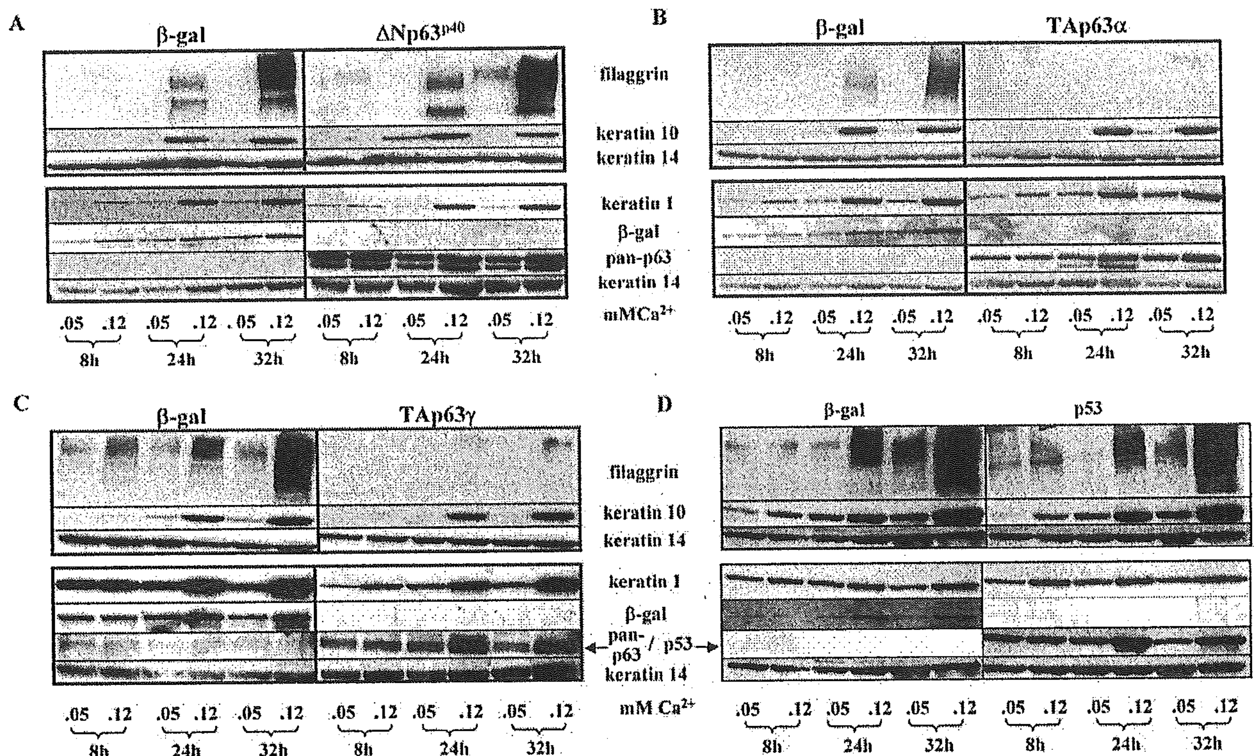


Fig. 4. p63 isotypes exert distinct effects on the keratinocyte differentiation program. Keratinocytes were infected with adenovirus encoding human ΔNp63^{p40}, TAp63α, TAp63γ, p53 or β-galactosidase (β-gal), as noted at the top of each panel. Seventeen hours post-infection cultures were either maintained in medium containing 0.05 mM Ca^{2+} or induced to differentiate by increasing extracellular [Ca^{2+}] to 0.12 mM. Whole cell lysates were harvested 8, 24 or 32 h post-Ca^{2+} trigger, as noted. (**A, B, C** and **D**) Top panels: Membranes were probed sequentially with antibodies directed to filaggrin, keratin 10 and keratin 14. Bottom panels: Replicate western blots of lysates described above were probed sequentially with antibodies directed to keratin 1, β-gal, p63 (pan-p63) or p53, and keratin 14. For each antibody within an experiment, the β-gal control and test sample blots were exposed to ECL reagent and film for the same length of time. Panels probed with β-gal and p53/p63 antibodies are included to demonstrate adenoviral driven expression of these genes. The p63 bands shown in these panels correspond to the specific p63 splice variant introduced by adenoviral transduction. Overexpression of ΔNp63^{p40} or p53 allowed for full expression of differentiation markers. Both TAp63α and TAp63γ interfere specifically with the induction of filaggrin, a late marker of differentiation.

including keratins 1 and 10 and filaggrin (Figure 4A). Likewise, normal induction of mRNA for these markers, as well as loricrin mRNA, was observed in ΔNp63^{p40}-overexpressing cells using RT–PCR (Figure 5). The previously described block in induction of keratin 10 and filaggrin by ΔNp63α (14) is mediated at the RNA level and is also observed for loricrin (Figure 5, top panels). These results suggest that the α-tail of ΔNp63α plays an important role in abrogating expression of differentiation-specific genes.

TAp63 isotypes block filaggrin expression but not early markers of differentiation

To determine whether the α-tail impacts differentiation outside the context of the ΔN subclass of isotypes, TAp63α was introduced via adenoviral transduction and effects on markers of differentiation were evaluated by western analysis and RT–PCR. In contrast to our previous results with ΔNp63α, TAp63α permitted keratin 10 induction at both the protein (Figure 4B) and mRNA (Figure 5) levels. Thus, the presence of the α-terminus is not sufficient to block early differentiation, but acts only in conjunction with the ΔN-terminus.

