

# 14-3-3 Protein Interacts with Huntingtin-associated Protein 1 and Regulates Its Trafficking<sup>\*S</sup>

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HAP1 (Huntingtin-associated protein 1) consists of two alternately spliced isoforms (HAP1A and HAP1B, which have unique C-terminal sequences) and participates in intracellular trafficking. The C terminus of HAP1A is phosphorylated, and this phosphorylation was found to decrease the association of HAP1A with kinesin light chain, a protein involved in anterograde transport in cells. It remains unclear how this phosphorylation functions to regulate the association of HAP1 with trafficking proteins. Using the yeast two-hybrid system, we found that HAP1 also interacts with 14-3-3 proteins, which are involved in the assembly of protein complexes and the regulation of protein trafficking. The interaction of HAP1 with 14-3-3 is confirmed by their immunoprecipitation and colocalization in mouse brain. Moreover, this interaction is specific to HAP1A and is increased by the phosphorylation of the C terminus of HAP1A. We also found that expression of 14-3-3 decreases the association of HAP1A with kinesin light chain. As a result, there is less HAP1A distributed in neurite tips of PC12 cells that overexpress 14-3-3. Also, overexpression of 14-3-3 reduces the effect of HAP1A in promoting neurite outgrowth of PC12 cells. We propose that the phosphorylation-dependent interaction of HAP1A with 14-3-3 regulates HAP1 function by influencing its association with kinesin light chain and trafficking in neuronal processes.

HAP1 (Huntingtin-associated protein 1) was first identified as a neuronal protein that binds huntingtin (htt),<sup>2</sup> the Huntington disease protein (1). The N-terminal region of htt contains a polyglutamine (poly(Q)) domain whose expansion (>37 glutamines) causes selective neuropathology in Huntington disease. Although the functions of HAP1 and htt remain to be fully characterized, mounting evidence has demonstrated that both

proteins are involved in intracellular trafficking (2–6). For example, HAP1 associates with the microtubule-dependent trafficking proteins dynactin p150 (7, 8) and kinesin light chain 2 (KLC2) (9). Dynactin p150 participates in retrograde transport mediated by dynein, whereas KLC is involved in anterograde transport by kinesin motors (10). Consistently, mutant htt is found to affect axonal transport by sequestering soluble dynactin p150 and kinesin (11–13) and also affects brain-derived neurotrophic factor (BDNF) vesicle transport, which requires HAP1 (2). The critical roles of htt and HAP1 are also supported by the fact that a lack of htt or HAP1 leads to embryonic or postnatal lethality in gene-targeted knock-out mice (14–16).

Although htt is ubiquitously expressed, HAP1 is enriched in neurons (1, 17). Rodent HAP1 consists of two alternately spliced isoforms (HAP1A and HAP1B), which differ only in their short C-terminal sequences (579–599 aa of HAP1A versus 579–629 aa of HAP1B) (1, 18). These unique C-terminal sequences apparently confer different properties on HAP1A and HAP1B. In nerve growth factor (NGF)-differentiated PC12 cells, HAP1A is enriched in growth cones and neurite tips, whereas HAP1B is mainly restricted to the cell bodies (6, 9, 19). Transfected HAP1A, but not HAP1B, forms cytoplasmic puncta in cultured cells (2, 18, 20). HAP1-immunoreactive puncta are also present in the cytoplasm of neurons in the brain (17, 18, 21). These puncta contain both HAP1A and HAP1B, as both proteins form heterodimers (18). Recently, we found that the interaction of HAP1A with KLC2 (9), an accessory protein for the kinesin motor that drives microtubule-dependent anterograde transport (22), is decreased by phosphorylation of the C terminus of HAP1A (6). Understanding the mechanism underlying this regulation is important for elucidating the involvement of HAP1 in intracellular trafficking.

In the course of our search for proteins that interact with HAP1, we also found that 14-3-3 binds HAP1 in the yeast two-hybrid system. The 14-3-3 family contains well conserved and ubiquitously expressed regulatory proteins. 14-3-3 proteins bind a large number of partners, including cytoskeletal and trafficking proteins, and are involved in the regulation of many crucial cellular processes, such as signal transduction and protein trafficking (23–28). In this study, we confirm the *in vitro* and *in vivo* association of 14-3-3 with HAP1. Moreover, we show that this interaction is enhanced by phosphorylation of the C terminus of HAP1A and can attenuate the association of HAP1A with kinesin light chain, leading to a decrease in the distribution of HAP1A in neurites and their outgrowths. These

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<sup>2</sup> The abbreviations used are: htt, Huntingtin; GFP, green fluorescent protein; KLC, kinesin light chain; NGF, nerve growth factor; HEK293, human embryonic kidney 293; IP, immunoprecipitation; BDNF, brain-derived neurotrophic factor; GST, glutathione S-transferase; aa, amino acids; PBS, phosphate-buffered saline.

findings offer new insight into the mechanisms that regulate HAP1 trafficking and function.

## EXPERIMENTAL PROCEDURES

**Antibodies**—We used the following primary antibodies: rabbit polyclonal antibodies to glutathione *S*-transferase (GST), HAP1A (18), kinesin light chain 2 (9), and 14-3-3  $\beta$  (K-19; Santa Cruz Biotechnology); 14-3-3 $\zeta$  (a gift from Dr. Alastair Aitken, University of Edinburgh, UK); guinea pig polyclonal antibody to HAP1 (EM78) (19); mouse antibodies to HAP1 (Transduction Laboratories); and the hemagglutinin (HA) epitope (YPYDVP-DYA) (Cell Signaling Technology). The following secondary antibodies were used: peroxidase- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch) and Alexa Fluor 488-conjugated secondary antibody or Alexa Fluor 350-conjugated secondary antibody (Invitrogen).

**Yeast Two-hybrid Screen**—We used the C-terminal sequences of HAP1A (441–599 amino acids) or HAP1B (441–629 amino acids), which were fused to the Gal4 DNA-binding domain, as bait to screen a rat brain cDNA library (1). Yeast strain Y190 was used for transformation. Positive clones were selected on Trp<sup>-</sup>, Leu<sup>-</sup>, and His<sup>-</sup> synthetic medium containing 25 mM 3-aminotriazole and further verified by a filter assay for  $\beta$ -galactosidase activity, as described previously (1).

**Mutagenesis**—To create point mutations in the C terminus of HAP1A, PRK expression vector encoding full-length HAP1A was used as a template. The following primers were used for PCR: forward primer 5'-CGGTGCAAGATCTCAAGCCGCTG-3' with reverse primer 5'-CGTCTAGATCATAGAGTTGCTGATCGGTA-3' for the S597A mutation, or with 5'-CGTCTAGATCATAGAGCTGATGATCGGTA-3' for the T598A mutation. The PCR products were then subcloned into the BglII and XbaI sites of HAP1A cDNA. All mutations were confirmed by DNA sequencing.

