The low affinity neurotrophin receptor, termed p75LNTR, plays a role in increasing the amount of nerve growth factor that becomes bound to the tyrosine kinase receptor, trkA (Barker, P. A., and Shooter, E. M. (1994) Neuron 13: 203-215), possibly by increasing the nerve growth factor concentration in the microenvironment surrounding the trkA receptor. Because protein acylation may be a means by which cell surface receptor distribution may be regulated, we have determined the acylation status of p75LNTR. We find that p75LNTR expressed in PC12, PCNA, or transfected COS cells is metabolically labeled with [3H]palmitic acid. Substitution of other cysteine residues present in the transmembrane or intracellular domain of the receptor has no effect on protein acylation, suggesting that only Cys\(^{TM}\) is esterified to palmitate.

The neurotrophins are a family of proteins believed to play crucial roles in the maintenance and development of the nervous system. To date, four members of this family have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Efnors et al., 1990; Hohn, 1990; Jones and Reichardt, 1990; Maisonnier et al., 1990; Rosenthal et al., 1990), and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992), all of which share about 50% amino acid homology. The neurotrophins bind to two forms of cell surface receptors. The trk family is composed of three related receptor tyrosine kinases that recognize the neurotrophins with a relatively high degree of binding specificity: trkA preferentially binds NGF, trkB prefers BDNF and NT-4/5, and trkC prefers NT-3 (for review see Barbaicd, 1993). The importance of each of these receptors in neuronal development has been demonstrated recently by the production of mice in which the receptors have been rendered null by homologous recombination (Klein et al., 1993, 1994; Smeyne et al., 1994).

The other class of neurotrophin receptor contains the low affinity neurotrophin receptor p75 (p75LNTR), which binds each of the neurotrophins with approximately equal avidity. p75LNTR belongs to a superfamily of related receptors that includes CD27, CD30, CD40, 4-1BB, CD40, the fas antigen, and the tumor necrosis factor receptors TNFR1 and TNFR2 (for review see Bazan, 1993). Each of these proteins has two to four domains within the extracellular region which contain 6 cysteines that form disulfide bridges critical for the maintenance of the domain structure. Some receptors of this group may mediate signal transduction events directly in response to ligand binding by a pathway involving activation of a sphingomyelinase and generation of ceramide (Dressler et al., 1992; Dbaibo et al., 1993), whereas others may play an indirect role, concentrating ligand at the cell surface to enhance binding to a signal transducing receptor (Tartaglia et al., 1993). The functional role of the p75LNTR has remained elusive. Several studies have indicated that, together with trkA, p75LNTR contributes to the formation of high affinity NGF binding sites believed to be required for NGF action on neurons (Hempstead et al., 1989, 1991; Battlement et al., 1993). However, other work suggests that activation of the trkA receptor alone is sufficient to mediate survival and neuritic outgrowth responses to NGF by PC12 cells and neurons (Weskamp and Reichardt, 1991; Ibanez et al., 1992; Rolelli et al., 1993). Mice in which the p75LNTR gene has been rendered null by homologous recombination show apparently normal central nervous system development but display a progressive loss of sensory and sympathetic nerve endings from peripheral targets (Lee et al., 1992). Interestingly, when assayed for neurotrophin-mediated survival in vitro, BDNF-, NT-3-, and NT-4/5-responsive neurons isolated from the p75LNTR null mice behave identically to those isolated from wild type litter mates, but NGF-responsive neurons show a shift in their dose response, with the p75LNTR null mice showing decreased responsiveness at low NGF concentrations (Davies et al., 1993; Lee et al., 1994). Recent studies indicate that these disparate findings might be reconciled if p75 acts to increase the amount of NGF which ultimately becomes bound to the trkA receptor. Mahadeo et al. (1994) have shown that p75LNTR increases the rate of association into the high affinity component, and recent findings from Verdi et al. (1994) indicate that p75LNTR acts to enhance the responsiveness of MAH cells expressing trkA. We have recently provided direct biochemical data which indicate that the presence of p75LNTR acts to enhance binding of NGF to trkA in a dose-dependent manner, being most relevant at low NGF concentrations (Barker and Shooter, 1994). Based on these and previous data, we have suggested that p75LNTR may act to concentrate NGF locally in the microenvironment surrounding the trk receptor and thus enhance the ability of trkA.
to bind and respond to NGF. Such an action would likely depend on some cell surface colocalization of the two receptors either by direct association or by concentration within a cell surface subdomain.