As endogenous expression of TAp63γ is modulated with differentiation, we investigated whether an imbalance in the TAp63γ isotype would influence the keratinocyte differentiation response to elevated [Ca^{2+}]. Similar to TAp63α, TAp63γ had no effect on the induction of keratins 1 and 10 (Figure 4B and C). However, both TA isotypes blocked induction of filaggrin protein (Figure 4B and C). RT–PCR analysis for differentiation-specific markers revealed that this block in filaggrin by both TAp63 isotypes is mediated at the RNA level (Figure 5). These findings suggest that the TA domain has a specific effect on filaggrin expression. Overexpression of p53, which shares ~22% homology with the p63 TA domain, has no discernible effect on differentiation marker expression (Figures 4D and 5).

Both ΔNp63 isotypes block Ca^{2+}-induced growth arrest

The ability of ΔNp63α to abrogate growth arrest mediated by elevated Ca^{2+} has been demonstrated previously (14). Despite differences between the ΔNp63 isotypes with regard to differentiation potential, both ΔNp63α and ΔNp63^{p40}, when overexpressed, impair the Ca^{2+}-induced growth arrest that is associated with differentiation. As shown in Figure 6A, keratinocytes overexpressing Ad-β-gal demonstrate a 37% decrease in the *S*-phase population under 0.12 mM Ca^{2+} conditions, whereas cells overexpressing ΔNp63^{p40} show only an 11% decrease in *S*-phase population. Overexpressing Ad-TAp63γ or Ad-TAp63α does not alter the Ca^{2+}-induced growth arrest, with *S*-phase population reductions in 0.12 mM Ca^{2+}

