The receptor for activated C-kinase-I (RACK-I) anchors activated PKC-β on melanosomes

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Accepted 11 March 2004
Journal of Cell Science 117, 3659-3668 Published by The Company of Biologists 2004
doi:10.1242/jcs.01219

Summary

Protein kinase C (PKC), a family of at least eleven isoforms, mediates numerous cell functions. In human melanocytes, α, β, δ, ε and ζ isoforms of PKC are expressed, but uniquely PKC-β activates tyrosinase, the key and the rate-limiting enzyme in melanogenesis, by phosphorylating specific serine residues on its cytoplasmic domain. To investigate the mechanism by which only PKC-β phosphorylates tyrosinase, we examined the expression of receptor for activated C-kinase-I (RACK-I), a receptor specific for activated PKC-β, on the surface of melanosomes, the specialized organelle in which melanogenesis occurs. Immunoblot analysis of purified melanosomes revealed that RACK-I is readily detectable. Immunoprecipitation of RACK-I from purified melanosomes, followed by immunoblot analysis using antibody against PKC-β, revealed abundant PKC-β, whereas PKC-α was not detected when immunoblot analysis was performed using antibody against PKC-α. Activation of PKC in melanocytes increased the level of PKC-β co-immunoprecipitated with RACK-I, while the level of melanosome-associated RACK-I decreased when melanocytes were treated chronically with the 12-0-tetradecanoyl-phorbol 13-Acetate (TPA), a condition known to deplete PKC and reduce tyrosinase activity. Immunoprecipitation with RACK-I antibody co-precipitated fewer PKC-β in the presence of UV-activated 1, 1′-decamethylenebis-4-aminoquinaldinium di-iodide (DECA), known to disrupt the interaction between activated PKC-β and RACK-I. Treatment of intact melanocytes with DECA also decreased tyrosinase activity. Moreover, suppression of RACK-I expression by transfecting melanocytes with siRNA against RACK-I reduced the basal tyrosinase activity and blocked TPA-induced increases in tyrosinase activity. Taken together, these results demonstrate that RACK-I anchors activated PKC-β on the melanosome membrane, allowing PKC-β to phosphorylate tyrosinase.

Key words: RACK-I, PKC-β, Melanocyte, Tyrosinase, Melanosomes, Pigmentation

Introduction

PKC is a family of at least 11 isoforms (Nishizuka, 1992) and mediates a wide range of membrane-generated signaling cascades underlying biological events, such as cellular proliferation and differentiation (Clemens et al., 1992; Rogalsky et al., 1992; Ohno et al., 1994), transformation (Housey et al., 1988; Choi et al., 1990; Nishizuka, 1984) and immune reactions (Dieter, 1992; Ishii et al., 1996; Haleem-Smith et al., 1995; Genot et al., 1995). All isoforms of PKC reside in the cytoplasm as an inactive form (Nishizuka, 1992). When the cell surface is perturbed by receptor binding to its specific ligand (Nishizuka, 1992; Nishizuka, 1984) or by ultraviolet irradiation (Punnonen and Yuspa, 1992), diacylglycerol is generated from the plasma membrane. Diacylglycerol then activates PKC and the activated kinase translocates to a particular fraction within the cell (Nishizuka, 1992). There is a differential expression of PKC isoforms among cell types (Nishizuka, 1984; Brandt et al., 1987; Ohno et al., 1987), with each cell type characteristically expressing multiple PKC isoforms. It is presumed that each isoform of PKC has a unique biological function within each cell type (Dekker and Parker, 1994), but there are few documented examples. Moreover, the mechanism by which a specific function is conferred in a specific cell type is not well elucidated.

It has been hypothesized that specific compartmentalization of a PKC isoform within the cell directs its unique biological function. In IICa cells, PKC-α was shown to be associated with the nuclear membrane (Leach and Raben, 1993), whereas PKC-ε was associated with the Golgi apparatus in NIH 3T3 cells (Lehel et al., 1995a; Lehel et al., 1995b) and in melanocytes PKC-β was shown to be associated with melanosomes (Park et al., 1999). Extensive studies revealed that, while structural differences among PKC isoforms might contribute to their associations with particular subcellular fractions, receptors for activated C-kinase (RACK) primarily direct the translocation of each PKC isoform to a specific cellular compartment (Mochly-Rosen, 1995; Mochly-Rosen and Gordon, 1998; Dempsey et al., 2000). RACKs are a family of proteins with molecular weights of 28-33 kD (Mochly-Rosen et al., 1991), and subsequent cloning of RACKs revealed that they are homologs of the β subunit of G proteins (Ron et al., 1994). RACKs serve neither as a substrate nor as an inhibitor for PKC (Mochly-Rosen, 1995; Mochly-Rosen and Gordon, 1998; Dempsey et al., 2000) but RACK increases PKC substrate phosphorylation by stabilizing the active form of

PKC (Mochly-Rosen, 1995). Microinjection of excess RACKs to oocytes was shown to block insulin-induced maturation of oocytes, presumably be sequestering all activated PKC (Smith and Mochly-Rosen, 1992).

It is thought that each PKC isoform has its unique RACK. To date, however, corresponding RACKs have been identified only for PKC-β and PKC-ε. RACK-I was shown to interact specifically with PKC-β (Mochly-Rosen et al., 1995) and when further studies distinguished PKC-βI from PKC-βII, RACK-I was shown to be selective for PKC-βII (Ron et al., 1999; Stebbins and Mochly-Resen, 2001). In Chinese hamster ovary cells, RACK-I binds specifically to activated PKC-β and transports it to a specific compartment to phosphorylate the target protein (Ron et al., 1999). Peptides whose amino acid sequence mimic the C2 and V5 regions of PKC-β, the regions containing binding sites for RACK-I (Ron et al., 1995; Stebbins and Mochly-Rosen, 2001), inhibited PKC-β function by competitively inhibiting the interaction between PKC-β and RACK-I (Stebbins and Mochly-Rosen, 2001). RACK-II or B′-COP was shown to be specific for PKC-ε (Csukai et al., 1997). More recently, the human gene GNB2L1 encoding a novel RACK was identified (Wang et al., 2003). However, specificity of this novel RACK for a particular PKC isoform is yet to be determined. Pseudo-RACK-II was also identified where it was shown to occupy the binding site of PKC-ε to RACK-II until PKC-ε is activated (Schechtman et al., 2004).