Many cellular proteins have been shown to be modified post-translationally by the covalent attachment of an amide bond with an amino-terminal glycine (Wilcox et al., 1987), whereas palmitoylation is a post-translational modification (Bonatti et al., 1989) generally occurring by thioester formation with one or more intracellular cysteine residues (Sefton and Buss, 1989). Unlike myristoylation, no consensus sequence for palmitoylation has yet been identified, and the cellular machinery responsible for this modification remains poorly characterized. Palmitic acid that becomes covalently linked to protein is turned over rapidly (Alverez et al., 1990; Paige et al., 1993), and alterations of the steady-state level of palmitoylation could therefore act as a regulatory mechanism of protein function. Protein palmitoylation may play several important roles within the cell, including mediating protein-membrane attachment, involvement in protein-protein interactions, and determination of cellular localization (O'Dowd et al., 1989; Skene and Virag, 1989; Shenoy-Scarica et al., 1993). Because palmitoylation has been suggested to play roles in protein localization and protein-protein contact, we have tested the possibility that p75\(^{LNT}\) is acyl-modified. Our results show that the p75\(^{LNT}\) is palmitoylated post-translationally, that this modification occurs via formation of a thioester bond, and that the thioester is formed with a single cysteine residue, Cys\(^{279}\), which is conserved in rat, chick, and human forms of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**9,10-[\(^{3}H\)]Palmitic acid (30–60 Ci/mmol), 9,10-[\(^{3}H\)]myristic acid (30–60 Ci/mmol), and EnHance were purchased from DuPont NEN. \(^{35}S\)Cysteine and \(^{35}S\)dATP and Enhanced Chemiluminescence reagent were from Amersham Corp. Sequenase 2.0 was from U. S. Biochemical Corp. Nitrocellulose was from Schleicher & Schuell. Bovine calf serum and equine serum were from HyClone Laboratories. 2.5 S NGF was from Bioproducts for Science. MC192, a monoclonal antibody directed against the extracellular domain of the rat p75 receptor, was produced in ascites fluid in BALB/c mice and purified using immobilin-pure columns (Pierce) as directed by the manufacturer. The polyclonal antisera directed against p75\(^{LNT}\), designated op75-p1, was raised against a peptide containing amino acids 383–408 of the rat p75\(^{LNT}\). Anti-mouse IgG, agrose, and anti-rabbit agarose were obtained from Sigma. Horseradish peroxidase-conjugated anti-rabbit antibodies were obtained from Jackson Laboratories.

**Cell Lines and Culture—**PC12 cells (Greene and Tischler, 1976) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 6% bovine calf serum, 1 mM glutamine, 1 mM sodium pyruvate, and 50 mg/ml gentamycin in 7% CO\(_2\) at 37 °C. PCNA cells (Radeke et al., 1987) were maintained in DMEM containing 10% calf serum, \(^{10}\)M hydroxyaniline, \(4 \times 10^{-3}\)M aminopterin, and a 1.6 x 10^{-3} M thymidiine, 1 mM glutamine, 1 mM sodium pyruvate, and 50 mg/ml gentamycin in 7% CO\(_2\) at 37 °C. COS7 cells were maintained in DMEM containing 10% calf serum, 1 mM glutamine, 1 mM sodium pyruvate, and 50 mg/ml gentamycin in 7% CO\(_2\) at 37 °C.

Construction of p75\(^{LNT}\) Expression Constructs with Intracellular Mutations and Their Expression in COS7 Cells—For expression studies, the rat p75\(^{LNT}\) open reading frame was cloned into the cytomegalovirus promoter-based expression vector pCMX (Davis et al., 1991) to produce plasmid pPB182. Mutant p75\(^{LNT}\) isoforms in which each of 4 cysteine residues located in the transmembrane or intracellular domain were individually changed to alanine were constructed by polymerase chain reaction-based overlap extension mutagenesis using specific synthetic oligonucleotides (PCR-Mate, Applied Biosystems) essentially as described previously (Barber et al., 1994). The fidelity of the mutated regions was confirmed by dideoxynucleotide sequencing with Sequenase as per the manufacturer's instructions, and cDNA containing the intended mutation was cloned into the appropriate restriction sites of pBB162. Plasmids pPB209, pPB210, pPB211, and pPB212 encode rat p75\(^{LNT}\) containing substitutions C257A, C279A, C379A, and C416A, respectively. For transfection, 10\(^6\) COS7 cells were plated on 100-mm plates 24 h prior to transfection. Cells were transfected with 10 mg of plasmid DNA using the DEAE-dextran-chloroquine method and used for metabolic labeling 6 h after transfection.

