Identification of Peripherin as a Akt Substrate in Neurons*

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Activation of Akt-mediated signaling pathways is crucial for survival and regeneration of injured neurons. In this study, we attempted to identify novel Akt substrates by using an antibody that recognized a consensus motif phosphorylated by Akt. PC12 cells that overexpressed constitutively active Akt were used. Using two-dimensional PAGE, we identified protein spots that exhibited increased immunostaining of the antibody. Mass spectrometry revealed several major spots as the neuronal intermediate filament protein, peripherin. Using several peripherin fragments, the phosphorylation site was determined as Ser66 in its head domain in vitro. Furthermore, a co-immunoprecipitation experiment revealed that Akt interacted with the head domain of peripherin in HEK 293T cells. An antibody against phosphorylated peripherin was raised, and induction of phosphorylated peripherin was observed not only in Akt-activated cultured cells but also in nerve-injured hypoglossal motor neurons. These results suggest that peripherin is a novel substrate for Akt in vivo and that its phosphorylation may play a role in motor nerve regeneration.

Akt (also known as protein kinase B) is a Ser/Thr kinase that plays essential roles in various cellular processes such as cell survival, proliferation, and differentiation (1). In the nervous system, Akt is suggested to be involved in neurogenesis (2, 3), neuronal survival (4), axon or dendrite formation (5, 6), synaptogenesis (7, 8), and synaptic transmission (9). The most evident role of Akt is neuroprotective action. For instance, several previous papers have demonstrated a strong protective effect of Akt on damaged neurons in vivo (10–13). Of particular interest, Akt was proven to have a crucial role in neuronal survival after peripheral nerve injury (10). In the peripheral nervous system, in which most neurons can survive and regenerate after injury, glial cells secrete various trophic factors to promote survival and regeneration of nerve-injured neurons. Astrocytes and microglia, which are located around the neuronal cell bodies, are thought to secrete various factors toward injured neurons (14, 15). Furthermore, in the distal stump of axons far from neuronal cell bodies, Schwann cells also secrete trophic factors (16). Such factors released from those glial cells include a wide range of growth factors such as nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and fibroblast growth factor-2 (17–19). They are known to activate the phosphatidylinositol 3-kinase-Akt pathway in injured neurons via their respective receptors (20–22). In fact, our previous study showed that Akt activity was markedly induced in motor neurons after nerve injury (10). We also revealed that activated Akt accelerated axonal elongation, as well as neuronal survival. It is well established that activated Akt exerts its function by phosphorylating its substrates; however, the substrates that specifically exist in neurons are largely unidentified. Thus, identification of novel neuronal substrates is pivotal to gain further insight into the function of Akt in neuronal regeneration.

In this study, we attempted to identify novel Akt substrates in neurons by a proteomic approach, using a unique antibody that recognizes the consensus motif phosphorylated by Akt. Here we demonstrate that peripherin, which is a peripheral nervous system neuron-specific intermediate filament protein, is a novel Akt substrate, and that Ser66 of peripherin is the phosphorylation site. Peripherin phosphorylation is apparently induced in motor neurons after nerve injury, suggesting that the Akt-mediated peripherin phosphorylation may play a role in motor nerve regeneration.

EXPERIMENTAL PROCEDURES

Materials—Anti-phospho-Akt substrate antibody (antibody 9611; Cell Signaling Technology, Danvers, MA), anti-phospho-Akt antibody (antibody 4051; Cell Signaling Technology), anti-peripherin antibody (antibody MAB1527 for Western blotting; antibody AB1530 for immunohistochemistry; Chemicon, Temecula, CA), anti-glutathione S-transferase (GST)2 antibody (antibody sc-138; Santa Cruz Biotechnology, Santa Cruz, CA), anti-His antibody (antibody 1922416; Roche Applied Science), anti-hemagglutinin (HA) antibody (antibody 1583816; Roche Applied Science; and antibody sc-138; Santa Cruz Biotechnology), anti-FLAG antibody (antibody F3166; Sigma), and anti-glyceraldehydes-3-phosphate dehydrogenase (antibody 4300; Ambion, Huntington, UK) were used as primary antibod-

