Choline acetyltransferase (ChAT, 1 acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) catalyzes synthesis of the neurotransmitter acetylcholine (ACh) in cholinergic neurons. These neurons are distributed widely throughout both central and peripheral nervous systems controlling a range of physiological functions. Dysfunction of cholinergic neurotransmission is found in a number of neurologic and psychiatric disorders including Alzheimer disease, amyotrophic lateral sclerosis, Huntington’s chorea, and schizophrenia. The regulatory mechanisms underlying ACh biosynthesis have not been resolved. Under a variety of experimental conditions, the rate-limiting step in ACh synthesis appears to be availability of the substrate choline rather than the catalytic activity of ChAT as the latter is considered to be present in the neuron in kinetic excess (1).

Distribution of ChAT within the neuron has been examined using subcellular fractionation techniques with characterization based upon immunological and biochemical approaches. In a number of species, about 80–90% of ChAT in nerve terminals is present as a cytosolic protein, a portion of which may be ionically associated with synaptic membranes, while the remaining 10–20% of the total enzyme appears to be bound nonionically to plasma membrane (2–5). Differential subcellular compartmentalization can serve as an important regulatory mechanism providing spatial restrictions for substrate or enzyme availability, as well as for the proximity of enzyme modulatory proteins and molecules. To date, the mechanism(s) governing the subcellular distribution of ChAT have not been elucidated, and the role that different pools of enzyme play in regulation of neurotransmitter biosynthesis is unclear.

While ChAT is encoded by a single gene, multiple mRNAs for the protein are produced by alternative splicing and differential utilization of three different exons within the noncoding region of the gene (6–9). In human, four isoforms of ChAT mRNAs (termed R, N1, N2, and M) have been identified (9). The physiological significance of ChAT mRNA polymorphism is not known, although different transcript isoforms may vary in their stability or translational efficiency or may be differentially expressed in response to trophic factors and hormones. With the exception of human, the multiple transcripts for ChAT identified within a given species all appear to translate to a common protein with apparent molecular mass around 68 kDa (for review, see Ref. 10). In humans, while the R and N transcripts encode a common 69-kDa protein, the M transcript translates to two protein isoforms with apparent molecular masses of 82 and 69 kDa due to the presence of two translation initiation sites in the M-ChAT mRNA (9, 11). The 82-kDa form of ChAT differs from 69-kDa ChAT only in terms of a 118-amino acid extension on its amino terminus.

Cloning of ChAT from mammalian species, including human, has greatly facilitated studies of mechanisms underlying expression and regulation of the enzyme. The advent of green fluorescent protein (GFP) technology has provided new opportunities for investigating the subcellular compartmentalization of proteins. Because of its inherent fluorescence properties, direct visualization of GFP from the bioluminescent jellyfish _Aequorea victoria_ expressed as a fusion protein with other recombinant proteins can serve as an effective marker for protein localization and trafficking in living cells (12, 13). In the present paper, we report for the first time the analysis of ChAT protein localization in living cells in real-time using confocal laser microscopy. Importantly, the 82- and 69-kDa forms of ChAT are differentially distributed in cells with the larger form localized specifically to nucleus and the smaller form present in cytoplasm.

**EXPERIMENTAL PROCEDURES**

* cDNA Constructs—Human N1- and M-ChAT cDNAs were obtained from Dr. H. Misawa, Department of Neurology, Tokyo Metropolitan Institute for Neuroscience, Tokyo. Plasmids for ChAT-GFP conjugates were made by ligating coding regions of the ChAT cDNAs into KpnI and BamHI cloning sites of pEFGP-N1 (CLONTECH) producing chimeric genes encoding NH₂-terminal fusions to GFP; flanking in-frame 3’ BamHI (used to replace the COOH-terminal stop codon) and 5’ KpnI restriction sites were introduced into both M- and N1-ChAT by PCR.

* The abbreviations used are: ChAT, choline acetyltransferase; ACh, acetylcholine; GFP, green fluorescent protein; NLS, nuclear localization signal.