**Metabolic Labeling and Immunoprecipitation—**10\(^5\) PCNA cells or 5 x 10\(^5\) PC12 cells were placed on 100-mm plates 1 day prior to the metabolic labeling. To analyze COS7 cell expression of wild-type and mutated forms of p75\(^{LNT}\), 10\(^5\) cells were plated on day 1, transfected on day 2, and metabolically labeled on day 4. For labeling, each cell type was washed twice in DMEM supplemented with 0.1% bovine serum albumin (DMEB) and then given DMEB containing either 200 (COS7 cells) or 400 (PC12 and PCNA cells) \(\mu\)Ci/ml \(^{3}H\)palmitic acid. Cells were maintained in this medium for 4 h and then rinsed twice with ice-cold Tris-buffered saline (20 mM Tris pH 8.0, 137 mM NaCl) and lysed with 0.75 ml of RIPA lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). The lysates were scraped into prechilled microcentrifuge tubes, vortexed, and centrifuged for 10 min to remove insoluble material. Cleared lysates were incubated with 5 mg of MC192 for 2 h, then supplemented with 45 ml of agarose-conjugated goat anti-rabbit IgG (Sigma) and incubated for an additional 2 h. Beads were pelleted and washed in lysis buffer three times and then suspended in 100 ml of Laemmli sample buffer lacking reducing agents and boiled for 5 min. For experiments in which PC12 cells were treated with NGF, cells were first metabolically labeled for 4 h and then exposed to NGF at either 5 or 200 ng/ml for 1, 5, or 20 min. In separate experiments, cells were pretreated with 5 ng/ml NGF in DMEB for 3 or 7 days and then metabolically labeled with \(^{3}H\)palmitic acid in the presence of NGF for 4 h.

**Immunoblotting and Fluorography—**For immunoblot, 20-ml aliquots of the immunoprecipitates described above were supplemented with dithiothreitol to a final concentration of 50 mM, boiled 5 min, and then separated on 10% Laemmli acrylamide gels containing a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) and incubated for an additional 2 h. Beads were pelleted and washed in lysis buffer three times and then suspended in 100 ml of Laemmli sample buffer lacking reducing agents and boiled for 5 min. For experiments in which PC12 cells were treated with NGF, cells were first metabolically labeled for 4 h and then exposed to NGF at either 5 or 200 ng/ml for 1, 5, or 20 min. In separate experiments, cells were pretreated with 5 ng/ml NGF in DMEB for 3 or 7 days and then metabolically labeled with \(^{3}H\)palmitic acid in the presence of NGF for 4 h.

**FIG. 1.** p75LNTR is palmitoylated in PC12 cells, PCNA cells, and COS cells. Cells were metabolically labeled with \(^{3}H\)palmitic acid for 4 h and then lysed in RIPA buffer. p75\(^{LNT}\) was immunoprecipitated with MC192 and analyzed by nonreducing SDS-PAGE followed by fluorography. COS cells were transfected with either plasmid driving expression of rat p75\(^{LNT}\) or a cytomegalovirus promoter (pPB182) or control plasmid lacking the p75 open reading frame (pPB138) 2 days prior to metabolic labeling. The position of p75\(^{LNT}\) is indicated by an arrow. Data represent one of three experiments.
Palmitoylation of the p75\textsuperscript{LNR} \\

Gels were then prepared for fluorography, dried, and exposed to x-ray film as described above.

RESULTS

To determine if p75\textsuperscript{LNR} is acyl-modified, PC12 cells were metabolically labeled with \[^{3}H\]palmitic acid, lysed with detergent-containing buffer, and p75\textsuperscript{LNR} was immunoprecipitated using MC192, a monoclonal antibody directed against the receptor's extracellular domain. Fig. 1 shows that MC192 immunoprecipitates a labeled protein from PC12 cells which comigrates with a 66-kDa molecular mass standard. This molecular mass value for nonreduced p75\textsuperscript{LNR} is consistent with previous reports. Similar analyses performed on PCNA cells, a fibroblast cell line stably transfected with the rat p75\textsuperscript{LNR} gene (Radeke et al., 1986) also showed specific labeling of a 66-kDa protein (Fig. 1). To confirm that the labeled 66-kDa protein was in fact p75\textsuperscript{LNR}, COS7 cells transiently transfected with the rat p75\textsuperscript{LNR} gene were analyzed by immunoprecipitation followed by fluorography.