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2The abbreviations used are: GST, glutathione S-transferase; HA, hemagglutinin; Hek, human embryonic kidney; WT, wild type; CA, constitutively active; DN, dominant negative; MOI, multiplicity of infection; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; pI056K, p70 S6 kinase; NF, neurofilament; CHAPS, 3-[N-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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ies. As secondary antibodies, horseradish peroxidase-conjugated antibodies (Amersham Biosciences) and Alexa Fluor-conjugated antibodies (Molecular Probes, Eugene, OR) were used for Western blotting and immunohistochemistry, respectively. All of the inhibitors were obtained from Calbiochem (La Jolla, CA).

Cell Culture—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) and 0.05 mg/ml penicillin/streptomycin. Both cell types were grown on cell culture dishes coated with collagen in RPMI 1640 containing 10% fetal bovine serum, 10% horse serum, and 0.05 mg/ml penicillin/streptomycin. Both cell types were cultured at 37 °C under 5% CO2.

In Vitro Kinase Assay—2.5 μg of GST fusion proteins were incubated with or without 100 ng of recombinant His-tagged CA-Akt (His-CA-Akt) (Upstate Biotechnology) in 20 μl Tris-HCl, pH 7.5, 10 mM MgCl2, 20 μM ATP, and 30 kBq [(γ-32P]ATP (PerkinElmer Life Sciences) for 30 min at 30 °C. The reaction mixtures were subjected to SDS-PAGE, and phosphorylation of the fragments was detected by autoradiography. For Western blot analysis, 1 μg of GST fusion proteins was reacted with 100 ng of His-CA-Akt, and one-tenth of the reaction mixtures was analyzed.

Phosphorylation-specific Antibody—A rat monoclonal antibody that specifically recognized phosphorylated peripherin...
Prepared ascites were used for immunological assays. The phosphorylation-specific antibody was screened by enzyme-linked immunosorbent assay using hybridoma supernatants, and clone 2C2 was selected. Finally, 2C2 hybridoma cells were injected into the abdominal cavity of nude mice, and prepared ascites were used for immunological assays.

Detection of Peripherin Phosphorylation in Cultured Cells—HEK 293T cells seeded on 60-mm culture dishes were grown to ~80% confluence and transfected with pcDNA3-peripherin and pcDNA3-HA-WT-Akt using Lipofectamine 2000 (Invitrogen). After 8 h, the cells were seeded into 12 well culture dishes and cultured for another 24 h. The cells were then serum-starved for 10 h, treated with insulin (Sigma), and subjected to Western blot analysis using the anti-pPer antibody. If necessary, inhibitors were added to the cultured medium 30 min before insulin treatment. As for the phosphorylation of endogenous peripherin, PC12 cells infected with AxCALNLLacZ (MOI 100), AxCALNHLA-WT-Akt (MOI 100), AxCALNHLA-CA-Akt (MOI 100), or AxCALNHLA-DN-Akt (MOI 100) together with AxCANCRe (MOI 30) for 48 h were examined.

Immunoprecipitation—HEK 293T cells seeded on 6-well culture dishes were transfected with pcDNA3-HA-WT-Akt together with pcDNA3 empty vector or FLAG-tagged head domain of peripherin (FLAG-Per 1–103) subcloned into pcDNA3 using Lipofectamine 2000. After 32 h, the cells were serum-starved for 10 h and treated with 100 nm insulin for 20 min. The cells were then washed in Tris-buffered saline briefly and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Na3VO4, and 10 mM NaF). After centrifugation at 10,000 × g for 10 min at 4 °C, the soluble fractions were collected and reacted with anti-FLAG antibody followed by precipitation using protein G-Sepharose 4B (Sigma). Immunoprecipitates were rinsed four times with lysis buffer and eluted by adding 2× SDS sample buffer.