**Immunoprecipitation**—Whole-brain extracts from wide-type or HAP1 knock-out mouse pups (16) at postnatal day 1–2 were prepared by homogenizing in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100) containing protease inhibitor mixture (Sigma). Homogenates were centrifuged at 16,000 rpm at 4 °C for 15 min, and 500  $\mu$ l of supernatant (at 1 mg/ml) was first clarified by incubation with 40  $\mu$ l of 50% protein A-Sepharose beads (Sigma) at 4 °C for 1 h to reduce nonspecific binding. After pelleting the beads, the supernatant was then incubated with antibody to HAP1 (EM78) overnight at 4 °C, and HAP1-containing immunocomplex was recovered by incubating with 15  $\mu$ l of 50% protein A-Sepharose beads for 1 h at 4 °C, followed by brief centrifugation. The immunoprecipitates were washed three times with RIPA lysis buffer and subjected to Western blotting with antibody to 14-3-3 (K-19; Santa Cruz Biotechnology). For immunoprecipitation of transfected proteins, HA-tagged 14-3-3 $\zeta$  was cotransfected into HEK293 cells with HAP1A, HAP1B, or mutant HAP1A (T598A) for 36 h. The cells were lysed in Nonidet P-40 buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 1% Nonidet P-40) containing protease inhibitor mixture (Sigma) and phosphatase inhibitors (5 mM NaF and 2 mM Na<sub>3</sub>VO<sub>4</sub>) by gentle rocking for 1 h at 4 °C. The lysates were clarified by centrifugation at 3000 rpm for 10

min. The supernatant was then subjected to immunoprecipitation with anti-HAP1 (EM78) antibody, as described above.

**In Vitro Binding**—GST fusion proteins containing full-length human 14-3-3 $\zeta$  cDNA (GST-14-3-3), KLC2 (amino acids 124–411) (9), or p150 dynactin (amino acids 1023–1223) (8) were described previously. These GST fusion proteins were produced in bacterial strain BL21 (Amersham Biosciences) and purified with glutathione-agarose beads (Sigma). HEK293 cells were transfected with HAP1A, HAP1B, or mutant HAP1A. After 24 h, cells were lysed in buffer containing 1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, pH 7.4, protease inhibitor mixture, and phosphatase inhibitors for 1 h at 4 °C. After centrifugation at 3000 rpm for 10 min, the supernatant was preclarified by incubating for 1 h with 40  $\mu$ l (1:1 slurry) of glutathione-agarose beads and then incubated for 1 h with 2  $\mu$ g of GST protein or GST-14-3-3 $\zeta$  coupled to 40  $\mu$ l of glutathione-agarose beads in 500  $\mu$ l of lysis buffer. After incubation, the beads were washed three times with the lysis buffer, and protein bound to the beads was resolved in SDS-PAGE sample buffer and underwent Western blotting with antibodies to HAP1 (EM78) and GST. For the *in vitro* binding assay with GST-KLC2 or GST-dynactin p150, HEK293 cells were either transfected with HAP1A alone or cotransfected with HAP1A and HA-14-3-3 $\zeta$  (HA-14-3-3) for 24 h. Cells were then lysed in 0.2% Triton X-100 in PBS containing protease inhibitor mixture and phosphatase inhibitors for 1 h at 4 °C. Cell lysates were centrifuged at 13,000 rpm for 10 min, and the supernatant was preclarified by incubating with 40  $\mu$ l (1:1 slurry) of glutathione-agarose beads for 1 h and then incubated overnight with 2  $\mu$ g of GST fusion proteins coupled to 40  $\mu$ l of glutathione-agarose beads in 500  $\mu$ l of lysis buffer. The beads were washed three times with lysis buffer, and protein bound to the beads was subjected to Western blotting with mouse anti-HAP1 and rabbit anti-GST antibodies.

**In vitro** binding of 14-3-3 to synthesized peptides was performed using the method described previously (29, 30). Human 14-3-3 $\zeta$  protein was expressed as a GST fusion protein and purified from *Escherichia coli* strain BL21. Fluorescence detection via microplates and determination of the equilibrium dissociation constant ( $K_d$ ) have already been described in detail in our previous studies (29, 30). Both nonphosphorylated (CGHPPASGTSYRSSTL) and phosphorylated Thr-598-containing (CGHPPASGTSYRSSpTL) peptides of HAP1A were examined. For comparison, nonphosphorylated SWTY (RGRSWTY) and phosphorylated SWpTY (RGRSWpTY) were also included in the study.

**Phosphorylation Studies**—HEK293 cells transfected with HAP1A for 24 h were incubated with serum-free medium overnight, and the cells were treated either with okadaic acid (0–2  $\mu$ M for 60 min) to increase phosphorylation or with staurosporine (0–1  $\mu$ M for 60 min) to inhibit phosphorylation. Cells were lysed in buffer containing 1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, pH 7.4, protease inhibitor mixture, and phosphatase inhibitors. The cell lysates were subjected to *in vitro* binding with 2  $\mu$ g of GST or GST-14-3-3.

**Immunofluorescent Labeling**—Transfected HEK293 cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Rat brain cortical neurons were isolated from

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E17–18 rat fetus and cultured as described previously (19). After three rinses with PBS, cells were permeabilized and blocked with 0.2% Triton X-100, 3% bovine serum albumin, 2% normal goat serum/PBS for 30 min at room temperature. The cells were then incubated with primary antibodies diluted in 3% bovine serum albumin, 2% normal goat serum/PBS overnight at 4 °C. After incubation, cells were washed three times with PBS and then incubated with secondary antibodies conjugated with Alexa Fluor 488 (green), rhodamine (red), or Alexa Fluor 350 (blue) in the blocking buffer for 30 min at 4 °C. For immunostaining of mouse brain, sections (10- $\mu$ m) containing the hypothalamic region from adult (4-month-old) mice were mounted on gelatin-precoated microscope slides and then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After three rinses with PBS, the sections were permeabilized and blocked with 3% bovine serum albumin, 2% normal goat serum, 0.5% Triton X-100 in PBS for 1 h at room temperature. The brain sections were then incubated with primary antibodies diluted in the blocking buffer at 4 °C overnight. After three rinses with PBS, the sections were incubated with Alexa Fluor 488- and rhodamine-conjugated secondary antibodies in the blocking buffer for 1 h at room temperature. The nuclei were stained with Hoechst dye. Light micrographs were taken using a Zeiss microscope (Axiovert 200 MOT) with the  $\times 63$  oil immersion objective lens and a digital camera (Hamamatsu ORCA-100) and processed with the Openlab modular software (Improvision, Inc). Confocal imaging was performed using the  $\times 60$  oil immersion objective lenses on a Zeiss LSM 510 confocal microscope system.

**Quantification of Relative HAP1A Signal at Growth Cones**—PC12 cells were cultured on coverslips precoated with poly-D-lysine. After transfection of HA-14-3-3 or GFP for 5 h, NGF was added to a concentration of 50 ng/ml for 24 h. Cells were fixed and subjected to immunofluorescent labeling with mouse anti-HA (Cell Signaling) and rabbit anti-HAP1A antibodies. Confocal images were captured using the  $\times 63$  oil immersion objective lens. The same setting was used to image all the cells examined. Two regions of interest were created for each cell as follows: the soma and the growth cone farthest from the soma (9). The relative HAP1A signal at the growth cones was represented as the ratio of growth cone HAP1A signal to soma HAP1A signal. A total of 30 cells from each group was selected and measured.