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The cDNA construct M119A was generated by PCR-mediated site-directed mutagenesis of human M-ChAT with codon 119 ATG (Met) changed to GCC (Ala). The truncated deletion mutant of M-ChAT, (~1–9), was generated by PCR to mutate codon 9 ARG (Arg) to ATG (Met), and remove nucleotides encoding the first nine amino acids of 82-kDa ChAT. Integration of the coding sequence, mutations, and confirmations was confirmed by deoxy DNA sequencing.

Subcellular Fractionation and Assay of ChAT Activity—HEK 293 expressing each construct (48 h after transfection) were subfractionated following the method of Antalis and Godbolt (15). Cells were washed twice with phosphate-buffered saline (PBS), scraped into PBS, and pelleted by centrifugation at 700 × g for 5 min at 4 °C. Cells were resuspended in 5 volumes of lysis buffer (10 mM Tris-HCl, pH 7.5, 0.05% Nonidet P-40, 3 mM MgCl2, 10 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin) and centrifuged at 400 × g for 5 min at 4 °C to yield a pellet containing crude nuclei and the postnuclear supernatant. Nuclear pellets were washed once in lysis buffer, then three times in wash buffer (10 mM HEPES, pH 6.8, 300 mM sucrose, 3 mM MgCl2, 25 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). Nuclei were extracted by resuspension in wash buffer containing 0.5% Triton X-100 and 700 units/ml of DNase I, then incubated for 45 min on ice to digest DNA (16). Membranes were pelleted from the postnuclear supernatant at 35,000 rpm for 1 h in a Beckman TLN 120 rotor at 4 °C, with the resulting supernatant yielding the cytosolic proteins. Membrane pellets were washed three times in lysis buffer containing 350 mM NaCl, then solubilized in lysis buffer containing 1% Nonidet P-40 with sonication (3 × 15 s) to yield the membrane proteins. Subcellular fractions were retained for analysis of ChAT specific activity using a radioenzymatic assay described previously (17).

Preparation of Recombinant ChAT—Recombinant 69- and 82-kDa human ChAT was prepared from baculovirus-infected High 5 cells expressing the proteins. Recombinant viruses were prepared from plasmids encoding either N1- or M119A-ChAT cDNA in pBlueBac4.5 (Invitrogen) and linearized Bac-N-Blue DNA (Invitrogen) in High 5 cells using the liposome transfection method (Invitrogen); following plasmid purification, high titer viruses were prepared. At 40 h after infection, cells were washed three times with PBS and lysed for 30 min at 4 °C in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM 2-mercaptoethanol, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin (2 μg/ml), apro- tinin (200 μg/ml), pepstatin (2 μg/ml), 0.5% (w/v) Triton X-100. After centrifugation (30 min; 15,000 × g), the resulting supernatant was used as a source of 69 and 82-kDa human ChAT.

Anti-ChAT Antibodies and Immunoblot—Polyclonal antibodies were produced in rabbits using a 13-amino acid peptide localized to the carboxyl terminus of human ChAT protein (CEKATRPSSQHGP) (9) conjugated to maleimide-activated keyhole limpet hemocyanin as immunogen (Genemed Synthesis Inc.). ChAT-specific immunoglobulins were affinity-purified on a column of the ChAT carboxy-terminal peptide coupled to NHS-Sepharose (4 mg/ml of medium) according to manufacturers (Amersham Pharmacia Biotech) instructions. Rabbit antiserum (25 ml) was loaded onto the prepared affinity column (2.5 ml), which had been prequillified with PBS at a rate of 0.5 ml/min. After loading, the column was washed with 10 column volumes of PBS supplemented with 500 mM NaCl and 0.1% Nonidet P-40. Immunoglobulins were eluted with 100 mM glycine-HCl, pH 2.7.