Biochemical analyses were performed to determine the nature of the acylation linkage. Palmitoylation occurs most commonly via a thioester bond with cysteine and less often by formation of a hydroxyester with serine (Kaufman et al., 1984). Biochemical analyses were performed to determine the nature of the acylation linkage. Palmitoylation occurs most commonly via a thioester bond with cysteine and less often by formation of a hydroxyester with serine (Kaufman et al., 1984).

The thioester bond is susceptible to cleavage with hydroxylase.

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**Fig. 2.** Biochemical analysis of the palmitoylation attachment indicates a thioester linkage. Panel A, PCNA cells were metabolically labeled with \[^{3}H\]palmitic acid for 4 h and then lysed in RIPA followed by immunoprecipitation of p75\textsuperscript{LNR} using MC192. Identical aliquots of the immunoprecipitate were run in separate lanes of a nonreducing SDS-polyacrylamide gel. Lanes were cut from the gel and treated as indicated in the figure and then fixed for fluorography.

**Fig. 3.** Cysteine at position 279 is required for p75\textsuperscript{LNR} palmitoylation. Top panel, amino acid sequence of the rat p75\textsuperscript{LNR} intracellular domain. Residues contained within the transmembrane domain are within the open box, and the one transmembrane and three intracellular domain cysteine residues are highlighted. Bottom panel, separate p75\textsuperscript{LNR} constructs were created in which each of the 4 cysteines indicated in the top panel were replaced by alanine. These were each subcloned into pCMX and then expressed in COS cells. Two days after transfection, cells were metabolically labeled with \[^{3}H\]palmitic acid for 4 h, and p75\textsuperscript{LNR} was immunoprecipitated using MC192. In panel A, samples of immunoprecipitates were analyzed by nonreducing SDS-PAGE followed by fluorography. In panel B, samples identical to those shown in panel A were separated by reducing SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with crp75-pl, an antibody directed against the intracellular domain of p75\textsuperscript{LNR}. The position of p75\textsuperscript{LNR} is indicated by an arrow. For both A and B, data represent one of two identical experiments.
mine treatment at neutral or basic pH or with reducing agents such as dithiothreitol and β-mercaptoethanol. In contrast, a hydroxyster formed through serine is labile only to hydroxylamine treatment at basic pH. To examine the linkage to p75LNTR, PCNA cells were metabolically labeled with [3H]palmitic acid, and p75LNTR was immunoprecipitated using MC192. Aliquots were then run in separate lanes of SDS-PAGE gels which were subsequently separated and exposed to hydroxylamine at either basic or neutral pH. Fig. 2A shows that treatment of samples with hydroxylamine leads to a substantial reduction in the amount of incorporated [3H]palmitic acid detected by fluorography regardless of the pH, indicating that the linkage of the acyl moiety to the protein was unlikely to occur via a hydroxyster. To test directly the possibility that the fatty acid linkage was due to a thioester, labeled p75LNTR derived from PCNA cells was separated into aliquots that were heated either in the absence of reducing agents or with dithiothreitol or β-mercaptoethanol. Fluorography of SDS-PAGE gels containing these samples shows that treatment of labeled p75LNTR with either of these reducing agents leads to a virtually complete loss of the incorporated radioactivity (Fig. 2B), indicating that its covalent attachment to the protein is mediated by formation of a thioester with cysteine.

Fig. 3A shows the positions of the 4 cysteine residues located within the transmembrane and intracellular domain of the rat p75LNTR. To identify the specific residue(s) required for this acyl modification, the transmembrane and intracellular cysteines were individually mutated to alanine. Each of the receptor isoforms containing these mutations was then expressed in COS7 cells and subjected to metabolic labeling with [3H]palmitic acid. Mutation of Cys379 or Cys402 to alanine did not affect labeling of the p75LNTR (Fig. 3B), and replacing transmembrane residue 257 with cysteine similarly did not affect the ability of the receptor to become labeled with [3H]palmitic acid. However, replacing Cys379 with alanine completely abolished the incorporation of [3H]palmitic acid into p75LNTR. Together with the finding that the attachment of an acyl moiety to p75LNTR occurs via a thioester bond, these results indicate that the palmitoyl moiety present on p75LNTR is attached to the protein by esterification of the intracellular cysteine at position 279.