Immunohistochemistry—Adult male Wistar rats weighing ~150 g were anesthetized with pentobarbital (40 mg/kg) and positioned supine, and their right hypoglossal nerves were crushed with forceps. The rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer 5 days after surgery. The brains were quickly removed, post-fixed overnight at 4 °C in the fixative, and immersed in 0.1 M phosphate buffer containing 25% sucrose for an additional day. Sections were cut on a cryostat (18 μm in thickness), washed once in PBS, and treated with 10 μg/ml proteinase K for 10 min. After two washes in PBS, the sections were blocked with PBS containing 10% normal goat serum for 1 h and subsequently reacted with primary antibodies (anti-peripherin antibody; 1:1000, anti-pPer antibody; 1:1000) in PBS containing 1% normal goat serum overnight at 4 °C. After three washes in PBS, the sections were incubated with secondary antibodies for 1 h and finally washed three times in PBS. The sections were visualized by fluorescent microscopy (AX70; Olympus, Tokyo, Japan).

**RESULTS**

Identification of Peripherin as an Akt Substrate in Neurons—To identify novel neuronal substrates for Akt, we utilized the anti-phospho-Akt substrate antibody. Akt preferentially phosphorylates Ser or Thr in the RXRXX(S/T) motif, and the antibody specifically recognizes this motif only when Ser or Thr is phosphorylated. PC12 cells infected with adenovirus expressing LacZ or CA-Akt were subjected to Western blot analysis using this antibody, and proteins exhibiting more intense signal in the CA-Akt-expressing preparation were searched. Our preliminary experiment using ordinary SDS-PAGE demonstrated stacked positive bands where isolation of the individual positive band was impossible (data not shown). We therefore performed two-dimensional PAGE to also separate proteins by their isoelectric points, and the two-dimensional gels were analyzed by Western blotting using the antibody. We initially used a wide pH range gel for the first dimension and found numerous spots were intensely stained in the CA-Akt-expressing preparation; in particular in the region in which the isoelectric point was 5.0–5.5 and molecular mass was ~60 kDa (Fig. 1A). We therefore focused on this region and separated proteins more precisely by using narrow pH range gels for the first dimension (Fig. 1B). Six spots that exhibited the intense positive immunostaining were identical to the protein spots in the protein-stained gels (spots 1–4, 8, and 9 in Fig. 1C). Judging from their sequential spot patterns, we assumed that spots 1–4 were the same proteins, each of which might have different post-translational modifications. Similarly, the spots 8 and 9 were assumed to be the same protein. As representative samples, spots 1 and 9 were punched out from the gel and analyzed by MALDI-TOF mass spectrometry to identify the corresponding proteins. The subsequent data base search revealed that both spots were identical to peripherin. All spots (spots 1–4, 8, and 9) were confirmed as peripherin by Western blot analysis using the anti-peripherin antibody (Fig. 1D).

Akt Phosphorylates Ser66 of Peripherin in Vitro—Peripherin, whose expression is mostly restricted to neurons in the peripheral nervous system, is a member of type III intermediate filament proteins (31). Because peripherin has not been identified as an Akt substrate, we performed further analysis. First, we aimed to determine the phosphorylation site by Akt in vitro using recombinant proteins. Although no typical consensus sequence for the Akt substrate, RXRXX(S/T), was found in peripherin, five potent sequences existed (Fig. 2A). Because several previous papers indicated that Akt could possibly recognize some similar sequences as its target (details are described under “Discussion”), we examined the possibility that Akt was able to recognize and phosphorylate some similar sequences. Four types of GST fusion proteins that contained one or two potent sequences were generated and reacted with recombinant CA-Akt protein in the presence of [γ-32P]ATP (Fig. 2B). Autoradiography showed that one fragment containing 51–100 amino acids of peripherin (GST-Per 51–100) was exclusively phosphorylated by CA-Akt among four fragments. Because GST-Per 51–100 contained two potent sequences, SARLGS66

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A. Immunoblot patterns of PC12 cells expressing LacZ (left panel) or CA-Akt (right panel) were subjected to two-dimensional PAGE using wide pH range gels (pH 3–10). The gels were then analyzed by Western blotting using anti-phospho-Akt substrate antibody. B. Protein staining (B) and immunoblotting (C) of the gels (pH 5.10–5.45; molecular mass (MW), 44–68 kDa) of the CA-Akt-expressing preparation. Extracted proteins from PC12 cells expressing HA-CA-Akt were separated by two-dimensional PAGE using a narrow pH range gel. The gel was stained with SYPRO Ruby, a protein detection reagent (B) or transferred to a nitrocellulose membrane followed by immunoblotting using anti-phospho-Akt substrate antibody (C). Six spots (spots 1–4, 8, and 9) in B were recognized by anti-phospho-Akt substrate antibody in C. D. peripherin (Per) spots in the HA-CA-Akt-expressing preparation. The same membrane used in C was reprobed with anti-peripherin antibody. All of the peripherin spots are indicated by arrows (spots 1–10).

