Protein Kinase C-β Activates Tyrosinase by Phosphorylating Serine Residues in Its Cytoplasmic Domain*

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We have previously shown that protein kinase C-β (PKC-β) is required for activation of tyrosinase (Park, H. Y., Russakovsky, V., Ohno, S., and Gilchrest, B. A. (1993) J. Biol. Chem. 268, 11742–11749), the rate-limiting enzyme in melanogenesis. We now examine its mechanism of activation in human melanocytes. In vivo phosphorylation experiments revealed that tyrosinase is phosphorylated through the PKC-dependent pathway and that introduction of PKC-β into nonpigmented human melanoma cells lacking PKC-β lead to the phosphorylation and activation of tyrosinase. Preincubation of intact melanosomes with purified active PKC-β in vitro increased tyrosinase activity 3-fold. By immunoelectron microscopy, PKC-β but not PKC-α was closely associated with tyrosinase on the outer surface of melanosomes. Western blot analysis confirmed the association of PKC-β with melanosomes. Only the cytoplasmic (extra-melanosomal) domain of tyrosinase, which contains two serines but no threonines, was phosphorylated by the serine/threonine kinase PKC-β. These two serines at positions 505 and 509 both are present in the C-terminal peptide generated by trypsin digestion of tyrosinase. Co-migration experiments comparing synthetic peptide standards of all three possible phosphorylated tryptic peptides, a diphosphopeptide and two monophosphopeptides, to tyrosinase-phosphorylated in intact melanocytes by PKC-β and then subjected to trypsin digestion revealed that both serine residues are phosphorylated by PKC-β. We conclude that PKC-β activates tyrosinase directly by phosphorylating serine residues at positions 505 and 509 in the cytoplasmic domain of this melanosome-associated protein.

Melanin production is principally responsible for skin color and plays an important role in prevention of sun-induced skin injury (1). Melanin is produced in melanocytes, neural crest-derived cells residing at the basal layer of epidermis; tyrosine and L-dihydroxyphenylalanine (L-dopa) serve as precursors for this complex biopolymer (1, 2). These precursor amino acids are oxidized by a rate-limiting enzyme tyrosinase (EC 1.14.18.1) (3, 4), and subsequent reactions involving tyrosinase and tyrosinase-related proteins result in deposition of melanin pigment in specialized organelles termed melanosomes (1).

Mammalian tyrosinase is a copper-dependent glycoprotein spanning the melanosomal membrane with a molecular mass ranging from 60 to 75 kDa, depending on its state of glycosylation (4). Human tyrosinase has been cloned and sequenced (5), and the deduced amino acid sequence reveals that tyrosinase is composed of 511 amino acids with >90% of the enzyme, the part containing the copper-binding sites (5), localized inside the melanosome. The remainder of the tyrosinase protein consists of a short amino acid sequence that spans the membrane of melanosome, followed by a tail of approximately 30 amino acids that reside in the melanocyte cytoplasm. Therefore, tyrosinase can be divided into inner, transmembrane, and cytoplasmic domains. Patients with oculocutaneous albinism, who lack tyrosinase activity, frequently display mutations in the copper binding region of tyrosinase, as well as other regions in the inner domain (6, 7). Point mutations at codons encoding for amino acids at positions 489 or 501 that result in premature termination of tyrosinase and loss of 21 or 22 amino acids at the C terminus lead to oculocutaneous albinism (6), suggesting that the cytoplasmic domain is similarly critical to melanogenic function. A second role for the cytoplasmic domain in proper subcellular trafficking of tyrosinase has been inferred from the observation that the platinum mutation in mice, which results in deletion of the 27 terminal amino acids of the cytoplasmic domain, causes tyrosinase to be incorporated into the plasma membrane rather than the melanosomal membrane (8–11). However, a recent report has suggested that only six amino acids residing near the melanosomal membrane within the cytoplasmic domain are responsible for the subcellular trafficking (11).

Although the biochemistry of melanogenesis has been extensively characterized, molecular mechanisms involved in regulation of tyrosinase activity are still poorly understood. In murine melanoma cells, agents that increase the intracellular level of cAMP, such as α-melanocyte stimulating hormone (α-MSH), dibutyryl cAMP, and forskolin, are known to be potent inducers of pigmentation (12–17). These agents increase tyrosinase mRNA, protein, and activity (13–15), as well transcription of tyrosinase (12). Inhibitors and activators of tyrosinase have also been postulated (16, 17), although neither an inhibitor nor an activator has yet been identified. Because tyrosinase is a glycoprotein, inhibition of glycosylation was specifically postulated to play a physiologic role in modulating tyrosinase activity (18).

In human melanocytes, even less is known about regulation of tyrosinase activity. Unlike murine melanoma cells, the regulation of tyrosinase activity appears to occur mostly at the posttranslational level. For example, the level of tyrosinase

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mRNA does not correlate with the total melanin level in cultured human melanocytes (19, 20), and human melanoma cells often fail to produce melanin despite having abundant tyrosinase protein (21, 22). However, the posttranslational mechanism by which tyrosinase activity is putatively regulated is not well understood. Again, an inhibitor of tyrosinase in human skin has been suggested (23), but it is yet undocumented.

