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Phosphorylation of Human *N*-Myristoyltransferase by N-Myristoylated SRC Family Tyrosine Kinase Members

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***N*-Myristoyltransferase (NMT) is an essential eukaryotic enzyme that catalyzes the cotranslational and/or posttranslational transfer of myristate to the amino terminal glycine residue of a number of important proteins especially the non-receptor tyrosine kinases whose activity is important for tumorigenesis. Human NMT was found to be phosphorylated by non-receptor tyrosine kinase family members of Lyn, Fyn and Lck and dephosphorylated by the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin. Deletion of 149 amino acids from the N-terminal end resulted in the absence of phosphorylation suggesting that the phosphorylation sites are located in the N-terminal end of NMT. Furthermore, a site-directed mutagenesis study indicated that substitution of tyrosine 100 with phenylalanine served NMT as a poor substrate for the Lyn kinase. A synthetic peptide corresponding to the amino-terminal region encompassing tyrosine 100 of NMT served as a good substrate for the Lyn and Fyn kinases. Our studies also indicated that NMT was found to interact with Lyn through its N-terminal end in a phosphorylation-dependent manner. This is the first study demonstrating the cross-talk between NMT and their myristoylated protein substrates in signaling pathways.**

***Key Words:* *N*-myristoyltransferase; phosphorylation; dephosphorylation; non-receptor tyrosine kinases; interaction; site-directed mutagenesis; calcineurin.**

All Src family members, as well as nearly 100 other cellular, viral, and oncoproteins contain a consensus sequence for attachment of the 14-carbon fatty acid, myristate catalyzed by the enzyme *N*-myristoyltransferase (NMT) (1–4). This protein modification normally occurs cotranslationally after the initiating methionine has been removed by an aminopeptidase to allow acyl group transfer to the first position glycine prior to protein folding (5, 6). Posttranslational myristoylation has been reported for a 68 kDa protein *D. discoideum* (7). Myristoylation of *P. syrinagae* effector protein occurs after the proteins have entered the plant host cell and serves to target the bacterial proteins to the host plasma membrane (8). Cleavage of the pro-apoptotic BID protein by caspase 8 generates a post-translational myristoylation site that enables the BID p15 fragment to localize at the mitochondria and induce cell death (9). For the Src oncoprotein to effect transformation, it must be co-translationally modified with a myristoyl-group catalyzed by the enzyme NMT. Many of the cellular proteins that are myristoylated are known to be involved in signal transduction and oncogenesis (1). The importance of myristoylation of proteins in tumorigenesis has been suggested by studies demonstrating that myristoylation of the viral oncogene product v-Src is required for membrane association and cell transformation (10). Activation of Src has been reported in human colon carcinoma (11), colon tumor derived cell lines (11) and colonic polyps with a high potential for developing cancer (12). Blockage of the myristoylation of c-Src in colonic cell lines depressed colony formation, cell proliferation, and localization of c-Src to the plasma membrane (13). Previously, we have demonstrated that the elevated levels of NMT activity and NMT protein in animal and human colon cancer tissues suggested a role for NMT in tumor progression (14, 15).

Abbreviations used: NMT, *N*-myristoyltransferase; hNMT, human NMT; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; SDM, site-directed mutagenesis.

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Protein phosphorylation is one of the best-understood and well-studied mechanisms for cellular regulation. In this study we demonstrate the phosphorylation of NMT by its own myristoylated substrates, the Src family members, and dephosphorylation by the protein phosphatase, calcineurin. In addition, we demonstrate the phosphorylation-dependent binding of NMT to Src family members suggesting that there is cross talk between enzyme and substrate. Also, we describe the phosphorylation of NMT, catalytic characterization, stoichiometry, deletion mutagenesis and site-directed mutagenesis to map out the essential tyrosine residues responsible for phosphorylation and interaction with Src tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials

[9,10-^{3}H]Myristic acid (39.3 Ci/mmol) and [1-^{14}C]myristic acid (54.7 Ci/mmol) purchased from DuPont NEN and [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals, Inc. (California). *Pseudomonas* acyl-CoA synthetase, coenzyme A, benzamidine, phenylmethylsulfonyl fluoride, and leupeptin were obtained from Sigma. Peptide substrates based on the N-terminal ends of cAMP-dependent protein kinase (GNAAAAKKRR) and Src (GSSKSKPKR) were synthesized by the Alberta Peptide Institute, Canada. The peptide NEN**Y**VEDDDNMFRFD, corresponding to the amino-terminal region of hNMT (amino acids 97–112) (15) was also synthesized by the Alberta Peptide Institute, Canada. The peptide was coupled to bovine serum albumin at a peptide to protein ratio of 14:1. The Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, was purified as described (16).

