Par-4 Inhibits Choline Uptake by Interacting with CHT1 and Reducing Its Incorporation on the Plasma Membrane

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CHT1 is a Na+- and Cl−-dependent, hemicholinium-3 (HC-3)-sensitive, high affinity choline transporter. Par-4 (prostate apoptosis response-4) is a leucine zipper protein involved in neuronal degeneration and cholinergic signaling in Alzheimer’s disease. We now report that Par-4 is a negative regulator of CHT1 choline uptake activity. Transfection of neural IMR-32 cells with human CHT1 conferred Na+-dependent, HC-3-sensitive choline uptake that was effectively inhibited by cotransfection of Par-4. Mapping studies indicated that the C-terminal half of Par-4 was physically involved in interacting with CHT1, and the absence of Par-4/CHT1 complex formation precluded the loss of CHT1-mediated choline uptake induced by Par-4, indicating that Par-4/CHT1 complex formation is essential. Kinetic and cell-surface biotinylation assays showed that Par-4 inhibited CHT1-mediated choline uptake by reducing CHT1 expression in the plasma membrane without significantly altering the affinity of CHT1 for choline or HC-3. These results suggest that Par-4 is directly involved in regulating choline uptake by interacting with CHT1 and by reducing its incorporation on the cell surface.

Cholinergic neurotransmission plays a major role in the regulation of many physiological functions (1–3). For example, basal forebrain cholinergic neurons are involved in learning and memory processes, and hypoactivity of the cholinergic system may be directly responsible for the cognitive deficit in Alzheimer’s disease (AD) (4–8). Reductions in choline acetyltransferase (ChAT) activity and a profound loss of cholinergic neurons in the nucleus basalis magnocellularis have been observed in AD brain (5–12). High affinity choline uptake is generally believed to be the rate-limiting step in acetylcholine synthesis in cholinergic neurons and is essential for cholinergic transmission in the central nervous system (13–16). Reduced choline uptake has been implicated in degenerating neurons in AD (15, 17), although spared cholinergic nerve terminals in AD may show a compensatory increase in choline uptake (18, 19).

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‡ The abbreviations used are: AD, Alzheimer’s disease; ChAT, choline acetyltransferase; HC-3, hemicholinium-3; hCHT1, human choline transporter-1; Par-4, prostate apoptosis response-4; NGF, nerve growth factor; PBS, phosphate-buffered saline; TFW, trophic factor withdrawal; SGLT1, sodium glucose cotransporter-1.

MATERIALS AND METHODS

Primary Neuronal Cultures, Analysis of Cholinergic Neuronal Phenotype, and Trophic Factor Withdrawal—Dissociated hippocampal, cortical, and cerebellar neuronal cultures were prepared from postnatal day 1 mouse pups using methods similar to those described previously (27). Briefly, hippocampal, cortical, and cerebellar tissues and the pillow-like basal forebrains were removed and incubated for 15 min in Ca2+- and Mg2+-free Hank’s balanced saline solution (Invitrogen) containing 0.2% papain. Cells were dissociated by trituration and plated onto polyethyleneimine-coated plastic or glass-bottom culture dishes containing minimal essential medium with Earle’s salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 1 mM pyruvate, 20 mM KCl, 10 mM sodium bicarbonate, and 1 mM HEPES (pH 7.2). Following cell attachment (3–6 h post-plating), the culture medium was replaced with Neurobasal medium with B-27 supplements (Invitrogen). Experiments were performed in 7-day-old cultures. Expression of the cholinergic neuronal phenotype in basal forebrain cultures was determined by measurement of the specific activity of ChAT in the cultures as described in our previous study (31). ChAT activity is expressed as picomoles of acetylcholine synthesized per mg of total protein in a 30-min period. In addition, as a measure of physiological responsiveness of the basal forebrain cholinergic neurons, nerve growth factor (NGF)-mediated changes in ChAT activity were monitored, as it has been shown that NGF enhances the specific activity of this cholinergic phenotypic marker (31). Primary stocks of mouse 2.5 S NGF were made at 100 μg/ml in 0.02% acetic acid. Working solutions of NGF were prepared at 10 μg/ml in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin. Trophic factor withdrawal (TFW) was initiated by washing cultures four times with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM
IMR-32 cells were washed twice with Krebs-Ringer HEPES buffer (130 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 5 mM Tris, and 10 mM glucose (pH 7.4) using isotonic replacement of NaCl with LiCl as described previously (33, 34). In brief, transfected cells were incubated in buffer containing 140 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 5 mM Tris, and 10 mM glucose (pH 7.4) for 30 min at 4°C. To examine the effect of Par-4 on the choline uptake activity of hCHT1, IMR-32 cells were cotransfected with CHT1 and Par-4; the hCHT1 uptake activity of hCHT1 was analyzed at increasing concentrations of hCHT1; and specific hCHT1 uptake was determined by subtracting the nonspecific uptake (as determined by the choline uptake in the absence of 1 µM HC-3) from the total uptake. $K_v$ for choline was calculated by nonlinear least-square fits using the Hill equation (SigmaPlot Version 8.0, SPSS Inc.).