2C). The S66A mutation entirely prevented Akt phosphorylation, whereas the S79A mutation did not cause any alterations. These results demonstrate that Akt phosphorylates Ser\(^{66}\) of peripherin in vitro. The sequence containing Ser\(^{66}\) is highly conserved among mammalian species (Fig. 2D).

Ser\(^{66}\) of Peripherin Is Phosphorylated in Akt-activated Cultured Cells—To evaluate peripherin phosphorylation in vivo, a monoclonal antibody (anti-pPer antibody) was raised against the synthetic peptide ARLGpS\(^{66}\)FRAPRC. Specificity of this antibody was tested by Western blot analysis using GST-Per 51–100 in vitro (Fig. 3A). The anti-pPer antibody could detect GST-Per 51–100 only when the fragment was reacted with CA-Akt, and the intense immunoreactivity entirely disappeared when the S66A mutant was used. Using this antibody, peripherin phosphorylation was examined in HEK 293T cells. HEK 293T cells were transfected with WT-Akt and peripherin, because they have no endogenous peripherin, subsequently stimulated with insulin to activate Akt, and peripherin phosphorylation was detected by Western blot analysis. First, HEK 293T cells were treated with increasing doses of insulin, and peripherin phosphorylation was examined (Fig. 3B). Both Akt activation, which was evaluated by the phosphorylation state of Akt (32), and peripherin phosphorylation occurred in a dose-dependent manner. Next, we observed changes in peripherin phosphorylation over time after insulin treatment (Fig. 3C). Peripherin was phosphorylated in a time-dependent manner, which paralleled Akt activation. To further demonstrate that Akt kinase activity regulated peripherin phosphorylation, we used several inhibitors to modulate Akt activity. Both Akt activation and peripherin phosphorylation were almost prevented by pretreating cells with LY294002 (phosphatidylinositol 3-kinase inhibitor, which inhibited upstream signaling of Akt). In contrast, peripherin phosphorylation was not prevented by Me\(_x\)SO (the vehicle for control), U0126 (MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase) inhibitor, which prevented mitogen-activated protein kinase signaling), or rapamycin (mTOR inhibitor, which prevented one of the downstream signaling of Akt). We also examined the phosphorylation of endogenous peripherin in PC12 cells by Western blot analysis (Fig. 3E). Peripherin phosphorylation was hardly detected in PC12 cells infected with adenovirus expressing LacZ, WT-Akt, or DN-Akt. In contrast, peripherin phosphorylation was clearly observed in cells expressing CA-Akt. Although some minor additional bands were observed at different molecular masses in this blotting using PC12 cells, we assumed those bands would be nonspecific because their intensity was not affected by WT-, CA-, or DN-Akt expression. Together with the results obtained by HEK 293T cells, these results demonstrate that Ser\(^{66}\) of peripherin is phosphorylated in an Akt-mediated pathway in cultured cells.

Akt Interacts with the Head Domain of Peripherin in Vivo—It is likely that Akt may directly phosphorylate Ser\(^{66}\) of peripherin in vivo. To provide further support for this possibility, we examined whether these two proteins could interact in vivo using a co-immunoprecipitation experiment. Full-length peripherin, most of which may form intermediate filament in cells, is almost detergent-insoluble (33), and we assumed that peripherin might not be solubilized in a typical lysis buffer for immu-
noprecipitation. Therefore, we used a deletion form of peripherin for the immunoprecipitation experiment. Because our preliminary experiment showed that the head domain of peripherin (1–103 amino acids), which contained Ser66, could be solubilized entirely in radioimmunoprecipitation assay buffer (data not shown), we used the head domain instead of full-length peripherin in this assay. HEK 293T cells transfected with FLAG-Per 1–103 and WT-Akt were treated with or without insulin and subjected to immunoprecipitation using the anti-FLAG antibody (Fig. 4). Akt was co-precipitated with FLAG-Per 1–103, indicating that they could interact in vivo. It was of note that this interaction was not dependent on Akt activity because insulin treatment did not enhance the interaction. A similar activity-independent binding has also been reported on several other Akt substrates (34, 35).