Several studies have indicated that myristoylation is mainly a cotranslational event, whereas palmitoylation occurs mainly post-translationally. To determine if the incorporation of fatty acid into p75LNTR is a post-translational process, COS7 cells were transfected with the p75LNTR expression plasmid and metabolically labeled with either [3H]palmitic acid or [3H]myristic acid. The incorporated [3H]palmitic acid or [3H]myristic acid in the absence or presence of 100 μg/ml cycloheximide (CHX) as described under "Experimental Procedures." p75LNTR immunoprecipitated from these cells was analyzed either by fluorography (panel A) or by immunoblot using mAb75-p1 (panel B). The position of p75LNTR is indicated by an arrow.

**Fig. 4. Acyl modification of p75LNTR is post-translational and occurs solely through Cys379.** COS cells transfected 2 days earlier with p162 were metabolically labeled with either [35S]cysteine or [3H]palmitic acid and that incorporation of both [3H]palmitic acid and [3H]myristic acid does not occur in p75LNTR containing the C279A mutation. The incorporated [3H]myristic acid can be removed from p75LNTR by sulphydryl reducing agents, indicating attachment is via a thioester through Cys379. Incorporation of myristic acid via a thioester is atypical; myristoylation normally occurs via cotranslational amide formation with an amino-terminal glycine. Thus, the low level of myristic acid incorporation into p75LNTR observed in these experiments likely reflects that the palmitoyltransferase responsible for the post-translational addition of fatty acid into p75LNTR is relatively nondiscriminating with respect to acyl chain length, consistent with previous observations (Schmidt et al., 1979; Wedegaertner et al., 1993).

**DISCUSSION**

This study establishes that p75LNTR becomes modified post-translationally by the addition of palmitic acid. This covalent modification of the receptor does not depend on a specific cell context, as it can be observed in PC12 cells, in stably transfected mouse fibroblasts, in human A875 cells (data not shown), or in COS7 cells transiently transfected with p75LNTR expression constructs. Treatment of the labeled protein with hydroxylamine under neutral or basic pH or with β-mercaptoethanol or dithiothreitol results in loss of the acyl group from the receptor, suggesting that its incorporation is dependent upon formation of a thioester. Site-directed mutagenesis of transmembrane or intracellular cysteine residues indicates that Cys379, located 6 amino acids within the intracellular domain, is the sole palmitoylation site within the receptor. Substitution of alanine for cysteine at this position reduces labeling of the...
physiologically since the level of palmitoylation of some of these proteins is regulated by ligand binding to the cognate receptor (Degtyarev et al., 1993; Mumby et al., 1994; Wedegaertner and Bourne, 1994). Exposure of PC12 cells to NGF at either 5 or 200 ng/ml does not appear to alter the level of palmitoylation of p75L

Among species. Some conversion of labeled fatty acids may occur during the course of metabolic labeling, and the possibility that the incorporated acyl group is myristic rather than palmitic acid must be considered. Under physiological conditions, myristoylation is a co-translational event that occurs via an amide formation through a thioester linkage (Resh, 1994). In contrast, we have shown that the addition of the acyl moiety to p75L

A B

Palmitoylation of the p75L

2. p75 is inefficiently myristoylated via Cys279. COS cells transfected 2 days earlier with either p162 or p210 (which contains the C279A substitution) were metabolically labeled with either [3H]palmitic acid or [3H]myristic acid, p75L

which is inefficiently myristoylated via [3H]myristic acid, is most likely to be palmitoylated. Interestingly, when these proteins are specifically mutated to lack palmitoylation but retain myristoylation, they no longer become activated following CD59 stimulation (Shenoy-Scaria et al., 1993). The mechanism(s) by which palmitoylation contributes to these diverse processes remains unclear. The acyl chain might play a role in protein-protein interactions, either by providing a crucial structural constraint or by directly contributing to a binding interface. An attractive alternative possibility is that palmitoylation may play a role in directing the cellular localization of various proteins, either by directing vectorial transport or by directing proteins to particular membrane subdomains. This latter notion has been supported by recent studies which indicate that palmitoylation of p59

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