Immunoprecipitation/Western Blot Analysis—The methods used have been described previously (35). In brief, to examine Par-4/CHT1 complex formation, aliquots of cell lysates containing 200 µg of protein were incubated for 1 h at 4°C with appropriate dilutions of mouse anti-Par-4 antibodies in buffer containing 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, and 5% normal goat serum in PBS, and membranes were permeabilized by incubation for 1 h with a mixture of Texas Red-labeled anti-rabbit and fluorescein-labeled anti-mouse antibodies. Membranes were blocked with 5% milk, followed by 1-h incubation in the presence of anti-Par-4 or rabbit anti-hCHT1 antibodies in immunoprecipitation buffer and solubilized by heating in Laemmli solubilization buffer containing 2-mercaptoethanol at 100°C for 4 min. 50 µg of solubilized proteins were separated by electrophoresis on a 4–12% gradient SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. For Western blot analysis, the nitrocellulose sheet was blocked with 5% milk, followed by 1-h incubation in the presence of 1:100 dilution of mouse anti-Par-4 or rabbit anti-hCHT1 antibodies in immunoprecipitation buffer and solubilized by heating in Laemmli solubilization buffer containing 2-mercaptoethanol at 100°C for 4 min. The sensitivity of immunoblotting was determined by preincubating the blot with 0.05 µg/ml of horseradish peroxidase-conjugated secondary antibody, and immunoblotted proteins were detected by chemiluminescence using the ECL system (Amersham Biosciences). To further confirm that Par-4/CHT1 interaction was specific, similar immunoprecipitation/Western blotting methods were employed to examine whether Par-4 interacts with a similar transporter protein, the sodium/choline cotransporter SGLT1, using a rabbit anti-SGLT1 polyclonal antibody (Chemicon International, Inc.). Equal loading was verified by probing the blots with anti-β-actin antibody. Western blot images were acquired and quantified using Kodak Image Station 2000R and Kodak Digital Science 1D Version 3.6 software.

**Immunohistochemistry and Confocal Laser Scanning Microscopy**—The affinity-purified rabbit anti-hCHT1 polyclonal antibody was a generous gift from Dr. Takashi Okuda (University College London, London, United Kingdom). This antibody has been reported to be able to detect the CHT1 protein on Western blots as two major ~45- and 80-kDa bands that represent oligomers and/or heterogeneous glycosylation of the CHT1 protein (32, 33). In CHT1-transfected IMR-32 cells, the antibody recognizes three major bands of ~50, 60, and 65kDa. The 65kDa band corresponds closely to the mass of the major band of hCHT1 primary structure (see “Results”). IMR-32 cells expressed little or no endogenous CHT1 (see “Results”). The anti-Par-4 antibody was a mouse monoclonal antibody raised against full-length rat Par-4 (Santa Cruz Biotechnology). This antibody recognizes both human and rodent Par-4 proteins at ~38 kDa. For immunocytochemical analysis of hCHT1 and Par-4 expression, the cultured cells were fixed for 30 min in 4% paraformaldehyde and PBS, and membranes were permeabilized by incubation in 0.2% Triton X-100 and PBS. Cells were incubated for 1 h in blocking serum (5% normal goat serum in PBS). Cells were then exposed to primary antibodies (1:1000 dilution of rabbit anti-hCHT1 polyclonal antibody and 1:100 dilution of mouse anti-Par-4 monoclonal antibody) overnight at 4°C, followed by incubation for 1 h with a mixture of Texas Red-labeled anti-rabbit and fluorescein-labeled anti-mouse secondary antibodies (Vector Labs, Inc., Burlingame, CA). Images of Par-4 and/or CHT1 immunofluorescence were acquired using a confocal laser scanning microscope (dual wavelength scan) with a ×60 oil immersion objective. All images were acquired using the same laser intensity and photodetector gain to allow quantitative comparisons of relative fluorescence intensity in the same cell and sites of colocalization of immunoreactivities were determined using Fluoview Version 2.0 software.