Skin pigmentation, the result of synthesis and dispersion of melanin, is a key physiological defense against sun-induced injuries, including photocarcinogenesis (Quevedo and Holstein, 1998). Synthesis of melanin, termed melanogenesis, occurs within a specific organelle, the melanosome, which resides within the cytoplasm of epidermal melanocytes (Orlow, 1998). Among the melanogenic proteins associated with melanosomes, tyrosinase has been shown to be the key and the rate-limiting enzyme (Pawelek and Charaborty, 1998). Tyrosinase is activated when phosphorylated by protein kinase C-β (PKC-β) specifically at the serine residues of amino acid positions at 505 and 509 (Park et al., 1999).

Cultured human melanocytes express α, β, δ, ε and ζ isoforms of PKC (Oka et al., 1995). However, PKC-β specifically is associated with melanosomes (Park et al., 1999), the site of melanin synthesis; and transfection of otherwise non-pigmented human melanoma cells with PKC-βII cDNA activates the transmembrane protein tyrosinase (Park et al., 1993). Therefore, PKC-βII is specifically implicated in stimulating melanogenesis. To elucidate the mechanism by which PKC-β specifically associates with melanosomes, we examined whether RACK-I is present on melanosomes, where it might anchor activated PKC-β and allow interaction between tyrosinase and PKC-β.

Materials and Methods

Materials

Medium 199 and trypsin were purchased from GIBCO/BRL. Recombinant basic fibroblast growth factor (bFGF) was purchased from Amgen, tri-iodothyronine from Collaborative Research, and hydrocortisone from Calbiochem. Phorbol 12,13-Dibutyrate (PDBu), insulin, transferrin, dibutyryl cAMP (dbcAMP), and 1,1-decamethylenebis-4-aminoquinaldinium di-iodide (DECA) were from Sigma Chemical Co. Bovine pituitary extract (BPE) was from Clonetics. Nylon membranes were from Amersham. PKC antibodies were from Transduction Laboratories. Fetal calf serum was from HyClone Laboratories. Polyvinyl difluoride (PVDF) membranes were from BioRad Laboratories. Monoclonal antibody against RACK-I was purchased from Calbiochem. Fluorescent isothiocyanate (FITC), and rhodamine-conjugated anti-mouse IgGs were purchased from Jackson ImmunoReserarch Laboratories.

Cell culture

Primary human melanocytes were cultured from neonatal foreskins as previously described (Park et al., 1999). In brief, the epidermis was separated from the dermis after overnight incubation in 0.25% trypsin at 4°C. Primary cultures of melanocytes were established by seeding between 0.5×10^6 to 1×10^6 total epidermal cells per 100 mm dish in Medium 199 supplemented with 10 μg/ml insulin, 10^{-9} M tri-iodothyronine, 10 μg/ml transferrin, 1.4×10^{-6} hydrocortisone, 80 μM dbcAMP, BPE (35 μg/ml), bFGF (10 ng/ml), epidermal growth factor (100 ng/ml), and 1% to 2% fetal calf serum. All post-primary cultures were maintained in a low calcium (0.03 mM) version of this medium known to selectively support melanocyte growth (Naeyaert et al., 1991). Cells at first to third passage were routinely used for all experiments.

Immunohistochemistry

Melanocytes were plated into 4 or 2-well Labtek chambers. To immunostain the cells with a single antibody, 24 hours after plating cells were first fixed using 5% formalin in 2 mM $MgCl_2$ and 2 mM EGTA. Then cells were exposed to 0.2% Triton-X-100 for 2 minutes to permeabilized the membrane, followed by incubation with the primary antibody at 37°C for 1-2 hours. Then cells were incubated with secondary antibody conjugated with rhodamine or FITC for additional 1 hour at room temperature. Cells were then processed for confocal laser microscopy. To determine whether RACK-I co-localizes with PKC-β, a subconfluent culture of melanocytes was double immunostained using antibodies specific for RACK-I and PKC-β. To prevent secondary antibodies from cross reacting with both antibodies, monoclonal antibody raised in mouse was used against RACK-I and a polyclonal antibody raised in rabbit was used for PKC-β. Monoclonal antibody against RACK-I was first incubated at 37°C for 1-2 hours, followed by incubation with the polyclonal antibody against activated PKC-β at 37°C for additional 1-2 hours. Cells were then incubated with the secondary antibody against mouse IgG conjugated with rhodamine and the secondary antibody against rabbit IgG conjugated with FITC at room temperature for one hour. Cells were then processed for confocal laser microscopy. Co-localization of two proteins was determined by overlaying two fluorescence images (FITC-green and rhodamine-red), with computer assignment of yellow-orange to superimposed signals indicating co-localization of two proteins.

Co-immunoprecipitation

To co-immunoprecipitate PKC-β and RACK-I, subconfluent cultures of melanocytes were scraped into phosphate buffered saline (PBS) containing 1% Triton-X-100, protease inhibitors and okadaic acid. Membrane-bound proteins were extracted overnight at 4°C, then diluted with PBS Triton-X-100 concentration of 0.1%. Then the lysate was incubated with monoclonal antibody against RACK-I overnight at 4°C. Then secondary antibody was added for one hour at 37°C, then protein A-Sepharose beads were added and the mixture rotated for 2 hours at room temperature. The beads were then washed three times with PBS containing 0.1% Triton-X-100 and boiled in presence of β-mercapthoethanol. Immunoprecipitated proteins were separated in sodium dodecylsulfate-polycrylamide gel electrophoresis (SDS-PAGE).

Immunoblot analysis

Immunoblot analysis was performed as previously described (Park et al., 1999). Protein samples were subjected to 7.5-10% SDS-PAGE and transferred to a nitrocellulose or PVDF membrane electrophoretically. The membrane was pre-incubated in 100% Blotto (5 g nonfat dry milk in 100 ml PBS) for 3 hours at room temperature with shaking, followed by an overnight incubation with antiserum (0.5-1 μg/ml in 10% Blotto) at 4°C. At the end of the incubation, the membrane was washed extensively with PBS containing 0.5% Tween-20, and processed using the ECL kit. The membrane was then exposed to Kodak X-OMAT film. To assess the loading of proteins in each lane, the membrane was then stained with Commassie Blue staining.

