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Girdin is phosphorylated on tyrosine 1798 when associated with structures required for migration

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ABSTRACT

The mammalian protein Girdin interacts with several key molecules such as actin, and it functions as a regulator of the cytoskeleton. Silencing of Girdin mRNA results in defective migration in a variety of cultured cells. Moreover, knockout of Girdin causes phenotypes related to defective migration, including hypoplasia of olfactory bulbs and a widened rostral migratory stream (RMS) in mice. To elucidate the molecular basis underlying cellular migration, we generated site- and phosphorylation state-specific antibodies against human Girdin peptides carrying four putative phosphorylation sites (serine1386 [S1386], S1416, tyrosine1764 [Y1764] and Y1798) that had been identified by mutagenesis analyses or mass spectrometric studies. We found that these residues were phosphorylated in an epidermal growth factor (EGF)-dependent manner. Among the four antibodies we developed, the antibody that targeted Girdin when phosphorylated at Y1798 (pY1798) worked well for immunohistochemistry of paraffin-embedded tissues as well as for cultured cells. Immunocytochemistry of HEK293FT cells transfected with an EGF receptor expression plasmid exhibited punctate signals with pY1798. These signals colocalized with those of endocytosed EGF receptors after EGF stimulation. Signals from pY1798 were also observed on lamellipodia, filopodia, focal adhesion and stress fibers in NIH3T3 cells under conventional culture conditions. Immunohistochemistry of paraffin-embedded mouse brain at P14 using anti-pY1798 antibody displayed signals at the hilum-side (internal side) of the dentate gyrus of the hippocampus, the RMS, the accessory olfactory bulb and the olfactory bulb in which Girdin expression was detected. Primary culture of RMS neurons showed punctate signals of pY1798 at the tips of leading processes as well as in the cytoplasm, whereas no signals were observed when neurons were treated with Src inhibitor, PP2. Our data revealed the changes in the phosphorylation status of Y1798 in Girdin when it associated with migration-related structures *in vitro* and *in vivo*.

1. Introduction

Girdin (also known as ccdc88a/GIV) was first identified in 2005 and was shown to interact with Akt/PKB, actin, microtubules, and Gα members [1–4]. In 2005, our group revealed possible involvement of Girdin in cell migration and stress fiber formation by silencing Girdin in Vero cells [1]. Involvement of Girdin in migratory ability was also supported by studies using human umbilical vein endothelial cells (HUVEC) [5] and cancer cells [6]. Despite the differences of methodology between individual experiments, common observations *in vitro* were that Girdin contributed to actin reorganization, leading to cell migration. In addition, Girdin-deficient mice demonstrated specific phenotypes, including a widened rostral migratory stream (RMS) and hypoplasia of the olfactory bulb [7,8]. These findings suggested that Girdin played a role in cell migration *in vivo*, at least in subpopulations of neurons.

Abbreviations: EGF, epidermal growth factor; RMS, rostral migratory stream..

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Not only the expression levels of Girdin, but also its post-translational modifications may also modulate cell migration. In fact, phosphorylation of Girdin at serine 1416 (S1416) was observed at the lamellipodia of migrating Vero cells, and mutation of S1416 in Girdin significantly impaired cell migration *in vitro* [1]. However, mice bearing serine/alanine substitution at 1416 (SA mutant mice) had normal viability and no gross anatomical change [7]. In contrast, Girdin-deficient mice exhibited complete pre-weaning lethality with severe anomalies. The lack of macroscopic abnormalities in SA mutant mice implied that ablation of S1416 phosphorylation was insufficient to disrupt whole functions of Girdin. Thus, we expanded the range of investigation of Girdin phosphorylation. Serine 1386 (S1386), tyrosine 1764 (Y1764), and Y1798 were chosen for further analyses. S1386 was identified by mass-spectrometry [9] and was selected from top-scoring results in a phosphorylation site search engine, PhosphositePlus (Cell Signaling Technology, Danvers, MA, http://www.phosphosite.org). Y1764 and Y1798 were identified as phosphorylation sites by EGF receptor and Src kinase [10].

In this study, we generated new rabbit polyclonal site- and phosphorylation state-specific antibodies that recognized phosphorylated S1386, Y1764, or Y1798, and we investigated the roles of Girdin phosphorylation in cell migration.

2. Materials and methods

2.1. Generation of anti-Girdin antibodies

Anti-phospho-S1416 Girdin antibodies were previously described [1]. To develop new anti-site and phosphorylation state-specific Girdin antibodies at S1386, Y1764, and Y1798, peptide sequences (Fig. 1A human sequences) were designed and both phosphorylated-peptides and nonphosphorylated-peptides were synthesized. Cysteine residues were added to the N-terminus of the synthesized phosphorylated peptides. A carrier protein (bovine thyroglobulin) was subsequently added to the cysteine residue using N-[e-maleimidocaproyloxy]-succinimide ester (EMCS) (Dojindo, Kumamoto, Japan) as a cross-linking agent. The resultant immunogen solution (0.5 mL) was mixed with adjuvant solution (0.5 mL) and subcutaneously administered to each rabbit ($n \geq 3$ per each peptide sequence).