**Neurite Outgrowth Studies**—PC12 cells were cultured on poly-D-lysine-coated coverslips in DMEM/F-12 medium (Invitrogen) supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. HA-14-3-3 was transfected into PC12 cells with Lipofectamine 2000 (Invitrogen). Six hours later, PC12 cells were infected with adenoviral HAP1A that also independently expresses GFP (31). Sixteen hours later, NGF was added to a final concentration of 50 ng/ml, and cells were cultured for an additional 24 h. For controls, PC12 cells were either mock-transfected or infected with adenoviral HAP1A after mock transfection. To identify PC12 cells that express HA-14-3-3, cells were fixed and subjected to immunofluorescent labeling with anti-HA antibody. Adenoviral HAP1A-infected cells, which express GFP as well, were counted as HAP1A-positive

cells. Adenoviral GFP-infected cells that express GFP alone served as a control. Confocal images were taken with the  $\times 40$  oil immersion objective lens. Cells (300–350) from each group were examined to determine the percentage with neurites longer than 2 body diameters.

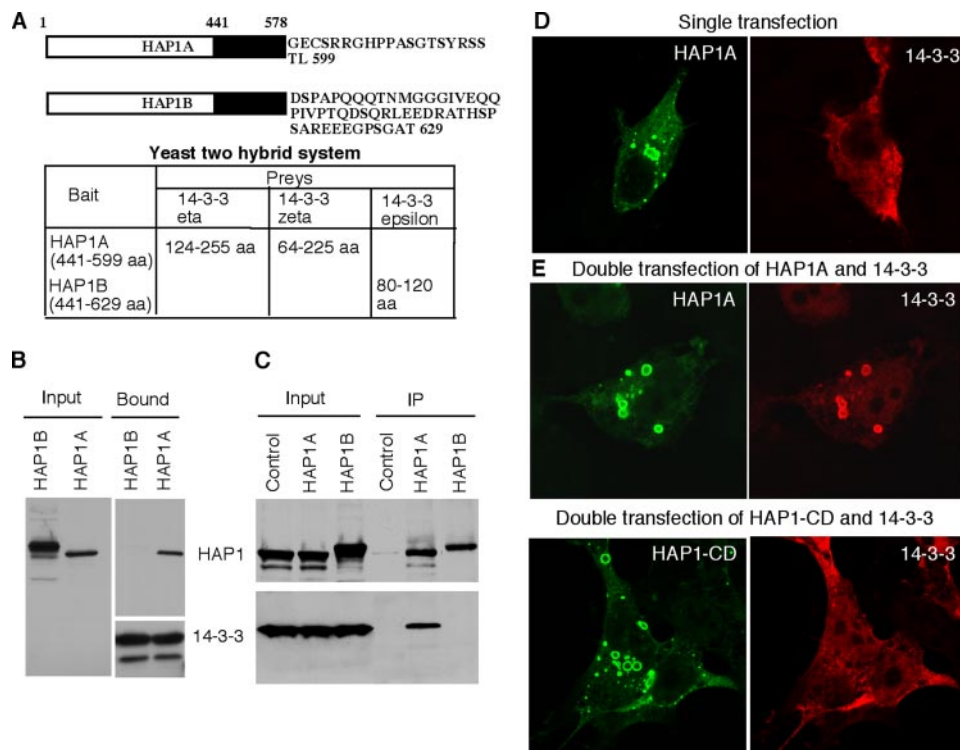
**Statistical Analysis**—Statistical significance ( $p < 0.05$ ) was assessed using the Student's  $t$  test whenever two groups were compared. When analyzing multiple groups, we employed analysis of variance with Scheffé's post hoc test to determine statistical significance. Calculations were performed using Prism (version 4.0) software.

## RESULTS

**Interaction of HAP1 with 14-3-3 in Vitro**—Rodent HAP1 consists of two alternately spliced isoforms, HAP1A and HAP1B, which differ only in their short C-terminal sequences (579–599 aa of HAP1A versus 579–629 aa of HAP1B; see Fig. 1A). To identify novel binding partners that may differentially interact with these HAP1 isoforms, we used HAP1 C termini (441–599 aa of HAP1A and 441–629 aa of HAP1B) as bait to screen a rat brain cDNA library in the yeast two-hybrid system. There were three positive clones identified (two from the HAP1A screen and one from the HAP1B screen) that encode partial sequences of three different 14-3-3 isoforms as follows:  $\zeta$  (64–225 aa),  $\eta$  (124–255 aa), and  $\epsilon$  (80–120 aa), respectively (Fig. 1A). The 14-3-3 proteins are a family of conserved regulatory molecules in which seven mammalian isoforms ( $\beta$ ,  $\gamma$ ,  $\eta$ ,  $\zeta$ ,  $\epsilon$ ,  $\tau$ , and  $\sigma$ ) are highly homologous and capable of binding to the same target (23, 32). The regions of 14-3-3 identified as interacting with HAP1 are highly homologous (>80% amino acid identity to the sequence) in all 14-3-3 isoforms, suggesting that HAP1 is likely to bind all the 14-3-3 proteins. We used 14-3-3 $\zeta$ , which is expressed in neuronal cells (33–35), for further characterization in subsequent studies.

To confirm the interaction of HAP1 with 14-3-3, we used GST fusion protein encoding 14-3-3 $\zeta$  (GST-14-3-3) and performed *in vitro* binding with lysates from HEK293 cells transfected with either HAP1A or HAP1B. Interestingly, HAP1A bound GST-14-3-3, whereas HAP1B did not (Fig. 1B). To determine whether the specific interaction of 14-3-3 with HAP1A also occurs in cells, we cotransfected HA-14-3-3 $\zeta$  (14-3-3) with either HAP1A or HAP1B in HEK293 cells and performed coimmunoprecipitation using anti-HAP1 antibody. Consistent with the GST-pulldown result, 14-3-3 was detected in HAP1A immunoprecipitates, but not in HAP1B immunoprecipitates (Fig. 1C). Thus, although 14-3-3 may bind various motifs of HAP1 in the yeast two-hybrid system, the interaction of HAP1 with 14-3-3 in mammalian cells appears to be dependent on the unique C-terminal sequences of HAP1A.