**[3H]Choline Uptake Assay**—[3H]Choline uptake assays were performed essentially as described previously (33, 34). In brief, transfected IMR-32 cells were washed twice with Krebs-Ringer HEPES buffer (150 mM NaCl, 2.7 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM MgCl$_2$, 0.5 mM CaCl$_2$, 0.1% glucose, 10-15 µM HC-3, and 10 µM HEPES (pH 7.4)) and incubated at 37°C for 2 h, followed by an additional incubation in Krebs-Ringer HEPES buffer containing 1 µM methyl-[3H]choline chloride (83 Ci/mmol; Amersham Biosciences) for 10 min. Choline uptake was terminated by washing cells three times with ice-cold Krebs-Ringer HEPES buffer. The level of accumulated [3H]choline was determined by solubilizing cells in 1% SDS and 0.2% NaOH, and radioactivity was measured with a liquid scintillation counter (Beckman Coulter LS 6500). The sensitivity of [3H]choline uptake to HC-3 was assessed by measuring [3H]choline uptake in the presence of 1 µM HC-3. In these experiments, experimental data were normalized by protein content, and specific [3H]choline uptake is represented as percent uptake above background (background choline uptake was determined in cells transfected with the empty vectors alone). The Na$^+$ dependence of [3H]choline uptake was assessed in buffer containing 140 mM NaCl, 2 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 5 mM Tris, and 10 mM glucose (pH 7.4) using isotonic replacement of NaCl with LiCl as described previously (33, 34). To test for competition for the choline uptake site, HEPES buffer containing 100 µM HC-3 was preincubated with or without 1 µM unlabeled HC-3 for 15 min at room temperature. Specific binding was determined by subtracting the nonspecific binding (as determined by the choline uptake in the presence of 1 µM HC-3) from the total uptake. $K_v$ for choline was calculated by nonlinear least-square fits using the Hill equation (SigmaPlot Version 8.0, SPSS Inc.).

**Cell-surface Biotinylation Assay**—These assays were used to analyze the cell-surface expression of CHT1 because biotinylated proteins are considered to be mostly expressed on the cell surface since they are greatly depleted of the intracellular marker actin (33). The methods used are described previously (33). In brief, cells were washed with Krebs-Ringer HEPES buffer and preincubated for 1–2 h at 37°C for equilibrium. Binding assays were performed at 4°C for 1–2 h and terminated by three washes with ice-cold Krebs-Ringer HEPES buffer. Saturation binding was determined using 0.1–10 nM [3H]-HC-3 with or without 1 µM unlabeled HC-3. Specific binding was determined by subtracting binding in the presence of 1 µM unlabeled HC-3 from total binding. Because HC-3 is hydrophilic and cell membrane-impermeable, the HC-3 binding activity represents the expression level of the transporter in the plasma membrane (33). $K_v$ for HC-3 was calculated by nonlinear least-square fits using the Hill equation (SigmaPlot Version 8.0).
RESULTS

Par-4 Is Present in Basal Forebrain Cholinergic Neurons and Colocalizes in the Same Neuron Positive for the Cholinergic Marker CHT1—Primary neuronal cultures of different brain regions (cortical, hippocampal, cerebellar, and basal forebrain) were established, and expression of the cholinergic neuronal phenotype in basal forebrain cultures was confirmed by measurement of the specific activity of ChAT in the cultures as described in our previous study (31). As shown in Fig. 1, primary basal forebrain neuronal cultures showed the most significant amount of basal ChAT activity (an average of −3832 pmol/mg of protein/30 min) compared with cortical, hippocampal, and cerebellar neuronal cultures. In addition, NGF-mediated changes in ChAT activity were also significantly greater in basal forebrain neuronal cultures than in cortical, hippocampal, and cerebellar cultures (Fig. 1b). These results are consistent with the overall cholinergic phenotype of the basal forebrain neuronal cultures. Western blot analysis showed that basal levels of Par-4 were relatively high in the basal forebrain, hippocampus, and cerebral cortex and low in the cerebellum (Fig. 1, c and d). We next examined whether Par-4 colocalizes in the same basal forebrain neuron positive for the cholinergic marker CHT1. Immunocytochemical analysis of cultured basal forebrain cholinergic neurons revealed that both Par-4 and CHT1 were predominantly located in cytoplasmic compartments (Fig. 1e). This observation is consistent with previous reports showing that, although CHT1 is constantly cycled through the plasma membrane and intracellular compartments, CHT1 is predominantly intracellular (33, 34). Double labeling immunocytochemistry using confocal laser scanning microscope further showed significant colocalization between Par-4 and hCHT1 in the perikaryon and neurites (Fig. 1e).