Recently, our laboratory has demonstrated that protein kinase C (PKC) plays an important role in regulating both human and murine pigmentation (21, 24–26). Diacylglycerol, the known physiologic activator of PKC that is often cleaved from cell membrane lipids when ligands bind their cell surface receptors (27) or following ultraviolet irradiation (28, 29), increased melanin content in cultured human melanocytes, an effect blocked by PKC inhibitors (25). In murine melanoma cells, depletion of PKC completely blocked α-MSH induced pigmentation (24); in intact guinea pig skin, topical application of diacylglycerols known to activate PKC caused tanning, whereas application of analogues lacking PKC activity did not (26). Subsequent experiments revealed that the β-isof orm of PKC specifically regulates human pigmentation, in that human melanoma cells expressing a normal level of tyrosinase but lacking PKC-β were amelanotic and that transfection with PKC-β-activated tyrosinase in these cells (21).

PKC is a serine/threonine kinase for which one of the best-characterized intracellular substrates is myristoylated alanine-rich C kinase substrate (30). Although tyrosinase contains possible phosphorylation sites for a serine/threonine kinase, it has never been determined whether phosphorylation is a mechanism by which tyrosinase activity is regulated. In the present study, we demonstrate that PKC-β acts directly on human tyrosinase by phosphorylating serine residues in its cytoplasmic domain.

**Experimental Procedures**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), L15, nonessential amino acids, glutamine, medium 199, and trypsin were purchased from Life Technologies, Inc. Recombinant basic fibroblast growth factor was purchased from Angen, triiodothyronine from Collaborative Research, and hydrocortisone from Calbiochem. Phorbol 12,13-dibutyrate (PDBu), 12-O-tetradecanoylphorbol-13-acetate (TPA), insulin, transferrin, purified tyrosinase, acidic phosphatase, synthetic melanin, and phosphoserine, phosphothreonine, and phosphotyrosine standards were from Sigma. Choleragen was from List Biologicals. Nylon membranes were from Amersham Pharmacia Biotech. PKC antibodies were from Seigaku International. Bovine calf serum and fetal calf serum were from HyClone Laboratories, Inc. All radiolabeled compounds were purchased from New England Nuclear. Cellulose thin-layer plates were purchased from Merck. Young rabbit brains were obtained from Seiji Matsuo et al. (36) from cultured human melanocytes. Cultures were washed with phosphate-buffered saline, scraped, pelleted, and resuspended in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA. Then, they were disrupted by freeze-thaw followed by nitrogen cavitation. The unbroken cells were removed by centrifugation at 1000 x g for 5 min; cavitation was repeated on the pellet; and the resulting supernatants were layered over an equal volume of 30% sucrose in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, and centrifuged in a swinging bucket rotor at 50,000 x g for 30 min. Electron microscopy was routinely performed on the purified melanosomes to assure the intactness of this organelle.

**Purification of PKC**—PKC was purified from young rabbit brain by a modification of procedures previously described (35). In brief, 40–50 g of wet tissue was homogenized and centrifuged to generate crude extract. Crude extract was applied to a DEAE-cellulose column, and proteins were eluted with a linear 0.3–300 mM NaCl gradient. Fractions containing PKC activity were pooled and applied to the 5-ml phenyl-Sepharose CL-4B column. The active fractions were pooled and applied to the 5-ml phenyl-Sepharose CL-4B column. The active fractions were pooled and applied directly to a 1-ml protamine-agarose column. Active fractions were pooled and 10% glycerol was added and applied to hydroxylapatite column. Fractions of 1.5 ml were collected and assayed for PKC activity. Active fractions corresponding to the three peaks of γ-, β-, and α-isoforms were analyzed by immunoblot analysis using monoclonal antibodies specific to each isoform. The active fractions corresponding to the β-isoform were further analyzed by immunoblot analysis using monoclonal antibodies against γ-, β-, and α-isoforms to rule out the possibility that this fraction was contaminated by other isoforms.

**Melanosomal Isolation**—Melanosomes were purified by a modification of the method of Seiji et al. (36) from cultured human melanocytes. Cultures were washed with phosphate-buffered saline, scraped, pelleted, and resuspended in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA. Then, they were disrupted by freeze-thaw followed by nitrogen cavitation. The unbroken cells were removed by centrifugation at 1000 x g for 5 min; cavitation was repeated on the pellet; and the resulting supernatants were layered over an equal volume of 30% sucrose in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, and centrifuged in a swinging bucket rotor at 50,000 x g for 30 min. Electron microscopy was routinely performed on the purified melanosomes to assure the intactness of this organelle.