When transfected into cells, HAP1B remains diffuse throughout the cytoplasm, whereas HAP1A forms puncta in the cytoplasm (18) (Fig. 1D). The punctate distribution of HAP1A allows us to examine its colocalization with other proteins. To test the critical role of the C-terminal region of HAP1A in binding to 14-3-3, we questioned whether 14-3-3 colocalizes with HAP1A puncta and, if so, whether this colocalization would depend on the C terminus of HAP1A. Transfection of 14-3-3 alone led to a diffuse distribution of transfected



**FIGURE 1. Interaction of HAP1 with 14-3-3.** *A*, different C-terminal sequences of rat HAP1A and HAP1B (441–599 and 441–629 amino acids, respectively) were used as bait in the yeast two-hybrid system. The *black box* represents the amino acid sequences shared by both isoforms. There were three 14-3-3 isoforms (preys) we identified that bind HAP1. *B*, *in vitro* GST pull-down of transfected HAP1A and HAP1B from HEK293 cells using GST-14-3-3. The pull-down was probed with guinea pig anti-HAP1 and rabbit anti-GST. The *input* is cell lysate for pull-down. Note that GST-14-3-3 could only pull down transfected HAP1A. *C*, immunoprecipitation of HAP1 and HA-14-3-3 from transfected HEK293 cells. HEK293 cells were cotransfected with 14-3-3 and HAP1A or HAP1B. HAP1A was precipitated by guinea pig anti-HAP1. The precipitates were probed by guinea pig antibody to HAP1 (*upper panel*) and mouse antibody to the HA tag (*lower panel*) to reveal HAP1 and 14-3-3, respectively. Note that 14-3-3 was only detected from HAP1A-immunoprecipitated proteins. *Input* is cell lysate for precipitation. Control is preimmune serum immunoprecipitation (*IP*). *D*, immunofluorescent staining of transfected cells expressing HAP1A or HA-14-3-3 alone with guinea pig antibody to HAP1 and mouse antibody to the HA epitope. HAP1A forms cytoplasmic puncta, whereas 14-3-3 is diffusely distributed within the cells. *E*, double transfection of HAP1A or mutant HAP1-CD (*green*) with HA-14-3-3 (*red*). The cells were stained with guinea pig antibody to HAP1 and mouse antibody to HA. Note that 14-3-3 colocalizes with HAP1A but not HAP1-CD puncta in the cytoplasm.

14-3-3 in the cytoplasm (Fig. 1*D*). Cotransfection of 14-3-3 with HAP1B showed the diffuse distribution of both proteins in the cytoplasm (data not shown). However, cotransfection of 14-3-3 with HAP1A caused an almost complete redistribution of 14-3-3 to HAP1A puncta (Fig. 1*E*). Deletion of the unique C-terminal sequence of HAP1A (HAP1-CD) still led to puncta formation, yet this deletion eliminated the colocalization of 14-3-3 with HAP1 puncta (Fig. 1*E*). These data therefore demonstrate that 14-3-3 interacts specifically with HAP1A in cells and that this interaction is dependent on the unique C-terminal sequences of HAP1A.

**Interaction of HAP1 with 14-3-3 *in Vivo***—Because hypothalamic neurons also show abundant HAP1 puncta in the cytoplasm (17, 31), we performed immunofluorescence double labeling of HAP1 and 14-3-3 in the mouse hypothalamus to verify the *in vivo* association of HAP1 with 14-3-3. Some HAP1 puncta clearly contain 14-3-3 staining in neurons (Fig. 2*A*). Because not all HAP1 puncta show 14-3-3 staining to the same extent, this colocalization may reflect a dynamic interaction between 14-3-3 and HAP1, which would be consistent with the regulatory role of 14-3-3 in protein complex assembly. Thus, it

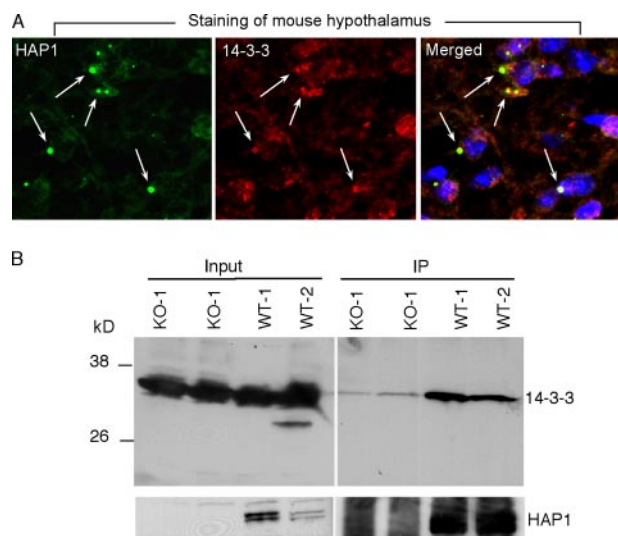
is necessary to determine whether soluble 14-3-3 also binds HAP1 *in vivo*. To this end, we performed HAP1 immunoprecipitation of mouse brain tissue. To rule out non-specific immunoprecipitation by the antibody we used, we included HAP1 knock-out mouse brain tissue (16) as a negative control to definitively establish whether the coprecipitation of 14-3-3 is dependent on HAP1. The antibody to HAP1 used for immunoprecipitation can precipitate both HAP1A and HAP1B, which form heterodimers in cells (18). The immunoprecipitates were probed with antibody to 14-3-3  $\beta$ , which recognizes all seven isoforms. The result shows that 14-3-3 was indeed coprecipitated with HAP1. Significantly, the background level of 14-3-3 in the immunoprecipitates from HAP1 knock-out mouse brain was low (Fig. 2*B*). Consequently, both the colocalization and immunoprecipitation assays demonstrate that 14-3-3 associates with HAP1 in the brain.

**Regulation of the Interaction of HAP1A with 14-3-3 by Phosphorylation**—The selective interaction of HAP1A with 14-3-3 in transfected cells suggests that the C-terminal region of HAP1A is required for their stable interaction in mammalian cells. We have recently found

that the Thr-598 residue in the C terminus of HAP1A is phosphorylated in cells (6). To determine whether this phosphorylation regulates the association of HAP1A with 14-3-3, we first examined the binding affinity of GST-14-3-3 $\zeta$  to nonphosphorylated HAP1A C-terminal peptides (HAP1a, CGHPPASGTSYRSSL) and to phosphorylated peptides (p-HAP1a, CGHPPASGTSYRSSLpTL), in which the Thr-598 residue is phosphorylated (Fig. 3*A*). We performed a competitive assay based on fluorescence anisotropy (29). The dissociation constant ( $K_d$ ) of p-HAP1a (0.16  $\mu$ M) to GST-14-3-3 is significantly lower than the dissociation constant of nonphosphorylated peptides (>17  $\mu$ M), indicating a higher affinity of p-HAP1a to 14-3-3. The binding affinity of p-HAP1a is similar to that of SWpTY peptides, which represent C-terminal sequences that bind 14-3-3 in a phosphorylation-dependent manner (29, 30). This result clearly indicates that phosphorylation of Thr-598 enhances the binding of the C-terminal peptides of HAP1A to 14-3-3.

