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Interactions between GIPC–APPL and GIPC–TRP1 regulate melanosomal protein trafficking and melanogenesis in human melanocytes

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Abstract

By virtue of the presence of multiple protein–protein interaction and signaling domains, PDZ proteins play important roles in assembling protein complexes that participate in diverse cell biological processes. GIPC is a versatile PDZ protein that binds a variety of target proteins in different cell types. In previous studies we showed that, in epidermal melanocytes, GIPC interacts with newly synthesized melanosomal protein TRP1 in the Golgi region and proposed that this interaction may facilitate intracellular trafficking of TRP1. However, since GIPC contains a single PDZ domain and no other known protein interaction motifs, it is not known how GIPC–TRP1 interaction affects melanosome biogenesis and/or melanin pigmentation. Here, we show that in human primary melanocytes GIPC interacts with AKT-binding protein APPL (adaptor protein containing pleckstrin homology, leucine zipper and phosphotyrosine binding domains), which readily co-precipitates with newly synthesized TRP1. Knockdown of either GIPC or APPL inhibits melanogenesis by decreasing tyrosinase protein levels and enzyme activity. In melanocytes, APPL exists in a complex with GIPC and phospho-AKT. Inhibition of AKT phosphorylation using a PI3-kinase inhibitor abolishes this interaction and results in retardation TRP1 in the Golgi. These data suggest that interactions between TRP1–GIPC and GIPC–APPL–AKT provide a potential link between melanogenesis and PI3 kinase signaling.

Keywords

PDZ proteins; GIPC; Adaptor APPL; Protein trafficking; Melanosomal proteins; Melanin pigmentation

Introduction

PDZ domains are among the most commonly found protein–protein interaction domains present in proteins in all organisms from bacteria to humans. PDZ domains can either bind to the C-termini of unrelated proteins or dimerize with other PDZ proteins. By virtue of the presence of multiple PDZ domains, other protein interaction and also signaling domains in a single polypeptide, PDZ proteins participate in the assembly of protein complexes involved in diverse biological processes such as organization of phototransduction pathway in *Drosophila* and signaling complexes at synapses and neuromuscular junctions, trafficking of receptors and other membrane proteins, and maintenance of epithelial cell morphology and

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polarity [1]. Thus, delineating the dynamics of complex protein networks in which PDZ proteins participate is necessary to understand their diverse roles [2].

PDZ protein GIPC was originally identified in association with a regulator of G protein signaling, RGS19-GAIP ($G_{\alpha i}$ interacting protein), on clathrin coated vesicles [3]. GIPC also interacts with a variety of intracellular and plasma membrane proteins [4–15] and human viral oncoproteins [16,17]. We showed that, in melanocytes, GIPC binds transiently to C-terminus of newly synthesized melanosomal membrane protein tyrosinase related protein 1 (TRP1[2]/gp75) in the Golgi region and proposed that this interaction is involved in trafficking of TRP1 along the endosomal pathway [18]. The 333 amino acid long GIPC polypeptide contains only a single PDZ domain and a C-terminal acyl carrier protein motif but no other protein interaction or signaling domains [3,18]. Therefore, it is not clear how GIPC–TRP1 interaction regulates TRP1 trafficking. Other PDZ proteins that contain a single PDZ domain, PICK1 (protein interacting with C kinase 1) and ERBIN (ERBB2/HER2 receptor interacting protein) also have, respectively, a coiled-coil domain and leucinerich repeats, which are thought to mediate their functions [19,20]. We hypothesized that functions of GIPC in TRP1 trafficking may be mediated by its interactions with other signaling proteins. Here, using affinity chromatography, we report identification of APPL as GIPC interacting protein in melanocytes and show that APPL forms complexes with GIPC and phospho-AKT as well as melanosomal membrane protein TRP1. Downregulation of either GIPC or APPL by small hairpin RNAs resulted in decreased tyrosinase activity and melanin pigment accumulation. Inhibition of phosphorylation of AKT by a phosphatidylinositol 3-kinase (PI3 kinase) inhibitor abolished APPL–AKT interaction and disrupted trafficking of newly synthesized TRP1. Our data suggest that APPL, through its interactions with GIPC, TRP1 and AKT, provides a potential link between PI3 kinase signaling and regulation of melanosomal protein trafficking and melanogenesis.

Materials & methods

Cell culture

Isolation and culture of melanocytes, keratinocytes and fibroblasts, culture of human melanoma cell lines was described earlier [18,21]. Normal human cells and other human tumor cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in media recommended by the supplier.

