Neuropathy target esterase (NTE), the human homologue of a protein required for brain development in Drosophila, has a predicted amino-terminal transmembrane helix (TM), a putative regulatory (R) domain, and a hydrophobic catalytic (C) domain. Here we describe the expression, in COS cells, of green fluorescent protein-tagged constructs of NTE and mutant proteins lacking the TM or the R- or C-domains. Esterase assays and Western blots of particulate and soluble fractions indicated that neither the TM nor R-domain is essential for NTE catalytic activity but that this activity requires membrane association to which the TM, R-, and C-domains all contribute. Experiments involving proteinase treatment revealed that most of the NTE molecule is exposed on the cytoplasmic face of membranes. In cells expressing a moderate level of NTE and all cells expressing ΔC-NTE, fluorescence was distributed in an endoplasmic reticulum (ER)-like pattern. Cells expressing high levels of NTE showed aberrant distribution of ER marker proteins and accumulation of NTE on the cytoplasmic surface of ER-derived tubuloreticular aggregates. Deformation of the ER was also seen in cells expressing ΔR-NTE or enzymatically inactive S966A-NTE but not ΔTM-NTE. The data suggest that NTE is anchored in the ER via its TM, that its R- and C-domains also interact with the cytoplasmic face of the ER, and that overexpression of NTE causes ER aggregation via intermolecular association of its C-domains.

Neuropathy target esterase (NTE) is the human homologue of a protein required for brain development in Drosophila (1, 2). mRNA encoding NTE is expressed in embryonic mouse neural tissue as a protein reactive with the organophosphates (OP), which cause a syndrome of axonal degeneration (4, 5). Using this assay, NTE has been shown to be firmly membrane-associated. Differential centrifugation of brain homogenates resulted in an enrichment of NTE in microsomal fractions containing elements of endoplasmic reticulum (ER), Golgi, and plasma membrane; attempts to further resolve NTE by density gradient centrifugation of microsomes were unsuccessful (9). In neural sections, immunoreactive NTE staining fills neuronal cell bodies, excluding the nucleus, and extends into the proximal axon; in addition, the rate of accumulation of NTE at a peripheral nerve ligation indicated that it is conveyed along axons by vesicular fast transport (10). These observations are consistent with an ER/Golgi location for NTE.

In keeping with the membrane-bound character of NTE, hydropathy analysis of its primary sequence predicts a transmembrane helix (TM) near the amino terminus (residues 10–32). Further examination of the sequence of the 1327 residues of NTE indicates two functional domains: 1) an amino-terminal putative regulatory domain of ~700 residues that includes areas with similarity to cyclic AMP-binding proteins; and 2) a carboxyl-terminal catalytic/esterase domain containing the active site serine residue (Ser-966), which reacts with OPs (7). Various carboxyl-terminal constructs of NTE have been expressed in Escherichia coli to define the minimum polypeptide with OP-sensitive phenyl valerate esterase activity. A hydrophobic recombinant protein called NEST (NTE amino acids 727–1216) had this property and, although lacking the amino-terminal TM of NTE, associated firmly with phospholipid membranes and required this association for its esterase activity (11, 12). In the present study, we expressed various green fluorescent protein (GFP)-tagged constructs of NTE in COS cells to relate the protein’s molecular features to its enzymatic activity and intracellular distribution in eukaryotic cells.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Other Products—**COS-7 cells were obtained from the European Collection of Animal Cell Culture (ECACC number 87021302). Antibodies to GFP and to calnexin were purchased from Zymed Laboratories Inc. and StressGen, respectively. TRITC and peroxidase conjugates of anti-rabbit IgG were from Sigma, and colloidal gold (10 nm)-conjugated anti-mouse IgG was from British Biocell International Ltd. [35S]methionine (43.5 TBq/mmol) was from PerkinElmer Life Sciences.

**DNA Cloning and Mutagenesis—**For generation of a full-length NTE carrying the GFP tag at the C terminus, two primers were designed to bring the human NTE cDNA in-frame into the pEGFP-N1 vector (Clontech) between SfiI and BamHI sites (forward primer, 5′-AGATCGTCGACCACTGAGAATC-3′; reverse primer, 5′-TGTCGAGATCCCAAGGCACTCTGAT-3′). A 4-kb PCR product was generated from the human
Fig. 1. NTE-GFP constructs used for transient transfection experiments. Full-length NTE (1,327 amino acids) was tagged with GFP at the carboxyl terminus. The predicted TM at residues 10–32 is represented with a vertical line, and the position of the active site, serine 986 (S966) is indicated. The putative regulatory domain (R) and the catalytic domain (C) are shown as striped and dotted areas, respectively. Also shown are the constructs ΔTM-GFP(Δ1–42), NEST-GFP(Δ1–680), ΔR-NTE-GFP(Δ155–673), and ΔC-NTE-GFP(Δ681–1,327).