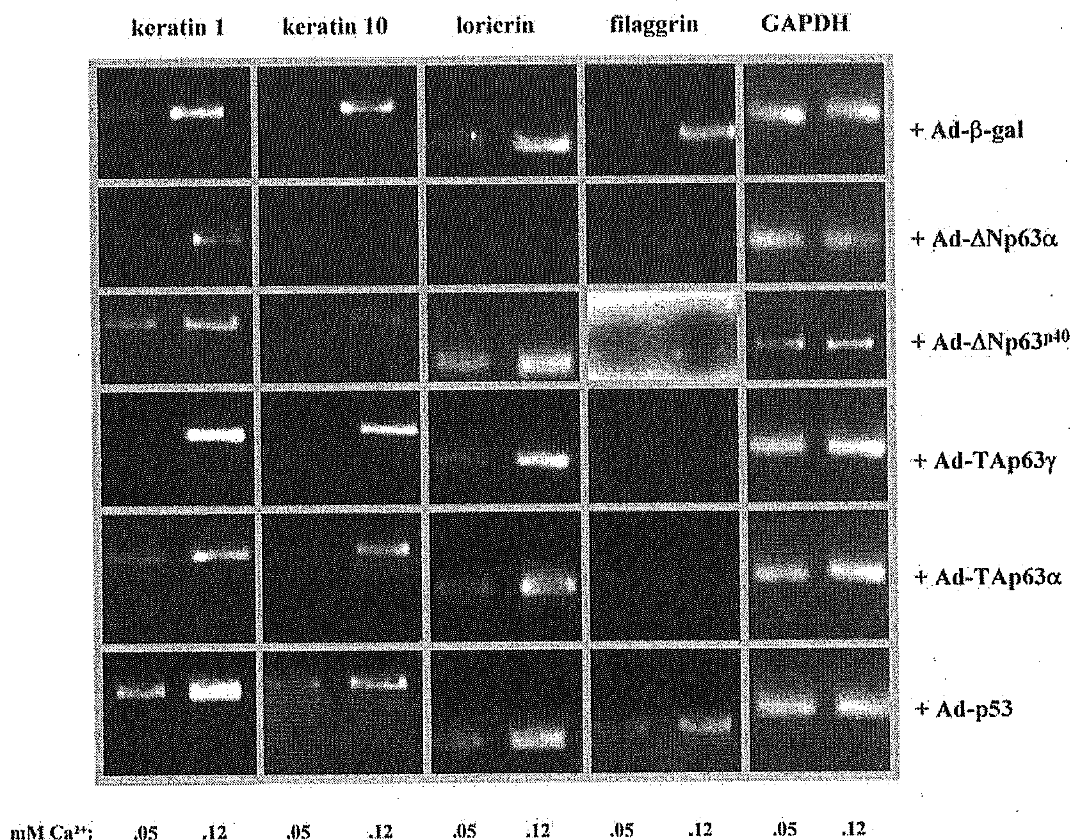


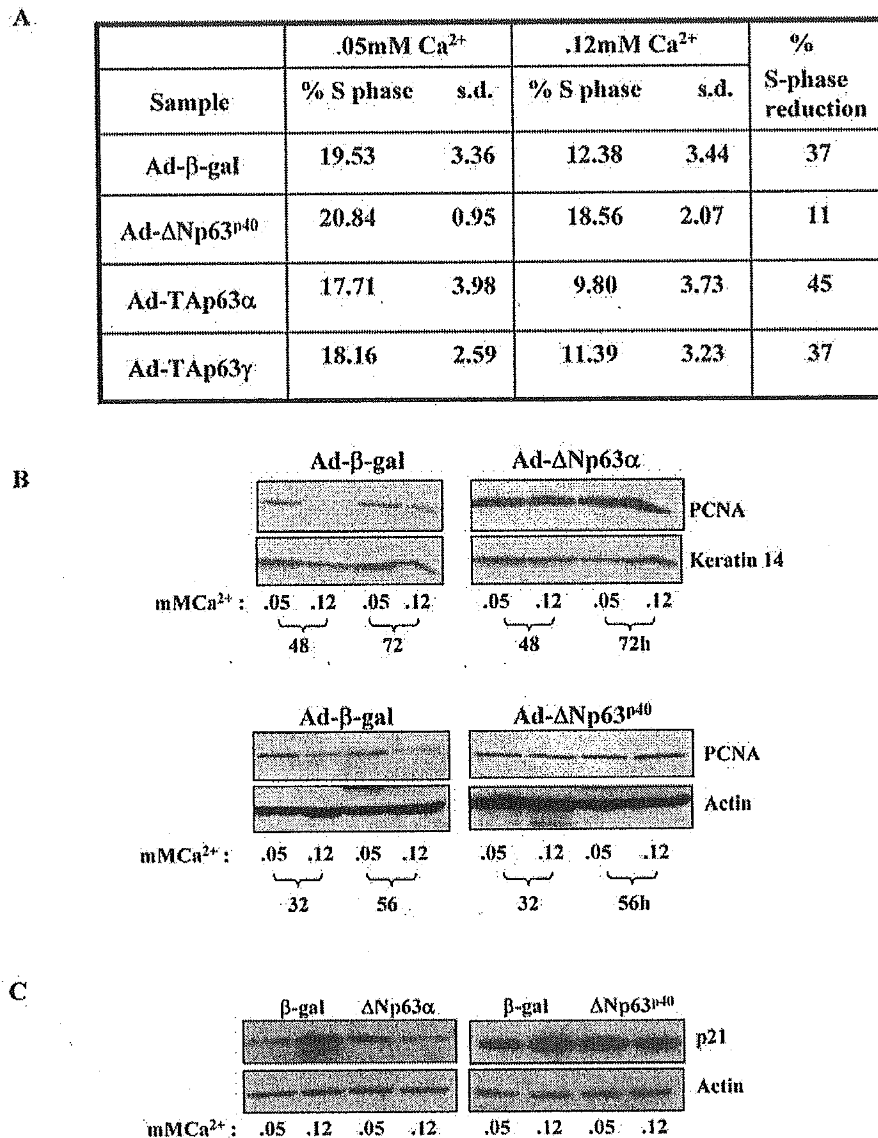
Fig. 5. Effects of p63 isotypes on differentiation-specific gene expression are regulated at the RNA level. Keratinocytes were infected with adenovirus encoding ΔNp63α, $\Delta Np63^{P40}$, TAp63α, TAp63γ, p53 or β-gal. Seventeen hours post-infection cultures were either maintained in medium containing 0.05mM Ca^{2+} or induced to differentiate by increasing extracellular $[Ca^{2+}]$ to 0.12 mM. RNA samples were harvested 24 h later, and semi-quantitative RT–PCR was performed using primers specific for keratin 1, keratin 10, loricrin, filaggrin and GAPDH. Induction of differentiation markers at the RNA level paralleled the results seen by western blotting. Overexpressed ΔNp63α blocked keratin 10 and filaggrin, but not keratin 1, consistent with the protein expression patterns previously described (14). Both TAp63α and TAp63γ blocked expression of filaggrin mRNA.

conditions of 37 and 45%, similar to that observed in the control cultures (Figure 6A). The decrease in S-phase cells in β-gal control cultures following Ca^{2+} treatment correlates with a decline in levels of Proliferating Cell Nuclear Antigen (PCNA) (Figure 6B). Consistent with the FACS data, western analysis demonstrated that keratinocytes overexpressing either ΔNp63α or $\Delta Np63^{P40}$ do not down-regulate PCNA levels in response to 0.12 mM Ca^{2+} (Figure 6B). The block in Ca^{2+}-induced growth arrest also corresponds to a block in the induction of the cyclin dependent kinase inhibitor $p21^{WAF1}$. Whereas β-gal control cultures exhibited an induction of $p21^{WAF1}$ under differentiating conditions, as previously described (27,30,41), both ΔNp63α and $\Delta Np63^{P40}$ block Ca^{2+}-triggered $p21^{WAF1}$ induction (Figure 6C). These results suggest that the ΔN portion of the p63 protein blocks cell cycle regulation independent of the α-terminus. This effect may be mediated at least in part via the regulation of $p21^{WAF1}$.

Discussion

That p63 is critical for development of normal squamous epithelium was demonstrated by the genetic deletion of all p63 isotypes in mice (42,43). Specific p63 isotypes are expressed during distinct stages of embryonic epidermal development (44) and roles attributed to these isotypes are beginning to be elucidated. Embryonic expression of TAp63 coincides with the onset of the epidermal stratification program and ectopic expression of TAp63α can initiate stratification of simple epithelium (44). Within the developed epidermis, ΔNp63α has been postulated to maintain keratinocytes in a proliferative state (1,13,14). Consistent with these findings, chemically induced tumors of mouse skin are shown here to express high levels of ΔNp63 (Figure 3). Additionally, we show that multiple p63 isotypes are expressed in normal primary neonatal keratinocytes and are individually modulated with *in vitro* differentiation. In addition to ΔNp63α, which has been regarded as the most abundant p63 isotype in keratinocytes, we observed a smaller ΔNp63 variant, $\Delta Np63_s$, that migrates on an SDS–PAGE gel between ΔNp63γ and $\Delta Np63^{P40}$. Along with the protein results, quantification of ΔN, α and γ mRNA levels suggest that the $\Delta Np63_s$ band reflects a novel p63 species or a post-translationally modified form of $\Delta Np63^{P40}$.