Melanosome isolation

Melanosomes were purified by a previously described method (Seiji et al., 1963) from cultured human melanocytes. Cultures were washed with PBS and scraped, in the buffer containing 250 mM sucrose in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 1 mM EDTA. Cells were disrupted by repeated freeze-thaw, followed by spinning 15 minutes at 1000 ***g*** to pellet the particulate fraction. The supernatants were separated from the particulate fraction and layered over an equal volume of 1.5 M sucrose in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 1 mM EDTA, and centrifuged in a swinging bucket rotor at 50,000 ***g*** for 30 minutes at 4°C. After of centrifugation, melanosomes were pelleted and the cytoplasmic fraction, which remains above the sucrose gradient, was carefully removed and the pelleted melanosomes were resuspended in 20 mM Tris (pH 7.2), 150 mM NaCl and 1 mM EDTA. As previously demonstrated using electron microscopy (Park et al., 1999), melanosomes purified by this method are intact and devoid of contamination by other subcellular organelles, such as mitochondria and membrane fragments. Furthermore, melanosomes purified in this manner are devoid of contaminating cytoplasmic proteins, such as PKC-α (Park et al., 1999), known to be comparably abundant with PKC-β in whole melanocyte preparations (Park et al., 1993).

Tyrosinase activity

Tyrosinase activity was measured according to Pomerantz (Pomerantz, 1964). In brief, 5×10^5 cells were briefly sonicated in 80 mM K_2PO_4 (pH 6.8) containing 1% Triton-X-100, and tyrosinase was extracted for 60 min at 4°C. We used 10-50 μg of cellular protein and incubated it with 250 nM L-tyrosine, 25 nM L-dihydroxyphenylalanine, 12.5 μg chloramphenicol and 5 μCi of L-[3,5-^{3}H] tyrosine (40-60 Ci/mmol) for 30-60 mins at 37°C. The reaction was stopped by addition of 500 μl of 10% TCA containing 0.2% bovine serum albumin. TCA soluble material was reacted with Norit A and released $[^3H]H_2O$ was measured using a scintillation counter. The activity was expressed as cpm of $[^3H]H_2O$ released/μg protein/hour minus the non-specific incorporation of radioactivity, determined by using lysate boiled for 30 minutes. Background was generally less than 10-15% of the sample.

RNA interference

To suppress the expression of RACK-I, siRNA sequences against RACK-I transcript were designed according to the selection criteria were based on the list published elsewhere (Elbashir et al., 2002). siRNA sequences were chosen using an Ambion target sequence finder, as well as Dharmacon and Whitehead Institute selection programs, available on www.ambion.com; www.dharmacon.com and www.wi.mit.edu/pubint/http://iona.wi.mit.edu/SiRNAext/ websites. The selected nucleotide sequences, 5′-AAACTGACCAG-GGATGAGACC-3′ and its complementary sequence, were first reverse transcribed separately into single-strand RNA and then into double-strand RNA using the Silencer^{TM} siRNA Construction Kit from Ambion. As a control, a scrambled sequence of 5′-AAAAGGCTGACCACCGATGAG-3′ was used.

Results

RACK-I is localized, in part, to melanosomes

To explore whether RACK-I is involved in anchoring activated PKC-β onto the melanosome surface, the distribution of RACK-I within melanocytes was first examined by immunohistochemistry under conditions known to support a readily detectable basal level of PKC-β-mediated melanogenesis (Park et al., 1993). A subconfluent culture of melanocytes was processed for double immunostaining using a monoclonal antibody against RACK-I and a polyclonal antibody against tyrosinase (obtained from Vincent Hearing at NIH), a protein known to localize to the melanosome surface in its mature state (Jimbow and Fitzpatrick, 1974; Maul and Brumbaugh, 1971), as described under Materials and Methods. Diffuse perinuclear staining for RACK-I was observed (Fig. 1A), and a similar distribution of tyrosinase, as previously described (Park et al., 1996) was also observed (Fig. 1B). When the two images were overlaid, yellow-orange color was observed in the perinuclear area (Fig. 1C), consistent with co-localization of tyrosinase and RACK-I on the melanosome surface.

Because immature and underglycosylated tyrosinase is found in the Golgi apparatus (Hearing, 1987) and the polyclonal antibody against tyrosinase is known to react with immature tyrosinase, co-localization of tyrosinase and RACK-I might reflect association of these proteins in the Golgi apparatus. To confirm that the RACK-I and tyrosinase co-localization occurs at the specific melanosome, melanosomes

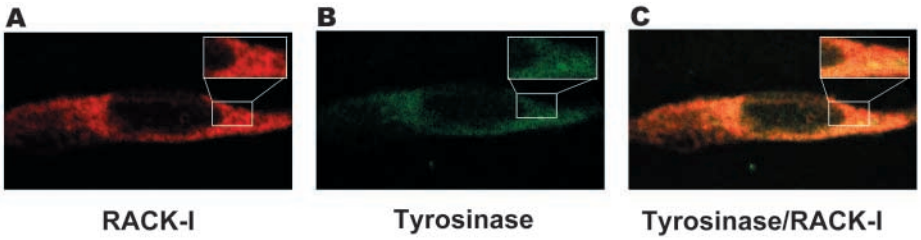

Fig. 1. Co-localization of RACK-I and tyrosinase. (A) Subconfluent culture of melanocytes plated on Labtek Chamber slides was first incubated with the monoclonal antibody against RACK-I, then additionally incubated using polyclonal antibody against tyrosinase. Subsequently, the cells were further incubated with rhodamine-tagged secondary antibody against RACK-I antibody (A) and FITC-tagged secondary antibody and against tyrosinase antibody (B) and examined by confocal microscopy. When the images were overlayed, the computer-assigned yellow-orange color indicates co-localization of the two proteins (C).

were purified from subconfluent cultures of melanocytes and immunoblot analysis using a monoclonal antibody against RACK-I was performed. RACK-I was readily detected in the highly purified melanosome preparations (Fig. 2A).