Ser66 of Peripherin Is Phosphorylated in Regenerating Hypoglossal Motor Neurons—Previous reports have revealed that Akt was activated in response to neuronal injury (10, 13, 36). In particular, Akt activation is crucial for nerve-injured motor neurons to regenerate (10). We therefore examined the phosphorylation of endogenous peripherin in nerve-injured hypoglossal motor neurons. We crushed the hypoglossal nerve, and then peripherin expression and phosphorylation were examined by immunohistochemistry 5 days after injury (Fig. 5). Peripherin expression was induced in the cell bodies and also axons of injured neurons (Fig. 5, A–C). The antibody we used recognizes the RXXRX(pS/pT) motif, which is preferentially recognized and phosphorylated by Akt. However, the sequence containing Ser66 (SARLGS66) is not typical for Akt substrates where only one Arg residue exists at the −5 position. Because a similar variation has been reported for several Akt substrates, the Arg at the −5 position may not always be necessary. For instance, the sequences of PSRTAS in ATP-citrate lyase, LSRRPS in cAMP response element-binding protein, GARRSS in 14-3-3ζ, PMRNTS in p21-activated protein kinase 1, and HVRAHS in Yes-associated protein can be phosphorylated by Akt both in vitro and in vivo (37–41). As for the +1 position, peripherin has a Phe residue that would be suitable as an Akt substrate because previous studies have shown that a large hydrophobic residue in the +1 position is preferable (42).

DISCUSSION

We performed a proteomic approach to identify novel Akt substrates in neurons using the anti-phospho-Akt substrate antibody. The present study revealed that Akt phosphorylates Ser66 of peripherin both in vitro and in vivo. The antibody we used recognizes the RXRXX(pS/pT) motif, which is preferentially recognized and phosphorylated by Akt. However, the sequence containing Ser66 (SARLGS66) is not typical for Akt substrates where only one Arg residue exists at the −3 position, although the typical one has Arg residues at both −5 and −5 positions. Because a similar variation has been reported for several Akt substrates, the Arg at the −5 position may not always be necessary. For instance, the sequences of PSRTAS in ATP-citrate lyase, LSRRPS in cAMP response element-binding protein, GARRSS in 14-3-3ζ, PMRNTS in p21-activated protein kinase 1, and HVRAHS in Yes-associated protein can be phosphorylated by Akt both in vitro and in vivo (37–41). As for the +1 position, peripherin has a Phe residue that would be suitable as an Akt substrate because previous studies have shown that a large hydrophobic residue in the +1 position is preferable (42).
These studies support the finding that Ser66 in peripherin is likely to be phosphorylated by Akt.

The RXRX(S/T) motif is fairly specific as an Akt substrate; however, this motif can also be the phosphorylation target by another evolutionally related kinase, p70 S6 kinase (p70S6K) (43). Because p70S6K is activated downstream of the phosphatidylinositol 3-kinase-Akt pathway (44), the result obtained from our screening raised an alternative possibility in which peripherin might be phosphorylated by p70S6K rather than Akt. This possibility might be disregarded by the results of the in vitro kinase assay, where only recombinant proteins were used in these experiments (Figs. 2, B and C, and 3A). However, that result does not entirely rule out a possibility that p70S6K may phosphorylate peripherin in vivo, because an in vitro kinase is not always an in vivo kinase (45, 46). To eliminate this possibility, we examined the effect of p70S6K inhibition on peripherin phosphorylation in vivo using cultured cells (Fig. 3D). We treated HEK 293T cells with rapamycin, which is known to inhibit p70S6K activation via inhibition of its upstream mTOR kinase (44). The phosphorylation state of peripherin was not changed by rapamycin treatment, whereas it was markedly decreased by phosphatidylinositol 3-kinase inhibitor LY294002 treatment when the cells were stimulated by insulin. Furthermore, we performed a co-immunoprecipitation experiment using the head domain of peripherin and revealed that peripherin was capable of interacting with Akt in vivo (Fig. 4). Taken together, these data suggest that Akt directly phosphorylates peripherin in vivo.