The *in vitro* binding of HAP1 peptides to 14-3-3 may not necessarily indicate the association of full-length proteins in cells. Thus, we examined the localization of 14-3-3 in HEK293

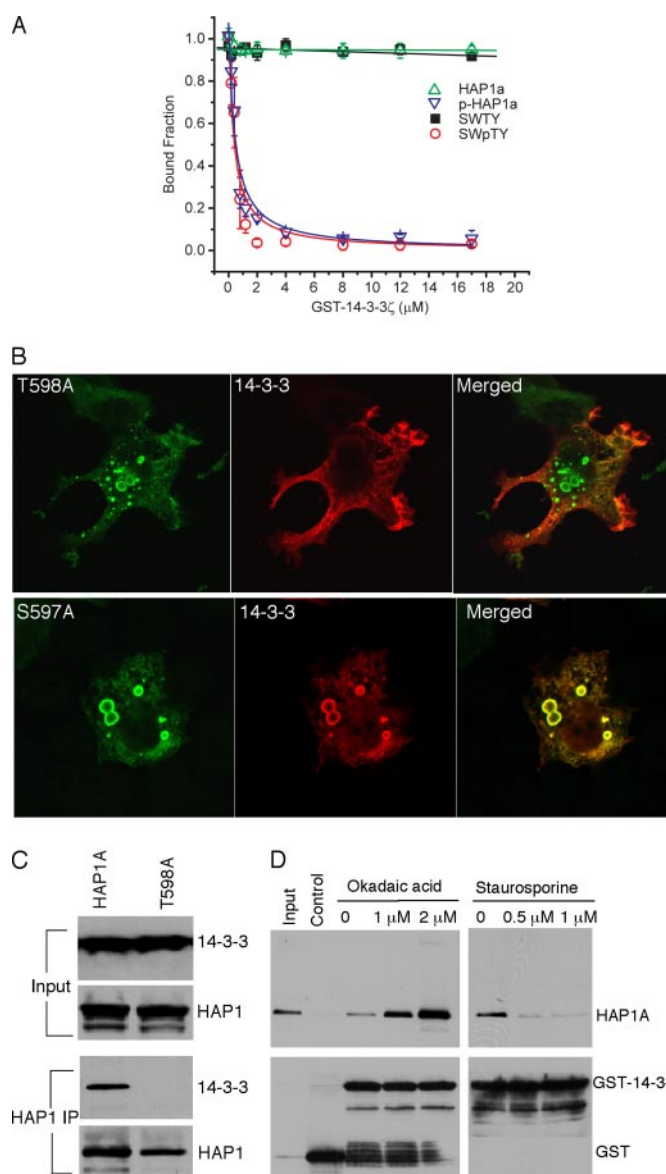
## Interaction of 14-3-3 with HAP1



**FIGURE 2. *In vivo* association of 14-3-3 with HAP1.** *A*, immunofluorescence double labeling of the mouse hypothalamus with guinea pig antibody to HAP1 and rabbit antibody to 14-3-3 $\zeta$ . HAP1 (green) and 14-3-3 $\zeta$  (red) colocalize in some cytoplasmic puncta (arrows) in the hypothalamus. The nuclei (blue) were stained with Hoechst dye and are shown in the merged image. *B*, immunoprecipitation of HAP1 and 14-3-3 from whole-brain tissues of wild type (WT,  $n = 2$ ) and HAP1 knock-out (KO,  $n = 2$ ) mouse pups at postnatal day 1–2. HAP1 was precipitated by guinea pig antibody to HAP1. The precipitates were probed by guinea pig antibody to HAP1 (lower panel) and rabbit antibody to 14-3-3  $\beta$  (upper panel), which recognize all seven of the 14-3-3 isoforms. Input is tissue lysate for precipitation (IP).

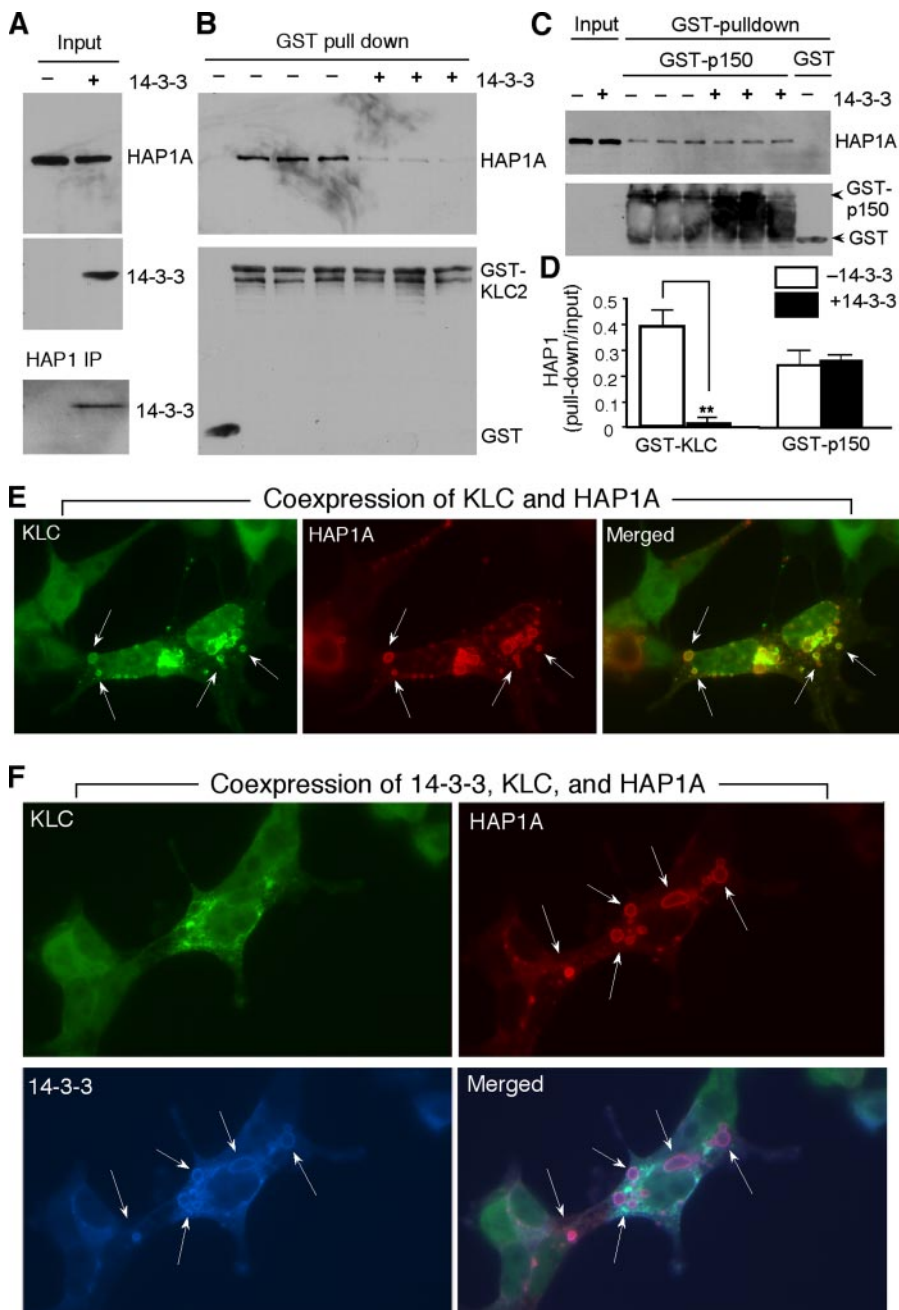
cells that express mutant HAP1A, in which the phosphorylated Thr-598 residue has been replaced with alanine (T598A). This mutation does not affect the formation of HAP1 puncta, but it does prevent the localization of 14-3-3 to HAP1 puncta, lending further support to the notion that the association of 14-3-3 with HAP1A is dependent on the phosphorylated Thr-598 residue (Fig. 3B). As a control, we substituted the adjacent serine (Ser-597) with alanine in HAP1A. This mutant HAP1A (S597A) also forms cytoplasmic puncta that colocalize with transfected 14-3-3. These data indicate that Thr-598 is a residue critical for the interaction between HAP1A and 14-3-3. To examine whether there would be a comparable specificity with soluble HAP1A, we performed HAP1 immunoprecipitation of HEK293 cells cotransfected with 14-3-3 and either wild type HAP1A or mutant T598A. The result establishes that T598A is not associated with 14-3-3 (Fig. 3C).