Methods

Purification of Src family kinases and NMT. Src family tyrosine kinase members of p56^{lyn} (Lyn), p55^{fyn} (Fyn) and p56^{lck} (Lck) were purified from bovine thymus according to the method described (17). A synthetic peptide corresponding in sequence to residues 6–20 of cdc2 (KVEKIGEGT**Y**GVVKK) was used as a tyrosine kinase substrate (18).

Construction of the human NMT (hNMT) vector and protein expression (amino acids 1–416; pT7-7-hNMT) have been previously described (19). Subcloning and expression of hNMT (amino acids 9–416) into the pTrcHisC vector (pTrcHisC:hNMT) (20) and the generation of N-terminal (amino acids 149–416) and C-terminal (amino acids 9–377) have also been described (21).

Other methods. Protein concentrations were determined by the method of Bradford (22) using bovine serum albumin as a standard. Purified proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (23) and visualized using Coomassie brilliant blue staining. Site-directed mutagenesis (SDM) was carried out using QuikChange site-directed mutagenesis kit (Stratagene Inc, LaJolla, CA) according to the method described earlier (21). A synthetic peptide corresponding in sequence to residues 6–20 of cdc2 (KVEKIGEGTYGVVKK) was used as positive control for Src tyrosine kinase family members and the phosphorylation reaction was carried out as described previously (18). NMT activity was carried out according to the method described earlier (24, 25).

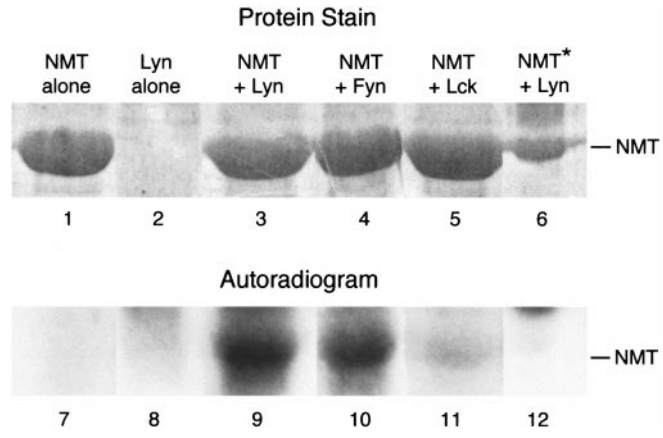

FIG. 1. Phosphorylation of hNMT by Src family tyrosine kinase members of Lyn, Fyn, and Lck. Recombinant hNMT (255 μg/ml) was phosphorylated by either Lyn (560 U/ml), Fyn (440 U/ml), or Lck (340 U/ml) for 45 min, as described under Experimental Procedures. After phosphorylation, aliquots of the samples were subjected to SDS–PAGE. Lanes: 1, hNMT alone; 2, Lyn alone; 3, hNMT and Lyn; 4, hNMT and Fyn; 5, hNMT and Lck; 6, same as lane 3 but the phosphorylated sample was incubated with an hNMT antibody followed by protein A Sepharose precipitation and the supernatent was resolved by SDS–PAGE. Lanes 7–12, respective autoradiographs of lanes 1–6.