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Cloning full-length and deletion mutant of APPL

A full-length APPL cDNA was amplified from messenger RNA isolated (Micro-FastTrack mRNA Isolation Kit, Invitrogen Corp., Carlsbad, CA) form human melanoma cell line SK-MEL-23. c.22 by reverse transcriptase-polymerase chain reaction (Two-step RT-PCR Kit, Roche Applied Sciences, Indianapolis, IN) using oligonucleotide primers designed to incorporate HindIII (5′-GACAAGCTTATGCCGGGGATCGACAAGCTG-3′) and KpnI (5′-ACTGGTACCTCTTATGCTTCTGATTCTCTCTT-3′) restriction endonucelase sites at 5′ and 3′ ends of APPL cDNA open reading frame. The amplified fragments were first cloned into TOPO TA cloning vector (Invitrogen Corp.) and subcloned into pFLAG-CMV5 (Sigma–Aldrich Corp., St. Louis, MO) to generate a C-terminal epitope-tagged APPL expression plasmid. C-terminal deletion mutants of APPL protein expression plasmids were constructed by amplifying the corresponding cDNA fragments using oligonucleotide

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[2]*Abbreviations used:* APPL, adaptor protein containing pleckstrin homology, leucine zipper and phosphotyrosine binding domains; GST, glutathione *S*-transferase; HMBA, hexamethylene bisacetamide; IP, immunoprecipitation; IB, immunoblotting; PI3 kinase, phosphatidylinositol-3 kinase; PTB, phosphotyrosine binding; SDS–PAGE, sodium dodecyl sulfate polyacryalmide gel electrophoresis; TRP1, tyrosinase related protein 1.

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primers (with 5′-HindIII site as above and with separate 3′-KpnI site primers containing stop codons at amino acid positions 707, 632, 501, 391, and 273) and cloning them into pFLAG-CMV5a vector. A C-terminal (stop codon at amino acid position 127) truncated GIPC expression plasmid was generated using a similar strategy. Presence of appropriate APPL and GIPC coding sequences were confirmed by automated DNA sequencing.

Transfection

SK-MEL-23.c22a human melanoma cells cultured in 60 mm dishes were co-transfected with various plasmids (up to a total 6 μg DNA/dish) using LipofectaminePlus reagent (GIBCO-BRL Life Technologies, Grand Islands, NY) according to manufacturer's instructions. pCMV2-FLAG-BAP (bacterial alkaline phosphatase; Sigma–Aldrich Corp.) plasmid was used for control transfections. Following 36 h transfection, cells were washed with cold PBS, harvested by gentle scrapping and lysed in 50 mM phosphate buffer, pH 7.0 containing150 mM NaCl, 1% Triton X-100 containing a cocktail of protease inhibitors (Roche Applied Sciences). Detergent lysates were cleared by centrifuging at 15,000×g for 20 min and used for immunoprecipitation. Cytosolic and membrane proteins were prepared as described earlier [18].

Human GIPC and APPL shRNA lentivirus

Short hairpin (sh) RNA plasmids, a collection of five each, targeting GIPC, APPL and one negative control shRNA plasmid were purchased from Open Biosystems (Huntsville, AL). Efficiency of knockdown by these shRNAs was tested by transfecting melanocytes. We generated GIPC and APPL shRNA and scrambled shRNA lentiviruses sing transfection of HEK293 cells with the envelope plasmid pSVG and a second generation packaging plasmid (pCMVΔ8.2) obtained from Trono Lab (École polytechnique Fédérale de Lausanne, Switzerland). Detergent lysates (equivalent to 50 μg protein) of shRNA lentivirus infected melanocytes were analyzed by Western blotting using anti-GIPC and anti-APPL antibodies [diluted 1:250 in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST)] [22]. Protein bands were detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation and immunoblotting

Protein concentration in cell lysates was estimated using bicin-chonnic acid (BCA) protein assay reagent kit (Pierce Biotechnology Inc., Rockford, IL) and aliquots of lysates containing equal amount of protein were incubated with GST-precleared rabbit anti-GIPC antibody [18], or anti-FLAG monoclonal antibody (mAb M2, Sigma–Aldrich Corp.) or pan-AKT antibody (Cell Signaling Technology Inc., Beverly, MA; catalog number 9272) at 4 °C for 12–16 h followed by addition of 60 μl protein A Sepharose for 1 h and washed the beads thoroughly (3–5 times with 1 ml/wash) with the lysis buffer. Proteins in total cell lysates and the immunoprecipitated proteins were separated by SDS–PAGE, transferred to a membrane and immunoblotted with appropriate antibodies, as described in the legends to the figures, and detected using ECL chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence microscopy

Cells were plated on glass coverslips and left untreated or treated with hexamethylene bisacetamide (HMBA), allowed to recover from HMBA as described earlier [18] and then treated with 100 nM wortmannin for 4 h. For immunofluorescence analysis of transiently transfected cells, the cells transfected in 60 mm tissue culture dishes were trypsinized after 24 h of transfection and plated on glass coverslips and allowed to attach for 24–36 h. The cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 10 min or ice-cold methanol for 1 min.