Although various types of Akt substrates have been identified in a variety of cell types to date, to our knowledge this is the first report that identified an intermediate filament protein as an Akt substrate. Peripherin is a member of type III intermediate filament proteins, which include vimentin, desmin, and glial...
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Fibrillary acidic protein (31). Intermediate filament proteins generally consist of a central coiled-coil α-helical rod domain flanked by a head and a tail domain (47), and Ser^{66} resides in the center of the head domain of peripherin. Generally, head and tail domains of intermediate filament proteins contain several phosphorylation sites, whereas rod domains do not (48). It is well known that a similar neuron-specific intermediate filament, the neurofilament (NF), which belongs to type IV intermediate filament proteins, has multiple phosphorylation sites within its head or tail domains (49). With regard to peripherin, it had been hypothesized that its head domain might contain multiple phosphorylation sites like other intermediate filament proteins (50), and in fact, it has been reported that the N terminus of peripherin, which contains a head domain and a half of rod domain, was phosphorylated in cultured neurons, although the exact phosphorylation sites have not been determined yet (33, 51). Therefore, Ser^{66} could be one of phosphorylation sites previously suggested in those papers.

Of the intermediate filament proteins, peripherin is probably one of the most unknown members in terms of physiological function. However, there are several studies demonstrating its characteristic expression pattern. In contrast to NF proteins, which are widely expressed in various kinds of neurons, peripherin expression is rather restricted to peripheral nervous system neurons (50, 52–54). It is of interest that peripherin expression is transiently induced in injured neurons (55–58). Helfand et al. (60) recently revealed that peripherin might be required for both formation and elongation of neurites in PC12 cells, although there remains controversy (59). Therefore, it is likely that peripherin may be involved in neuronal regeneration, probably by contributing to rearrangement of intermediate filaments in neurons during nerve regeneration processes. Although the functional significance of the Akt-mediated peripherin phosphorylation remains unclear, Akt, which is necessary for nerve regeneration, would transfer divergent signals to the substrates, including peripherin, for proper nerve regeneration.

The present immunohistochemical study demonstrated that peripherin phosphorylation occurred endogenously in hypoglossal neurons after nerve injury (Fig. 5). These data suggest that not only peripherin expression but also peripherin phosphorylation may be associated with neuronal regeneration. Although it remains unclear how peripherin phosphorylation plays a role in motor nerve regeneration, immunohistochemical localization of phosphorylated peripherin may provide clues to address this issue. In adult rats, peripherin expression was induced in both cell bodies and axons of injured neurons (Fig. 5, A, D, G, and J). However, phosphorylated peripherin was only observed in cell bodies of injured neurons (Fig. 5, E and H) and could hardly be detected in injured axons (Fig. 5K). This cell body-specific localization is reminiscent of phosphorylated NF-L. It has been shown that Ser^{55} within the head domain of NF-L is phosphorylated by protein kinase A (61, 62). This occurred immediately after its synthesis in neuronal cell bodies, and thereafter the phosphorylation of NF-L disappeared, along with its translocation into axons (61). In addition, transgenic mice in which Ser^{55} was replaced with Asp to mimic permanent phosphorylation showed aberrant NF-L inclusions in neuronal perikarya (63). Because NF-L phosphorylation by protein kinase A can lead to disassembly of the filament (64), it is assumed that phosphorylation may block premature assembly of NF-L in cell bodies before transport into axons (49, 61, 63). As mentioned above, Ser^{66} of peripherin is located at the head domain, and some phosphorylation of head domains often causes disassembly of intermediate filaments (48). If Ser^{66} phosphorylation is able to trigger disassembly of peripherin fil-

![Image of Peripherin phosphorylation in injured hypoglossal motor neurons](https://example.com/image.png)
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Peripherin, Akt may control the dynamics of intermediate filament in regenerating axons by phosphorylating newly synthesized peripherin and prevent them from assembly in neuronal cell bodies until the appropriate timing.