RESULTS

To address the issue of subcellular localization of ChAT, in particular whether the 82- and 69-kDa forms of human ChAT are differentially distributed within the cell, we prepared constructs incorporating either the human M- or N1-ChAT cDNAs into a vector with the gene encoding GFP (Fig. 1A). Immun-
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Fig. 2. Fluorescence images displaying subcellular distribution of human M- and N1-ChAT GFP fusion constructs in transiently transfected HEK 293 cells. A, M-ChAT-GFP fluorescence is distributed diffusely in cytoplasm with accumulation of intense fluorescence throughout the nucleus. In contrast, N1-ChAT-GFP fluorescence is located exclusively in cytoplasm. Cells expressing GFP alone showed homogeneous distribution of the protein throughout all subcellular compartments (data not shown). B, the relative subcellular distribution of fluorescence for each construct is characterized further by analysis of pixel intensities extracted along an adjustable black line that crosses the image of each cell. Comparison of the fluorescence intensity histograms generated for N1-ChAT and M-ChAT illustrate further the differential cellular distribution of enzyme. The images and line scan analysis shown are representative of those collected for multiple cells from at least six independent experiments. The subcellular ChAT fluorescence for each construct showed no difference in distribution in HEK 293 cells for periods between 24 h and 5 days after transfection. C, biochemical analysis of the subcellular distribution of ChAT activity from HEK 293 cells expressing human M- and N1-ChAT-GFP fusion constructs. Following subfractionation of cellular proteins into nuclear, cytoplasmic, and membrane-associated fractions, ChAT activity was measured. Data are expressed as the percentage of total ChAT activity found in each subcellular fraction. ChAT activity was distributed about equally between nuclear and cytoplasmic compartments for the M-ChAT construct that expresses both the 82- and 69-kDa forms of ChAT. In contrast, ChAT activity in cells expressing the N1-ChAT construct (only 69-kDa ChAT) was localized almost exclusively to cytoplasm. Statistical analysis is included in the legend to Fig. 3C.

blots of homogenates of HEK 293 cells transiently expressing M- or N1-ChAT cDNAs are shown in Fig. 1B; both 69- and 82-kDa ChAT are expressed from M-ChAT, whereas only 69-kDa ChAT is expressed from N1-ChAT. Importantly, both ChAT-GFP fusion proteins retain catalytic activity with respect to synthesis of ACh in an in vitro assay (Fig. 1C).

Confocal microscopy of HEK 293 cells expressing M- or N1-ChAT-GFP conjugates revealed a striking difference in the fluorescence patterns for subcellular distribution of ChAT protein produced from the two constructs (Fig. 2A). GFP-mediated fluorescence associated with M-ChAT was observed in both nuclear and cytoplasmic compartments of the cells, whereas that associated with N1-ChAT was localized to cytoplasm and absent from the nucleus. Cells expressing GFP alone revealed a homogeneous subcellular distribution for the protein (data not shown; Ref. 12). The distribution of ChAT enzymatic activity measured radioenzymatically following subcellular fractionation of transiently transfected HEK 293 cells was consistent with and paralleled observations made for the GFP fluorescence patterns for M- and N1-ChAT (Fig. 2B). Specifically, ChAT activity was distributed about equally in nuclear (48%) and cytoplasmic (44%) fractions in cells expressing M-ChAT cDNA. In contrast, 90% of ChAT activity was present in cytoplasmic fractions of cells expressing N1-ChAT cDNA with minimal enzyme activity associated with the nuclear fraction (2%). In both cases, a small fraction (4–6%) of total ChAT activity was measured in the membrane fraction.