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After blocking with 3% BSA in PBS, cells were incubated at 4 °C for 1 h with primary antibodies [affinity purified anti-DIP13α (1:20) or preimmune IgG (1:20) or Cy3-conjugated mAbM2 (1:100)]. After washing and incubation with rhodamine-conjugated goat anti-rabbit IgG (1:100, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h, cells were finally stained with fluorescein isothiocyanate (FITC)-conjugated anti-gp75 mAb TA99 (1:40) for 1 h at RT. Immunofluorescence was captured using a laser scanning confocal image system (LSM510, Carl Zeiss, Jena, Germany).

Results

Identification of GIPC interacting protein APPL

To identify GIPC interacting proteins in melanocytes, we performed glutathione *S*-transferase (GST)-GIPC-Sepharose affinity chromatography. GST-Sepharose precleared biotinylated melanocyte cell extracts were incubated with GST-GIPC-Sepharose beads. Bound proteins by were eluted by digestion with thrombin, which cleaves the immobilized fusion protein at a site between GST and GIPC, revealed two prominent polypeptide bands of ~25 and 50 kDa (Supplementary Data, Fig. 1A). A scaled-up preparation of GIPC bound proteins from unlabeled melanoma cell lyaste followed by in-gel trypsin digestion of the ~50 kDa band and reverse-phase HPLC nano-electrospray tandem mass spectrometry (performed at Harvard Microchemistry Facility) yielded 30 overlapping peptides with amino acid sequences identical to peptide sequence deduced from of human adaptor protein APPL cDNA (Accession number AF169797). These peptides spanned N-terminal portion of the predicted amino acid sequence of human APPL cDNA encoding an 83 kDa protein (Supplementary Data, Fig. 1B). Protein encoded by this cDNA was originally identified as serine/threonine kinase AKT2/protein kinase B (PKB) interacting protein [23]. APPL polypeptide has three potential thrombin cleavage sites (Pro–Arg/Lys–Xaa and Gly–Arg/Lys–Xaa) [24] at Arg_{314}, Arg_{477} and Arg_{594}. The predicted molecular mass (53.5 kDa) of the peptide fragment generated by cleavage at Arg_{477} in the sequence Gly–Arg–Arg corresponds to the apparent molecular mass of the polypeptide obtained by thrombin digestion of GIPC bound proteins.

APPL was also identified, independently, as DIP13α, a protein that interacts with the tumor suppressor DCC, deleted in colon cancer [25] and as a protein (APPL1) that links Rab5 to nuclear signal transduction via an endosomal compartment [26]. In PC12 cells, APPL was shown to recruit GIPC to peripheral endosomes and regulate trafficking of nerve growth factor receptor TrkA [27]. APPL contains a leucine zipper domain (LZ), coiled-coil domains (CC), pleckstrin homology domain (PH) and a phosphotyrosine binding domain (PTB) and a BAR (BIN1/Amphiphysin/RVS167) domain at the N-terminus of APPL [26].

GIPC interacts with APPL in melanocytes and melanoma cells

To investigate GIPC–APPL interactions in melanocytes *in vivo*, we cloned APPL cDNA from human melanocytes into FLAG-epitope expression plasmid. We transfected melanoma cells with APPL-FLAG plasmid and immunoprecipitated the cell lysates with control rabbit IgG or anti-GIPC antibody and analyzed the immune complexes by western blotting with anti-FLAG mAb M2. Anti-GIPC antibody, but not control IgG, immunoprecipitated 83 kDa APPL (Fig. 1A). Endogenous APPL also co-precipitated with endogenous GIPC in melanocytes and two pigmented melanoma cell lines SK-MEL-19 and -23 confirming this interaction *in situ* in melanocytic cells (Fig. 1B). In all normal melanocytes (NMC), primary (WM75, WM35, WM98-1) and metastatic (451Lu, 1205, SK-MEL-23) melanoma cell lines tested there was nearly same amount of APPL was detectable showing that its expression is not affected by melanocyte transformation and melanoma tumor progression (Supplementary Fig. S2).

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Association and co-localization of APPL with TRP1

Since we identified GIPC as a TRP1-interacting protein in melanocytes and both APPL and TRP1 can bind to the single PDZ-domain containing GIPC through their C-terminal PDZ binding motifs ([18] and Supplementary Fig. S3), we asked whether APPL is also associated with TRP1. We used mAbTA99 to immunoprecipitate lysates of TRP1-negative melanoma cells transiently transfected with TRP1 and FLAG-APPL. Western blotting with anti-FLAG M2 showed co-precipitation of 83 kDa FLAG-APPL with TRP1 in anti-TRP1 immune complexes but not in control IgG complexes (Fig. 2A). However, very little or no APPL could be co-precipitated with TRP1 at steady state in melanocytes or melanoma cells, similar to our observation on co-precipitation of GIPC with TRP1. These data suggest that similar to the interaction of GIPC with TRP1 [18], APPL also binds to newly synthesized TRP1. Since both TRP1 and APPL bind to GIPC thorough its PDZ-binding motif [18,28] and GIPC can form oligomers [29], co-precipitation of APPL with newly synthesized TRP1 suggests that in melanocytes APPL can form transient multi-protein complex containing TRP1–GIPC and GIPC–APPL.