It is also possible that peripherin phosphorylation may modulate interactions with other proteins. Previous reports suggest that peripherin has interactive proteins. Peripherin was shown to interact with the small heat shock protein αB-crystallin (66). Although it has not been addressed which part of peripherin may be responsible for these interactions, these interactive properties may be regulated by Akt-mediated phosphorylation.

In conclusion, we have identified peripherin as a novel neuronal substrate for Akt both in vitro and in vivo, and Akt-mediated phosphorylation was induced in regenerating motor neurons. Because Akt is known to play a pivotal role in neuronal regeneration, peripherin would be one of the significant substrates for Akt during nerve regeneration processes. To gain a better understanding of Akt function in regenerating neurons, in particular the functional significance of Akt for the cytoskeletal rearrangement in neurons after nerve injury, further studies, such as how Ser66 phosphorylation is capable of changing physiological property of peripherin, are required.

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REFERENCES

- Ament, A. M., and Levison, S. W. (2004) J. Neurochem. 89, 1092–1100
- BRECKEN, 1998 Microsurgery 18, 397–405
- Terenghi, G. (1999) J. Anat. 194, 1–14
- Grothe, C., and Nikkhah, G. (2001) Anat. Embryol. (Berl.) 204, 171–177
- Boyd, J. G., and Gordon, T. (2003) Mol. Neurobiol. 27, 277–324
- Patapoutian, A., and Reichardt, L. F. (2001) Curr. Opin. Neurobiol. 11, 272–280
- Besset, V., Scott, R. P., and Ibanez, C. F. (2000) J. Biol. Chem. 275, 39159–39166
- Karajannis, M. A., Vincent, L., Direnzo, R., Shmelkov, S. Z., Fang, Z., Feldman, E. I., Bohlen, P., Zhu, Z., Sun, H., Kussie, P., and Rafii, S. (2006) Leukemia 20, 979–986
- Cohen, A. D., Takeuchi, F., and Roth, R. A. (1996) J. Biol. Chem. 271, 21920–21926
- Sato, Y., Tanaka, K., Lee, G., Kanega, Y., Sakai, Y., Kaneko, S., Nakabayashi, H., Tamaoki, T., and Saito, I. (1998) Biochem. Biophys. Res. Commun. 244, 455–462
- Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikukawa, U., and Kasuga, M. (1998) Mol. Cell. Biol. 18, 3708–3717
- Miyake, S., Makimura, M., Kanega, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1320–1324
- Kanega, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S., and Saito, I. (1995) Nucleic Acids Res. 23, 3816–3821
- Konishi, H., Namikawa, K., and Kiyama, H. (2006) GLIA 53, 723–732
- Kishiyo, Y., Kagawa, M., Iaito, I., and Sado, Y. (1995) Cell Struct. Funct. 20, 151–156
- Ushijima, R., Sakaguchi, N., Kano, A., Maruyama, A., Miyamoto, Y., Sekimoto, T., Yoneda, Y., Ogino, K., and Tachibana, T. (2005) Biochem. Biophys. Res. Commun. 330, 880–886
- Coulombe, P. A., Ma, L., Yamada, S., and Wawersik, M. (2001) J. Cell Sci. 114, 4345–4347
- Cofer, P. J., Jin, J., and Woodgett, J. R. (1998) Biochem. J. 335, 1–13
- Giasson, B. I., and Mushynski, W. E. (1998) J. Neurochem. 70, 1869–1875
- Datta, S. R., Dukde, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
- Kim, A. H., Kurosihara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) Mol. Cell. Biol. 21, 893–901