CHT1 Confers Na+-dependent, HC-3-sensitive Choline Uptake in IMR-32 Cells: Inhibition by Par-4 in Transfected IMR-32 Cells and in Basal Forebrain Neurons—Since Par-4 is present and colocalizes with CHT1 in cholinergic neurons, we next tested the hypothesis that Par-4 regulates the specific choline uptake activity of CHT1 in transfected human neuroblastoma IMR-32 cells. The IMR-32 cell line exhibits many cholinergic and adrenergic properties and has been used as a model for studying cholinergic and adrenergic neurotransmission (36). The results from representative Western blot analysis showing increased levels of expression of Par-4 and/or ChT1 in transfected IMR-32 cells are given in Fig. 2 (a and b). Note that anti-CHT1 antibody detected the CHT1 protein on Western blots as three major bands of ~80, 68, and 45kDa. The 68-kDa band corresponds closely to the molecular mass predicted from hCHT1 primary structure, whereas various other bands may represent oligomers and/or heterogeneous glycosylation of the CHT1 protein. In examining the functional properties of CHT1, we found that transfection of CHT1 resulted in an ~2.3-fold increase in specific [3H]choline uptake compared with vector-transfected control cells (Fig. 2c). The increased [3H]choline uptake was completely abolished in the presence of 1 μM HC-3. Equimolar replacement of Na+ with Li+ also abolished the choline transport activity of the CHT1-transfected cells (Fig. 2c). The CHT1-mediated [3H]choline uptake was concentration-dependent and saturable (Fig. 2d). Since IMR-32 cells express little or no endogenous CHT1, the background levels of choline uptake observed in vector-transfected control cells and in Par-4-transfected cells were not significantly affected by HC-3 or replacement of Na+ with Li+. These results demonstrate that transfection of CHT1 confers Na+-dependent, HC-3-sensitive choline transport activity in IMR-32 cells.

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Coexpression of Par-4 resulted in a significant (~41%) reduction of Na+-dependent, HC-3-sensitive choline uptake mediated by CHT1 (Fig. 2c). A dose-response analysis with increasing concentrations of [3H]choline also showed that specific choline uptake was significantly reduced in cells coexpressing Par-4 and CHT1 compared with cells transfected with CHT1 alone (Fig. 2d). Kinetic analysis indicated that cells coexpressing Par-4 and CHT1 showed a significant reduction in Vmax with no apparent alteration in CHT1 affinity for choline (Fig. 2d and Table 1). To further confirm that the inhibitory effect of Par-4 on CHT1 transporter activity was not an artifact of transfection and overexpression, proteins, we examined whether induction of Par-4 would decrease CHT1-mediated choline uptake in primary basal forebrain neurons. We previously reported that apoptotic insults, such as TFW, induce aberrant Par-4 expression in primary neurons (24, 28, 31, 35). As shown in Fig. 2 e and f, a significantly decreased CHT1 transporter activity was observed in primary cholinergic neurons 8 h following TFW, the time point of maximal induction of Par-4 expression after TFW. The decrease in CHT1 activity was largely blocked by pretreatment of neurons with the antisense Par-4 oligonucleotide. These results show that induction of Par-4 was largely responsible for the observed decrease in CHT1 activity following TFW in basal forebrain neurons. The data suggest that inhibition of CHT1 activity by Par-4 is a physiologically relevant and pathologically meaningful event.