GIPC interacts with newly synthesized melanosomal membrane protein TRP1 in the Golgi region [18]. Therefore, we asked whether APPL, through its interactions with GIPC and TRP1, is also associated with membranes. Immunoblotting of cytosolic and membrane fractions of a pigmented TRP1-positive melanoma cell line SK-MEL-19 cells showed that whereas the bulk of endogenous APPL was present in the cytosolic soluble fraction, a small amount of APPL protein was also present in the membrane fraction (Fig. 2B). Sucrose density gradient analysis of postnuclear supernatants showed that membrane associated APPL is found predominantly in the low-density fractions (data not shown). Confocal immunofluorescence microscopy of pigmented melanoma cells stained with FITC conjugated anti-TRP1 mAbTA99 and affinity purified, polyclonal APPL antibody showed characteristic Golgi and punctate cytoplasmic staining for TRP1 and a uniform and predominantly diffuse cytoplasmic distribution of APPL staining (Fig. 2C). Intense staining for APPL and TRP1 in a few vesicles in the region adjacent to the nucleus could also be seen (Fig. 2C lower panels). Together with co-precipitation data, these observations suggest that APPL localized to the intracellular vesicles in melanocytes is associated with TRP1.

Knockdown of GIPC and APPL inhibits melanogenesis

To understand the role of these interactions between melanosomal protein TRP1, GIPC and APPL, we performed lentiviral shRNA mediated knockdown of GIPC and APPL in pigmented SK-MEL-23 (clone 22) melanoma cells. We tested five different shRNAs (Open Biosystems, Huntsville, AL) and used two different shRNAs that produced reproducible knockdown as determined by immunoblotting (Supplementary Fig. S4 and Fig. 3A). This minimized potential off-target effects of shRNAs. Knockdown of either GIPC or APPL resulted in a significant inhibition (up to 70%) of melanogenesis as evident both by visual appearance of cell pellets and quantitation of alkali soluble intracellular melanin (Fig. 3B). Consistent with decreased melanin content, western blot analysis showed reduced tyrosinase protein and enzyme assays showed tyrosinase activity in GIPC and APPL-knockdown cells (Fig. 3C and D). Knockdown of GIPC or APPL did not result in detectable change in other melanogenic proteins TRP1 and TRP2. These data suggest that GIPC and APPL play a role in melanogenesis by regulating tyrosinase activity by influencing the accumulation of tyrosinase protein.

APPL forms a ternary complex with AKT and provides a potential link between PI3 Kinase signaling and TRP1 transport

APPL was originally identified as an AKT2 interacting protein [23]. To test whether AKT2 also binds to GIPC–APPL in melanocytes, we first transiently transfected with FLAG-APPL

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or a control FLAG-BAP plasmids and immunoprecipitated FLAG-tagged proteins with anti-FLAG mAb M2 and analyzed the immunoprecipitated proteins by immunoblotting with anti-AKT2 and anti-GIPC antibodies. Both endogenous AKT2 and GIPC precipitated with FLAG-APPL, but not with FLAG-BAP control (Supplementary Fig. S5), showing that APPL can form a ternary complex with AKT and GIPC. To test whether APPL forms such complex endogenously, we performed a reverse immunoprecipitation analysis of primary melanocyte and SK-MEL-19 melanoma cell lysates with anti-AKT2 antibody. Immunoblotting of precipitated proteins with anti-APPL and anti-GIPC antibodies showed that both APPL and GIPC co-precipitated with AKT more readily from melanoma cells than in melanocytes (Fig. 4B) although APPL co-precipitates with GIPC equally efficiently from both melanocytes and melanoma cells (Fig. 4A). Since APPL co-precipitated with AKT more efficiently from melanoma cells than from normal melanocytes, we asked whether this difference could be due to levels of pAKT. Immunoblot analysis of cell lysates using anti-AKT and anti-phosphoAKT2 (pAKT2) antibody showed that although both melanocytes and melanoma cells have nearly equal amounts of total AKT, melanoma cells had significantly higher levels of pAKT. Analysis of several independent cultures of melanocytes and melanoma cell lines also confirmed these observations (data not shown). These data suggest that binding of AKT to APPL in melanocytic cells is directly proportional to the levels of pAKT. This is supported by the observation that APPL fails to co-precipitate with AKT in melanocytes and melanoma cells treated with wortmannin [30], an inhibitor of PI3 kinase, a principal kinase that phosphorylates AKT (Fig. 4C, lanes 3 and 4).