Previously we showed that overexpression of ΔNp63α blocks keratinocyte differentiation, consistent with the decline in ΔNp63α expression that normally occurs with differentiation. In contrast to ΔNp63α, $\Delta Np63_s$ maintains robust



A

Sample	.05mM Ca^{2+}		.12mM Ca^{2+}		% S-phase reduction
	% S phase	s.d.	% S phase	s.d.	
Ad-β-gal	19.53	3.36	12.38	3.44	37
Ad-ΔNp63^{p40}	20.84	0.95	18.56	2.07	11
Ad-TAp63α	17.71	3.98	9.80	3.73	45
Ad-TAp63γ	18.16	2.59	11.39	3.23	37

Fig. 6. Both ΔNp63 isotypes block Ca^{2+} induced growth arrest. Keratinocytes were infected with adenovirus encoding ΔNp63^{P40}, TAp63α, TAp63γ, ΔNp63α or β-gal. Seventeen hours post-infection cultures were either maintained in medium containing 0.05 mM Ca^{2+} or induced to differentiate by increasing extracellular [Ca^{2+}] to 0.12 mM. (A) Twenty hours post-medium change, cultures were pulsed with 10 μM BrdU for the final 4 h prior to harvesting, and the S-phase population of each sample was determined using FACS analysis. At least three replicates were performed for each condition. Data are presented as mean ± SD, of a minimum of three independent experiments. (B) Protein lysates were prepared from cultures of primary keratinocytes overexpressing ΔNp63α, ΔNp63^{P40} or β-gal and maintained in medium containing 0.05 or 0.12 mM Ca^{2+} for the times indicated. Unlike β-gal control cultures, the PCNA expression levels in keratinocytes overexpressing ΔNp63α or ΔNp63^{P40} remained elevated following culture in 0.12 mM Ca^{2+}. (C) Keratinocytes overexpressing ΔNp63α, ΔNp63^{P40} or β-gal were harvested for western analysis following culture at 0.05 or 0.12 mM Ca^{2+} for 24 h. Induction of the cell cycle arrest protein p21^{WAF1} was observed in samples derived from control cultures of differentiating keratinocytes, but not in samples derived from cultures overexpressing ΔNp63α or ΔNp63^{P40}.

expression in differentiated keratinocytes. To determine the potential contribution of an abbreviated ΔNp63 form and the role of the ΔN domain with minimal influence of C-terminal sequences, we evaluated the effects of introducing the truncated ΔNp63 isotype, ΔNp63^{P40}. Microarray analysis revealed potential functional differences with regard to differentiation between ΔNp63α and ΔNp63^{P40} and this finding was confirmed by RT–PCR and western analysis. Unlike ΔNp63α, overexpression of ΔNp63^{P40} was compatible with keratinocyte differentiation (Figures 4A and 5). Similarly, ΔNp63α harboring a mutation in the SAM domain is aberrantly expressed suprabasally in cells co-expressing markers of terminal differentiation (45). It has been previously shown that ΔNp63α blocks differentiation-associated induction of the cdk inhibitor p21^{WAF1} (30), a critical step in cell cycle withdrawal (46). Like ΔNp63α, ΔNp63^{P40} blocks p21^{WAF1} induction, demonstrating that this function of ΔNp63 is not influenced by the α-tail. These findings suggest that the ΔN-terminus maintains proliferative capacity of keratinocytes in the presence of 0.12 mM Ca^{2+}, and the C-terminal α-terminus, within the context of the ΔNp63 subclass of isotypes, modulates differentiation of keratinocytes. A summary of the biological effects of

Table II. Summary of effects on keratinocyte proliferation and differentiation observed following overexpression of individual p63 isotypes

Isotype introduced	Biological effects				
	Growth arrest	Keratin 1	Keratin 10	Loricrin	Filaggrin
ΔNp63α	Blocks	NE	Blocks	Blocks	Blocks
ΔNp63^{P40}	Blocks	NE	NE	NE	NE
TAp63α	NE	NE	NE	NE	Blocks
TAp63γ	NE	NE	NE	NE	Blocks

Cultures were infected with adenovirus encoding individual p63 isotypes and analyzed as described in Materials and methods. Table summarizes results presented in Figures 3–5. NE, no effect.

overexpressing individual isotypes is presented in Table II. Whether the effects of the α-tail on differentiation can be attributed to the SAM domain or other C-terminal sequences remains to be determined.

That overexpression of ΔNp63^{P40} permits induction of differentiation markers in a population of proliferating cells is unusual, but not unprecedented. Replication of suprabasal keratinocytes expressing keratin 10 has been suggested to be an early alteration occurring during mouse skin carcinogenesis (47). In squamous papillomas loss of the epithelial growth factor (EGF) receptor allows for premature migration of proliferating keratinocytes into the suprabasal compartment and subsequent co-localization of BrdU incorporation and keratin 10 expression (48). Similarly, keratinocytes lacking Rb co-express keratins 5 and 10 and incorporate BrdU suprabasally (49). In addition, keratin 6, a marker of proliferation, is co-expressed with keratin 10 in psoriasis (50).

Taken together, the results reported here suggest that maintenance of the balance of p63 isotypes is critical for keratinocyte growth regulation and begin to tease apart the functions of the individual domains within the context of a biological system. The TA and ΔNp63 isotypes have been shown to possess overlapping as well as unique functions (51,52) and are capable of regulating each other (53). Although it is generally perceived that the ΔNp63 isotypes work in a dominant negative manner towards p53 and the TAp63 isotypes, several studies have shown that in the right cellular context ΔNp63 isotypes harbor transcriptional activation capacity (14,54,55). This function is attributed to additional transcriptional activation domains within the molecule (54,55), which might be regulated by interaction with co-factors. It is unclear to what extent this activity might influence the biological response of keratinocytes to differentiation signals. Current studies are aimed at elucidating potential co-factors contributing to the specific ability of ΔNp63α to block keratinocyte differentiation. The elucidation of unique domain functions should help to clarify the contribution of p63 overexpression in cancers and potentially lead to therapeutic approaches to modulate the growth and differentiation properties of tumors with altered expression of p53 family members.