To characterize the distribution of RACK-I within melanocytes, cytoplasmic, melanosomal and other subcellular components were fractionated from subconfluent cultures of melanocytes as previously described (Seiji et al., 1963). Immunoblot analysis using the monoclonal antibody specific for RACK-I revealed that, under basal conditions, RACK-I was readily detected in both cytoplasmic and melanosomal fractions (Fig. 2B). A slightly higher level of RACK-I was found in the cytoplasmic than in the melanosomal fraction, and RACK-I was undetectable in the particulate fraction that contains nuclear and membrane fragments. Association of RACK-I with melanosomes, presumably anchoring activated PKC-β, in the control cultures is consistent with our previous report that 40-50% of total PKC is active in melanocytes under the basal condition due to the effect of serum, bovine pituitary extract and growth factors present in the medium (Park et al., 1993), including sufficient active PKC-β to maintain a basal level of melanogenesis (Park et al., 1993).

If specific interaction of RACK-I with activated PKC-β is required before translocation of the complex to melanosomes, then depletion of PKC in melanocytes would reduce the level of RACK-I associated with melanosomes. To test this hypothesis, paired cultures of subconfluent melanocytes were treated chronically with 10^{-7} M 12-0-tetradecanoyl-phorbol 13-acetate (TPA) for 2 weeks, a condition known to deplete PKC by greater that 90% and to reduce tyrosinase activity and total melanin level (Park et al., 1996; Park and Gilchrest, 1999).

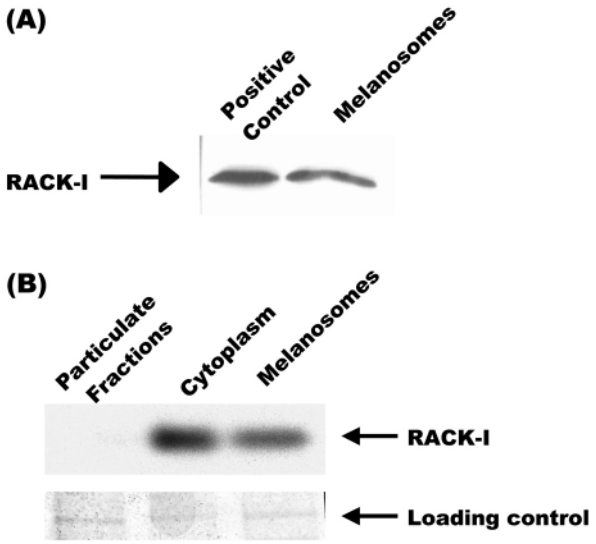

Fig. 2. Distribution of RACK-I within melanocytes. (A) Purified melanosomes from subconfluent melanocyte cultures were subjected to immunoblot analysis using a monoclonal antibody against RACK-I. As a positive control, fibroblasts over expressing RACK-I were used, as suggested by the vendor. (B) Melanosomes were separated from the cytoplasmic and particulate fractions of melanocytes as previously described (Seiji et al., 1963). Proteins from cytoplasmic, melanosome and particulate fractions were subjected to immunoblot analysis using a monoclonal antibody specific for RACK-I. As the loading control, the membrane was stained with Coomassie Blue.

Melanosomes and cytoplasm were then fractionated as previously described (Seiji et al., 1963). Immunoblot analysis using monoclonal antibody against RACK-I showed that while the total level of RACK-I in vehicle- and TPA-treated cells were similar (Fig. 3A), depletion of PKC reduced the level of RACK-I associated with melanosomes (Fig. 3C), whereas the level of RACK-I was higher in the cytoplasm of TPA-treated sample (Fig. 3B). These results strongly support the hypothesis that RACK-I translocates to melanosomes after binding activated PKC-β.

RACK-I and activated PKC-β are complexed in cultured human melanocytes

To determine whether RACK-I and activated PKC-β co-localized in melanocytes, subconfluent cultures plated onto Labtek chamber slides were first treated with 2 μM PDBu or vehicle (DMSO) for 90 minutes to activate PKC, the time point shown to activate PKC maximally in melanocytes (Park et al., 1993). Cells were then sequentially incubated with monoclonal antibody against RACK-I and PKC-β. To demonstrate that RACK-I co-localizes only with activated PKC-β, a paired plate was treated with 2 μM PDBu and sequentially incubated with antibodies against RACK-I and PKC-α. Then, the cells were incubated with rhodamine-tagged secondary antibody against RACK-I monoclonal antibody and FITC-tagged secondary antibody against PKC-α or -β polyclonal antibody. In vehicle-treated cells, both PKC-α and PKC-β were detected diffusely throughout the cytoplasm (Fig. 4,A1,B1), as expected. Upon PKC activation by treatment with PDBu, PKC-β translocated to the perinuclear area (Fig. 4,A2), whereas PKC-α translocated exclusively to the nucleus (Fig. 4,B2). Similar to the results from earlier immunostaining, RACK-I was concentrated in the perinuclear area (Fig. 4,A3,B3). Overlaying PKC-β and RACK-I images resulted in computer assignment of yellow-orange color in the perinuclear area (Fig. 4,A4), implying PKC-β and RACK-I co-localization. In contrast, overlaying of PKC-α and RACK-I images did not result in yellow-orange color assignment (Fig. 4,B4).

To confirm whether PKC-β and RACK-I are complexed and anchored on melanosomes, melanosomes were purified from subconfluent cultures of melanocytes, and subjected to immunoprecipitation using a monoclonal antibody against RACK-I. The immunoprecipitated proteins were then separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was cut at the 50 kD molecular weight standard marker and then the upper part of the membrane was reacted with a monoclonal antibody specific for PKC-β (78-80 kD) and the bottom part of the membrane with an antibody against RACK-I (28-33 kD). Immunoblot analysis revealed readily detectable levels of PKC-β and RACK-I (Fig. 5A), indicating that antibody against RACK-I could also immunoprecipitate PKC-β. As a control, melanosomes were subjected to immunoprecipitation using monoclonal antibody against RACK-I and immunoblot analysis using a monoclonal antibody against PKC-α was performed. PKC-α did not co-immunoprecipitate with RACK-I (Fig. 5B). This result is consistent with the previous reports that RACK-I is specific for PKC-β (Mochly-Rosen et al., 1995; Ron et al., 1999; Stebbins and Mochly-Resen, 2001) and that only PKC-β is associated with melanosomes (Park et al., 1999).