RESULTS

Phosphorylation and Dephosphorylation of hNMT

When purified hNMT (residues 1–416) was incubated with the Src family tyrosine kinase members of Lyn, Fyn and Lck in the presence of [γ-^{32}P] ATP, the hNMT was phosphorylated (Fig. 1). Furthermore, these results suggest that hNMT is a good substrate for Lyn and Fyn; however, it is a poor substrate for Lck. The phosphorylation of hNMT was essentially abolished (Fig. 1, lane 12) when the Lyn phosphorylated hNMT was removed from the sample with an hNMT antibody followed by protein A Sepharose precipitation. These results further suggest that hNMT was phosphorylated by the Lyn kinase. Phosphorylation of hNMT by Lyn resulted in the incorporation of about 0.5 pmol phosphate per mol of hNMT at 60 min and did not reach plateau levels after 60 min. (Fig. 2A). Similar stoichiometry of hNMT phosphorylation was observed with Fyn (data not shown).

Dephosphorylation of phosphorylated hNMT was examined using calcineurin from bovine brain. The dephosphorylation reaction was carried out with Ni^{2+}-stimulated calcineurin at 20 μg/ml and 40 μg/ml (Fig. 2B). At lower concentration (20 μg/ml) of calcineurin, the rate of reaction is reduced; however, the final dephosphorylation of hNMT after 60 min is the same using either 20 or 40 μg/ml calcineurin (Fig. 2B). These results suggest that hNMT dephosphorylation could be mediated by calcineurin.

