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SYT14L, especially its C2 domain, is involved in regulating melanocyte differentiation

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ABSTRACT

Background: The formation of dendrites by melanocytes is highly analogous to that process in neural cells. We previously reported that a C2 domain-containing protein, copine-1, is involved in the extension of dendrites by neural cells. However, the effect of C2 domain-containing proteins in dendrite formation by melanocytes has not yet been elucidated.
Objective: The aim of this study was to screen novel C2 domain-containing proteins related to dendrite outgrowth in melanocytes and to investigate their precise roles in melanocyte dendrite formation during differentiation.
Methods: We transduced mouse melan-a melanocytes with a recombinant adenovirus expressing a C2 domain library. Dendrite elongation, melanin content, tyrosinase activity and Western blot analyses were conducted to elucidate the possible underlying mechanisms of action in melanocytes.
Results: Sixteen sets of C2 domain-containing proteins were identified whose over-expression resulted in dendrite lengthening. Among those, we focused on the C2 domain of SYT14L (truncated mutant of SYT14L) in this study. Forced expression of full length SYT14L or the C2 domain of SYT14L induced a significant elongation of dendrite length accompanied by the induction of melanocyte differentiation-related markers, including melanin synthesis, tyrosinase catalytic activity and the expression of tyrosinase (TYR), tyrosinase related protein-1 (TRP-1) and TRP-2. In addition, over-expression of either the C2 domain or the full length form of SYT14L significantly increased the phosphorylation of ERK and CREB.
Conclusion: These results suggest that SYT14L, especially its C2 domain, may play an important role in regulating melanocyte differentiation through the modulation of ERK and (or) CREB signaling.

1. Introduction

Dendrites are thin structures that arise from the cell body and are a morphologic characteristic of melanocytes and neural cells. Growth factors and hormones that affect melanocyte dendricity have been identified, but little is known about the molecular mechanisms of dendrite formation [1].

The dendrites of melanocytes serve as conduits for the transfer of melanosomes from melanocytes to keratinocytes, which is critical to the distribution of pigment in the skin by keratinocytes [2]. Thus, the number and length of melanocyte dendrites have a significant influence on melanocyte function and skin color [2].

The C2 domain is known as a Ca^{2+}-binding motif approximately 130 residues in length that was originally identified in Ca^{2+}-dependent isoforms of protein kinase C (PKC). The C2 domain displays the remarkable property of binding to a variety of different ligands and substrates, including Ca^{2+}, phospholipids, inositol polyphosphates and intracellular proteins [3].

In a previous study, we showed that C2 domain-containing proteins are involved in dendrite formation and the differentiation of neuronal cells [4]. Dendrite formation in both melanocytes and neural cells requires actin polymerization, Rho family GTPases, including RhoA and Rac1, and cyclic adenosine monophosphate (cAMP), indicating that the roles of those molecules and other growth factors in dendrite formation of neuronal cells is highly

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similar to that process in melanocytes [1,5–7]. In other words, identical or similar regulatory mechanisms can operate in melanocytes that work in neuronal cells in terms of dendrite elongation. However, the effects of C2 domain-containing proteins on dendrite formation by melanocytes have not been clearly elucidated so far.

In this study, we screened a C2 domain library for factors that stimulate dendrite formation by melanocytes. Ultimately, we focused on the C2 domain of SYT14L (truncated mutant of SYT14L) among the C2 domain libraries. We demonstrate that SYT14L enhances melanocyte dendricity and differentiation via regulation of the ERK and CREB pathways. Additionally, we observed that the full length form of SYT14L has a similar effect on melanocyte dendrite formation as its truncated form, the C2 domain, demonstrating that the C2 region of SYT14L is important in this process.

2. Materials and methods

2.1. Screening of melanocyte differentiation-related genes

The C2 domain library was manufactured using the sequences of 145 kinds of C2 domains by the gateway adenovirus system [8]. That adenovirus library was then infected to melan-a melanocytes. Final candidates were classified and selected by the degree of their effects on dendrite extension. Data are expressed as a percentage of dendrite-elongated cells out of all GFP-expressing cells. All experiments were done at least three times with similar results, and the data are expressed as means ± SD.