**Phosphoamino Acid Analysis by Thin Layer Electrophoresis**—Tyrosinase was phosphorylated in the presence of 10−7 M TPA and [γ32P]orthophosphate and subsequently immunoprecipitated as described (21). 1–2 μg of partially enriched lysate for purified PKC was routinely used. Protein samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane electrothermally. The membrane was preincubated in 100% blotto (5 g of nonfat dry milk in 100 ml of phosphate-buffered saline) for 3 h at room temperature with shaking, followed by an overnight incubation with antisera (0.5–1 μg/ml in 10% blotto) at 4 °C. At the end of the incubation, the membrane was washed extensively with phosphate-buffered saline containing 0.5% Tween-20 and processed using the ECL kit. The membrane was then exposed to Eastman Kodak Co. X-OMAT film.
scribed above. The SDS gel containing the immunoprecipitated tyrosinase was electrophoreted onto a PVDF membrane and exposed to autoradiography to identify radiolabeled tyrosinase. The tyrosinase band was cut and diced into small pieces that were incubated with 5.9 μl of sodium hydroxide for 1 h at 110°C. The sample was evaporated and dissolved in electrophoresis buffer (formic acid/acetic acid (2.5:7.8, pH 1.9)) before phosphoamino acid analysis by thin-layer electrophoresis. The product of acid hydrolysis was spiked with phosphoserine, phosphothreonine, and phosphotyrosine standards and separated on a cellulose thin-layer plate using electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. After the plate was dry, radioactivity was detected by autoradiography, and the location of the phosphoamino acid standards was found by ninhydrin staining.

Immunoelectron Microscopy—Immunoelectron microscopy was performed by first fixing melanocytes with 0.5% glutaraldehyde in 0.9 mg of sodium barbital-sodium acetate buffer (pH 7.4) for 15 min at room temperature. Then cells were fixed for 15 min at room temperature with 0.5% osmium tetroxide-1.5% potassium ferrocyanide solution in the same buffer. The samples were then dehydrated in a graded series of ethanols starting with 70% and then dried, radioactivity was detected by autoradiography, and the location of the phosphoamino acid standards was found by ninhydrin staining after polymerization at 60°C for 18 h. Thin sections were cut by inverting the grids onto droplets of the antibody diluted in 0.1M Tris buffer (pH 7.4)

To examine whether tyrosinase is phosphorylated through the PKC-dependent pathway and are consistent with our previous report that a portion of melanocyte PKC is active under basal culture conditions (21).

We have previously shown that nonpigmented MM4 human melanoma cells (NP-MM4) lack PKC-β but contain tyrosinase and PKC-α protein at levels comparable to those of the pigment-producing parental melanoma line or normal human melanocytes (21). We have also previously shown that introduction of PKC-β into these amelanotic MM4 cells by transient transfection activates tyrosinase (21), although no mechanism was established. To examine whether tyrosinase is phosphorylated specifically by the PKC-β, NP-MM4 cells were permanently transfected with PKC-β and SV2Neo cDNAs or SV2Neo cDNA alone. NP-MM4 cells permanently transfected with PKC-β cDNA (PKC-β NP-MM4) expressed a readily detectable level of the β isoform (Fig. 2A) and their tyrosinase was active (Fig. 2B), as previously reported for transiently transfected NP-MM4 cells (21). NP-MM4 cells transfected only with SV2Neo cDNA (control NP-MM4) had undetectable levels of both PKC-β protein and tyrosinase activity. Paired cultures of control NP-MM4 cells and PKC-β NP-MM4 cells were then incubated in phosphate-free DMEM in the presence of [32P]orthophosphate for 90 min, followed by treatment with TPA (1 × 10−7 M) for an additional 90 min. Tyrosinase was immunoprecipitated from both cell types. Incorporation of [32P]phosphate into tyrosinase was readily detected in TPA-treated PKC-β NP-MM4 cells, whereas [32P]phosphate was undetectable in the TPA-treated control NP-MM4 cells (Fig. 3A). However, phosphorylation of myristoylated alanine-rich C kinase substrate was apparent in TPA-

**RESULTS**

**Tyrosinase Is Phosphorylated by PKC-β**—Because PKC-β was previously shown to activate tyrosinase in human melanocytes (21), we sought to determine whether the activation involves phosphorylation of tyrosinase by PKC-β. Paired cultures of human melanocytes were preincubated with phosphate-free DMEM in the presence of [32P]orthophosphate (8500–9120 Ci/mmol) for 90 min; one culture received TPA (1 × 10−7 M) for 90 min to activate PKC, and the other culture remained untreated. At the end of the incubation period, cells were harvested, and tyrosinase was immunoprecipitated using a polyclonal antibody specific for tyrosinase (33). Untreated melanocytes showed a low level of [32P]phosphate incorporation into tyrosinase, whereas melanocytes treated with TPA showed increased [32P]phosphate incorporation (Fig. 1). These results indicate that tyrosinase is a phosphorylase that is phosphorylated through the PKC-dependent pathway and are consistent with our previous report that a portion of melanocyte PKC is active under basal culture conditions (21).

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**FIG. 1. In vivo phosphorylation of tyrosinase in melanocytes**

Paired cultures of subconfluent melanocytes were treated with either 1 × 10−7 M TPA or vehicle alone (MeSO4) for 90 min in presence of [32P]orthophosphate in phosphate-free medium. Cells were harvested and tyrosinase was immunoprecipitated as described under “Experimental Procedures.” Incorporation of radiolabeled phosphate into tyrosinase was determined by separating total protein by SDS-PAGE (1–2 × 105 cpm/lane), followed by autoradiography. One representative experiment from five separate experiments is presented.