Interaction of GIPC simultaneously with newly synthesized TRP1 and with the AKT-binding signaling protein APPL raised the possibility that APPL, through its interaction with GIPC, provides a link between PI3 kinase/AKT signaling pathways and biosynthetic transport of TRP1. To investigate whether PI3 kinase signaling is involved in TRP1 trafficking, we tested the effect of wortmannin on biosynthetic transport of endogenous TRP1 (Fig. 4D) using a strategy we described to synchronize biosynthesis of endogenous TRP1 [18]. Previously, we showed that treatment of melanoma cells with the differentiation-inducer hexamethylene bisacetamide (HMBA) selectively and reversibly inhibits transcription of *TRP1* gene [21] and new TRP1 protein accumulates steadily during recovery after wash-out of the inhibitor [18]. We used this reversible inhibition of TRP1 to synchronize the biosynthesis of endogenous TRP1 to test the effect of wortmannin. SK-MEL-19 melanoma cells expressing TRP1, constitutively and during recovery from HMBA inhibition, were treated with wortmannin for 4 h and stained with anti-TRP1 mAb TA99. In control melanoma cells, a characteristic intense juxtanuclear and punctate cytoplasmic distribution of constitutively expressed TRP1 could be seen (Fig. 4D, panel a). Treatment with wortmannin did not markedly affect the intracellular distribution of preexisting TRP1 (panel b). In cells recovering from inhibition by HMBA, newly synthesized TRP1 showed juxtanuclear patchy and cytoplasmic punctate staining similar to the protein in control cells (panel c). Presence of wortmannin in the medium during recovery from HMBA resulted in the accumulation of the newly synthesized protein in the juxtanuclear region with little or no detectable staining in the periphery (panel d).

Taken together with the observations that wortmannin treatment abolishes interaction of APPL with AKT and inhibition of PI3 kinase results in retardation of TRP1 in the Golgi, these data suggest that the dynamic interactions between the PDZ protein GIPC with TRP1 and the signaling complex APPL-AKT provide link between PI3 kinase signaling and biosynthetic trafficking of TRP1 providing a cell biological mechanisms for regulation of melanosome biogenesis and melanin pigment accumulation.

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Discussion

PDZ proteins, which generally contain multiple protein interaction domains and also signaling domains, are ideal candidates for linking cell biological processes including membrane trafficking, with signaling [1]. In this study we show that the single PDZ domain protein GIPC binds multi-domain adaptor protein APPL and propose that this interaction of GIPC with AKT-binding APPL provides, in melanocytes, a potential link between PI3 kinase signaling and regulation of intracellular trafficking of the melanosomal membrane protein TRP1. Interaction with APPL seems to compensate for the absence of additional protein interaction and signaling domains on the single PDZ domain containing protein GIPC. Such interactions are thought to increase the connectivity of existing proteins and endow them with new functions [31]. Binding of GIPC to newly synthesized TRP1 and to APPL, a protein with coiled-coil and signaling domains, is consistent with a well established role for long coiled-coil proteins (found on the Golgi and endosomes) in vesicular trafficking [32]. Also, a role for APPL in linking signaling and intracellular membrane trafficking is consistent with the newly described function of APPL in linking Rab5, a key regulator of endocytosis, to nuclear signal transduction. Moreover, the presence of BAR domain, a structure that is related to arfaptins known to be recruited to Golgi, in APPL also supports a role for this protein in membrane trafficking [26]. The concept that there is convergence between signaling and membrane trafficking, two distinct cell biological processes, is gaining support with the identification of an increasing number of functional interactions between components involved in these two processes [26,33–36].

GIPC interacts with a variety of plasma membrane [10,37] and intracellular [38] proteins and viral oncoproteins [16,17]. The exact functions of these diverse interactions or how the GIPC-target protein interactions are translated to cell biological processes are not clear. GIPC was originally thought to be a component of a G protein-coupled signaling complex involved in the regulation of vesicular trafficking [3]. In melanocytic cells GIPC interacts transiently with newly synthesized melanosomal membrane protein TRP1 in the early biosynthetic compartments, presumably endoplasmic reticulum and Golgi, suggesting that GIPC plays a role in trafficking of TRP1 [18]. It is also of interest to note that in all human cancer cell types tested (data not shown), including melanoma, APPL readily co-precipitated with GIPC, suggesting that the GIPC–APPL complex participates in cellular functions common to most cancer cells. For example, in prostate cancer cells, GIPC may sequester APPL and prevent its binding to DCC and induction of apoptosis [25]. Conversely, in other cancer cells, interaction of GIPC with APPL may prevent GIPC from binding to its target proteins and attenuate its cellular functions, for example, mediating growth inhibitory signaling by transforming growth factor beta type III receptor [10].

The versatile and cell type-specific actions of GIPC are highlighted by the diverse nature of GIPC-interacting proteins, their mode of interaction with GIPC, and the cell biological consequences of such interactions. In transfected HEK293 cells and endogenously in PC12 cells, GIPC has been reported to bind GAIP and the tyrosine kinase receptor TrkA and this association seems to provide a link between nerve growth factor signaling through TrkA and G protein signaling pathways [12]. Both GAIP and TrkA bind to the single PDZ domain of GIPC, albeit at different sites. Whereas GAIP–GIPC–TrkA complex is proposed to be involved in retrograde transport of the TrkA receptors, transient interaction of GIPC and APPL with newly synthesized TRP1 indicates a role for the GIPC–APPL complex in the biosynthetic transport of TRP1. Thirdly, through its interaction with TrkA, GIPC was proposed to provide a link between G protein signaling and MAP kinases in PC12 cells, whereas interaction of GIPC with AKT-binding adaptor APPL, in melanocytes, appears to link its actions with PI3 kinase signaling pathways.