Supplementary material

Supplementary material is available at: http://carcin.oxfordjournals.org/

Acknowledgements

We gratefully acknowledge Dr Anil Rustgi for ΔNp63γ cDNA, Dr Shuntaro Ikawa for murine TAp63γ cDNA and Dr David Sidransky for adenovirus encoding ΔNp63^{P40}. We thank Drs Stuart Yuspa and Henry Hennings for providing tumor samples, Drs Luowei Li and Adam Glick for helpful advice regarding FACS methods, Jesse Quintero for assistance with Real-Time PCR assays and Jamie Zellers for assistance with tumor staining. We are grateful to Drs Stuart Yuspa and Wen Jin Wu for critical reading of the manuscript. This research was supported in part by the Intramural Research Program of the NIH (Center for Cancer Research, National Cancer Institute).

Conflict of Interest Statement: None declared.

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Received January 28, 2005; revised June 28, 2005; accepted July 28, 2005

* Manuscript

Functional Analysis of Human Tyrosinase-Related Protein 1 (TYRP1) by Site-Directed Mutagenesis on Tyrosinase-Mediated Melanin Production and Cytotoxicity

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Key words: Tyrosinase, tyrosinase-related protein, melanin, cytotoxicity, mutagenesis

Running head: Functional analysis of human TYRP1

Abbreviations: TYRP, tyrosinase-related protein; DOPA, 3, 4-dihydroxy-phenylalanine; DHICA, 5, 6-dihydroxyindole-2-carboxylic acid; DCT, DOPAchrome tautomerase; DMEM, Dulbecco's modified Eagle's medium; FBS,

fetal bovine serum; moi, multiplicity of infection; pfu, plaque-forming unit; PAGE, polyacrylamide gel electrophoresis

Abstract

Tyrosinase, tyrosinase-related protein 1 (TYRP1) and DOPAchrome tautomerase (DCT) are three major melanogenic glycoproteins involved in melanin biosynthesis. Their catalytic activities and sites of action in the melanin production pathway are unique to each other. Our recent study showed that human TYRP1 and DCT increase tyrosinase-mediated melanin production, though they alone do not possess any melanogenic activity and that they suppress cell death by ectopic expression of tyrosinase in melanocytic cells [1]. To further define the functional domain of TYRP1 for tyrosinase-mediated melanin production and cytotoxicity, we constructed recombinant adenoviruses which express missense or deletion mutant of human TYRP1. By RT-PCR and Western blot analyses, amounts of transcript and/or protein product of mutant TYRP1s were comparable with those of wild type. All the deletion mutants (dl137/267, dl260/322 and dl260) lost the two biologic activities of TYRP1, modulation of tyrosinase-mediated melanin production and cell death. The missense mutants H192L and H377L, which affected N-terminal and C-terminal copper-binding domains respectively, lost the additive activity of TYRP1 to the tyrosinase-mediated melanin production. However, one of them (H192L) showed suppression against tyrosinase-mediated cytotoxicity as effectively as wild-type TYRP1. On the other hand, the missense mutants located between 38th and 517th codon (S38C, C110Y, S354C and D517Y) retained the melanogenic activity, whereas they lost the anti-cytotoxic one. These results suggest that 1) the functional domains of TYRP1 for the melanogenic activity and

the inhibition of tyrosinase-mediated cytotoxicity are different and distinct, and 2) the anti-cytotoxic activity of TYRP1 is not associated with the specific domain, but with the whole protein structure.

Introduction

Melanosomes contain melanogenic proteins, i.e., tyrosinase and its related proteins (TYRP1 and DCT), and several other structural and/or transport-associated proteins such as gp100 and P proteins [2]. Among tyrosinase family proteins, tyrosinase works in the initial and rate-limiting process of melanogenesis by converting tyrosine to DOPA and DOPAquinone [3]. Murine TYRP1 and DCT have been reported to possess enzymatic activities of DHICA oxidase [4] and DOPAchrome tautomerase [5, 6], respectively. Although human and murine TYRP1 cDNAs show about 93% homology with each other [7, 8], DHICA oxidase activity was not detected in the human TYRP1 [9] and, thus, the two proteins play at least partially different biologic and biochemical roles in each organism.

The transmembrane region of human TYRP1 is highly hydrophobic, and, along with its cytoplasmic tail, the region is essential for its proper trafficking to melanosomes and incorporation into the melanosome-limiting membrane [10, 11]. There are two copper-binding domains that are highly conserved among tyrosinase, TYRP1 and DCT. Each domain contains three histidine residues that are required for copper binding, and the domains are important to the catalytic activity of tyrosinase [12]. Furumura *et al* [13] also demonstrated that murine TYRP1 does not bind to other metals, such as zinc and iron, and that the difference in the affinities of metal binding between tyrosinase and TYRPs is partly responsible for differences in their respective catalytic functions.

Loss of TYRP1 function in mutations is the cause of oculocutaneous albinism type 3 [14]. Kobayashi *et al* [15] found that tyrosinase is degraded more rapidly

in melanocytes expressing mutant TYRP1 than in those expressing wild-type equivalent. The stability of tyrosinase in the mutant TYRP1-expressing melanocytes could be recovered by the introduction of the wild-type TYRP1 (wtTYRP1) into the mutant melanocytes. They attributed this stabilization to the protection of tyrosinase from degradation by the melanin intermediates or to the involvement of TYRP1 in the glycosylation and trafficking of tyrosinase. Taken together, these observations suggest that TYRP1 possesses the ability to stabilize and enhance tyrosinase activity, possibly through direct or indirect interaction with tyrosinase.