These observations suggested that accumulation of GFP fluorescence in the nucleus was associated with the 82-kDa form of ChAT, since both M- and N1-ChAT constructs express the 69-kDa form of the enzyme. To test this hypothesis, we generated a mutant of M-ChAT in which the second translation initiation site, methionine 119, was replaced by an alanine residue (designated M119A) so that only the 82-kDa form of the enzyme was translated (Fig. 1A): this was confirmed when expression of this construct in HEK 293 cells resulted in production of only 82-kDa ChAT (Fig. 1B). Determination of the relative specific activity of the mutant form of ChAT (M119A) revealed that it did not differ significantly from that determined for the smaller isoform (Fig. 1C). Importantly, confocal microscopy of the ChAT-GFP mutant M119A fusion protein shows that the 82-kDa form of ChAT is localized predominately in the nucleus, with a low level of fluorescence distributed diffusely throughout the cytoplasm (Fig. 3). In parallel with these observations, subcellular fractionation studies revealed that about 75% of total M119A-ChAT activity was found in the nuclear fraction, with the remaining approximately 20% of enzyme activity in the cytoplasm and a small fraction associated with membranes (Fig. 3). These data suggest that 82-kDa ChAT is preferentially targeted to, and compartmentalized in, the nucleus.

In eukaryotic cells, the selective transport of proteins from cytoplasm to nucleus is mediated by short amino acid sequences, characteristically rich in basic residues, commonly referred to as nuclear localization signals (NLS) (19). NLS-containing proteins interact with cytoplasmic NLS receptors and a number of other soluble factors, which collectively function to dock and catalyze translocation of the protein through the nuclear pore complex in an energy-dependent manner (20, 21). A core NLS contains four or more arginine (Arg) and lysine (Lys) residues within a hexapeptide and is frequently flanked by acidic residues or helix-breakers such as proline (Pro) and glycine (Gly). A bipartite NLS consists of two clusters of basic amino acids separated by a spacer region of ten or more non-conserved residues. Analysis of the amino terminus of the 82-kDa form of ChAT from amino acid residues 1–33 (MGLRTAKKRGLGGGGKWKREEGGGTRGRREVRP) reveals elements of both core and bipartite NLSs.

To investigate the structure-function relationship of the predicted core NLS located in the immediate amino terminus of 82-kDa ChAT, a truncated mutant of M-ChAT in which amino acid residues 1–9 (MGLRTAKKR) were deleted was constructed (designated −(1–9); truncated 82-kDa ChAT) (Fig. 1A). Consistent with this region functioning as an NLS, when the GFP fusion protein of this mutant was expressed transiently in HEK 293 cells, the mutant ChAT was no longer localized to the nucleus. Moreover, the fluorescence expression pattern of this construct mirrored that observed for the N-ChAT construct. Corresponding with the absence of nuclear localization of this ChAT mutant was loss of ChAT enzymatic activity associated with the nuclear compartment (10% of total) (Fig. 3) and an increase in enzyme present in the cytoplasmic fraction (84%); 5% of ChAT was associated nonionically with membranes.
Expression and subcellular distribution of M-, N-, M119A, and -(1–9)-GFP fusion constructs were also examined in another non-neuronal cell line, Chinese hamster ovary cells, and in neuronal pheochromocytoma line PC12 cells. Results similar to those reported for HEK 293 cells were found in PC12 and Chinese hamster ovary cells, indicating that at least in the cells tested, the findings are consistent in both neuronal and non-neuronal phenotypes (data not shown).

Finally, we determined whether differential subcellular compartmentalization of ChAT altered ability of cells expressing the different forms of enzyme to synthesize ACh. It was predicted that if the major source of the ChAT substrates choline and acetyl-CoA are cytoplasmic, then ACh synthesis would be reduced in cells expressing the mutant ChAT M119A when compared with the other constructs. ACh synthesis assays were performed in intact transfected HEK 293 cells incubated for 30 min at 37 °C with [3H]choline (0.5 μM). Interestingly, these assays revealed no differences between cells expressing N- (69 kDa) or M- (69 and 82 kDa) ChAT or either of the mutant cDNAs M119A (82 kDa) or -(1–9) (truncated 82 kDa and 69 kDa) ChAT in terms of incorporation of [3H]choline into [3H]ACh. In cells expressing roughly equivalent amounts of enzyme, [3H]ACh synthesis was found to be 104% (M), 98% (M119A), and 105% (-(1–9)) of that measured in cells transfected with N1-ChAT cDNA (100%) (data are average of two separate experiments).