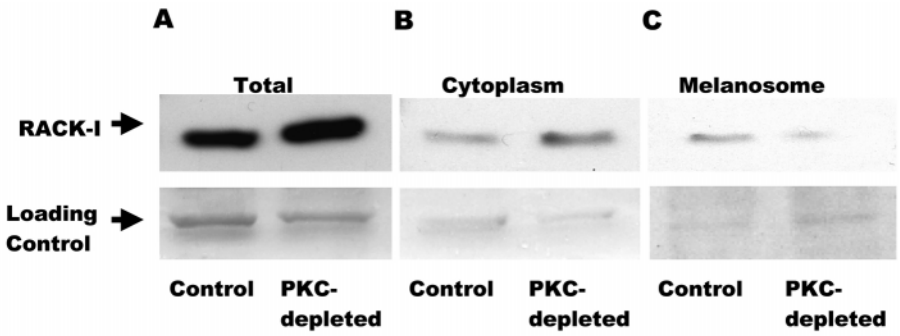

Fig. 3. Depletion of PKC reduced the level of RACK-I associated with melanosomes. Paired cultures of subconfluent melanocytes were treated with vehicle alone or 10^{-7} M TPA for 2 weeks, the time period known to delete PKC in pigment cells (Park et al., 1996; Park and Gilchrest, 1999). Cells were harvested and the total level of RACK-I in vehicle- and TPA-treated samples was determined. Then cytoplasmic (B) and melanosome (C) fractions were separated and immunoblotted for RACK-I. Membranes were stained with Coomassie Blue as loading control.

TPA increases the level of PKC-β associated with RACK-I

To determine if activation of PKC by TPA elevates the level of activated PKC-β associated with RACK-I, subconfluent cultures of melanocytes were treated with 10^{-7} M TPA or vehicle alone for 90 minutes. Cells were then harvested and equal amounts of protein from vehicle- and TPA-treated lysates were subjected to immunoprecipitation using a monoclonal antibody against RACK-I. Then the immunoprecipitated proteins were separated and transferred onto nitrocellulose membranes and immunoblot analysis was performed. The upper part of the blot was immunoreacted with polyclonal antibody against PKC-β and the bottom part of the blot was immunoblotted using monoclonal antibody against RACK-I. The level of immunoprecipitated RACK-I was similar in vehicle-treated compared with TPA-treated cells, as expected, but the level of PKC-β associated with RACK-I was higher in TPA-treated cells than in vehicle-treated cells (Fig. 6), as expected if PKC-β activation enhanced its binding to RACK-I.

DECA decreases the level of activated PKC-β associated with RACK-I and tyrosinase activity

It has been shown that DECA, when activated with UV-light, binds to the C2 region of PKC-β and prevents it from interacting with RACK-I (Rotenberg and Sun, 1998). Therefore, within our working hypothesis, treatment of cultured human melanocytes with activated DECA should interfere with the association between RACK-I and PKC-β, reducing tyrosinase phosphorylation by PKC-β and therefore reducing tyrosinase activity. To confirm that DECA disrupts the interaction between activated PKC-β and RACK-I as expected, paired cultures of subconfluent melanocytes were first treated with 50 μM DECA or vehicle alone for 30 minutes, and UV-irradiated as previously described to activate the DECA (Rotenberg and Sun, 1998). Then all culture plates were treated with 10^{-7} M TPA for 90 minutes to activate PKC. Cells were then harvested and equal amounts of protein from vehicle- or DECA-treated lysates were separated and subjected to immunoprecipitation using antibody against RACK-I. Immunoprecipitated proteins were the separated and immunoblot analysis was performed using polyclonal antibody against PKC-β for the upper part of the membrane and the monoclonal antibody against RACK-I for the bottom part of the membrane. The level of activated PKC-β co-immunoprecipitated with RACK-I was significantly reduced in DECA treated cells (Fig. 7A), confirming that DECA interferes with binding of activated PKC-β to RACK-I in melanocytes,

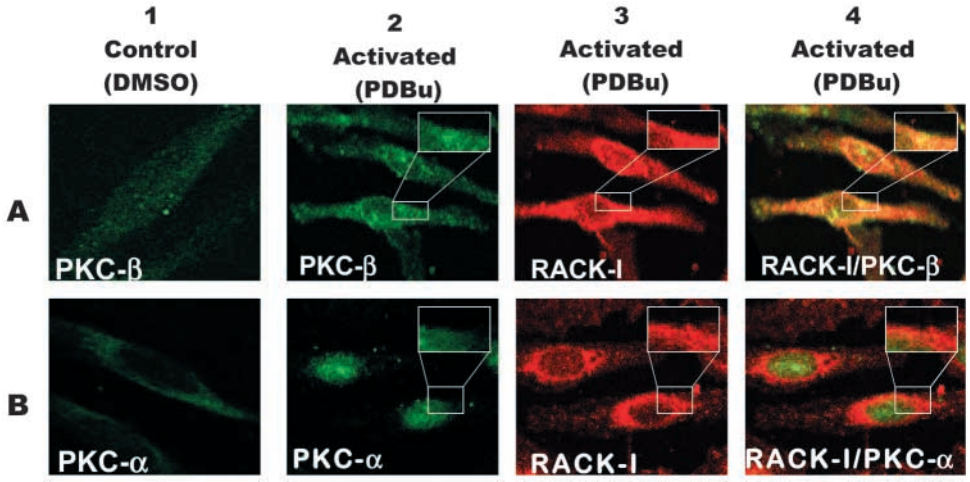

Fig. 4. Co-localization of activated PKC-β and RACK-I. To determine whether activated PKC-β co-localizes with RACK-I, paired melanocyte cultures plated onto Labtek Chamber slides were treated with phorbol dibutyrate (PDBu) for 90 minutes to activate PKC. Control wells received DMSO only (A1 and B1). Then they were subjected to double immunostaining using polyclonal antibody against PKC-β (A2) or PKC-α (B2) and monoclonal antibody against RACK-I (A3 and B3). Then cells were further incubated with FITC-conjugated secondary antibody against PKC-β or PKC-α antibodies and rhodamine-conjugated secondary antibody against RACK-I antibody and analyzed as in Fig. 1. The images were overlaid to determine co-localization (indicated in yellow) between PKC-β and RACK-I (A4) and PKC-α and RACK-I (B4).

as previously reported in other cell types (Rotenberg and Sun, 1998).