In our recent study, expression of wtTYRP1 modulated tyrosinase-associated biologic functions in melanocytic cells, resulting in increased melanin production and suppression of tyrosinase-mediated cytotoxicity [1]. To further study the biological roles of TYRP1 in relation to tyrosinase function, we constructed substitution and deletion mutants of human TYRP1 and inserted them into adenovirus vector. By expressing the TYRP1 mutants in SK-mel-24 amelanotic melanoma cells, we studied their effects on tyrosinase-mediated melanin production and cytotoxicity. We found that several missense TYRP1 mutants retained the enhancement activity of TYR-mediated melanin production, but lost the inhibitory effect against TYR-mediated cell death. Our results suggest that the two biologic activities of TYRP1 are functionally distinct and mapped to different sites of the molecule.

Materials and Methods

Construction of plasmids carrying TYRP1 mutants

The N-terminal half and the C-terminal one of 1.7kb human TYRP1 cDNA

were separately amplified from pRHOHTα [16] by the primers containing *Sal*I and *Bam*HI sites and by *Bam*HI and *Eco*RI sites. After digestion with *Sal*I plus *Bam*HI and *Bam*HI plus *Eco*RI, respectively, two cDNA fragments were ligated with *Sal*I- and *Eco*RI-cleaved pUC19. The resulting recombinant plasmid, pUC-TYRP1, was used as the source for all TYRP1 mutants. Deletion mutants, dl137/267, dl260/322 and dl260, were constructed by cleaving pUC-TYRP1 with restriction enzyme pairs *Afl*II and *Bam*HI, *Eco*RV and *Bst*BI and *Eco*RV and *Bst*XI, respectively. After both ends had been filled with Klenow fragment (Takara Shuzo, Co., Shiga, Japan), each of the products was ligated by T4 DNA ligase and propagated in DH5α. The six point mutants, S38C, C110Y, H192L, S354C, H377L and D517Y, were constructed by an LA PCR in vitro Mutagenesis Kit (Takara Shuzo, Co., Shiga, Japan). Briefly, primers used in the constructs of point mutations were S38C: 5'-GGGCAACACATACCACATCT-3', C110Y: 5'-AGGACGGCACGTCCCATAGT-3', H192L: 5'-GACTGAGTAATAGAGTGT CC-3', S354C: 5'-CCACTGTGTTTCGGAAACAG-3', H377L: 5'-GATGAGCCAA ATTGAGAAG-3', D517Y: 5'-GCATTGATACTGATAAGTG-3', and common primers, RV: 5'-CAGGAAACAGCTATGAC-3', and M4: 5'-GTTTTCCCAGTCAC GAC-3' and a mismatched primer which destroys restriction site, MUT5: 5'-CAGGTCCACTATAGATGATC-3'. Firstly, two pUC-TYRP1 fragments were amplified separately using either primer RV and each point mutation primer or MUT5 and M4. The amplified products were mixed, denatured and annealed. The resulting products were further amplified by PCR using primers RV and M4, and digested with two restriction enzymes, *Sal*I and *Eco*RI, one of which should recognize the site that had been destroyed by MUT5. After mutated nucleotides

had been confirmed by nucleotide sequencing, they were cloned into the pAd-BglII [17]. The structures of the mutants obtained and the predicted amino acid changes are shown in Table 1 and Fig.1.

Construction of recombinant adenovirus

Recombinant adenovirus was constructed as described previously [17, 18, 19]. Briefly, after mutagenized TYRP1 cDNAs had been cleaved off from pUC19 by *Eco*RI and *Sal*I and blunted by T4 polymerase, they were inserted into the *Hin*dIII site, which had also been blunted by T4 polymerase, of pAd-BglII downstream of human cytomegalovirus early promoter [17]. Parts of the inserted cDNAs in pAd-BglII were verified by nucleotide sequencing. The resulting pAd-TYRP1 mutants (each 0.5 □g) were separately co-transfected into 293 cells (3 X 10^5/well of 24-well-plate) with pJM17 (0.5 □g) [20] by using SuperFect (Qiagen K.K., Tokyo, Japan). Recombinant adenovirus was cloned from 293 cell culture, which showed a cytopathic effect characteristic of adenovirus infection. Cloning of adenovirus and titration of viral stocks were carried out in 293 cells as described [18]. The recombinant adenoviruses used in the present study were Ad-LacZ (β-galactosidase), Ad-TYR (human tyrosinase) and Ad-TYRP1 (human wtTYRP1) [19, 21].

Cells and cell culture

The human cell lines used in this study were: SK-mel-24, an amelanotic melanoma cell line [22] and 293, an adenovirus type 5 DNA-transformed human embryonic kidney cell line [23]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Gibco-BRL, Tokyo, Japan), penicillin G and streptomycin. The anti-cytotoxic

effect of wild-type and mutant TYRP1s against tyrosinase was examined by counting the number of viable cells after co-infection of Ad-TYR plus Ad-TYRP1 or Ad-TYR plus Ad-TYRP1 mutant. Briefly, SK-mel-24 cells were infected with Ad-LacZ, Ad-TYR, Ad-TYR plus Ad-TYRP1 or Ad-TYR plus Ad-mutant TYRP1 at a total multiplicity of infection (moi) of 50 plaque-forming units (pfu)/cell and cultured in the DMEM with 1% FBS. After 7-day-culture, cells were detached and the number of viable cells was counted by hemocytometer as described previously [1].