1 The abbreviations used are: BSA, bovine serum albumin; L-dopa, L-dihydroxyphenylalanine; PKC, protein kinase C; NP-MM4, nonpigmented melanoma; PDAs, phorbol 12,13-dibutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco's modified essential medium; α-MSH, α-melanocyte stimulating hormone; PKA, cAMP dependent protein kinase; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride; Fmoc, fluorenylmethyloxycarbonyl.
PKC-β Phosphorylates Tyrosinase

FIG. 2. Expression of PKC-β and activation of tyrosinase. To determine whether PKC-β is required for tyrosinase phosphorylation, NP-MM4 cells were permanently transfected with the expression vector containing PKC-β cDNA and SVNeo or with SVNeo alone as control. A, paired cultures of control and PKC-β-transfected cells were harvested, and the levels of PKC-β protein were determined by Western blot analysis. B, tyrosinase activity was determined by Pomerantz assay.

PKC-β Phosphorylates Tyrosinase by PKC-β Transfected NP-MM4 Cells. A, a set of subconfluent control NP-MM4 and PKC-β-transfected NP-MM4 cultures were treated with 10−7 M TPA or vehicle alone (Me2SO) for 90 min in the presence of [32P]orthophosphate. Cells were harvested and tyrosinase-immunoprecipitated (1–2 × 106cpm/lane), and incorporation of radiolabeled phosphate was visualized by autoradiography. Both cells types are known to have comparable levels of tyrosinase (21). One representative experiment from three independent experiments is presented. B, to eliminate the possibility that PKC is not properly activated in control-NP-MM4 cells, these cells were treated with either TPA or vehicle alone for 90 min in the presence of [32P]orthophosphate. The 80-kDa myristoylated alanine-rich C kinase substrate (30) was immunoprecipitated using an antibody specific for this substrate protein and separated in a 7.5% SDS-PAGE (0.5–1 × 106cpm/lane). A phosphorylated band of the expected size was readily detected.

FIG. 3. Phosphorylation of tyrosinase in control and PKC-β transfected NP-MM4 cells. A, to examine whether PKC-β can directly phosphorylate tyrosinase, purified mushroom tyrosinase was incubated with purified PKC-β in vitro. A, the purity of PKC-β isolated from mouse brain was confirmed by performing immunoblot analysis using antibodies against the α, β, and γ isofoms (labeled at top) of PKC. B, purified melanosomes were preincubated with purified PKC-β (5 ng) in the presence of phospholipid and TPA, required for PKC-β activation, or in the absence of phospholipid and TPA, a condition inadequate for PKC-β activation. Tyrosinase activity in each condition was determined using Pomerantz assay as described under “Experimental Procedures.”

PKC-β Co-Localizes with Tyrosinase at the Melanosomal Membrane—It has been shown in many other cell types that compartmentalization of a specific PKC isoform is associated with a unique biological function (40, 41). To determine whether PKC-β was physically associated with tyrosinase at the site of melanin synthesis, the melanosomes, we performed immunoelectron microscopy. Paired cultures of melanocytes were immunostained with either tyrosinase and PKC-β antibodies, tagged with gold beads of different size, or as a control with tyrosinase and PKC-α antibodies. The antibodies were labeled with 10-nm diameter gold beads for tyrosinase and 5-μm beads for PKC-β or PKC-α, as described under “Experimental Procedures.” Tyrosinase was restricted to melanosomes, as expected, and 10-nm gold beads were observed on the cytoplasmic side of the melanosomal membrane, as also expected, given that the tyrosinase antibody employed is directed against the cytoplasmic domain of the enzyme (33) (Fig. 5A). Interestingly, PKC-β was localized to the cytoplasmic side of the melanosomal membrane, and always close to tyrosinase, but PKC-α was instead observed diffusely in the melanocyte cytoplasm, unrelated to melanosomes (Fig. 5A). This suggests that PKC-β compartmentalizes at the melanosome, thus determining its specific function in regulating tyrosinase activity.

Because the electron density of melanosomes might obscure the presence of immunogold staining within the organelle, we could not determine from above experiments whether PKC-β is also present inside melanosomes. Melanosomes were therefore purified from intact melanocytes and separated into two groups. One was untreated, and the other was treated with trypsin to remove any membrane-associated proteins, including PKC-β. Whole melanocyte lysate, loaded as a positive control, contained readily detectable levels of PKC-β, as did untreated melanosomes, whereas trypsin-treated melanosome preparation had no detectable PKC-β (Fig. 5B). These results suggest that PKC-β is not present inside melanosomes, but instead closely associates with the cytoplasmic surface of melanosomes, consistent with the electron micrographs and with current literature indicating that PKC-β is a cytoplasmic protein (27).