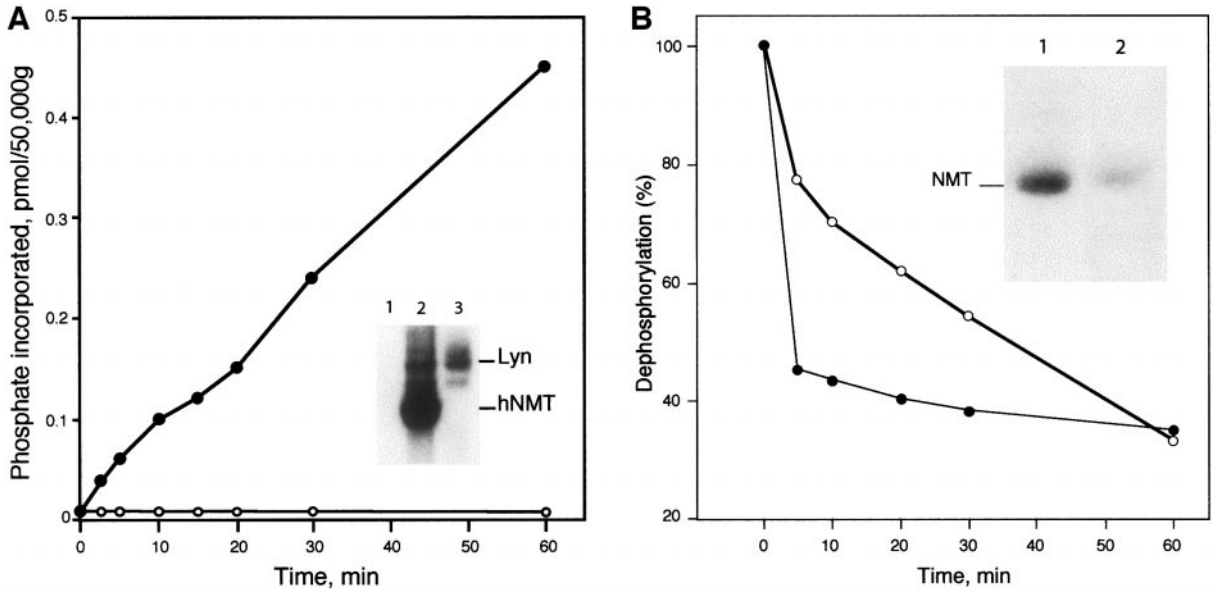

FIG. 2. (A) Time course of phosphorylation of hNMT by Lyn. Human NMT (400 μg/ml) was phosphorylated in the absence (open circles) and presence Lyn (560 U/ml) (closed circles) in a standard reaction mixture. At various time intervals indicated, aliquots were removed for analysis of phosphate incorporation. (Inset) After incubation for 60 min, aliquots were subjected to SDS–PAGE and autoradiography. Lane 1, hNMT alone; lane 2, hNMT and Lyn; lane 3, Lyn alone. (B) Time course of dephosphorylation of the phosphorylated hNMT by calcineurin. Phosphorylated hNMT (282 μg/ml) was incubated at 30°C in a reaction mixture containing 100 mM Tris–HCl (pH 7.0), 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, 20 μg/ml calcineurin, (open circles) and 40 μg/ml (closed circles) calcineurin with 10 μg/ml calmodulin, and 0.1 mM Ni^{2+}. The calcineurin sample was incubated with 1 mM Ni^{2+} at room temperature for 1 h prior to the dephosphorylation reaction. At various time intervals indicated aliquots were removed for quantification of dephosphorylation. (Inset) Autoradiogram: lane 1, 0 min; lane 2, 60 min dephosphorylation.

Effect of Phosphorylation on hNMT Activity

The kinetic properties of the non-phosphorylated and phosphorylated hNMT were compared with Src or cAMP-dependent protein kinase-derived peptide substrates. Under all conditions, essentially similar activities were found in the non-phosphorylated and phosphorylated enzyme. Initial experiments suggested that phosphorylated hNMT exhibited an increased V_{max} compared to non-phosphorylated hNMT. Subsequent studies indicated that the NP-40, which was present in kinase elution buffer (17), stimulated the hNMT activity several fold independent of phosphorylation. We have attempted to remove the NP-40 from the kinase preparation by employing various methods: dialysis, fast protein liquid chromatography, anion exchange chromatography on Mono Q or absorbent beads (Bio-Rad). The kinase protein recovered from all these treatments possesses a drastically reduced kinase activity $<$10% towards a cdc2-based peptide substrate (data not shown). In addition, detergents have been shown to stimulate the NMT activity in brain tissues (24), which is not a surprising observation. These limitations have made it difficult to address the effect of hNMT phosphorylation on its ability to myristoylate either Src or cAMP-dependent protein kinase-derived peptide substrates. Therefore, we have focussed on the characterization of hNMT phosphorylation by, and binding to, the Lyn kinase.

Identification of the Major Lyn Phosphorylation Site on hNMT

We have expressed the wild type hNMT protein (49 kDa, residues 9–416), an N-terminal deletion mutant (32 kDa, residues 149–416) and a C-terminal deletion mutant (45 kDa, residues 9–377) (20, 21). The first 8 amino acids of hNMT are not present since the 5′ cloning site used was located between the codons for the 8th and 9th amino acids (20). The wild type hNMT, N-terminal and C-terminal deletion mutant proteins were subjected to phosphorylation by Lyn tyrosine kinase as above. The wild type hNMT protein was phosphorylated by Lyn (as shown in Fig. 1; assigned value of 100%), and the N-terminal deletion mutant protein served as a poor substrate for the Lyn kinase (15% as compared to wild type hNMT) (data not shown). The C-terminal deletion mutant showed a 1.8-fold increase in phosphorylation by Lyn (i.e., 184%, as compared to wild type hNMT) (data not shown). These results suggest that the N-terminal end of hNMT may contain phosphorylation sites for the Lyn kinase. The increase in phosphorylation of the hNMT C-terminal deletion mutant indicated an inhibitory motif may be present in the C-terminal end; and that this end may be masked in wild type hNMT.

The phosphorylation site prediction program (26) (http://www.cbs.dtu.dk/services/NetPhos/) was used to

TABLE I

Site-Directed Mutagenesis of Selected Tyrosine Residues in NMT

Position of Tyr[1]	Sequence[2]	NMT activity (%)[3,4]	Ability of Lyn to phosphorylate mutant NMT[4,5]	Score[6]
WT		100	100	—
37	SKRS**Y**QFWD	100	45	0.582
70	RQEP**Y**TLPQ	67	49	0.749
93	LKEL**Y**TLLN	30	45	0.535
100	LNEN**Y**VEDD	1.7	16.6	0.863

[1] Point mutation of Tyr to Phe at the indicated residues using site-directed mutagenesis according to the method described under Experimental Procedures. WT is the full-length wild-type NMT (amino acids 1–416) with no mutations. The number indicates the Tyr residue mutated in the context of the full-length NMT protein.

