtained at indicated time intervals after UVB exposure, from wild type and GRASP<sup>−/−</sup> mice. Samples were counterstained with nuclear marker DAPI (blue). Scale bars = 50 μM. (G) Quantitation and comparison of the p53<sup>+</sup> cells of the basal layer of epidermis at indicated times intervals following UVB exposure in wild-type and GRASP<sup>−/−</sup> mice respectively. Values in (G) represent the mean +/- S.E.M. of two littermate controlled mice for each time point and genotype. Scale bars = 50 μM.
Figure 3.6
Figure 3.7: GRAPs co-IPs with the tumor suppressors p53 and PTEN.

Co-immunoprecipitation of Myc-GRASP and indicated PTEN mutants in HEK293 cells
Figure 3.S1: The steady-state levels of the p53 transcript and the induced levels of the p53 protein after UVB exposure are comparable between the wild-type and GRASP^/- mice.

(A) Quantitative realtime-PCR (q-RTPCT) was performed to determine p53 transcript levels in skin samples obtained from wild-type (unshaded bars) and GRASP^/- (shaded bars) mice at indicated times after UVB exposure. Values in (A) represent the mean +/- S.E.M. of three littermate controlled mice for each time point and genotype. (B) Immunoblot analysis of p53 protein was performed in skin lysates obtained from wild-type and GRASP^/- mice at indicated times after UVB exposure.
General Conclusion

Chapter 4
Since the discovery of GRASP as a retinoic acid induced gene in P19 cells, significant progress has been made in understanding the physiological role of this scaffold protein. GRASP was known to interact with numerous neuronal proteins and influence the membrane expression of certain membrane receptors; however the molecular underpinnings of this GRASP-dependent trafficking route was not known. Furthermore, GRASP was shown to be highly expressed in the post-natal murine brain but paradoxically, mice with germline deletion of GRASP did not exhibit any obvious defects. Our studies have now helped shed light on the physiological role of GRASP by a combination of in vitro and in vivo approaches and provided a solid framework for future research.

Previous work from our laboratory has demonstrated a strong interaction of GRASP with Grp1, a guanine nucleotide exchange factor (GEF) for Arf6. In this study using in vitro approaches we show that GRASP through its interaction with Grp1 regulates the Arf6-dependent membrane trafficking pathway. GRASP can localize into endosomal structures and potentially recruits Arf6-GEF like Grp1 to initiate Arf6-activation. Arf6-activation is not only crucial for membrane recycling of certain class of receptors but is also known to regulate phosphoinositide metabolism and actin-dynamics. Both, balance of membrane phosphoinositide species and surface actin-
dynamics are crucial for various cellular activities like cytokinesis, cellular migration, axonal growth or synapse formation. Taken together, our studies suggests the exciting possibility of GRASP involvement in these above mentioned Arf6-dependent cellular events which will be the focus of future research.

We also generated mice with germline deletion of GRASP (GRASP\(^{-/-}\)). This provides an ideal in vivo system to test the physiological role of GRASP by loss of function studies. Under basal conditions we observed no obvious differences between the wild-type and GRASP\(^{+-}\) mice, recapitulating previously published reports. We detected expression of GRASP in the epidermal layers of murine skin and identified a robust induction of the GRASP gene in both the epidermal and dermal layers of the skin following ultraviolet-B (UVB) exposure. In comparison to wild-type mice, we identified an attenuated proliferative and apoptotic response in GRASP\(^{-/-}\) mice, following UVB exposure. Our results strongly indicates the role of GRASP in regulating the sustained nuclear localization of p53 potentially through the regulating of the PTEN-Akt-PI3K axis and suggests the absence of this regulation led to the aberrant response to UVB exposure in GRASP\(^{-/-}\) mice. The exact mechanism of GRASP mediated nuclear localization of p53 will be the focus of future research. Misregualtion of p53-dependent pathways is a major etiological factor in majority of
the human cancers, our studies opens a new avenue for therapeutic intervention against cancer.

In conclusion, our studies suggest the likely role of GRASP as a 'late gene' that potentially functions to regain homeostasis in a given biological system following exposure to stress. This suggestion is strengthened by considering previous reports wherein altered behavioral response of the GRASP\(^{-/-}\) mice were observed only under conditions of induced chemical stress namely morphine administration.
Bibliography

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