2.2. Plasmid constructions

Full-length clones were generated from cDNA as templates from SK-MEL-24 melanoma cells, via the PCR-based gateway cloning method, as described previously [8]. The resulting PCR products were cloned into destination vectors, pDEST-AD-GFP, using the gateway cloning system (Invitrogen, Carlsbad, CA).

2.3. Cell culture

Melan-a immortal mouse melanocytes were a kind gift of Dr. Dorothy C. Bennett (St George's Hospital Medical School, London, UK). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen), streptomycin–penicillin (100 mg/ml each) and 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), at 37 °C in an atmosphere containing 5% CO_2.

2.4. Adenovirus amplification and transfection

Adenoviruses expressing green fluorescent protein (GFP, the negative control), the C2-deleted form (ΔC2), the C2 domain (C2) and the full length form (full) of SYT14L were prepared and propagated in HEK 293A cells as described previously [8]. After purification by cesium chloride gradient centrifugation, adenoviruses were dialyzed into storage buffer. For adenoviral infection, melan-a melanocytes were plated in 6-well or 24-well plates at a density of 2×10^4 cells/cm^2, then were grown for 72 h in TPA-free medium to observe the dendrite elongation supplemented with the GFP (negative control), ΔC2, C2 and full length forms of SYT14L. TPA was added only in the medium of the positive control (GFP + TPA) because it is a potent stimulator for dendrite extension in melanocytes.

2.5. Imaging analysis and quantification of dendrite outgrowth

Quantification of dendrite outgrowth was determined by confocal microscopy (Olympus Fluoview FV-1000). Several randomly chosen fields were photographed from cells infected by the C2 domain-containing virus. The number of cells having dendrites longer than double the cell body diameter was counted as dendrite-outgrown cells, and is represented as a percentage of all GFP-expressing cells. Cell aggregates were not counted and cells bearing atypical processes, such as hair-like branched outgrowths, also were not scored.

2.6. Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1 mM PMSH and 0.1% NP-40) containing a protease inhibitor cocktail. Whole-cell lysates were incubated on ice for 30 min and then were cleared at 13,000 rpm for 20 min at 4 °C. Cell lysates were separated by SDS-PAGE using 10% gels, transferred onto nitrocellulose membranes and were then incubated with appropriate primary antibodies overnight at 4 °C with gentle agitation. Blots were then incubated with peroxidase-conjugated secondary antibodies for 30 min at room temperature and were visualized by enhanced chemiluminescence (Intron, Daejeon, Korea). The following primary antibodies were used in this study: TYR, TRP-1, TRP-2, MITF, phospho-CREB, ERK and phospho-ERK (all from Cell Signaling Technology, Danvers, MA), AKT and phospho-AKT (both from Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (Sigma, St. Louis, MO).

2.7. Determination of melanin content and tyrosinase activity

To determine melanin content, cells were dissociated with TrypLE™ (Invitrogen) and were harvested. After centrifugation, pellets were dissolved in 1 ml 1 N NaOH at 100 °C for 30 min. The samples were then vigorously vortexed to solubilize the melanin pigment. The optical density of each supernatant was measured at 405 nm, and results are expressed as a percentage of the control. To measure tyrosinase activity, cells were lysed in RIPA buffer, and lysates were clarified by centrifugation. After quantification, 250 μg total protein in 100 μl lysis buffer were transferred into 96-well plates, and 100 μl 1 mM L-DOPA was added. After incubation for 1 h at 37 °C, absorbance was measured at 490 nm. Tyrosinase activity is expressed as a percentage of the control.

2.8. Statistical analysis

Data were evaluated statistically using Student's *t*-test. Statistical significance was set at $p < 0.05$. Data are reported as means ± standard deviations (SD) of three independent experiments.

3. Results

3.1. Screening of an adenoviral C2 domain library to identify factors that affect dendrite formation

A large number of C2 domains that have important biological functions have been identified so far. We cloned 145 of those genes into an adenoviral expression system. Mouse melan-a melanocytes were infected with the recombinant adenovirus expressing the C2 domain library, which was secured and manufactured in a DNA-delivering adenovirus (Fig. 1A). After infection for 72 h, melanocytes whose dendrites were longer than double the cell body diameter were scored as dendrite-outgrown cells (Fig. 1B). Among the cells infected with the C2 domain-containing adenovirus, 129 kinds of the C2 virus showed minor morphological changes. In contrast, 16 kinds of the C2 domain-containing virus lengthened