[2] The amino acid sequence surrounding the Tyr (Y), where the mutated Tyr is underlined.

[3] Mutant NMT proteins (9–12 μg/assay) were subjected to an NMT assay using a Src-derived peptide substrate (500 μM), and are expressed as percent activity as compared to the wild-type NMT (100%).

[4] All assays were carried out at least in triplicate with similar results.

[5] The ability of the Lyn tyrosine kinase (560 U/ml) to phosphorylate the mutant NMT proteins (350–480 μg/ml) expressed as percent activity towards wild-type NMT (100%).

[6] Phosphorylation scores were calculated based on the phosphorylation site prediction program (26) (http://www.cbs.dtu.dk/services/NetPhos/). Scores above 0.5 are deemed to be possible phosphorylation sites and the higher the score, the more likely a particular site will be phosphorylated.

indicate which tyrosine residues within the hNMT sequence are the most likely to be phosphorylated. This program looks at the sequence context of each tyrosine residue (i.e. the 4 residues before and after each tyrosine) and assigns an output score between zero and one. Scores above 0.5 are deemed to be possible phosphorylation sites and the higher the score, the more likely a particular site will be phosphorylated. This approach predicts phosphorylation sites in independent sequences with a sensitivity of 69 to 96% (26). This analysis indicated that of the 17 tyrosine residues in the hNMT protein, the 13 most C-terminal residues were unlikely phosphorylation sites, with scores all less than 0.1. The most likely tyrosine phosphorylation sites were the 4 residues present within the first 100 amino acids at the N-terminal end of hNMT at residues 37, 70, 93 and 100, respectively (Table I).

Site-directed mutagenesis studies were employed to substitute tyrosine with phenylalanine to understand the role of these amino acids in the hNMT sequence. The results indicate that substitution of Tyr37 with Phe was found to have no effect on hNMT activity; however, a 55% reduction in the ability of the Lyn kinase to phosphorylate this mutant hNMT was observed (Table I). Substitution of Tyr70 and Tyr93 with Phe, resulted in 33 and 70 percent reduction in hNMT activity and 51 and 55 percent reduction in the ability of Lyn to phosphorylate these hNMT mutant proteins (Table I). Whereas, substitution of Tyr100 with Phe not only resulted in the loss of hNMT activity, but also made this mutant hNMT a poor substrate for Lyn kinase (Table I). These results suggested that Tyr100 (LNEN**Y**VEDD) may be the major site at which Lyn phosphorylates hNMT. It is interesting to note that the inability of Lyn to phosphorylate these mutant hNMT proteins, correlated well with the high scores assigned using the phosphorylation prediction program (Table I). For example, Tyr100 has a high phosphorylation prediction score of 0.863 and its respective mutant served as a poor substrate for Lyn kinase (16.6%) and also had a decreased hNMT activity towards a Src peptide (1.7%). However, mutation of each of thirteen other tyrosine residues in the hNMT sequence with low phosphorylation prediction scores (0.007 to 0.079) did not affect their ability to serve as substrates for Lyn kinase (data not shown). Therefore, phosphorylation prediction analysis and site-directed mutagenesis studies suggest that Tyr100 may be the major phosphorylation site in hNMT.

Phosphorylation of NMT-Peptide by Src Family Tyrosine Kinases

The phosphorylation predictions and site-directed mutagenesis studies clearly indicated that Tyr100 might be the major phosphorylation site on hNMT. To address whether phosphorylation can be achieved on a small peptide independent of the full-length NMT protein and also to demonstrate specific phosphorylation on Tyr100, a peptide from the N-terminal region of hNMT corresponding to amino acids 97–112 (containing Tyr100) was synthesized and coupled to BSA. NMT-Peptide-BSA and BSA were subjected to phosphorylation by Src tyrosine kinase family members of Lyn, Fyn and Lck. The results of the experiment indicate that the NMT peptide could be phosphorylated by the Src family tyrosine kinases Lyn and Fyn, but not Lck (Fig. 3). Similar results were observed when we used the full length NMT protein (Fig. 1). BSA was not phosphorylated by any of these Src family tyrosine kinases. These results further suggest that Tyr100 is the principle phosphorylation site on hNMT for Lyn and Fyn.