PKC-β Phosphorylates the Cytoplasmic Domain of Tyrosinase—Tyrosinase has a large inner domain, residing within melanosomes, a transmembrane domain, and a cytoplasmic domain that extends approximately 30 amino acids beyond the...
melanosomal membrane (5). The published amino acid sequence of human tyrosinase reveals a total of 60 serine and threonine residues, but only two serines and no threonines in the cytoplasmic domain (5). Because our data confirmed that PKC-\(\beta\) in melanocytes is a cytoplasmic protein, as in other cell types (27, 41), we hypothesized that PKC-\(\beta\) phosphorylates only the cytoplasmic domain of tyrosinase. To test this hypothesis, paired cultures of melanocytes were preincubated with \[^{32}P\]orthophosphate, and PKC was activated to allow phosphorylation of tyrosinase. Melanosomes were purified and divided into two groups, one of which was exposed to trypsin for 2 h to digest the outer cytoplasmic domain of tyrosinase (4), known to have multiple trypsin-sensitive arginine and lysine sites (42) at amino acid positions of 483–484, 485–486, 491–492, 493–494, and 500–501, beyond the melanosomal membrane. Trypsin-treated control melanosomes were then immunoprecipitated using PEP-5, a polyclonal antibody specific for the inner domain of tyrosinase (33). Immunoprecipitated proteins were run on a gel and \[^{32}P\]orthophosphate label incorporation was readily detected in untreated full-length tyrosinase, as expected, but not in samples exposed to trypsin prior to immunoprecipitation (Fig. 6A), indicating that the remaining intramelanosomal portion of tyrosinase is not phosphorylated. When the supernatant of the trypsin-treated melanosome was concentrated and run on a 17% gel, a radiolabeled cytoplasmic peptide, consistent with the largest predicted tryptic digestion fragment from the cytoplasmic domain of tyrosinase, was recovered (Fig. 6B). Other smaller predicted fragments were not detected, as expected, because they were too small to be retained in the gel. To be certain that there was tyrosinase in all immunoprecipitated samples, Western blot analysis was performed in parallel. Equal amounts of tyrosinase protein reactive with antibody directed against epitopes in the inner domain of tyrosinase were present in both preparations (Fig. 6C), excluding the possibility that lack of label was due to complete loss of trypsin-treated tyrosinase during immunoprecipitation. Taken together, these results indicate that PKC-\(\beta\) activates tyrosinase by phosphorylating the cytoplasmic domain of the enzyme.

**Phosphoamino Acid Analysis of Human Tyrosinase**—The deduced amino acid sequence of human tyrosinase contains two serines but no threonines in the cytoplasmic domain (5). To confirm the expectation that only serine residues are therefore phosphorylated by PKC, phosphoamino acid analysis of immunoprecipitated radiolabeled tyrosinase was performed. Tyrosinase was phosphorylated in vitro by preincubating melanocytes with \[^{32}P\]orthophosphate, followed by activation of PKC with TPA. Subsequently, \[^{32}P\]tyrosinase was immunoprecipitated, separated by SDS-PAGE, and transferred to a PVDF membrane. Immunoprecipitated tyrosinase was further identified by incubating the PVDF membrane with L-dopa, which is incorporated by active tyrosinase into melanin and then deposited on the PVDF membrane. The protein band corresponding to human tyrosinase was then cut out and hydrolyzed to individual amino acids. The resulting mixture of amino acids was
PKC-β Phosphorylates Tyrosinase

Because the cytoplasmic domain of tyrosinase contains only serine and no threonine residues, phosphoamino acid analysis was performed to determine whether only serines are phosphorylated by PKC-β. Subconfluent cultures of melanocytes were treated with TPA in presence of [32P]orthophosphate to phosphorylate and radiolabel tyrosinase. Tyrosinase was then immunoprecipitated and transferred to a PVDF membrane. Tyrosinase was then visualized by reacting the PVDF membrane with 1-dopa. The band containing the radiolabeled tyrosinase was cut out and incubated with 5.9 M hydrochloric acid for 1 h at 110 °C. The sample was evaporated, mixed with phosphoamino acid standards, and subjected to two-dimensional thin-layer electrophoresis. Radioactivity was detected by autoradiography, whereas the location of the phosphoamino acid standards was found by ninhydrin staining. (pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine). This figure is representative of five analyses.

Both Serine Residues on the Cytoplasmic Domain Are Phosphorylated—To determine whether one or both serines on the cytoplasmic domain are phosphorylated, radiolabeled tyrosinase was first partially purified from human melanosomes. Tyrosinase was extracted from melanosome and separated from other melanosomal proteins by nonnaturating gel electrophoresis. The protein was then identified by the appearance of a brown-colored band after the in situ tyrosinase activity assay (33). Because the product of 1-dopa oxidation by tyrosinase is highly hydrophobic and might interfere with the digestion of tyrosinase or with recovery of the tryptic peptides from the gel, the sample was run in multiple lanes, one of which was excised and stained with 1-dopa. The remaining portion of the gel was subjected to autoradiography, and the area corresponding to the stained section of the 1-dopa treated lane was excised and treated directly with trypsin. Of the predicted tryptic fragments of the human tyrosinase cytoplasmic domain (5), Glu-Asp-Tyr-His-Ser(P)-Leu-Tyr-Gln-Ser-P-His-Leu, contains both of the serines. All three possible phosphoserine-containing peptides, one phosphorylated on both serines and two phosphorylated on each one of the serines, were synthesized and used as phosphorylated standards in subsequent co-migration experiments. To remove salts, unincorporated [32P]orthophosphate, and other unrelated peptides, the tryptic digest was first subjected to an HPLC purification step. Nonlabeled synthetic phosphopeptides were then added to the digest as markers, because the quantity of [32P]-labeled peptides was too low to detect by UV absorption.