Par-4 Interacts with CHT1 in Transfected IMR-32 Cells and in Primary Neurons—The observations that Par-4 colocalizes with CHT1 in cholinergic neurons and that coexpression of Par-4 in IMR-32 cells results in a significant decrease in CHT1-mediated, Na+-dependent, HC-3-sensitive choline uptake indicate that Par-4 might directly modulate CHT1 transporter activity. Indeed, this hypothesis was supported by data from our co-immunoprecipitation studies showing that Par-4 physically interacted with CHT1 in transfected IMR-32 cells and in primary neurons. In transfected IMR-32 cells coexpressing Par-4 and CHT1, when immunoprecipitation was performed using the specific antibody against CHT1, a 38-kDa Par-4 band was clearly detected on the immunoblots (Fig. 3a). Reverse-order immunoprecipitation/Western blot analysis of the same transfected cell lines showed similar Par-4/CHT1 complex formation (Fig. 3b). Similar Par-4/CHT1 complex formation was confirmed in primary basal forebrain neurons at physiological concentrations of these proteins (Fig. 3, c and d). These results demonstrate that Par-4/CHT1 complex formation is physiologically relevant. To confirm that Par-4/CHT1 interaction is specific, we examined whether Par-4 would interact with the sodium/glucose cotransporter SGLT1. SGLT1 is a member of the Na+/glucose cotransporter family, shares a relatively significant amino acid homology with CHT1, and is reportedly expressed in the kidney, intestine, and brain (37). Immunoprecipitation/Western blot experiments using tissue extract from the hippocampus and basal forebrain showed that SGLT1 was detected in proteins from total lysate, but not from those immunoprecipitated with anti-Par-4 or anti-CHT1 antibody, indicating that neither Par-4 nor CHT1 interacted with SGLT1 (Fig. 3, e and f).
Fig. 1. Par-4 colocalizes with CHT1 in basal forebrain cholinergic neurons. a, analysis of primary neuronal cultures from the indicated brain areas showing the predominant cholinergic phenotype of basal forebrain neurons as measured by specific ChAT activity. ChAT activity is expressed as picomoles of acetylcholine synthesized per mg of total protein in a 30-min period. Values are the means ± S.E. of determinations made in six separate cultures. ****, p < 0.001 compared with the values in hippocampal, cortical, and cerebellar cultures. b, analysis of primary neuronal cultures from the indicated brain areas showing the predominant cholinergic phenotype of basal forebrain neurons as measured by NGF-mediated changes in ChAT activity. Primary neurons were cultured in the presence or absence (control) of 100 ng/ml NGF for 7 days. The NGF-mediated change in ChAT activity is expressed as percent increase in enzyme activity compared with non-NGF-treated control cultures. Values are the means ± S.E. of determinations made in six separate cultures. ***, p < 0.01 compared with the values in hippocampal, cortical, and cerebellar cultures. c, representative Western blot analysis showing that Par-4 was present in primary basal forebrain neurons cultured for 7 days. d, statistical analysis of relative levels of Par-4 in primary neurons from the indicated brain regions upon Western blotting. Note that Par-4 levels were significantly higher in basal forebrain neurons than in cerebellar, hippocampal, and cortical neurons. Values are the means ± S.E. of determinations made in six separate Western blots. ****, p < 0.001 compared with the value in cerebellar neurons; ***, p < 0.01 compared with the value in basal forebrain neurons (analysis of variance with Scheffe’s post-hoc tests). e, Par-4 colocalizes with CHT1 in basal forebrain cholinergic neurons. Representative confocal laser scanning microscopic images of primary neurons from basal forebrain are shown. Panel 1, Par-4 immunoreactivity as indicated by green fluorescence; panel 2, CHT1 immunoreactivity as indicated by red fluorescence; panel 3, merging of images from panels 1 and 2 showing areas of colocalization of Par-4 and CHT1 as indicated by yellow fluorescence. Note that Par-4 colocalized with CHT1 predominantly in the perikaryon and neurites.
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**FIG. 2.** Effect of Par-4 on the specific [3H]choline uptake activity of CHT1 in transfected IMR-32 cells and in primary cholinergic neurons. *a* and *b*, shown are the results from representative Western blot analysis indicating the levels of expression of Par-4 and/or CHT1 in transfected IMR-32 cells used in this study. Proteins from the indicated cell lines (50 μg of protein/lane) were immunoreacted with anti-Par-4 (*a*) or anti-hCHT1 (*b*) polyclonal antibody. The cell lines used were as follows: the untransfected parent cell line (Untransfected), the cell line transfected with empty vectors (Vector Transfected), cell clone C9 transfected with Par-4 (Par-4 Transfected), cell clone C10 transfected with hCHT1 (CHT1 Transfected), and cell clone C16 cotransfected with Par-4 and hCHT1 (Par-4/CHT1 Co-transfected). Similar data were obtained in other Par-4- and/or CHT1-transfected cell clones employed in this study. IMR-32 cells express little or no endogenous CHT1 and Par-4. Equal loading of proteins in each lane was confirmed by probing the blots for β-actin.

**c**, transfection of CHT1 conferred Na⁺-dependent, HC-3-sensitive choline uptake that is largely blocked by coexpression of Par-4 in IMR-32 cells. Specific [3H]choline uptake by the indicated IMR-32 cell lines was measured and is presented as percent uptake above background (background choline uptake was determined in cells transfected with the empty pcDNA3.1 and pREP4 vectors alone). The sensitivity of [3H]choline uptake to HC-3 was assessed by measuring [3H]choline uptake in the presence of 1 mM HC-3. The Na⁺ dependence of [3H]choline uptake was assessed using isotonic replacement of NaCl with LiCl. Similar data were obtained in at least three separate clones of each of the transfected cell lines. Values are the means ± S.E. of determinations made in six separate cultures. ****, *p* < 0.0001 compared with values of choline uptake in the presence of HC-3 or following the removal of Na⁺ in the CHT1-transfected cell group; ###, *p* < 0.01 compared with the corresponding value in the CHT1-transfected cell group. *d*, the [3H]choline uptake activity of CHT1 was analyzed at increasing concentrations of [3H]choline, and specific [3H]choline uptake was quantified by subtracting nonspecific uptake (as determined by the choline uptake in the presence of 1 μM HC-3) from total uptake. Values are the means ± S.E. of determinations made in six separate cultures. Similar data were obtained in at least three separate clones of each of the transfected cell lines. ****, *p* < 0.0001 compared with corresponding values in cells transfected with CHT1 alone. *e* and *f*, aberrant Par-4 expression induced by TFW inhibited CHT1 choline transporter activity in primary
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Kinetic analysis showed that coexpression of Par-4 with CHT1 resulted in significant reductions in $V_{\text{max}}$ in choline uptake assays and in $B_{\text{max}}$ in $[^{3}H]\text{HC-3}$ binding studies, with no apparent alterations in $K_{m}$ for choline or in $K_{D}$ for HC-3 binding. These results indicate that Par-4 reduced the choline uptake by inhibiting cell-surface expression of CHT1 without significantly altering the affinity of CHT1 for choline or HC-3.