Phosphorylation-Dependent Binding of NMT to the Lyn Tyrosine Kinase

The N-terminal, C-terminal and wild type NMT proteins were subjected to phosphorylation by Lyn followed by immunoprecipitation with anti-hNMT antibody. The immune complexes were washed and subjected to kinase assay using a cdc2 peptide substrate to determine if Lyn remained bound to the hNMT im-

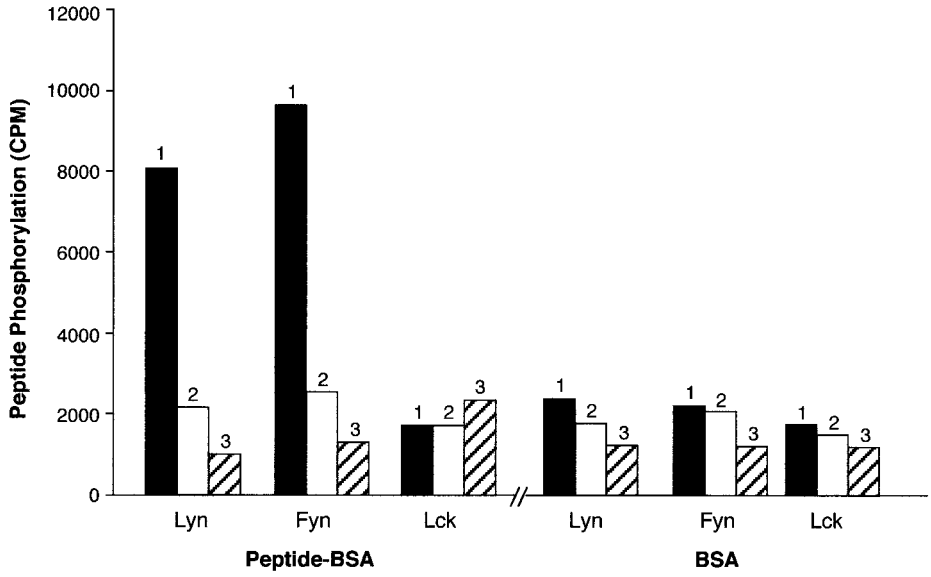

FIG. 3. The incorporation of phosphate into the peptide corresponding to the amino-terminal region of hNMT by Src family tyrosine kinase members of Lyn, Fyn, and Lck. NMT-peptide-BSA (1260 μg/ml) and BSA (1260 μg/ml) was phosphorylated by either Lyn (560 U/ml), Fyn (440 U/ml), or Lck (340 U/ml) in a standard reaction mixture as described under Experimental Procedures. Bars: 1, NMT-peptide or BSA and tyrosine kinase; 2, tyrosine kinase alone; and 3, NMT-peptide or BSA alone.

mune complex. Lyn kinase activity was found to be bound to wild type NMT (Fig. 4). In contrast, the N-terminal deleted NMT, which lacks the first 148 amino acids including Tyr100, failed to bind Lyn kinase activity. In the C-terminal deleted NMT protein, close to 50% kinase activity was observed (Fig. 4).