RNA isolation and RT-PCR

The PCR primer sets for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GA1 and GA2 were synthesized as described in the previous report [24]. For amplification of TYRP1 mutant cDNA, four primers were used; TYRP1-4 (n.p. 4~23), 5'-AGTGCTCCTAAACTCCTCTC-3', and TYRP1-343 (n.p. 324~343), 5'-GACGGCACGTCCCACAGTTG-3', and TYRP1-616 (n.p. 616~635), 5'-GGTAGGACAGGAAAGCTTTG-3', and TYRP1-1555 (n.p 1536~1555), 5'-ACT GATCAGTGAGGAGAGGC-3'. The primer pair TYRP1-4 and TYRP1-343 is predicted to amplify 339-bp fragment from all the mutants. The reaction mixture of 50 μl consisted of 2.0 μl of cDNA product, 5mM Tris-HCl pH 8.0, 10 mM NaCl, 0.01 mM EDTA, 10 μl DTT, 0.1%(w/v) Triton X-100, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 0.25 U of Taq DNA polymerase (Promega, USA). This reaction mixture was started hot and processed for amplification by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 2 min in a Takara PCR Thermal Cycler (TAKARA SHUZO Co., LTD, Japan). PCR products were analyzed by electrophoresis on 1%

agarose gels followed by ethidium bromide staining.

Western blotting

For detection of adenovirus-mediated expression of TYRP1 and TYRP1 mutants, Western blotting was carried out as described previously [21]. Five X 10^5 cells were infected with Ad-TYRP1 or one of the Ad-TYRP1 mutants at the moi of 20 pfu/cell and cultured for 60 hrs. After cells had been collected and lysed in 10mM KCl, 1.5 mM $MgCl_2$, 10mM Tris (pH 7.4), 0.5% SDS, 1mM PMSF, they were sonicated for 10 sec by Branson's sonicator. Protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL). Samples containing 5.0 □g protein were separated in 5-20% gradient SDS/polyacrylamide gel electrophoresis (PAGE) (Ready Gel, Bio- Rad Laboratories, Tokyo, Japan) at 100V for 80 min. The separated proteins were transferred onto nitrocellulose membranes (Protran, Schleicher and Schuell) by electroblotting at 100V for 60 min. The membrane was probed with the primary antibody as follows: 1/2000-diluted rabbit anti-sera for TYRP1 in 5 ml of PBS-T (0.1% Tween 20 in phosphate-buffered saline [PBS]) at room temperature for 60 min. Antihuman TYRP1 polyclonal antibody was raised in rabbits by injecting C-terminal amino acid residue, CNQPLLTDQYQSYAEE, of human TYRP1 into domestic rabbits [19]. The blot was conjugated to a secondary antibody. The specific complexes were detected by an ECL chemiuminescence reagent (Amersham Pharamacia Biotech, Bickinghamshire, U.K.).

Measurement of melanin

After 5 X 10^5 cells had been seeded in 6 cm dishes and cultured for 24 hrs, they were infected with Ad-LacZ, Ad-TYR, Ad-TYRP1, and Ad-TYRP1 mutants at

a total moi of 20 pfu/cell and maintained for a further 60 hrs. Cells were detached and collected in Eppendorf tubes. Then, cells were dissolved in 1 ml of alkaline solution (1 M NaOH in DMSO) and incubated at 80°C for 2 hrs. After centrifugation at 500 X g for 10 min, the protein concentration and melanin in the supernatant were measured. The amount of melanin was determined by a spectrophotometer at 420 nm by using synthetic melanin (Sigma Aldrich Japan, Tokyo, Japan) as a standard.

Results

Construction of recombinant adenovirus carrying TYRP1 mutants

To address the biological and biochemical roles of each domain of human TYRP1 in the sorting, melanogenesis and anti-cytotoxicity against tyrosinase, we constructed recombinant plasmids containing nine different TYRP1 mutants. These were designated S38C, C110Y, H192L, S354C, H377L, D517Y, dl137/267, dl260/322 and dl260 aligned with wtTYRP1 (Fig. 1 and Table 1). The mutant cDNAs were inserted into pAd-BglII and recombinant adenoviruses were constructed as described in the Materials and Methods. The mutants S38C and C110Y have a single amino acid substitution in the cysteine-rich region. The mutants H192L, S354C and H377L have a single amino acid substitution in the copper-binding domain A, cysteine-rich region and copper-binding domain B, respectively. The mutant D517Y has a single amino acid substitution in the cytoplasmic tail region. The mutant dl137/267 deleted 130 amino acids in the copper-binding domain A and a cysteine-rich region. The mutant dl260/322 deleted 63 amino acids in a cysteine-rich region. The mutant dl260 deleted all

amino acids at the C-terminus and the TGA stop codon appeared at the 3' end after the 260th codon.

Mutant TYRP1s were expressed in amelanotic melanoma cells

Nucleotide changes and resulting amino acid substitutions or deletions might affect the efficiency of transcription, translation or stability of mRNA or protein product. To examine whether the mutant TYRP1s were transcribed and expressed as efficiently and stably as wtTYRP1, we analyzed mutant TYRP1 mRNAs and proteins by RT-PCR and Western blotting, respectively. RT-PCR amplified 339 kb-sized bands from RNAs isolated from all the SK-mel-24 cells expressing each of the TYRP1 mutants (Fig. 2, A). The primer set TYRP1-616 and TYRP1-1555 amplifies TYRP1 sequences containing cysteine-rich region (Fig. 1), and, as predicted, it detected smaller bands from cells expressing the deletion mutants dl260/322 and dl260 (Fig. 2, B). Because dl137/267 lacked the sequences corresponding to TYRP1-616, no specific band was amplified by PCR using TYRP1-616 and TYRP1-1555.