| Cell lines          | Choline uptake | $[^{3}H]\text{HC-3 binding}$ |
|--------------------|---------------|-------------------------------|
|                    | $V_{\text{max}}$ | $K_{m}$ | $B_{\text{max}}$ | $K_{D}$ |
| CHT1               | 102.3 ± 5.1   | 2.5 ± 0.3 | 125.4 ± 8.9 | 7.2 ± 1.0 |
| CHT1 + Par-4       | 41.5 ± 2.1    | 2.8 ± 0.4 | 40.0 ± 3.9  | 7.5 ± 1.3 |

$^{a}p < 0.001$ compared with corresponding values in CHT1-transfected cell groups.

### TABLE I

|                         | Choline uptake | $[^{3}H]\text{HC-3 binding}$ |
|-------------------------|---------------|-------------------------------|
|                        | $V_{\text{max}}$ | $K_{m}$ | $B_{\text{max}}$ | $K_{D}$ |
| Choline uptake          | 102.3 ± 5.1   | 2.5 ± 0.3 | 125.4 ± 8.9 | 7.2 ± 1.0 |
| Par-4                   | 41.5 ± 2.1    | 2.8 ± 0.4 | 40.0 ± 3.9  | 7.5 ± 1.3 |

$^{a}p < 0.001$ compared with corresponding values in CHT1-transfected cell groups.

### DISCUSSION

Cholinergic neurotransmission is involved in the regulation of many complex motor, autonomic, behavioral, and cognitive functions (1–3). Profound loss of cholinergic function has been implicated in several neurodegenerative diseases, such as AD (4–8, 38, 39). Because cholinergic neurons lack the capacity to synthesize choline de novo, their function depends upon choline uptake. More important, Na$^{+}$-dependent, HC-3-sensitive, high affinity choline uptake is unique to cholinergic neurons and is generally believed to be the rate-limiting step in acetylcholine synthesis (13). These observations suggest that studies of the regulatory machinery of choline uptake should have significant implications regarding our understanding of normal cholinergic neurotransmission and pathogenesis of cholinergic hypoactivity in AD. The cloning and functional characterization of CHT1 genes from tissues of different species (Caenorhabditis cholinergic neurons). In e, basal forebrain neuronal cultures were pretreated for 2 h with either the antisense Par-4 oligonucleotide (AS; 25 μM) or no DNA (Control). Cultures were then subject to TFW for 8 h and processed for Par-4 immunofluorescence analysis by confocal laser scanning microscopy. Values are the means ± S.E. of determinations made in six separate cultures. $^{***}p < 0.001$ compared with values in control and antisense Par-4 oligonucleotide + TFW treatment groups. In f, basal forebrain neuronal cultures were pretreated for 2 h with either the antisense Par-4 oligonucleotide (25 μM) or no DNA. Cultures were then subject to TFW for 8 h, and the $[^{3}H]$choline uptake activity of CHT1 in basal forebrain neurons was then analyzed at 10 μM $[^{3}H]$choline. Specific $[^{3}H]$choline uptake was quantified by subtracting nonspecific uptake (as determined by the choline uptake in the presence of 1 μM HC-3) from total uptake. Values are the means ± S.E. of determinations made in six separate cultures.

$^{***}p < 0.001$ compared with values in control and antisense Par-4 oligonucleotide + TFW treatment groups (Analysis of variance with Scheffe’s post-hoc tests).
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Fig. 3. Par-4 interacts with CHT1 in transfected IMR-32 cells and in primary basal forebrain neurons. a and b. Par-4 interacts with CHT1 in transfected IMR-32 cells. In a, protein samples from IMR-32 cells cotransfected with CHT1 and Par-4 (clones C16, C12, and C29) were immunoprecipitated (IP) with anti-hCHT1 antibody, followed by Western blot analysis with anti-Par-4 antibody (lanes 4–6). Preimmune rabbit serum was used as a control (lanes 7–9). The Par-4/CHT1 complex was clearly observed in all three clones of transfected cells (lanes 4–6). The Par-4 protein in the transfected cells was detected on Western blots as a major band of ~38 kDa. In b, reverse-order immunoprecipitation/Western blot analysis of the same transfected IMR-32 cells showed similar Par-4/CHT1 complex formation (lanes 4–6). c and d, interaction between endogenous CHT1 and Par-4 in basal forebrain neurons. In c, proteins from total lysates of primary basal forebrain neurons were immunoprecipitated with rabbit anti-hCHT1 antibody (lane 2), followed by Western blotting with anti-Par-4 antibody. Preimmune rabbit serum was used as a control (lane 3). In d, reverse-order immunoprecipitation/Western blot analysis of the same basal forebrain neurons showed similar endogenous Par-4/CHT1 complex formation. e and f, neither Par-4 nor CHT1 interacts with the sodium/glucose cotransporter SGLT1. In e, proteins from total lysates of the hippocampal and basal forebrain tissues were immunoprecipitated with rabbit anti-Par-4 antibody (lanes 3 and 4), followed by Western blotting with anti-SGLT1 antibody. Similar data were obtained by reverse-order immunoprecipitation/Western blot analysis of the same transfected cells (data not shown). In f, proteins from total lysates of the hippocampal and basal forebrain tissues were immunoprecipitated with anti-CHT1 antibody (lanes 3 and 4), followed by Western blotting with anti-SGLT1 antibody. Similar data were obtained by reverse-order immunoprecipitation/Western blot analysis of the same transfected cells (data not shown). All Input lanes show 10% of the total protein used in immunoprecipitation experiments.