In the HPLC elution profile of the [32P]-labeled tyrosinase tryptic peptide mixture, the more polar diphosphopeptide eluted earlier than the monophosphopeptides, which were not resolved. Because the radioactivity of individual fractions was so low and to avoid handling losses, the fractions containing the phosphoamino acid analysis of immunoprecipitated tyrosinase. Because the cytoplasmic domain of tyrosinase contains only serine and no threonine residues, phosphoamino acid analysis was performed to determine whether only serines are phosphorylated by PKC-β. Subconfluent cultures of melanocytes were treated with TPA in presence of [32P]orthophosphate to phosphorylate and radiolabel tyrosinase. Tyrosinase was then immunoprecipitated and transferred to a PVDF membrane. Tyrosinase was then visualized by reacting the PVDF membrane with 1-dopa. The band containing the radiolabeled tyrosinase was cut out and incubated with 5.9 M hydrochloric acid for 1 h at 110 °C. The sample was evaporated, mixed with phosphoamino acid standards, and subjected to two-dimensional thin-layer electrophoresis. Radioactivity was detected by autoradiography, whereas the location of the phosphoamino acid standards was found by ninhydrin staining. (pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine). This figure is representative of five analyses.

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In the HPLC elution profile of the [32P]-labeled tyrosinase tryptic peptide mixture, the more polar diphosphopeptide eluted earlier than the monophosphopeptides, which were not resolved. Because the radioactivity of individual fractions was so low and to avoid handling losses, the fractions containing the carrier phosphopeptides were collected as a single fraction and subjected to two-dimensional peptide mapping.

The mixture was first separated by thin layer electrophoresis on cellulose in formic acid/acetic acid/water (pH 1.9), followed by thin-layer chromatography at right angles. The thin-layer chromatography plate was dried and exposed to x-ray film. Ninhydrin staining of the plate revealed two spots, corresponding to the diphosphopeptide and the faster moving unresolved mixture of monophosphopeptides (Fig. 8A). Autoradiography revealed an area of radioactivity coincident with the diphosphopeptide (Fig. 8B). No radioactivity was associated with the monophosphopeptides. The radioactive signal associated with the diphosphopeptide was weak, requiring long exposures to detect. Nevertheless, these results demonstrate that tryptic digestion of in vivo [32P]-labeled tyrosinase produces a peptide that co-migrates with the synthetic tyrosinase-related peptide, Glu-Asp-Tyr-His-Ser(P)-Leu-Tyr-Gln-Ser-P-His-Leu, in three separation procedures: HPLC, thin layer electrophoresis, and thin-layer chromatography. Together with earlier results, this confirms that both serine residues of tyrosinase, at amino acid positions of 505 and 509, are phosphorylated by PKC-β.

To demonstrate that the cytoplasmic domain of tyrosinase can act as a substrate for PKC-β, a peptide containing the putative phosphorylation sites in tyrosinase (Glu-Asp-Tyr-His-Ser-Leu-Tyr-Gln-Ser-P-His-Leu, termed TP 501–511), was synthesized and incubated with [32P]ATP in the presence of active recombinant PKC-β. The peptide was then eluted from the phosphocellulose membrane with a 50 mM ammonium bicarbonate solution and analyzed by thin layer electrophoresis. The eluent was spiked with TP 501–511 and a portion of the eluent (approximately 10 μl) was subjected to one-dimensional thin layer electrophoresis (pH 1.9). It was reasoned that if the peptide (TP 501–511) had become phosphorylated, it would migrate more slowly toward the anode than the nonphosphorylated peptide on thin layer electrophoresis. Fig. 8C shows the electrophoretic mobility of the peptide TP 501–511 phosphorylated in vitro with [32P]ATP. Autoradiography of the plate reveals only a single radioactive spot, whereas ninhydrin staining yields two spots (Fig. 8C, dashed ovals), one corresponding to TP 501–511 and the other to the phosphorylated TP 501–511 peptide.
migrated more slowly toward the anode than TP 501–511, consistent with incorporation of phosphate groups into the synthetic peptide.

**DISCUSSION**

It has been surprisingly difficult to decipher the regulation of human melanogenesis despite rapid evolution of adequate culture methods for human melanocytes over the past two decades. The confusing role of phorbol esters, initially added to media to stimulate melanocyte growth in the absence of then unidentified key mitogens (43), but eventually discovered to severely down-regulate tyrosinase activity through PKC depletion (21), has only recently been appreciated. In addition, many potent inducers of murine pigmentation, such as α-MSH, either fail to induce human pigmentation or are far less effective, a situation also likely to be attributable at least in part to media artifacts, such as reliance on cholera toxin or other agents to elevate basal cAMP levels (21, 43). For example, after several negative or ambiguous studies, it was finally reported that under defined culture conditions, α-MSH can induce tyrosinase mRNA, protein, and activity in human melanocytes (44, 45), but its overall contribution to regulation of human melanogenesis is appears nevertheless to be far less than in the murine system. Importantly, posttranslational modification appears to play a more critical role in regulating tyrosinase activity in human than in murine melanocytes, with mRNA and protein levels poorly correlated with the enzyme activity (19, 20), and amelanotic human melanoma cells often expressing ample tyrosinase protein (21, 22).