Next, we explored whether altered phosphorylation regulates the interaction of 14-3-3 with HAP1A. To increase intracellular phosphorylation, we treated HAP1A-transfected cells with okadaic acid, a phosphatase inhibitor. For purposes of comparison, we reduced cellular phosphorylation by treating the cells with staurosporine, a protein kinase inhibitor. Purified GST-14-3-3 was then used to pull down transfected HAP1A. Okadaic acid treatment greatly enhanced the interaction between HAP1A and 14-3-3 in a dose-dependent manner, whereas staurosporine decreased this interaction (Fig. 3D). Given the effect of Thr-598 mutation on HAP1A binding to 14-3-3, these data also support the idea that the interaction between HAP1A and 14-3-3 is dependent on the phosphorylation of Thr-598 in HAP1A, and our findings also help to explain the selective binding of 14-3-3 to HAP1A in mammalian cells.



**FIGURE 3. Phosphorylation-dependent interaction of HAP1A with 14-3-3.** *A*, *in vitro* binding of GST-14-3-3 $\zeta$  to synthesized nonphosphorylated (HAP1a) and phosphorylated (p-HAP1a) peptides of C-terminal HAP1A. Nonphosphorylated SWTY and phosphorylated SWTY peptides (SWpTY) were also included for comparison. The sequences of these peptides are described under "Experimental Procedures," and their binding to GST-14-3-3 $\zeta$  was assessed via competitive binding fraction assays. The  $K_d$  values for SWTY, SWpTY, HAP1a, and p-HAP1a are  $>17$ ,  $0.13 \pm 0.03$ ,  $>17$ , and  $0.16 \pm 0.03$   $\mu$ M, respectively. *B*, immunofluorescent double labeling of mutant HAP1 (T598A and S597A) (green) and HA-14-3-3 (red) in transfected HEK293 cells with guinea pig antibody to HAP1 and mouse antibody to HA. Substitution of the phosphorylated Thr-598 residue with alanine prevents the association of 14-3-3 with HAP1A puncta. By contrast, 14-3-3 colocalizes with mutant HAP1A (S597A) in the cytoplasmic puncta (lower panel). *C*, immunoprecipitation (IP) of transfected HAP1A or mutant HAP1A T598A with guinea pig antibody to HAP1. The precipitates were probed with mouse antibody to HA and guinea pig antibody to HAP1. Transfected HA-14-3-3 was coprecipitated with HAP1A but not T598A. *D*, *in vitro* GST-14-3-3 pulldown of transfected HAP1A from HEK293 cells treated with okadaic acid or staurosporine at the concentrations indicated. Control is GST pulldown. The blots were probed by rabbit antibodies to HAP1A (upper panel) and GST (lower panel). Input is cell lysates for pulldown.

**14-3-3 Suppresses HAP1A Association with Kinesin Light Chain**—14-3-3 was found to regulate the subcellular distribution of many target proteins, potentially via alteration of pro-



**FIGURE 4. Overexpression of 14-3-3 suppresses the association of HAP1A with kinesin light chain.** *A*, HEK293 cells were transfected with HAP1A alone or HA-14-3-3 and HAP1A. The cell lysates used for GST-KLC pull-down were immunoprecipitated with guinea pig anti-HAP1 to show coprecipitation of HAP1A and 14-3-3. *B*, *in vitro* GST-KLC pull-down of transfected HAP1A from HEK293 cells that were cotransfected either with (+,  $n = 3$ ) or without (–,  $n = 3$ ) 14-3-3. Note that less HAP1A was pulled down by GST-KLC (upper panel) in the presence of transfected 14-3-3. The blots were also probed with antibody to GST to reveal GST and GST-KLC (lower panel). *C*, GST or GST-dynactin p150 (GST-p150) pull-down of HAP1A in the presence (+) or absence (–) of transfected 14-3-3. The blots were probed with antibody to GST to reveal GST and GST-p150, in which lower smear bands may be degraded products. *D*, densitometry analysis of the ratios of HAP1A pull-down to input (mean  $\pm$  S.E.,  $n = 3$  experiments; \*\*,  $p < 0.01$ ). Note that transfected 14-3-3 reduces the binding of HAP1A to GST-KLC. *E*, immunofluorescent labeling of HEK293 cells cotransfected with KLC2 (green) and HAP1A (red). Note that KLC2 colocalizes with cytoplasmic HAP1A puncta (arrows in merged image). *F*, coexpression of KLC2, HAP1A, and HA-14-3-3 in transfected HEK293 cells. The cells were stained with rabbit antibody to KLC2 (green), guinea pig antibody to HAP1 (red), and mouse antibody to HA (blue). Note that in the presence of 14-3-3, less KLC2 localizes to HAP1A puncta, in which 14-3-3 labeling is intense (arrows).

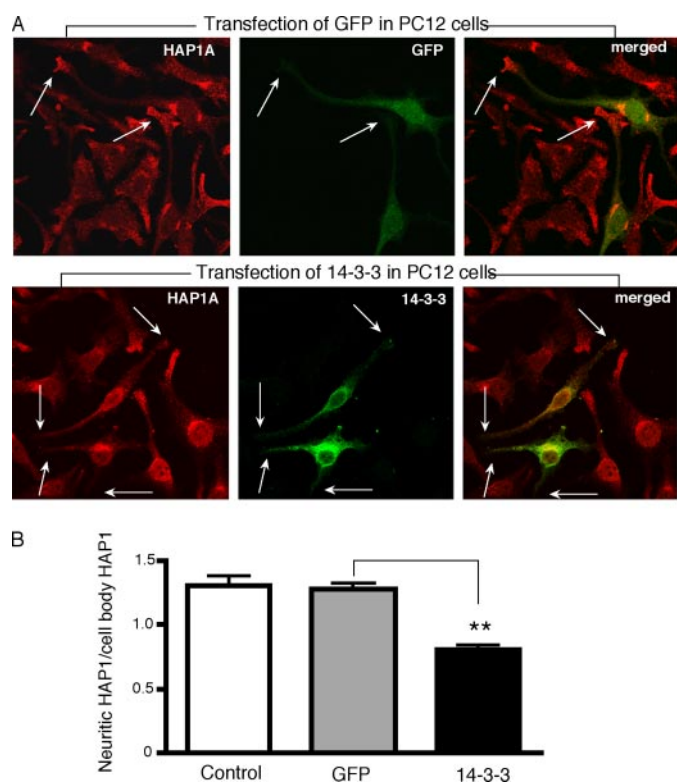
tein-protein interactions (23, 32). We noted previously that phosphorylation of Thr-598 in HAP1A reduces the interaction of HAP1A with KLC and decreases its localization to neuronal processes (6). These findings prompted us to investigate