elegans, rat, human, mouse, Torpedo, and Limulus) mark a significant breakthrough in this effort (13, 20, 21, 33, 34, 40–48). The data from this study demonstrate that Par-4, a leucine zipper protein previously found to play a significant role in neuronal degeneration and cholinergic signaling, is a novel and effective regulator of CHT1-mediated, Na+-dependent, HC-3-sensitive choline uptake.

Although Par-4 was initially identified as playing an important role in hippocampal and cortical neuronal cell death in AD (24), an increase in Par-4 expression was subsequently shown to alter elements of cholinergic signaling even under non-apoptotic conditions. For example, overexpression of Par-4 results in a significant non-apoptotic reduction of ChAT activity in transfected neural cells (31), suggesting that Par-4 may be involved in the regulation of cholinergic function. The results from the present study indicate the following. 1) Par-4 was present in primary basal forebrain cholinergic neurons. 2) Transfection of IMR-32 cells with CHT1 conferred Na+-dependent, HC-3-sensitive choline uptake that was effectively inhibited by cotransfection of Par-4. This inhibitory effect of Par-4 was physiologically relevant because it was also observed in primary basal forebrain neurons. 3) Par-4 colocalized with CHT1 in basal forebrain neurons, and co-immunoprecipitation studies showed that Par-4 interacted with CHT1 in transfected IMR-32 cells and in basal forebrain neurons. 4) Neither Par-4 nor CHT1 interacted with SGLT1, a member of the Na+/glucose cotransporter family that shares a significant amino acid homology with CHT1, indicating that Par-4/CHT1 interaction is specific. 5) The CHT1 interaction domain was mapped to the C-terminal half of Par-4 using a C-terminal half deletion mutant of Par-4 (Par-4ΔCHT). 6) Coexpression of CHT1 with Par-4ΔCHT, which abolishes the interaction between Par-4 and CHT1, precluded the loss of the choline uptake function of CHT1 induced by Par-4, indicating that Par-4/CHT1 complex formation is necessary for the inhibitory effect of Par-4 on CHT1 activity. 7) Par-4 reduced the choline uptake by inhibiting CHT1 expression on the cell surface without significantly altering the affinity of CHT1 for choline or HC-3. These results suggest that Par-4 plays a direct role in regulating the choline uptake activity of CHT1 in neural cells.

The precise mechanisms by which Par-4/CHT1 complex formation alters choline uptake need to be carefully examined. It is noteworthy that, although aberrant induction of Par-4 expression sensitizes neural cells to a variety of apoptotic insults, overexpression of Par-4 alone does not significantly alter cell
growth rate and is not sufficient to induce spontaneous apoptosis in transfected IMR-32 cells. Therefore, it is unlikely that the reduction in choline uptake induced by overexpression of Par-4 in IMR-32 cells was due to an alteration in growth rate or induction of the cell death process. Although CHT1 is predominantly intracellular (34), CHT1 shuttles constitutively between the plasma membrane and intracellular compartments, and synaptic vesicles may function as a reservoir to deliver CHT1 to the plasma membrane following neuronal excitation (34). Since, under physiological conditions, Par-4 is located primarily in cytoplasmic compartments and is enriched in synaptic terminals (49), one possibility is that Par-4/CHT1 complex formation results in an increased retention of CHT1 in intracellular compartments, thereby reducing the incorporation of CHT1 on the plasma membrane. Our data clearly support this hypothesis. The fact that Par-4 interacts with CHT1 in transfected IMR-32 cells as well as in primary neurons demonstrates the physiological relevance of Par-4/CHT1 complex formation. The observation that neither Par-4 nor CHT1 interacted with the CHT1 homolog protein SGLT1 indicates that Par-4/CHT1 interaction is specific. Indeed, mapping studies further showed that the C-terminal half of Par-4 was directly involved in interacting with CHT1. Kinetic analyses and data from our HC-3 binding assays clearly showed that Par-4/CHT1 interaction in cotransfected IMR-32 cells resulted in significant reductions in V_{max} (in choline uptake assays) and in B_{max} (in [3H]HC-3 binding studies). These results indicate that Par-4/CHT1 complex formation results in an increased retention of CHT1 in intracellular compartments, thereby reducing the incorporation of CHT1 on the plasma membrane. Another possibility is that Par-4 binds to a critical functional domain of CHT1, resulting in an altered CHT1 conformation and therefore reduced binding affinity of CHT1 for choline and/or the substrate transport activity of CHT1. However, kinetic analyses found no apparent alterations in K_{m}, for choline or in K_{f} for HC-3 binding, indicating that Par-4/CHT1 complex formation does not significantly affect the affinity of CHT1 for choline or HC-3.