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Protein kinase B (AKT), the primary target of activation by class I PI3 kinase, is a central point for many signal transduction pathways and regulates several cellular processes. In addition to binding to its substrate proteins, AKT also interacts with many non-substrate proteins [39], including APPL, which can modulate its activity. Thus, in melanocytes, binding of GIPC to APPL–AKT complex might allow regulation of GIPC-mediated processes by PI3 kinase/AKT signaling pathways. Our data, from several experiments, show that in melanocytic cells APPL preferentially binds activated, phosphorylated AKT. They are: (a) APPL co-precipitated with AKT2 more readily in melanoma cells in which there is significantly more activated AKT than in melanocytes, (b) inhibition of activation of AKT by PI3 kinase activity in melanoma cells by treatment with wortmannin decreased the amount of APPL precipitated by anti-AKT2 antibody, and (c) sucrose density gradient fractionation (data not shown) and immunofluorescence analyses showed that APPL is associated with intracellular vesicles.

A role for phosphatidyl inositol polyphosphates and PI3 kinases in endocytosis and in sorting and trafficking of proteins in endosomal/lysosomal compartments has been well documented [30,40–46]. PI3 kinase, which is stimulated by growth factors and cytokines, seems to play a role in both constitutive and regulated membrane trafficking events. However, the role of PI3 kinase in biosynthetic trafficking of proteins is not clear. Involvement of PI3 kinase in transport of newly synthesized TRP1 has been proposed earlier [47]. In MeWo human melanoma cells, inhibition of PI3 kinase activity with wortmannin interferes with the trafficking of TRP1 resulting in its redistribution within a novel endosomal/lysosomal compartment. Based on the staining for markers of endosomes, it was suggested that TRP1 transport from late endosomes to melanosomes is dependent on PI3 kinase. However, the exact mechanism of regulation of TRP1 trafficking by PI3 kinase is not known. A role for PI3 kinase in trafficking of melanosomal protein TRP1 is somewhat puzzling in view of the reports that showed that activation of AKT is not required for coat pigmentation in mice *in vivo* [48], and that PI3 kinase activity correlates with negative regulation of pigmentation in melanoma cells *in vitro* [49,50]. However, in this context, it is important to note that the exact role of TRP1 in human skin pigmentation is not clear. Nonetheless, it is reasonable to speculate that in epidermal melanocytes APPL-AKT complex may be involved, during UV-stimulated melanogenesis, in mediating the effects of PI3 kinase signaling [51].

In summary, in this paper we demonstrate that pAKT-binding adaptor APPL co-precipitates with newly synthesized TRP1 and treatment with wortmannin results in retardation of biosynthetic transport of TRP1. Based on these observations we propose that binding of GIPC recruits APPL–pAKT complex to biosynthetic compartments (ER and/or Golgi) and that APPL provides a link between PI3 kinase signaling and vesicular trafficking events in melanosome biogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.abb.2011.01.021.

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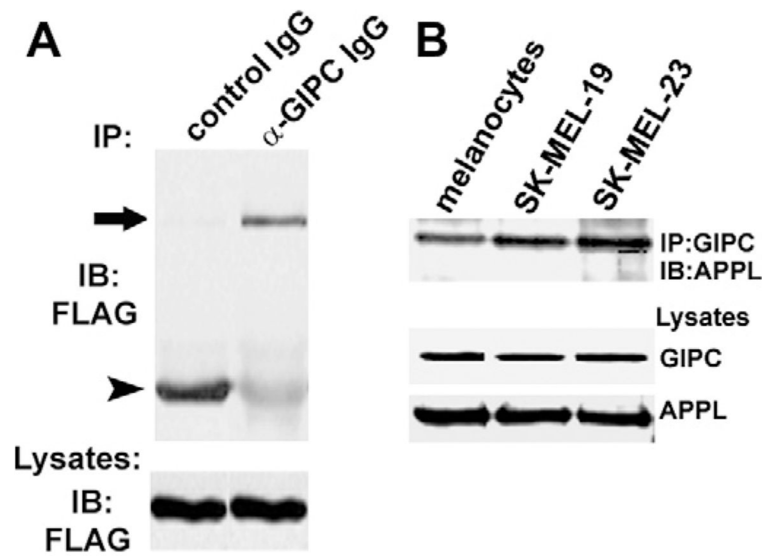

Fig. 1.