Table 1

| NTE-GFP protein | Relative mean fluorescence level<sup>a</sup> | Esterase activity<sup>b</sup> | NTE-GFP | ΔTM-NTE-GFP | NEST-GFP | ΔR-NTE-GFP | NTE-GFP |
|-----------------|------------------------------------------|-----------------------------|---------|-------------|-----------|------------|---------|
| %               | µmol/min/mg protein                     | µmol/mg protein/10 min       |         |             |           |            |         |
| GFP (vector)    | 4.4 ± 0.5                               | 18.1 ± 4.2                  |         |             |           |            |         |
| NEST-GFP        | 45.5                                     | 90.96 ± 3.74                |         |             |           |            |         |
| ΔTM-GFP         | 73.5                                     | 230.6 ± 33.6                |         |             |           |            |         |
| ΔR-NTE-GFP      | 55.6                                     | 309.2 ± 17.5                |         |             |           |            |         |
| NTE-GFP         |                                            |                             |         |             |           |            |         |

<sup>a</sup> Relative fluorescence levels were calculated as percentage of those of full-length NTE-GFP based on mean levels determined by FACS analysis.

<sup>b</sup> Particulate fractions were isolated from COS cells 48 h after transfection with the indicated constructs, and NTE (phenyl valerate) esterase activities were determined as described under "Experimental Procedures." Data are the mean and standard deviation of three separate experiments.

NTE clone D16 (7) using Pfu DNA polymerase (Stratagene) and cloned into pEGFP-N1 to produce pNTE-GFP (Fig. 1).

To generate a construct deleting the first 42 amino acid residues, which include the TM at the N terminus, we designed a forward primer (5′-GGCCAAAGATCTCAGGGCCGCATGGTG-3′) with a reverse primer (5′-ATGCCAAGGATCTCAGGGCCGCATGGTG-3′) to bring in a Kozak consensus, an EcoRI site, and a translation start codon. The primer was paired with a primed reverse primer (5′-TGTCCAGGATCTCAGGGCCACATCTGGTG-3′) with a BamHI site to remove the original stop codon to amplify a 4-kb PCR fragment from NTE clone D16. The PCR fragment was cloned into the EcoRI/BamHI sites of vector pEGFP-N1.

To construct an expression vector for the catalytic domain of human NTE lacking the first 680 residues of the full-length protein, a forward primer (5′-GGCCAAAGATCTCAGGGCCGCATGGTG-3′) was designed to make use of an internal HindIII site of human NTE cDNA and bring in a Kozak consensus for optimal transcription. This forward primer, with a reverse primer (5′-TGTCCAGGATCTCAGGGCCACATCTGGTG-3′), was used in a PCR reaction to amplify a 2-kb DNA fragment, which was cloned into the HindIII and BamHI sites of the vector pEGFP-N1 to make pNTE-GFP (Fig. 1).

To generate a construct lacking the first 42 amino acid residues (ΔNTE-GFP; Fig. 1) the appropriate region of human D16 NTE cDNA was amplified by PCR with the same forward primer used to make ΔNTE-GFP (above) and a new reverse primer (5′-TGTGAGGATCTCAGGGCCACATGGTG-3′). The PCR product was cut with NheI and BamHI and cloned into the vector backbone of the construct pNTE-GFP to replace the full-length NTE-encoding sequence.

Mutation of the active site serine (Ser-966) to alanine by site-directed mutagenesis was achieved by using QuikChang™ site-directed mutagenesis kit (Stratagene) and the primers 5′-GGCCAAAGATCTCAGGGCCACATCTGGTG-3′ and 5′-GGCCAAAGATCTCAGGGCCACATCTGGTG-3′. The mutation was verified by DNA sequencing and enzymatic activity assay.