- Yu, F., Sugawara, T., Maier, C. M., Hsieh, L. B., and Chan, P. H. (2005) Neurobiol. Dis. 20, 491–499
- Du, K., and Mntominy, M. (1998) J. Biol. Chem. 273, 32377–32379
- Powell, D. W., Rane, M. J., Chan, Q., Singh, S., and McLeish, K. R. (2002) J. Biol. Chem. 277, 21639–21642
- Zhou, G. L., Zhuo, Y., King, C. C., Fryer, B. H., Bokoch, G. M., and Field, J. (2003) Mol. Cell. Biol. 23, 8058–8069
- Basu, S., Totty, N. F., Irwin, M. S., Sudol, M., and Downward, J. (2003) Mol. Cell 11, 11–23
- Berwick, D. C., Hers, L., Heesom, K. J., Moule, S. K., and Tavare, J. (2002) J. Biol. Chem. 277, 33895–33900
- Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemings, B. A., and Cohen, P. (1996) FEBS Lett. 399, 333–338
- Leighton, I. A., Dalby, K. N., Caudwell, F. B., Cohen, P. T., and Cohen, P. (1995) FEBS Lett. 375, 289–293
- Asnaghi, L., Bruno, P., Priulla, M., and Nicolin, A. (2004) Pharmacol. Res. 50, 545–549
- Lovestone, S., Reynolds, C. H., Latimer, D., Davis, D. R., Anderton, B. H., Gallo, I. M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., Woodgett, J. R., and Miller, C. C. J. (1994) FEBS Lett. 370, 149–153
- Strelkov, S. V., Herrmann, H., and Aebi, U. (2003) Bioessays 25, 243–251
- Omary, M. B., Ku, N. O., Tao, G. Z., Toivola, D. M., and Liao, J. (2006) Trends Biochem. Sci. 31, 383–394
- Grant, P., and Pant, H. C. (2000) J. Neurocytol. 29, 843–872
- Leonard, D. G., Gorham, J. D., Cole, P., Greene, L. A., and Ziff, E. B. (1988) J. Cell Biol. 106, 181–193
51. Huc, C., Escurat, M., Djabali, K., Derer, M., Landon, F., Gros, F., and Portier, M. M. (1989) *Biochem. Biophys. Res. Commun.* **160**, 772–779
52. Portier, M. M., de Nechaud, B., and Gros, F. (1983) *Dev. Neurosci.* **6**, 335–344
53. Parysek, L. M., and Goldman, R. D. (1988) *J. Neurosci.* **8**, 555–563
54. Brody, B. A., Ley, C. A., and Parysek, L. M. (1989) *J. Neurosci.* **9**, 2391–2401
55. Oblinger, M. M., Wong, J., and Parysek, L. M. (1989) *J. Neurosci.* **9**, 3766–3775
56. Troy, C. M., Muma, N. A., Greene, L. A., Price, D. L., and Shelanski, M. L. (1990) *Brain Res.* **529**, 232–238
57. Terao, E., Janssens, S., van den Bosch de Aguilar, P., Portier, M., and Klosen, P. (2000) *Neuroscience* **101**, 679–688
58. Beaulieu, J. M., Kriz, J., and Julien, J. P. (2002) *Brain Res.* **946**, 153–161
59. Troy, C. M., Greene, L. A., and Shelanski, M. L. (1992) *J. Cell Biol.* **117**, 1085–1092
60. Helfand, B. T., Mendez, M. G., Pugh, J., Delsert, C., and Goldman, R. D. (2003) *Mol. Biol. Cell* **14**, 5069–5081
61. Sihag, R. K., and Nixon, R. A. (1991) *J. Biol. Chem.* **266**, 18861–18867
62. Nakamura, Y., Hashimoto, R., Kashiwagi, Y., Aimoto, S., Fukusho, E., Matsumoto, N., Kudo, T., and Takeda, M. (2000) *J. Neurochem.* **74**, 949–959
63. Gibb, B. J., Brion, J. P., Brownlee, J., Anderton, B. H., and Miller, C. C. (1998) *J. Neurochem.* **70**, 492–500
64. Nakamura, Y., Takeda, M., Angelides, K. J., Tanaka, T., Tada, K., and Nishimura, T. (1990) *Biochem. Biophys. Res. Commun.* **169**, 744–750
65. Leung, C. L., Sun, D., and Liem, R. K. (1999) *J. Cell Biol.* **144**, 435–446
66. Djabali, K., de Nechaud, B., Landon, F., and Portier, M. M. (1997) *J. Cell Sci.* **110**, 2759–2769