whether 14-3-3 can alter the association of HAP1A with KLC, based on the assumption that 14-3-3 might compete with KLC for binding HAP1A. To explore this, we performed GST-KLC pull-down with lysates of HEK293 cells containing transfected HAP1A and 14-3-3. As expected, transfected 14-3-3 was precipitated with HAP1A (Fig. 4A). We also incubated the cell lysates with GST or GST-KLC to pull down transfected HAP1A (Fig. 4A). Consistent with our previous report (9), GST-KLC interacted with HAP1A, whereas GST did not. However, the presence of transfected 14-3-3 reduced the interaction of HAP1A with GST-KLC (Fig. 4B), and this effect was confirmed by quantitative assessment of the HAP1 ratio of pull-down to input (Fig. 4D). We did not observe the presence of 14-3-3 in GST-KLC pull-down (data not shown), suggesting that 14-3-3 binds soluble HAP1A and inhibits its binding to GST-KLC. We also tested the effect of 14-3-3 on the interaction of HAP1A with GST-dynactin p150, a trafficking protein that also binds HAP1 (7, 8). Under the same conditions as those for the GST-KLC pull-down, we did not observe any significant effects of transfected 14-3-3 on the interaction of HAP1A with GST-p150 dynactin (Fig. 4, C and D).

The inhibitory effect of 14-3-3 on the association of HAP1A with KLC may also cause the dissociation of KLC from HAP1A puncta in cells. To test this idea, we examined the localization of HAP1A and KLC in the presence of transfected 14-3-3 in HEK293 cells. In agreement with previous reports (36, 37), transfected KLC is diffuse in the cytoplasm of cells (data not shown). When coexpressed with HAP1A in the absence of transfected 14-3-3, KLC is localized to HAP1A puncta (Fig. 4E). When 14-3-3 was also cotransfected with KLC and HAP1A, 14-3-3 was almost completely colocalized with HAP1 puncta. Importantly, there was very little KLC localized to HAP1 puncta (Fig. 4F). This result gives credence to the idea that overexpressed 14-3-3 can suppress the association of HAP1A with KLC.

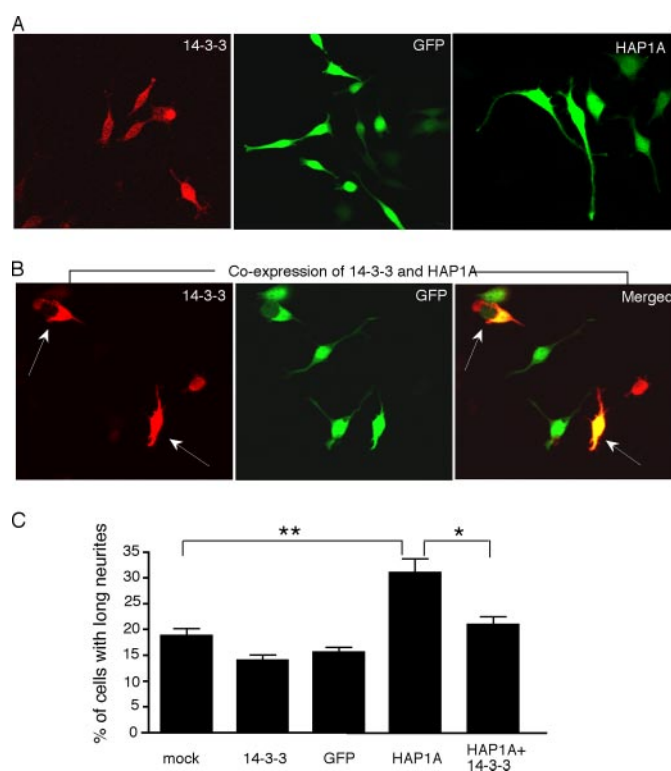
Downloaded from www.jbc.org at Johns Hopkins University on March 7, 2007

## Interaction of 14-3-3 with HAP1



**FIGURE 5. 14-3-3 regulates the subcellular distribution of HAP1A.** *A*, immunofluorescent labeling of endogenous HAP1A in NGF-treated PC12 cells. In cells overexpressing GFP (green in upper panel), HAP1A (red) was enriched in nerve terminals (upper panel). In cells overexpressing 14-3-3 (green in lower panel), HAP1A (red) colocalized with 14-3-3 and accumulated in cell bodies. *B*, quantitative analysis of the ratio of HAP1A signal in neurite tips to HAP1A signal in cell bodies. Note that the neuritic accumulation of HAP1A was diminished in PC12 cells transfected with 14-3-3 ( $n = 30$ ,  $p < 0.01$ ).

*Overexpression of 14-3-3 Alters the Subcellular Distribution of HAP1A*—It has been reported previously that HAP1A transports bidirectionally on microtubules and accumulates in nerve terminals (9, 19, 38, 39). KLC is known to participate in anterograde transport mediated by kinesin motors (10, 40). The finding that 14-3-3 suppresses the interaction between HAP1A and KLC led us to investigate whether 14-3-3 regulates the distribution of HAP1A in the processes of PC12 cells. Because neuronal cells express several forms of 14-3-3, it is difficult to suppress 14-3-3 expression via short interfering RNA. Also, there is a competitive effect of 14-3-3 on HAP1 binding to KLC that can be assessed by overexpressing 14-3-3. Therefore, we transfected 14-3-3 into PC12 cells, which then underwent NGF treatment that promotes neurite outgrowth. We next selected cells with elongated neurites to see whether 14-3-3 alters the distribution of endogenous HAP1A in neuronal processes. In control cells transfected with GFP only, HAP1A was enriched in neurite tips, and its distribution was similar to that found in nontransfected PC12 cells (Fig. 5*A*). By contrast, in cells transfected with 14-3-3, HAP1A accumulated within the cell bodies, showing a reduced distribution in neurite tips compared with nontransfected cells (Fig. 5*A*). Quantitative analysis of the ratio of the HAP1A signal in neurite tips to the HAP1A signal in the cell body revealed that the neuritic accumulation of HAP1A was diminished in the presence of transfected 14-3-3 (Fig. 5*B*).



**FIGURE 6. 14-3-3 inhibits the effect of HAP1A in promoting neurite outgrowth.** *A*, images of PC12 cells transfected with HA-14-3-3 (red) or infected with either adenoviral GFP or adenoviral HAP1A that also expresses GFP (green). Mouse anti-HA was used to reveal 14-3-3 expression. *B*, PC12 cells were transfected with 14-3-3 and infected with adenoviral HAP1A. Arrows indicate cells that express both 14-3-3 and HAP1A and show shorter neurites than those that only express HAP1A. *C*, percentage (mean  $\pm$  S.E.) of PC12 cells that had neurites longer than two cell bodies. Each group contains 300–350 cells (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) from three different transfections.

This result suggests that overexpressed 14-3-3 decreases the trafficking of HAP1A to the neuronal processes and neurite tips.