PKC-β is the first documented control point for human tyrosinase activity. The protein level of PKC-β correlates with total pigment content in melanocytes and activation of PKC leads to increased tyrosinase activity, whereas depletion of PKC decreases tyrosinase activity (21). PKC-β is required to activate tyrosinase, in that when the lysate from amelanotic human melanoma NP-MM4 cells, which lack PKC-β expression but have abundant tyrosinase protein, was mixed with the lysate of normal melanocytes, tyrosinase in the NP-MM4 cells was activated. This activation was abolished when the melanocyte lysate was immunoprecipitated using a specific monoclonal antibody to remove PKC-β before being mixed with the NP-MM4 cell lysate. In addition, transient expression of PKC-β in NP-MM4 cells activates tyrosinase (21). A key role for PKC-β is not unique to human melanogenesis but also has been documented in the murine system. Pigmented S91 murine melanoma cells, known to progressively lose their ability to pigment both under basal conditions and after α-MSH stimulation if serially passaged (46), in parallel lose the expression of PKC-β, whereas the expression of PKC-α and tyrosinase remains unaffected (46). Furthermore, the induction of murine pigmentation by α-MSH or other cAMP elevating agents, such as isobutyl methyl xanthine or dibutyryl cAMP, requires PKC in that when PKC is depleted, induction of pigmentation by these agents is completely blocked (24). In human melanocytes, we have further recently shown that PKC-β is rate-limiting for cAMP-mediated elevation of tyrosinase activity in that cAMP-induced tyrosinase activation does not correlate temporally with increased tyrosinase protein, but rather parallels the later cAMP-mediated induction of PKC-β activity (25).

In cultured human melanocytes, and others have reported that at least the α, β, δ, and ε-isoforms of PKC are expressed (48, 49). The present immunoelectron microscopy studies show that the β-isoform co-localizes with tyrosinase at the surface of melanosomes, whereas the α-isoform is found diffusely in the cytoplasm and not specifically near melanosomes. These results suggest that compartmentalization of PKC-β with melanosomes may determine its ability to phosphorylate tyrosinase. The mechanism by which certain PKC isoforms may adopt a nonrandom localization within cells is incompletely elucidated. However, specific receptors for activated protein kinase C have been identified, suggesting that PKC isoform specific receptor for activated C kinase proteins may govern the intercellular distribution of PKC isoforms (50). It would thus be interesting to determine whether melanosomes express a PKC-β specific receptor activated C kinase that is melanosome-associated.

Our current and previously published work (21) establishes that PKC-β activates tyrosinase activity in human melanocytes. The data presented here further indicate that PKC-β-mediated phosphorylation exclusively involves the cytoplasmic domain of tyrosinase and that both serine residues in this domain are phosphorylated. Radioactivity from the tryptic digest of 32P-labeled tyrosinase co-migrates with the synthetic diposphopeptide Glu-Asp-Tyr-His-Ser(P)-Leu-Tyr-Gln-Ser(P)-His-Leu, corresponding to amino acids 501–511, using separation methods based on hydrophobicity (reversed phase HPLC), charge and size (thin layer electrophoresis), and polarity (thin layer chromatography). Furthermore, synthetic standard peptides corresponding to this portion of tyrosinase are phosphorylated by PKC-β in vitro, even though the conventional substrate sequence for PKC (51) is lacking, further supporting this conclusion.

Tyrosinase-related proteins 1 and 2 (TRP1 and TRP2), two proteins known to be important for melanogenesis, share 70–80% nucleotide sequence homology and 40–45% amino acid identity with tyrosinase (52). Like tyrosinase, they are transmembrane melanosomal proteins, with cytoplasmic domains of about 26–28 amino acids. In addition, TRP1 has one serine at amino acid position 502 and one threonine at amino acid position 516, and TRP2 has two serines at amino acid positions 510 and 511 and three threonines at amino acid positions 503, 508, and 516, (52). They are therefore potential PKC substrates, although they have never been reported to be phosphoproteins. However, TRP1 and TRP2 do not share any amino acid homology with tyrosinase in their cytoplasmic domains (5, 52), and therefore, the tryptic fragments generated from the cytoplasmic domains of TRP1 and TRP2 are vastly different from those of tyrosinase. Hence, the tryptic fragments of TRP1 and TRP2 would not co-migrate with the synthetic mono- and diposphopeptide standards that were used for mapping experiments in this report, excluding the possibility that our phosphorylation results reflect TRP1 and/or TRP2 contamination. It has also been demonstrated that only peptides of identical size and charge will co-migrate in the thin-layer chromatography employed in these experiments (39).