To determine whether DECA treatment of intact melanocytes decreases tyrosinase activity, paired cultures of melanocytes were first treated with 50 μM DECA or vehicle alone for 30 minutes, UV-irradiated to activate the DECA, and subsequently treated with 10^{-7} M TPA for 90 minutes to activate PKC. Cells were harvested and tyrosinase activity was also measured as previously described (Pomerantz, 1964). Tyrosinase activity was reduced in DECA-treated cells (P<0.003) by 45% compared to vehicle-treated cells (Fig. 7B), confirming the prediction that anchoring activated PKC-β onto melanosomes, mediated by RACK-I, is critical for phosphorylation of tyrosinase and thus for enzyme activation.

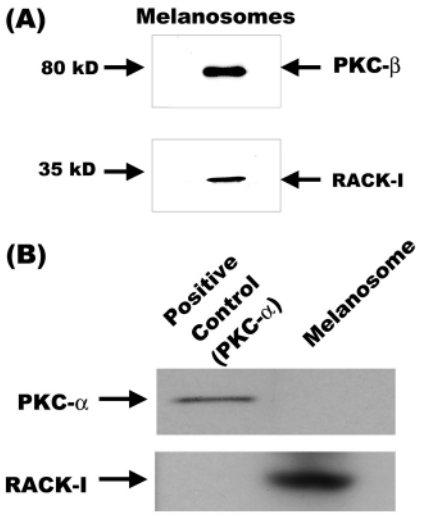

Fig. 5. RACK-I and PKC-β are complexed on melanosomes. (A) Purified melanosomes were subjected to immunoprecipitation using a monoclonal antibody against RACK-I. Immunoprecipitated proteins were then separated in 7.5% SDS-PAGE, transferred onto a nitrocellulose membrane, and the membrane was then cut at the molecular weight of 50 kD. The upper part of the membrane was immuno-reacted using a monoclonal antibody against PKC-β and the bottom part of the membrane was immuno-reacted using a monoclonal antibody specific for RACK-I. (B) Purified melanosomes were subjected to immunoprecipitation using a monoclonal antibody against RACK-I. Then the immunoprecipitated proteins were separated and immunoblot analysis was performed using specific antibody against PKC-α on the upper part of the membrane and monoclonal antibody against RACK-I. Purified recombinant PKC-α (5 ng) purchased from Panvera was used as a control.

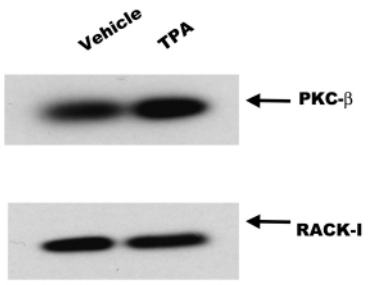

Fig. 6. TPA increases the amount of activated PKC-β associated with RACK-I. Subconfluent melanocyte cultures were treated with 10^{-7} M TPA or vehicle alone for 90 minutes. Equal amounts of proteins from vehicle- and TPA-treated cell lysates were subjected to immunoprecipitation using an antibody against RACK-I. The immunoprecipitated proteins were separated and subjected to immunoblot analysis as described for Fig. 5. Densitometric analysis of each band was performed. When the ratio of PKC-β to RACK-I in vehicle-treated samples was set arbitrarily at 1.0, the ratio in TPA-treated samples was 2.67.

Suppression of RACK-I expression with siRNA blocks TPA-induced increase in tyrosinase activity

To further demonstrate that RACK-I plays a critical role in activating tyrosinase by anchoring activated PKC-β onto melanosomes, RACK-I expression in melanocytes was suppressed using siRNA against RACK-I. Paired subconfluent melanocyte cultures were transfected with either 10 nM of control RNA or siRNA against RACK-I, and 72 hours after the transfection, cells were harvested and the protein levels of RACK-I, tyrosinase and PKC-β were determined using immunoblot analysis. The protein level of RACK-I was significantly reduced in cells transfected with siRNA against RACK-I, while protein levels of tyrosinase and PKC-β,

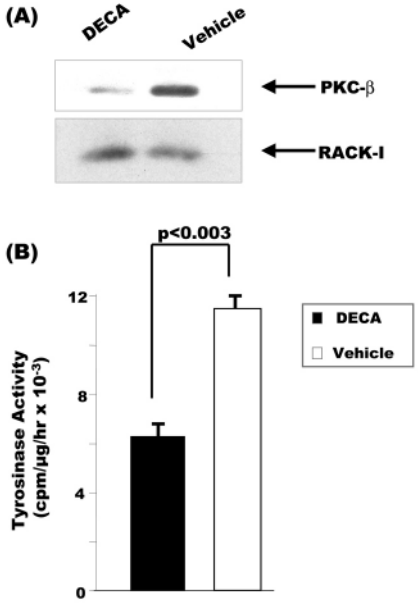

Fig. 7. DECA disrupts the interaction between PKC-β and RACK-I. (A) Subconfluent melanocyte cultures were treated with DECA or vehicle alone for 30 minutes. Then the cultures were UV irradiated (UVB, 12 mJ/cm^2) to activate DECA and treated with 10^{-7} M TPA for an additional 90 minutes. Then cells were harvested and equal amounts of proteins from DECA- or vehicle-treated cells were subjected to immuno-precipitation using an antibody against RACK-I, followed by immunoblot analysis using antibodies against PKC-β and RACK-I, as described for Fig. 5. Densitometric analysis of each band was performed. When the ratio of PKC-β to RACK-I was set arbitrarily at 1.0, the ratio in DECA-treated samples was 0.25. (B) Subconfluent melanocyte cultures were treated with DECA or vehicle alone, UV-irradiated to activate DECA and then treated with 10^{-7} M TPA as described above. Cells were harvested and tyrosinase activity was determined as previously described (Pomerantz, 1964). Statistical analysis using a paired Student's t-test was performed.

measured in parallel, were unaffected (Fig. 8A). Tyrosinase activity measured in paired cultures showed that transfecting siRNA against RACK-I markedly decreased (P<0.01) tyrosinase activity (Fig. 8B). These results further demonstrate that the presence of RACK-I is critical for tyrosinase activity.