Western blotting detected a band of approximately 69 kd (Fig. 3) from SK-mel-24 cells expressing wtTYRP1 (Fig. 3). The missense mutants, S38C, C110Y, H192L, S354C and H377L produced bands at approximately the same size as wtTYRP1 (Fig. 3). Deletion mutants dl137/267 and dl260/322 produced smaller bands of approximately 62kd and 52kd, respectively. The dl137/267 protein seemed to be less stable than the wild type and other mutants (Fig. 3). The protein products of dl260 and D517Y were not detectable by Western blot analysis. It is likely that these two mutants lack the antigenic recognition site by the polyclonal anti-TYRP1 antibody used in this study.

Effect of TYRP1 mutants on tyrosinase-mediated melanin production

Co-expression of TYRP1 along with tyrosinase produces a larger amount of melanin than single expression of tyrosinase [1]. To examine the effect of TYRP1 mutants on tyrosinase-mediated melanin production, we infected SK-mel-24 cells with Ad-TYR alone, Ad-TYR plus Ad-TYRP1 or Ad-TYR plus Ad-TYRP1 mutant, and measured the amount of melanin as described in the Materials and Methods. As shown in Fig. 4, the in-frame deletion mutants (dl137/267 and dl260/322) and frame shift deletion mutant (dl260) decreased tyrosinase-mediated melanin production. Missense mutants, H192L and H377L, which contained an amino acid substitution within copper-binding domains, also decreased melanin production. In contrast, missense mutants S38C, C110Y, S354C and D517Y increased intracellular melanin to almost the same level as wtTYRP1 (Fig. 4). Thus, it is suggested that both of the copper-binding domains are essential for the melanogenic activity of TYRP1.

Effect of TYRP1 mutants on tyrosinase-mediated cytotoxicity

We examined the biological effect of TYRP1 mutants on the inhibition of tyrosinase-mediated cytotoxicity. SK-mel-24 cells were infected with Ad-LacZ, Ad-TYR, Ad-TYR plus Ad-TYRP1 or Ad-TYR plus Ad-TYRP1 mutant at a total moi of 50 pfu/cell and the numbers of cells were counted on the 7th day after virus inoculation. All three deletion mutants lost their anti-cytotoxic activity against tyrosinase. Only H192L retained its anti-cytotoxic effect against tyrosinase (Fig. 5). Interestingly, all of the melanogenic mutants, S38C, C110Y, S354C and D517Y, failed to suppress the cytotoxic effect of tyrosinase (Fig. 5). Thus, the anti-cytotoxic activity of TYRP1 against tyrosinase was not mapped to a specific

domain or subregion of the molecule, but suggested to be dependent on the whole protein conformation. It was also suggested that the biologic activities of TYRP1, i.e., enhancement of tyrosinase-mediated melanin production and suppression of tyrosinase-mediated cell degradation are functionally different and distinct.

Discussion

Tyrosinase family proteins, tyrosinase, TYRP1 and DCT, are melanosomal glycoproteins that are structurally similar to each other and act as immune targets as melanoma differentiation antigens in melanoma patients [11, 25]. They possess different enzymatic activities catalizing the major steps in melanin biosynthesis [11] and have been reported to be trafficked on distinct routes after they are sorted via the *trans*-Golgi network [26, 27, 28]. Both TYRP1 and DCT enhance eumelanin biosynthesis, possibly by forming stable complexes with tyrosinase in the melanosomes. Co-expression of TYRP1 with tyrosinase results in a larger amount of melanin production than single expression of tyrosinase [1]. Human TYRP1 was reported to have some tyrosine hydroxylase activity and possibly to modulate TYR activity [29]. Zhao *et al* [30] found that transfection of TYRP1 into a melanoma cell line which lacks this protein but contains tyrosinase, resulted in an increase in tyrosinase activity and subsequently an increase in melanin synthesis. In addition to the melanogenic activity, our previous reports showed that co-expression of tyrosinase and TYRP1 prevented cell death of melanocytes, which is related to tyrosinase-mediated production of melanin intermediates[1, 22].

In the present study, by using missense and deletion mutants of TYRP1, we showed that the ability of TYRP1 to protect cells from tyrosinase-mediated cytotoxicity is functionally distinct from its additive effect on the tyrosinase-mediated melanin production. Four out of the six missense mutants of TYRP1 used in the present study had an amino acid change at the N-terminal cysteine-rich region or one of the copper-binding domains. Missense mutations of the copper-binding domains (H192L and H377L) resulted in a complete loss of the additive activity of tyrosinase-mediated melanin synthesis. The loss of the melanogenic activity of the TYRP1 mutants was not caused by the down-regulation of the protein expression, because Western blot analysis showed no significant decrease in the steady state levels of TYRP1 protein. Thus, these results suggest that both the copper binding domains of TYRP1 are essential for its melanogenic function. In an experiment using deletion mutants, the functional domains of TYRP1 were mapped within cysteine-rich region and copper-binding domains [31]. These domains may interfere with the formation of a proper disulfide bridge and copper binding of a TYRP1 molecule.

However, the mutants S38C and C110Y, which affected the cysteine-rich region, were unable to suppress tyrosinase-mediated cell death but retain the melanogenic activity. Recently, Sarangajan *et al* [32] reported that the transfection of human TYRP1 cDNAs with a mutation in the cysteine-rich region resulted in the formation of aberrant melanosomal structures. This suggests that mutations of TYRP1 affect both melanosomal transport and TYRP1 function. However, our mutants S38C and C110Y were unable to suppress tyrosinase-mediated cell death, suggesting again that proper localization of