*14-3-3 Inhibits the Function of HAP1A in Promoting Neurite Outgrowth*—In our previous studies, we found that HAP1A is enriched in neurites of differentiated PC12 cells and that overexpression of HAP1A can promote neurite outgrowth of PC12 cells (9, 19). Tang *et al.* (41) have reported that 14-3-3 overexpression can decrease the number of cells with long neurites. If 14-3-3 reduces the distribution of HAP1A in neurite tips, it may also inhibit the ability of HAP1A to promote neurite outgrowth. To test this hypothesis, we transfected PC12 cells with 14-3-3, followed by adenoviral HAP1A infection for 16 h. The HAP1A-adenoviral vector independently expresses GFP (31), allowing us to identify GFP-positive cells that also express HAP1A and to compare cells that express GFP only via infection by the adenoviral GFP vector. Also, the distribution of GFP in neurites is not affected by 14-3-3 and can accurately reflect the length of neurites (Fig. 6*A*). PC12 cells with neurites longer than two body diameters were counted as having long neurites. There were more cells overexpressing HAP1A (31%) that showed longer neurites than those expressing 14-3-3 (14%) or GFP (18.8%) alone (Fig. 6, *B* and *C*). Importantly, overexpression of 14-3-3 reduced the number (20.8%) of HAP1A-positive cells with long neurites. These data bolster the idea that 14-3-3 suppressed the

distribution of HAP1A in neurites and its related function of promoting neurite outgrowth.

## DISCUSSION

14-3-3 proteins are multifunctional regulators, and they bind a large number of proteins (42, 43). Their interactions with various partners allow 14-3-3 proteins to participate in a variety of cellular processes, including intracellular signaling, cell survival, and protein trafficking (23–28). In this study, we provide several lines of evidence that 14-3-3 binds to and regulates the function of HAP1. First, the interaction of HAP1 with 14-3-3 is dependent on the phosphorylation of Thr-598 in HAP1A. Second, the *in vivo* association of HAP1 with 14-3-3 is confirmed by immunoprecipitation and colocalization of endogenous proteins in mouse brain. Third, overexpression of 14-3-3 can inhibit the binding of HAP1A to KLC. As a result, overexpressed 14-3-3 reduces the neuritic distribution of HAP1A and affects its function in promoting neurite outgrowth.

The phosphorylation-dependent binding of 14-3-3 to its partners is a well known phenomenon (23, 24, 26). Although 14-3-3 often binds to phosphopeptides, additional interactions between the phosphopeptide ligand and residues present in the groove of 14-3-3 also contribute to binding specificity (43–46). Indeed, both HAP1A and HAP1B contain internal sequences (<sup>113</sup>RPGVSGP<sup>119</sup> and <sup>439</sup>RKFITDP<sup>445</sup>, respectively) that fit well with the consensus binding motif for 14-3-3, RXXXpSXP (24, 27), and both HAP1A and HAP1B bind 14-3-3 in the yeast two-hybrid system. It is known that 14-3-3 binds multiple sites on a single ligand and that one of them can function as a gatekeeper whose phosphorylation is necessary for 14-3-3 binding but may not be sufficient for full biological activity (48). Our recent study has found that the C-terminal RSSTL of HAP1A is a potential protein kinase A phosphorylation site (RSpS or RSSpT) (6, 49). Although the C-terminal sequences of HAP1A are not within the established optimal 14-3-3-binding motifs, mode I (RSXpSXP) and mode II (RX(Y/F)XpSXP) (24, 50), the amino acid Thr-598 in C-terminal HAP1A would appear to fit with a novel binding motif mode III ((pS/T)X<sub>1–2</sub>-COOH), which has been identified as a mediator of 14-3-3 interactions with several proteins (30, 51, 52). Because 14-3-3 can bind multiple sites in the same protein (46, 48, 53, 54), and because phosphorylation of amino acids within or outside the 14-3-3-binding sites can dramatically regulate protein interactions with 14-3-3 (43–46), the findings in this study reveal that the interaction of HAP1A with 14-3-3 is at least critically regulated by phosphorylation of the C terminus of HAP1A.

HAP1 has been found to interact with a number of proteins, including Huntingtin and microtubule-dependent transporters. It is likely that HAP1 is dynamically involved in different protein complexes to execute its trafficking function. 14-3-3 also interacts with a large number of proteins and appears to regulate the formation of different protein complexes, therefore influencing the function, distribution, and trafficking of various proteins. For example, 14-3-3 suppresses the binding of KCNK3 potassium channels (55) and kainate receptor subunit KA2 (56) to the coatamer protein complex I vesicle coat, allowing forward transport. We have shown previously that the phosphorylation of Thr-598 regulates the interactions of HAP1

with KLC2 (6). However, it remains unclear how this regulation occurs. Our findings in this study suggest that phosphorylation of HAP1A increases its interaction with 14-3-3, and that this increased interaction suppresses the association of HAP1A with KLC2. It is also possible that 14-3-3 binds KLC2 to regulate its binding to HAP1, because KLC2 is found to interact with 14-3-3 (57). However, we did not observe that GST-KLC pulled down 14-3-3 under experimental conditions allowing for the interaction of KLC with HAP1 (data not shown). Instead, overexpressed 14-3-3 occupies HAP1 puncta and reduces the localization of KLC to HAP1 puncta in cultured cells. The inhibitory effect of 14-3-3 on the HAP1-KLC interaction could account for the reduced distribution of HAP1A in neurites when 14-3-3 is overexpressed. As a result, the decreased association of KLC with HAP1A can also affect the function of HAP1A in promoting neurite outgrowth. It has been reported that overexpression of 14-3-3 suppresses NGF-induced neurite outgrowth in PC12 cells (41). Identification of the interaction between HAP1A and 14-3-3 provides mechanistic insight into this phenomenon.

In addition to microtubule-dependent transporter proteins, several receptors are also found to bind HAP1, including  $\gamma$ -aminobutyric acid, type A, receptors (31, 58), the type 1 inositol 1,4,5-trisphosphate receptor (59), and the androgen receptor (20). The interactions of HAP1 with microtubule-dependent transporters and these receptors are consistent with the theory that HAP1 participates in vesicular trafficking and/or endocytosis. An important question has been how these interactions are regulated such that HAP1 can participate in trafficking or transport of various targets. Because HAP1 is found to be involved in the trafficking of BDNF (2), we examined cultured rat cortical neurons in which BDNF is transported to neuronal processes. However, we did not find that overexpressed 14-3-3 could significantly alter the distribution of transfected BDNF in neurites (supplemental Fig. 1). In conjunction with the fact that overexpressed 14-3-3 does not affect the association of HAP1 with dynactin p150, 14-3-3 appears to specifically regulate the interaction of HAP1 with kinesin light chain. Thus, the findings in this study implicate that the association of HAP1 with various proteins is likely to be differentially regulated by 14-3-3 and other molecules.

Unlike htt and 14-3-3, which are ubiquitously expressed, HAP1 is expressed in different types of neurons at varying levels. Thus, it would be interesting to know whether 14-3-3-mediated regulation is also cell type-specific or involved in pathological conditions. Our findings in this study have led us to propose a model for future studies. In this model, the C-terminal phosphorylation of HAP1A increases its association with 14-3-3, resulting in a decreased association of HAP1A with kinesin. As a result, less HAP1A is transported to neuronal processes via anterograde transport. On the other hand, nonphosphorylated HAP1 has a decreased affinity to bind 14-3-3 and is likely to favor the binding of other trafficking proteins so that it is transported along neurites to accumulate in neurite tips (6).

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