To determine whether siRNA RACK-I could block a TPA-induced increase in tyrosinase activity, paired cultures of subconfluent melanocytes were transfected with 10 nM siRNA or control RNA and 72 hours later, cells were treated with either vehicle or 10^{-7} M TPA for 90 minutes, harvested, and tyrosinase activity was measured (Fig. 9). In melanocytes transfected with control RNA, TPA increased tyrosinase activity (P<0.02) as previously reported (Park et al., 1993). In contrast, TPA treatment failed to increase tyrosinase activity in melanocytes transfected with siRNA against RACK-I (Fig. 9). Basal tyrosinase activity was also reduced (P<0.03) by RACK-I siRNA (Fig. 9). These results further confirm our hypothesis that RACK-I plays a critical role in melanogenesis by anchoring activated PKC-β onto melanosomes.

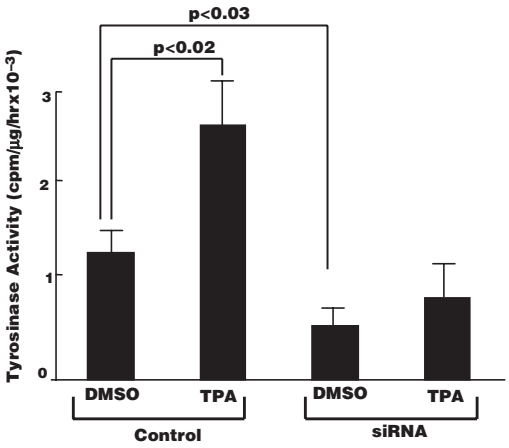

Fig. 9. RACK-I siRNA blocks the TPA-induced increase in tyrosinase activity. Paired melanocyte cultures were transfected with 10 nM control RNA or siRNA and 72 hours after transfection cells were treated with 10^{-7} M TPA or vehicle alone for 90 minutes. Then cells were harvested and tyrosinase activity was measured. Statistical analysis using a paired Student's *t*-test was performed.

Discussion

Our previous studies demonstrate that PKC-β, but not PKC-α, is associated with the outer surface of melanosomes (Park et al., 1999), consistent with the known location of this cytoplasmic enzyme (Nishizuka, 1992). Brief trypsinization of melanosomes removed PKC-β (Park et al., 1999). However, the mechanism by which activated PKC-β associates with melanosomes and phosphorylates tyrosinase remained to be elucidated. Our present results demonstrate that RACK-I on melanosomes anchors activated PKC-β, allowing the kinase to phosphorylate tyrosinase. The localization of RACK-I to melanosomes is strongly suggested by its co-localization, as demonstrated by confocal microscopy, with tyrosinase, a transmembrane protein known to be detected at the melanosome surface (Jimbow and Fitzpatrick, 1973; Maul and Brumbaugh, 1971). However, further support for this localization is provided by the readily detectable level of RACK-I in the purified melanosome preparation, as demonstrated by immunoblotting. The RACK-I detected in association with purified melanosomes cannot be attributed to contamination with cytoplasmic proteins because such melanosome preparations are devoid of mitochondria and other cellular organelles and membrane fragments by electron microscopy (Park et al., 1999), and because cytoplasmic proteins, such as PKC-α, whose abundance is similar to that of PKC-β in whole melanocytes (Park et al., 1993), are undetectable by immunoblot analysis (Park et al., 1999). That is, if the melanosome preparations were contaminated with cytoplasmic proteins, leading to a non-specific association of RACK-I with melanosomes, PKC-α as a second non-specific protein should also be detected by confocal microscopy and immunoblotting, but was not.

It has been suggested that the RACK family is responsible for compartmentalization of different PKC isoforms within the cell, thus conferring unique biological functions. RACK-I is a specific receptor for activated PKC-β (Mochly-Rosen, 1995) and subsequent studies reveal that RACK-I selectively interacts and anchors PKC-βII (Ron et al., 1999; Stebbins and Mochly-Resen, 2001). In neonatal cardiac myocytes, phorbol ester-induced cardiac myocyte hypertrophy can be inhibited by using a peptide known to block interaction between RACK-I and PKC-βII (Stebbins and Mochly-Rosen, 2001). Our results further confirm that RACK-I is the specific receptor for the activated PKC-β, not PKC-α.

RACK-I is mostly present in the cytoplasm and translocates to a specific compartment upon binding with activated PKC-β (Ron et al., 1999). Our results demonstrated that in

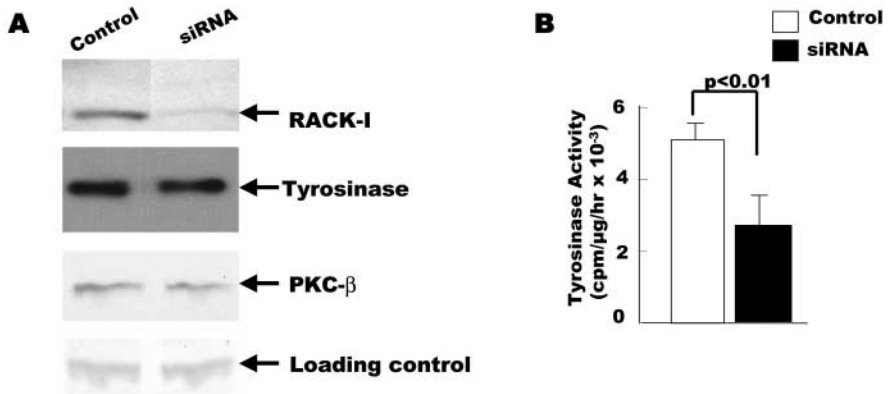

Fig. 8. RACK siRNA decreases tyrosinase activity. (A) A paired culture of melanocytes were transfected with 10nM control RNA or RACK-I siRNA. 72 hours later, cells were harvested and immunoblot analysis was performed for RACK-I, tyrosinase and PKC-β. (B) In parallel, tyrosinase activity was measured. Statistical analysis was performed using a paired Student *t*-test.

melanocytes, under the basal conditions without exogenous addition of a PKC activator, RACK-I is distributed at a similar level between the melanosome and cytoplasmic fractions. However, association of RACK-I with melanosomes in unstimulated melanocytes is probably due to the fact that at least 40-50% of PKC is active in melanocytes under basal condition (Park et al., 1993), likely due to the growth factors, bovine pituitary extract and serum in melanocyte medium that would activate PKC, including the PKC-β. This would lead to interaction between activated PKC-β and RACK-I and allow this complex to translocate to melanosomes. The hypothesis that RACK-I translocates to melanosomes after interacting with activated PKC-β in melanocytes is further supported by the observation that depletion of PKC in melanocytes by prolonged treatment with TPA reduced the level of RACK-I associated with melanosomes.