Cell Culture and Fluorescent Microscopy—COS cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax-I (Invitrogen), 10% fetal bovine serum, and 2% of both penicillin and streptomycin at 37 °C, 5% CO₂. Transfection was carried out using PolyFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. For fluorescent microscopic study, cells were plated at 4 × 10⁴/well in an 8-well Lab-Tek II chamber slide (Nalge Nunc International) and transfected with various constructs after 24 h of culture. 24–48 h after transfection, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature and permeabilized by methanol/acetone (50:50) at −20 °C for 10 min. After three washes with phosphate-buffered saline, the cells were blocked with 3% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature and probed with anti-calnexin (1:400) followed by TRITC-labeled anti-rabbit IgG (1:400) with three washes between and after antibody reaction. Slides were mounted with Vectorshield mounting medium (Vector Laboratories). Fluorescent images were acquired by confocal scanning using an argon-krypton laser and a Leica TCS-4D confocal imaging system.
C for 2 min. The supernatant fraction was further centrifuged at 100,000 g
through a 25-gauge hypodermic needle, and centrifuged at 100 g for 2 min. The supernatant fraction was further centrifuged at 100,000 g at 4 °C for 45 min in an Optima™ TLX ultracentrifuge using a TLA120 rotor (Beckman). After removing the soluble cytosolic fraction, the particulate fraction was washed once by resuspension and centrifugation and finally resuspended in the original volume of TE buffer. Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories). NTE (phenyl valerate) esterase assay was carried out as described previously (11). Soluble and particulate fractions were run on SDS-PAGE (4–20% gradient gels), blotted, and probed with anti-GFP antisera (1:1000) followed by peroxidase-launched anti-rabbit IgG (1:1000) with or without 1% Triton X-100. The digestion was terminated with 4 mM phenylmethanesulfonyl fluoride (PMSF). After incubation for a further 20 min, samples were run on SDS-PAGE and blotted onto a nitrocellulose membrane, which was then subject to autoradiography. As a control, parallel reactions were run using cDNA encoding MADM, a mammalian protein cloned in this laboratory (14), which (unlike NTE) has an amino-terminal signal peptide and a carboxy-terminal TM.

Subcellular Fractionation, Esterase Assay, and Western Blotting—COS cells were plated at 0.8 × 10⁶ in 10-cm dishes and cultured for 24 h before transfection. Forty-eight hours after transfection, cells were harvested by trypsinization. The cell pellet was resuspended in TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0), homogenized with 10 passages through a 25-gauge hypodermic needle, and centrifuged at 100,000 × g at 4 °C for 45 min in an Optima™ TLX ultracentrifuge using a TLA120 rotor (Beckman). After removing the soluble cytosolic fraction, the particulate fraction was washed once by resuspension and centrifugation and finally resuspended in the original volume of TE buffer. Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories). NTE (phenyl valerate) esterase assay was carried out as described previously (11). Soluble and particulate fractions were run on SDS-PAGE (4–20% gradient gels), blotted, and probed with anti-GFP antisera (1:1000) followed by peroxidase-launched anti-rabbit IgG (1:1000) with or without 1% Triton X-100. The digestion was terminated with 4 mM phenylmethanesulfonyl fluoride (PMSF). After incubation for a further 20 min, samples were run on SDS-PAGE and blotted onto a nitrocellulose membrane, which was then subject to autoradiography. As a control, parallel reactions were run using cDNA encoding MADM, a mammalian protein cloned in this laboratory (14), which (unlike NTE) has an amino-terminal signal peptide and a carboxy-terminal TM.

RESULTS AND DISCUSSION

Amino-terminal TM Facilitates Membrane Association, but Neither TM nor the R-domain Is Essential for a Catalytically Active Conformation of NTE in COS Cells—Constructs of NTE tagged at the carboxy terminus with GFP, including the full-length protein, deletion mutants with either the amino-terminal TM segment (∆TM), the putative regulatory (∆R) domain or the catalytic (∆C) domain removed, and a truncated polypep-
tide of similar length to bacterially expressed NEST (11) were made as shown in Fig. 1. FACS analysis revealed that, in the transfected cells, the relative mean levels of fluorescent protein expression were quite similar; mean values for cells transfected with NTE-GFP were about 2-fold higher than those with NEST-GFP, whereas cells transfected with the ΔH9004 TM- and ΔH9004 R-constructs showed intermediate values (Table I).

Phenyl valerate esterase assays on soluble and particulate fractions from the cells transfected with the various constructs indicated that all the activity was confined to the latter fraction (data not shown); this may reflect the fact that association with phospholipids is required for NTE esterase activity (11, 12). Esterase activities in particulate fractions from NTE-GFP- and ΔR-NTE-GFP-transfected cells were roughly proportional to the relative mean fluorescence intensity of the cells (Table I). The fact that ΔR-NTE has essentially identical catalytic activity as the full-length protein indicates that the R-domain is neither required for, nor does it substantially inhibit, NTE phenyl valerate esterase activity. Sequence homology within the R-domain with proteins that bind cyclic AMP (7) suggests that this nucleotide might modulate NTE catalytic activity. However, we have found no effect of cyclic AMP on phenyl valerate esterase activity in particulate fractions from NTE-transfected COS cells (data not shown).