**DISCUSSION**

We describe novel findings for the subcellular compartmentation of the 82-kDa form of the cholinergic neurotransmitter-synthesizing enzyme ChAT. This unique form of ChAT found in humans and primates is localized predominantly to the nucleus, whereas the 69-kDa form of the enzyme, which bears homology to that found in other species, is found in the cytoplasm. Results of mutation analysis also demonstrate that the immediate 5′ core NLS basic region of the 82-kDa form of ChAT appears to be both necessary and sufficient for nuclear localization of the enzyme. The other basic regions of the amino terminus of 82-kDa ChAT may either not be involved in nuclear localization or may participate in modulating the potency or extent of this subcellular compartmentalization, as has been reported for some other nuclear-targeted proteins (22–24).

The physiological function associated with localization of ChAT in the nucleus is unknown. To date, no other examples of nuclear compartmentalization of a neurotransmitter-synthesizing enzyme have been reported. Experiments designed to determine whether nuclear compartmentalization of ChAT reduced neurotransmitter synthesis in cells expressing 82-kDa ChAT suggest that localization of at least 75% of total cellular ChAT in nucleus, as seen in the case of the mutant M119A, did not affect the ability of cells to synthesize ACh. One possible explanation for this observation may be that the approximately 20% of ChAT found in cytoplasm of cells expressing this ChAT mutant is adequate to support neurotransmitter synthesis. Because protein synthesis occurs in the cytosolic compartment and the process of nuclear translocation is saturable (25), 82-kDa ChAT in cytoplasm either in transit to the nucleus or present in kinetic excess for the transport system may provide sufficient enzyme activity in the cytoplasm to catalyze ACh synthesis from the [3H]choline taken up by the cells. Alternatively, subcellular fractionation data presented in Fig. 2 demonstrate clearly that nuclear-localized ChAT is catalytically active by acetylating choline in an in vitro assay. As the ChAT substrates choline and acetyl-CoA are small molecular weight compounds, they may diffuse freely through nuclear pores to equilibrate within the nucleus with the result that ACh synthesis could occur within the nucleus. The functional significance of a nuclear pool of neurotransmitter is not clear as it would not likely enter into the recycling vesicular pool from which ACh is released by exocytosis to communicate information to target cells. Recent reports indicate, however, that there is a form of muscarinic ACh receptors that is localized to the nuclear envelope (26, 27); this is one example of a growing class of G-protein-coupled receptors compartmentalized to the nucleus. It is not clear how the receptor binding sites are oriented. If the ligand binds in the cytoplasm and signals into the nucleus, cytoplasmic ACh could serve as a pool of ligand, or ACh formed in the nucleus could diffuse into cytoplasm through nuclear pores adjacent to receptors. If, however, the receptor ligand-binding site is orientated into the nucleus, ACh synthesized in the nucleus could bind and initiate signaling events to the cytoplasm.

Alternatively, ChAT in the nucleus may acetylate other nuclear substrate(s), with potential candidate(s), including hist-
Differences between ChAT undergo proteolytic processing to yield a 67-kDa ChAT. A major reservoir of precursor enzyme whose proteolytic cleavage results in generation of a smaller form of the enzyme. A potential reservoir of precursor enzyme whose proteolytic cleavage restores correct folding of the protein into its higher, tertiary structures of proteins being revealed functionally only with correct folding of the protein into its higher, tertiary structure. Finally, the 82-kDa form of human ChAT may not have a functional role in nucleus per se, but could perhaps serve as a reservoir of precursor enzyme whose proteolytic cleavage results in generation of a smaller form of the enzyme. A potential precursor form of ChAT has been reported in Drosophila (30) and bovine (31) based upon the existence of a 73–75-kDa ChAT site. It appears, from the data that exist, that the M transcript that encodes the 82-kDa form of human ChAT also encodes 69-kDa ChAT from a second translation initiation site. It appears, from the data that exist, that the M transcript that encodes the 82-kDa form of human ChAT also encodes 69-kDa ChAT from a second translation initiation site.

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