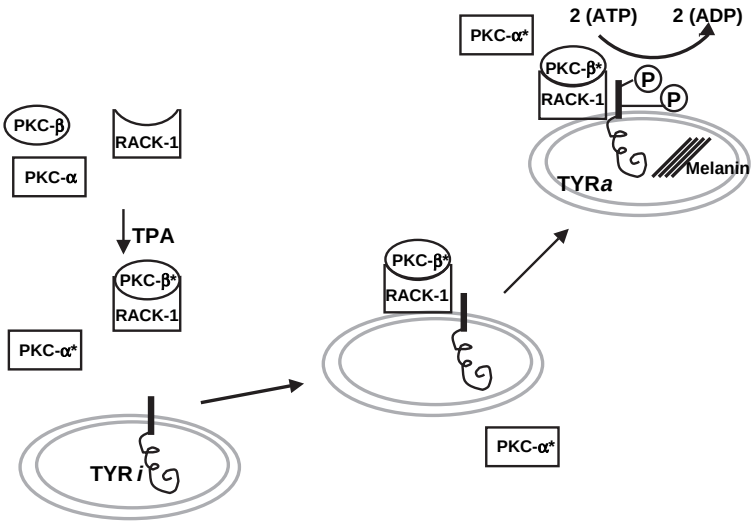

Fig. 10. Proposed mechanism by which RACK-I anchors activated PKC-β on melanosome membranes. In human melanocytes, when PKC is activated by TPA treatment, or a physiologic signal, activated PKC-β (PKC-β*), but not activated PKC-α (PKC-α*), binds RACK-I and translocates to the melanosome membrane. Activated PKC-β, anchored on the melanosome via RACK-I, then phosphorylates inactive tyrosinase (TYR*i*) at serine residues of amino acid positions at 505 and 509 of its cytoplasmic domain and activates tyrosinase (TYR*a*), leading to melanogenesis.

It is not clear how the complex of RACK-I and activated PKC-β translocates specifically to melanosomes. RACK-I has been suggested to be a mobile protein, involved in protein-protein interactions, capable of localizing at different sites (Liliental and Chang, 1998; Chang et al., 1998). It has been suggested that RACK-I targets membranes of organelles via binding to the pleckstrin homology (PH) domains (Souroujon and Mochly-Rosen, 1998) that bind phospholipids and proteins (Ron et al., 1999). WD40-containing proteins interact with PH-domain-containing proteins (Touhara et al., 1994) and RACK-I is a WD40-containing protein (Neer et al., 1994). It is possible that PKC-β and RACK-I complexes translocate to melanosomes because melanosome contains proteins with PH-domains, yet to be identified. Another possible mechanism for RACK-I and PKC-β complex translocation to specific organelles is through a direct interaction with the PKC-β substrate. RACK-I associates with the cytoplasmic domain of β-integrin, allowing PKC-β to phosphorylate either β-integrin or neighboring proteins (Liliental and Chang, 1998). Whether activated PKC-β and RACK-I complexes translocate to melanosomes via directed interaction with the cytoplasmic tail of tyrosinase, allowing PKC-β to phosphorylate tyrosinase, is yet to be tested.

It is well documented that inactive PKC resides within the cytoplasm and upon activation translocates to membranes within a cell (Nishizuka, 1992). Presumably, all isoforms are activated within melanocytes in response to PDBu or TPA, and then all isoforms move to membranes, including melanosomal membranes, but only the β isoform anchors there, due to its association with RACK-I. Our immunostaining results indicated that PKC-α does not interact with RACK-I, as anticipated (Mochly-Rosen et al., 1995), but rather translocates to the nucleus, consistent with the inability of PKC-α to support melanogenesis (Park et al., 1993). PKC-α presumably mediates events other than activation of tyrosinase in melanocytes, but this has not been studied to date.

Suppressing the expression of RACK-I by transfecting melanocytes with siRNA against RACK-I, or interfering with its binding to activated PKC-β using DECA, clearly demonstrates that RACK-I is integral to activating tyrosinase. Tyrosinase activity is reduced in cells transfected with siRNA against RACK-I, despite unchanged levels of tyrosinase and PKC-β; and activated DECA, known to prevent the interaction between RACK-I and activated PKC-β, also decreased the level of tyrosinase activity in melanocytes. Photo-activated DECA blocks PKC function by interfering with the formation of PKC-β-RACK-I complexes, specifically by binding to the PKC-β domain that interacts with RACK-I (Rotenberg and Sun, 1998). Binding sites on PKC-β for RACK-I were demonstrated to be in the C2 and V5 regions of PKC-β (Ron et al., 1995; Stebbins and Mochly-Rosen, 2001). Peptides whose amino acid sequence mimics the C2 and V5 regions (Ron et al., 1995; Stebbins and Mochly-Rosen, 2001) and the competitive inhibitor DECA (Rotenberg and Sun, 1998) both competitively inhibited PKC function.

In summary, our results demonstrate that when PKC-β is activated, it binds RACK-I, which then translocates to melanosomes. RACK-I-mediated anchoring of PKC-β onto melanosomes then allows phosphorylation of tyrosinase and subsequent melanin synthesis (Fig. 10). These new insights expand our appreciation of PKC biology and suggest novel approaches to regulating melanogenesis in skin.

This work was supported in part by a grant from the National Institutes of Health CA RO1 72763 (HYP) and a grant from the Carl J. Herzog Foundation (BAG). We would like to give special thanks to Melissa Murphy-Smith and Shaun Peterson for their excellent technical support and Vincent Hearing at NIH for providing the polyclonal antibody against tyrosinase.

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