Mean fluorescence levels in NEST-GFP-transfected COS cell populations were about half of those in NTE-GFP transfected...
cells, but esterase activity in particulate fractions from NEST-GFP-expressing cells was only $\sim 5\%$ of those from cells expressing the full-length protein (Table I). However, Western blotting of soluble and particulate fractions indicated that, whereas $\sim 90\%$ of immunoreactive GFP in NTE-GFP-transfected cells was confined to the latter fraction, only $\sim 30\%$ of GFP in NEST-GFP-transfected cells was particulate (Fig. 2). Thus normalized for the amount of NEST present, the esterase activity in particulate fractions of NEST-transfected COS cells is actually about one-third of that in NTE-transfected particulates. Similarly, $\sim 60\%$ of immunoreactive GFP was present in the particulate fractions of cells transfected with the $\Delta$TM construct (Fig. 2) and, thus, when data in Table I are normalized on this basis, the esterase activity in particulate fractions from $\Delta$TM-NTE-GFP-expressing cells is about half of that in NTE-GFP-transfected cells.

When expressed in *E. coli*, NEST is able to fold to a catalytically active conformation in association with the bacterial membrane (11). The present results suggest that recombinant constructs of NTE lacking the N-terminal TM can also adopt a catalytically active conformation once they become associated with membranes in eukaryotic cells; thus, the major function of the TM is to facilitate more efficient association with membrane. Interestingly, hydropathy analysis predicts at least one TM near the amino terminus of all the eukaryotic NTE homologues but none in YCHK, a 34-kDa TM near the amino terminus of all the eukaryotic NTE homologues (7).

Most of the NTE Molecule Is Exposed on the Cytoplasmic Surface of Intracellular Membranes—Because NTE lacks a signal sequence, it is likely to associate with intracellular membranes with the great majority of the molecule exposed to the cytoplasm. To confirm this possibility, we showed that proteinase K treatment (either in the absence or presence of Triton X-100) of sealed membrane vesicles from NTE-GFP-transfected COS cells reduced the size of the GFP-immunoreactive polypeptide from $\sim 180$ kDa (NTE-GFP) to $\sim 30$ kDa (similar to GFP itself), indicating that the membranes did not protect NTE from proteolysis (Fig. 3A). By contrast, a 70-kDa fragment of calnexin (*i.e.* its intraluminal domain; see Ref.13) was clearly protected until the membrane vesicles were disrupted by treatment with Triton X-100 (Fig. 3A). In a second approach to the question of the topology of NTE with intracellular membranes, we subjected NTE cDNA to *in vitro* transcription and translation in the absence or presence of pancreatic microsomes. The resulting polypeptide was not protected by microsomes from degradation by added proteinase K, suggesting a predominantly cytoplasmic disposition (Fig. 3B). By contrast, in a parallel control experiment, the polypeptide formed by transcription/translation of MADM (a mammalian membrane protein with an amino-terminal signal sequence TM; Ref. 13), which is imported into the lumen of the microsomes, was protected from proteinase K digestion (Fig. 3B).

*Fig. 5.* Ultrastructural membrane abnormalities in cells overexpressing NTE-GFP. Transmission electron micrographs of COS7 cells. *a*, a cell transfected with vector alone showing the presence of dense, osmiophilic droplets of the transfection fluid within the cytoplasm (arrowheads); bar, 2.5 $\mu$m. *b*, a cell overexpressing full-length NTE-GFP showing fine tubular structures (20–40 nm diameter) adjacent to the nucleus (*); bar, 500 nm. *c*, a cell overexpressing full-length NTE-GFP showing immunogold localization of GFP in a cluster of tubules adjacent to the nucleus (*); bar, 500 nm. *d* and *e*, disrupted cell overexpressing full-length NTE-GFP showing localization of GFP on the cytoplasmic face of membrane clusters; bar, 250 nm. *f*, a cell overexpressing $\Delta$R-NTE-GFP showing two complex tubuloreticular membrane structures (*curved arrows*) and a normal Golgi apparatus (*straight arrow*); bar, 2.5 $\mu$m. *f*, detail of region outlined by the box in panel *e* showing continuity between the tubuloreticular structures and the ER; bar, 500 nm.
ΔTM-NTE-GFP, fluorescence was distributed in a pattern distinct from that in cells expressing GFP itself and was partially cytoplasmic and partially coincided with that of the ER marker calnexin (Fig. 4). This pattern is consistent with the soluble/particulate distribution of these recombinant proteins detected by Western blotting and supports the view that both the C- and R-domains of NTE contribute to its association with the cytoplasmic face of the ER membrane.