GIPC interacts with APPL *in vivo*. (A) SK-MEL-23. c22a melanoma cells were transfecetd with plasmid pCMV2FLAG-APPL and then immunoprecipitated with either control IgG or anti-GIPC antibody and immunoblotted with anti-FLAG antibody. Arrow indicates 83 kDa APPL and arrowhead shows IgG heavy chain. (B) Detergent soluble proteins from fresh primary cultures of neonatal foreskin melanocytes, and pigmented melanoma (SK-MEL-19 and SK-MEL-23) cell lines were immunoprecipitated with rabbit anti-GIPC antibody, separated by SDS–PAGE, and immunoblotted with anti-APPL1 antibody (top panels). Immunoblotting of cell lysates with ant-GIPC (middle panels) and anti-APPL (bottom panels) antibodies shows the steady-state expression levels of the interacting proteins and equal loading of proteins.

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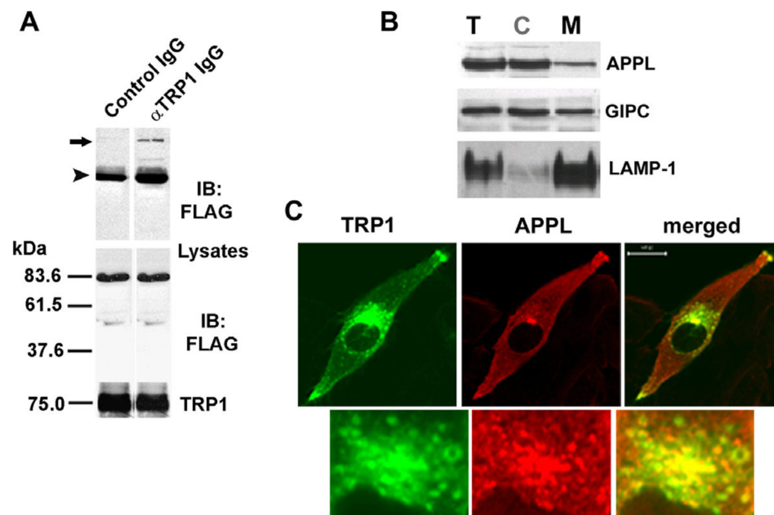

Fig. 2.

Co-precipitation of APPL (A) with TRP1 and intracellular distribution of APPL (B and C). TRP1-negative melanoma cells (SK-MEL-23 c.22a) were co-transfected with pCMV5a-TRP1 plasmid together with either empty vector pCMV5a (lane 1) or pcMV5a-APPL-FLAG (lane 2) plasmid. Forty eight hours after transfection, detergent soluble proteins were immunoprecipitated with anti-TRP1 mAb TA99 and immunoprecipitated proteins were analyzed (top panel) by immunoblotting analysis with anti-FLAG mAb M2. Arrow: APPL; arrowhead: IgG heavy chain. Levels of expression of transfected proteins in total cell lysates were analyzed by immunoblotting (middle and bottom panels) with anti-FALG mAb M2 and anti-TRP1 mAb TA99. Numbers on the left show molecular weights (kDa) of marker proteins. (B) Postnuclear supernatants (T), 100,000×*g* soluble (C) and crude membrane (M) fractions of human melanoma cells (SK-MEL-19) were analyzed by immunoblot analysis with antibodies to indicated proteins: APPL, GIPC and lysosome associated membrane protein, LAMP-1. (C) Immunofluorescence localization of TRP1 and APPL in human melanoma SK-MEL-19 cell line. Cells were plated on glass coverslips, fixed, permeabilized and incubated with Cy3 conjugated anti-FLAG mAb M2 (1:100) followed by FITC conjugated anti-TRP1 mAbTA99 (1:25). Images were captured on Zeiss LSM 510 confocal microscope and assembled using Adobe Photoshop 6.0 (Adobe Systems Inc., CA).

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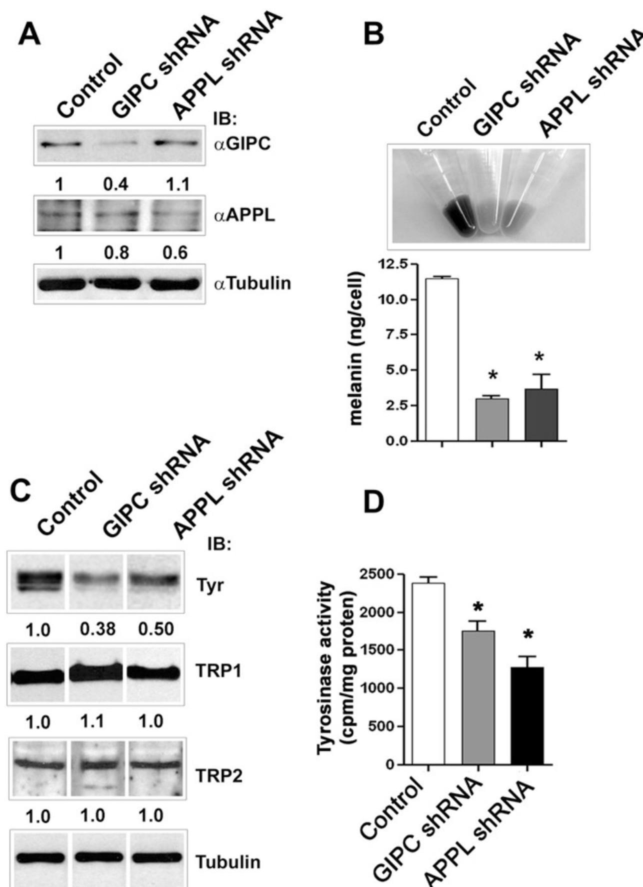

Fig. 3.