The identification of Par-4 as a novel and effective regulator of CHT1 activity is of great significance. Profound loss of cholinergic function has been linked to memory deficit in AD, and accordingly, overall high affinity choline transport is reduced in AD (1, 5–8, 15, 17). Although spared neurons in AD brain may show compensatory up-regulation of the high affinity choline transporter (18, 19), the data of the present study suggest that CHT1-mediated, Na^+-dependent, HC-3-sensitive, high affinity choline uptake is most likely to be significantly reduced in degenerating neurons wherein Par-4 is aberrantly overexpressed. Since aberrant induction of Par-4 expression has been observed in many degenerating neurons in AD patients (24) and since Par-4 expression is induced during the early phase of apoptotic neuronal death (upstream of mitochondrial dysfunction and caspase activa-

Western blot analysis of the same transfected cells showed the presence of the Par-4/CHT1 complex in cells coexpressing Par-4 and CHT1 (lane 3) and the absence of CHT1/Par-4 interaction in cells coexpressing Par4ΔCTH and CHT1 (lane 4). Similar data were obtained with at least two clones of each cotransfected cell line. All Input lanes show 10% of the total protein used in immunoprecipitation experiments. c, the [3H]choline uptake activity of CHT1 in the indicated cell lines was analyzed at 10 μM [3H]choline, and specific [3H]choline uptake was quantified by subtracting nonspecific uptake (as determined by the choline uptake in the presence of 1 μM HC-3) from total uptake. Values are the means ± S.E. of determinations made in six separate cultures. Similar data were obtained in at least three separate clones of each of the transfected cell lines. ***, p < 0.01 compared with corresponding values in cells transfected with CHT1 alone (analysis of variance with Scheffé’s post-hoc tests).

Fig. 4. Mapping of the interaction domain to the C-terminal half of Par-4: absence of Par-4/CHT1 interaction precludes the loss of the choline uptake function of CHT1 induced by Par-4. a, protein samples from IMR-32 cells cotransfected with CHT1 and Par-4 (clone C16) and those from IMR-32 cells cotransfected with CHT1 and Par-4ΔCTH (clone C8) were immunoprecipitated (IP) with anti-hCHT1 antibody, followed by Western blot analysis with anti-Par-4 antibody (lanes 3 and 4). Preimmune rabbit serum was used as a control (lanes 5 and 6). The Par-4/CHT1 complex was clearly observed in cells coexpressing Par-4 and CHT1 (lane 3), but was absent in cells coexpressing Par-4ΔCTH and CHT1 (lane 4). b, reverse-order immunoprecipitation/
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Fig. 5. Par-4 alters CHT1 levels in the plasma membrane: an essential role for Par-4/CHT1 interaction. a, the relative levels of CHT1 in the plasma membrane of the indicated cell lines measured by [3H]HC-3 binding assays using intact cells expressing CHT1 and those coexpressing Par-4/CHT1 or Par-4ΔCTH/CHT1. Specific [3H]HC-3 binding was quantified at increasing concentrations of [3H]HC-3 and calculated by subtracting nonspecific uptake (as determined by the choline uptake in the presence of 1 μM HC-3) from total uptake. Values are the means ± S.E. of determinations made in six separate cultures. Similar data were obtained in at least three separate clones of each of the transfected cell lines. ***, p < 0.001 compared with corresponding values in cells transfected with CHT1 alone (analysis of variance with Scheffe’s post-hoc tests). b, representative immunoblot showing reduced cell-surface expression of CHT1 as analyzed by cell-surface biotinylation assays. Immunoblots of 500 μg of biotinylated protein fractions using anti-CHT1 antibody demonstrated a significant decrease in cell-surface CHT1 immunoreactivity in cells cotransfected with Par-4 and CHT1 (lane 5) compared with CHT1 immunoreactivity in cells transfected with CHT1 alone (lane 4). Blots were stripped and reprobed with anti-hCHT1 polyclonal antibody and other related materials.  

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