2.2. Dot blot assay

Peptide samples (non-phospho-S1386, pS1386, non-phospho-Y1764, pY1764, non-phospho-Y1798, and pY1798) were reconstituted with distilled water to make 1 μg/μL peptide solutions. Five microliter of methanol was pipetted and placed on dry polyvinylidene difluoride (PVDF) membranes (Immobilon-P, IPVH00010, Millipore, Billerica MA), and 2 μL of peptide solution (2 μg peptide) was immediately added onto the methanol drop. The peptide-bound membrane was treated with 1:50 diluted rabbit antisera, and incubated at 4 °C overnight. Membrane-bound rabbit antibodies were detected with 1:5000 diluted polyclonal swine anti-rabbit immunoglobulins/HRP (P0399, Dako, Glostrup, Demark) and Amersham ECL Western Blotting Detection Reagents (RPN2106, GE Healthcare, Little Chalfont, UK).

2.3. Generation of Girdin phosphorylation-deficient constructs

Mutagenesis to generate full-length Girdin phosphorylation-deficient mutants was performed following the PCR-based mega-primer method using V5-tagged full-length wild-type human Girdin coding sequence on a pCAGGS plasmid (10403 bp) as a template, and using PrimeSTAR Max (Takara Bio, Otsu, Japan) as the DNA polymerase. Targeted serine residues (S1386 or S1416) were replaced with alanine residues and that targeted tyrosine residues (Y1764 or Y1798) were replaced with phenylalanine residues using following primers (Gothic letters represent altered codons).

Cloning sense; 5′-TACATCTTGGCTGGGAACTGGAACA-3′

Cloning antisense; 5′-AGCCAGAAGTCAGATGCTCAAGGGGCT-3′

S1386A sense; 5′-GACCCA **GCG** CCTCCTAGAAGGAGAGGC-3′

S1386A antisense; 5′-AGGAGG **CGC** TGGGTCATAAAATTTGTAT TGATCC-3′

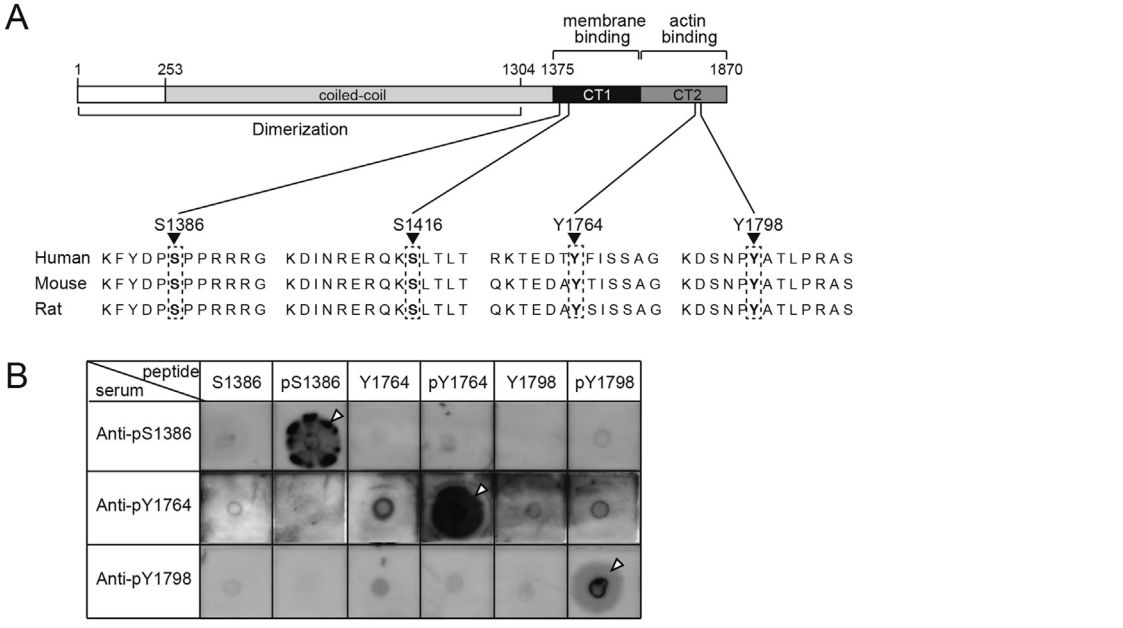

Fig. 1. Generation of site- and phosphorylation state-specific antibodies against four putative phosphorylation sites of human Girdin. **A**. Domain structures of Girdin, and positions of putative phosphorylation sites. Amino acid sequences of human, mouse and rat Girdin are aligned. **B**. Specificities of three newly generated antibodies (anti-pS1386, anti-pY1764, and anti-pY1798) were assessed using dot-blot assay. Peptides including S1386, Y1764, and Y1798 with or without phosphorylation were placed on PVDF membranes and reacted with each antibody. Note that each antibody reacted with the corresponding site-specific phosphorylated peptide (open arrow heads).