Effect of knockdown of GIPC and APPL on melanin pigmentation. (A) Western blot analysis of knockdown of GIPC and APPL in pigmented melanoma cell lines (SK-MEL-23 cl.22) at 72 h post-infection with scramble (control) or GIPC or APPL shRNA lentivirus. The numbers below each panel show relative band intensities and show selective knockdown of GIPC and APPL by respective shRNAs. A representative experiment of three independent transductions is shown. (B) Effect of GIPC and APPL knockdown on pigmentation visualized by pellet color (upper panel) and quantitation of alkali soluble melanin (pg/cell) (lower panel) (*$p < 0.05$). (C) Western blot analysis of tyrosinase, TRP1 and TRP2 in scrambled (control) and GIPC or APPL shRNA transduced cells. Numbers below each panel represent relative band intensities and representative results from two independent experiments are shown. (D) Tyrosinase activity in cells transduced with control and GIPC or APPL shRNA. Data from triplicate assays of two separate transductions is shown as 3H2O released (per mg protein) from tritiated tyrosine is shown (*$p < 0.05$).

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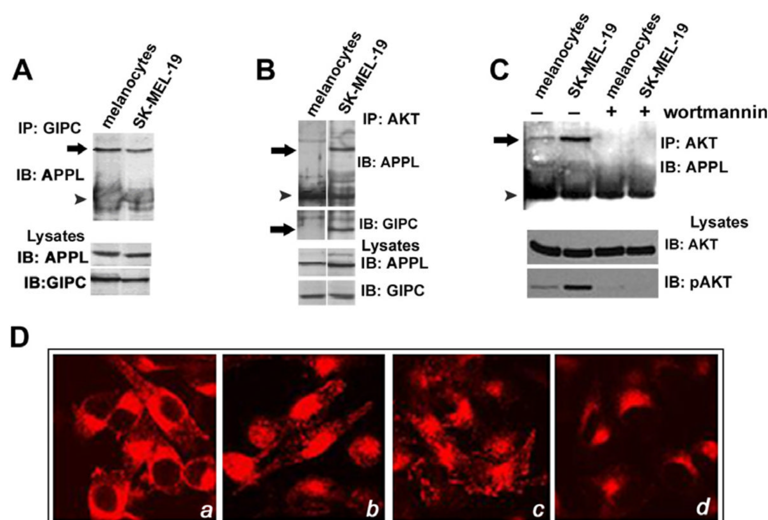

Fig. 4.

Role of APPL–AKT interaction and PI3 kinase signaling in TRP1 transport. (A and B) Differential binding of AKT to GIPC–APPL complex in melanocytes and melanoma. (A) Cell lysates were immunoprecipitated with anti-GIPC antibody and immunoblotted with anti-APPL antibody (A). In (B), melanocyte and melanoma lysates were immunoprecipitated with anti-AKT2 antibody and immunoblotted with anti-APPL (top panel) and anti-GIPC antibody (middle panel). Whereas APPL co-precipitates with GIPC equally efficiently in both melanocytes and melanoma cells (A) (Arrow: APPL; arrowhead: IgG heavy chain), APPL–GIPC complex (arrows in top and middle panels in B) associates with AKT more efficiently in melanoma cells than in melanocytes. (C) APPL binds preferentially phospho-AKT. Melanocytes and melanoma cells were treated with DMSO or wortmannin and cell lysates were immune-precipitated with anti-AKT2 antibody and immunoblotted with anti-APPL antibody (top panel). Arrow shows the co-precipitated APPL and arrowhead indicates IgG heavy chain. Levels of total AKT (middle panel) and phospho-AKT (bottom panel) are shown. (D) Effect of wortmannin on intracellular distribution of newly synthesized TRP1. Pigmented SK-MEL-19 melanoma cells were either left untreated (panels a and b) or treated with 5 mM HMBA (panels c and d). After 48 h, medium in all wells was replaced with regular growth medium for 20 h and then either vehicle (a and c) or 100 nM wortmannin (panel b) was added for 4 h. Cells were fixed with formaldehyde, permeabilized with methanol, stained with anti-TRP1 mAb TA99 followed by anti mouse IgG-TRITC and fluorescent images were captured on Zeiss LSM 510 confocal microscope. All images were captured at identical pinhole, detector gain and amplifier gain settings. Images were finally assembled using Adobe Photoshop 6.0 (Adobe Systems Inc., CA).