A central role for PKC-β in melanogenesis does not diminish the importance of other signal transduction molecules. cAMP-dependent protein kinase (PKA), the principal serine/threonine kinase mediating the actions of cAMP, also activates tyrosinase either directly or indirectly through phosphorylation. Addition of purified PKA to murine S91 cell lysates activates tyrosinase (17), presumably through phosphorylation of a PKA substrate protein, but in one early study (15), when these cells were treated with α-MSH in the presence of 32P-labeled phosphate, tyrosinase apparently was not phosphorylated. Indeed, only recently was an obligate role for PKA in α-MSH-induced murine melanogenesis firmly established (53). As noted above, the role of the PKA pathway in human melanogenesis is even less clear and appears intimately connected to the PKC pathway (53).

The critical role of the cytoplasmic domain of tyrosinase in its melanogenic activity established by the present experiments is also strongly suggested by mutations identified in patients.
with oculocutaneous albinism. Insertion of single nucleotides at codons determining the amino acid at position 489 or 501 causes premature termination of tyrosinase, deleting 20–22 amino acids from the cytoplasmic domain, including the serines at 505 and 509 (6). This abolishes tyrosinase activity, although the intramelanosomal domain of the tyrosinase protein is present and normal. Site-directed mutagenesis to alter or eliminate one or both of the serines in the cytoplasmic domain, followed by expression of the mutated DNA in pigment cells lacking wild-type tyrosinase, would rigorously demonstrate that phosphorylation of these residues is required for protein activity, but unfortunately, substantial technical difficulties in transfecting human melanocytes would need to be overcome before such experiments could be performed. In mice harboring the platinum mutation, in which the last 27 amino acids of tyrosinase are absent, pigment production is greatly reduced and, in addition, tyrosinase is directed to the cytoplasmic membrane (8–10). However, a more recent study has suggested that amino acids at positions 494–498 of the human tyrosinase are responsible for the subcellular trafficking (11). These findings and our report suggest that the cytoplasmic domain of tyrosinase is critical for both subcellular trafficking and the activation of the protein.

Our results strongly suggest that direct phosphorylation of tyrosinase by PKC-β leads to its activation. When purified human melanosomes are incubated with purified activated PKC-β, tyrosinase activity increases. This activation is thus likely due to the direct interaction between tyrosinase and PKC-β. Demonstration of direct activation of purified human tyrosinase by PKC-β was not attempted due to the difficulty of obtaining a sufficient amount of highly purified enzyme from poorly proliferative human melanocytes, particularly in light of its close association in the melanosome membrane with highly homologous TRP1 and TRP2 (52) and the hydrophobic polymer melanin. Moreover, PKC-β-mediated phosphorylation of isolated tyrosinase might well not lead to the same activation as when the protein is residing in the melanosome membrane, in a specific steric relationship to other cellular constituents complicating interpretation of negative results.

We have shown that phosphorylation occurs on the two serine residues in the cytoplasmic domain of tyrosinase, but the precise mechanism by which this phosphorylation activates tyrosinase has yet to be determined. Recent studies suggest that tyrosinase might interact with other melanogenic enzymes, such as TRP1 and TRP2, in the melanosomal membrane (54, 55), and tyrosinase activity was reported to be diminished when the enzyme complexes with TRP1 and TRP2 (55). Moreover, it has recently been suggested that a point mutation in the TRP1 gene that changes the coat color in mice disrupts the interaction of TRP1 with tyrosinase (54), suggesting that TRP1 may function as the long-sought tyrosinase inhibitor. In this context it would be of interest to determine whether phosphorylation of tyrosinase, and possibly also TRP1 by PKC-β might sterically hinder their association in the melanosome.

Our approach to identifying the exact phosphorylation sites using co-purification of 32P-labeled tryptic peptides along with unlabeled synthetic carrier phosphopeptides is novel and may have general applicability for analyzing phosphoryproteins for which the amino acid sequence is known. It has the advantage of not requiring sophisticated instrumentation, as do Edman degradation, mass spectroscopy, and extensive purification of the protein. A mixture of nonlabeled, predicted synthetic tryptic phosphopeptides added as carriers to a tryptic digest of the 32P-labeled protein allows purification to homogeneity of the peptide of interest by several separation methods, ensuring that the specific carrier peptide and the labeled peptide are identical. Moreover, the target protein does not have to be particularly pure, in that contaminating radiolabeled peptides derived from unrelated proteins will eventually be separated from the carrier peptides. In addition, intact cells can be used to phosphorylate the substrate of interest, increasing the probability that the observed phosphorylation sites are physiologically relevant. This is especially critical when portions of the substrate protein are inaccessible to kinase in vivo due to physical sequestration. For example, in vitro phosphorylation of isolated recombinant tyrosinase might be misleading because phosphorylation might occur on amino acids in the more than 90% of the tyrosinase protein normally sequestered within the melanosome from cytoplasmic kinases such as PKC-β.

In summary, we have demonstrated that tyrosinase is directly activated by PKC-β-mediated phosphorylation, and that the phosphorylation occurs on the two serine residues in the C-terminal cytoplasmic domain of tyrosinase. This first detailed insight into the regulation of human melanogenesis should increase understanding of normal and pathologic pigmentation in human skin and hair, as well as facilitate its manipulation.

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