An ER-like localization was observed for fluorescence in essentially all cells expressing ΔC-NTE-GFP but in only a minority of cells expressing full-length NTE-GFP (Fig. 4). In a majority of COS cells expressing NTE-GFP, intense fluorescence was observed in the juxtanuclear area, and those expressing ΔR-NTE-GFP commonly showed even more intense and bizarre patterns of fluorescence (Fig. 4). Calnexin appeared to colocalize with juxtanuclear areas of intense expression of NTE-GFP or ΔR-NTE-GFP in a pattern that was clearly abnormal (Fig. 4). Although some cells expressing ΔTM-NTE-GFP also showed relatively intense juxtanuclear fluorescence, calnexin distribution in these cells was relatively normal (Fig. 4). Similar morphological observations, and (those relating to esterase activity) were also made with transfected human HeLa and N2a mouse neuroblastoma cells using protein disulphide isomerase rather than calnexin as an ER marker (data not shown). Furthermore, the intense juxtanuclear fluorescence was not readily dispersed by treating the cells with brefeldin A (data not shown), an indication that NTE-GFP was not associated with the Golgi apparatus (16).

The ultrastructure of COS cells with intense juxtanuclear expression of NTE-GFP (Fig. 5b) was compared with that of GFP-vector control cells (Fig. 5a) to investigate reasons for the abnormal distribution of calnexin. Cells expressing high levels of NTE-GFP exhibited fine tubular structures (20–40 nm in diameter) contiguous with the ER, which was often distended to result in vesicles containing fine flocculent material (Fig. 5b). In many of these cells the tubular structures were aggregated in the cytoplasm to form irregular clusters up to 7 μm in diameter. Immunogold labeling of resin sections demonstrated the localization of NTE-GFP to these membrane clusters (Fig. 5c). Greater resolution was obtained by immunogold labeling of disrupted cell fractions (16), which clearly revealed the presence of NTE-GFP on the cytoplasmic face of these membrane clusters, whereas adjacent Golgi, mitochondrial, lysosomal, and nuclear membranes were not labeled (Fig. 5d). Electron microscopy of cells expressing ΔR-NTE-GFP revealed even more markedly abnormal membrane structures than those observed with the full-length protein. These tubuloreticular membrane structures were up to 3 μm in diameter and contiguous with the ER (Fig. 5, e and f).

Recently, we demonstrated that purified recombinant NEST catalyzes hydrolysis of membrane lipids in vitro (17). We wondered whether the abnormal membrane structures observed in COS cells overexpressing NTE and ΔR-NTE, but not in those with ΔC-NTE, might reflect hydrolysis of membrane lipid. However, the same aberrant membrane clusters were observed in cells expressing the enzymatically inactive S966A mutant forms of both NTE-GFP and ΔR-NTE-GFP (data not shown). Thus, disruption of the ER and formation of the tubuloreticular structures appear to reflect a non-enzymatic property of the catalytic domain of overexpressed NTE.

The abnormal ER morphology induced by overexpression of NTE is reminiscent of that described in COS cells overexpressing the inositol 1,4,5-triphosphate receptor (18), malformed cytochrome P450 (19), and microsomal aldehyde dehydrogenase (20). All of these proteins are anchored in the ER membrane by at least one TM and have large cytoplasmic domains. It has been suggested that the ER membranes aggregate by the head-to-head association of the cytoplasmic domains of these proteins (20). Our present experiments showing that recombinant membranes do not protect NTE from proteinase K digestion (Fig. 3) indicate that the protein is probably anchored in the ER membrane via its amino-terminal TM with residues 33–1327 exposed to the cytoplasm. This topology is also consistent with the pattern of immunogold labeling of disrupted cell fractions (Fig. 5d). When NTE is overexpressed, intermolecular association of the cytoplasmic hydrophobic C-domains could give rise to ER aggregation by the general mechanism noted above. The fact that overexpression of ΔTM-NTE-GFP does not cause gross redistribution of calnexin indicates that this ER aggregation requires NTE to be anchored via its TM. The leading role of the C-domain in the aggregation is emphasized by the relatively normal morphology of cells expressing ΔC-NTE. To some degree, the R-domain may hinder intermolecular association of C-domains, and this may reflect the exacerbated ER abnormality in cells expressing ΔR-NTE.

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