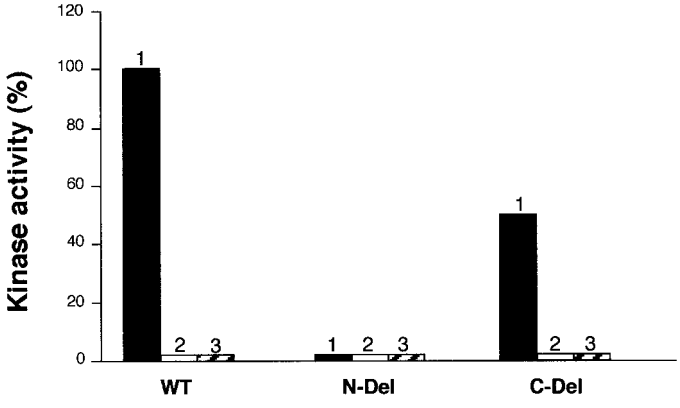

FIG. 4. Phosphorylation of various NMT mutant proteins. Deletion mutants were generated as described under Experimental Procedures. Human NMT wild type (192 μg/ml), N-terminal deletion mutant (104 μg/ml) and C-terminal deletion mutants (56 μg/ml) were incubated in the presence of either Lyn (560 U/ml) (bar 1) or the absence of Lyn (560 U/ml) (bar 2) or cold ATP (bar 3). NMT was immunoprecipitated with anti-hNMT antibody (5 μg) overnight followed by 2 h incubation with 40 μl of protein A Sepharose. The immune complexes were washed and the tyrosine kinase activity was measured as described under Experimental Procedures employing a cdc2 peptide as substrate. Kinase activity was expressed as the percent of phosphorylation observed with the Lyn kinase associated with wild-type hNMT under phosphorylation conditions.

DISCUSSION

Our observation that hNMT is phosphorylated by Src family tyrosine kinases (Lyn, Fyn and to a lesser extent Lck) and dephosphorylated by calcineurin indicates cross-talk between NMT and its N-myristoylated substrates. These results showing hNMT phosphorylation by Src family tyrosine kinases and dephosphorylation by calcineurin now offers a possible mechanism for NMT regulation. Deletion of 149 amino acids from the N-terminal end resulted in the absence of phosphorylation suggesting that the phosphorylation sites are located in the N-terminal end of NMT. A site-directed mutagenesis study indicated that substitution of tyrosine 100 with phenylalanine resulted in the loss of 98% NMT activity towards a Src-derived peptide substrate. When this mutant NMT protein was used as a Lyn kinase substrate, it was poorly phosphorylated (17% as compared to wild type NMT). A synthetic peptide corresponding to the amino-terminal region encompassing tyrosine 100 of NMT served as a good substrate for the Lyn and Fyn kinases. Our studies also indicate the phosphorylation-dependent interaction of hNMT with the Lyn tyrosine kinase. This interaction with Lyn could be completely abolished when we used an N-terminal deletion mutant hNMT protein. However, approximately 50% of the interaction was maintained with C-terminal deletion mutant protein, suggesting that an intact C-terminal domain may provide optimal conditions for phosphorylation, such as the correct folding. These experiments suggest a role

for the N-terminal end of hNMT in mediating the interaction with the tyrosine kinase.

Previous reports regarding the regulation of NMT indicated that the C-terminal region was important for the catalytic activity in yeast (27), human (28, 29), bovine (30) and plant (*Arabidopsis*) (31) NMT. The N-terminal region has been suggested as a targeting signal allowing the enzyme to access myristoyl-CoA pools produced by the activation of the exogenous myristate (27). The full-length enzymes from human and mouse NMT have a poly-lysine region (KKKKKKQKRKKEK) near the N-terminus (32, 33). Similar poly-lysine regions in other proteins involved in co-translational processing of nascent proteins have been identified as being ribosomal targeting signals, such as rat N-methionylaminopeptidase (AKKKRRKKKKS/GKS) (34) and the initiation factor eIF-2β subunit from yeast (ALKKKKKTKKVPID) (35) and human (MSKKKKKKKKPFML) (36). Therefore, cytosolic versus ribosomal subcellular localization and peptide substrate preferences provide a plausible reason for the presence of multiple NMT isozymes. Additionally, lower molecular weight forms of the NMT enzyme such as those found in bovine spleen, plants, yeast, and drosophila do not contain the putative poly-lysine ribosomal targeting signal found at the N-terminal end of longer forms (32, 33). Differential centrifugation of human CEM, MOLT-4, and HeLa cells has shown the majority of NMT activity could be found in the ribosomal subcellular fractions, and an approximately 60 kDa protein in these ribosomal fractions could be immunoblotted with anti-hNMT antibody (32). On the other hand, in plant NMT that does not possess this poly-lysine region, the majority of NMT activity has been associated with ribosomal subcellular fractions (31). These results suggest that there may be other targeting signals in short NMT isoforms. This is true in the case of *Drosophila* NMT, which has been purified through toll affinity chromatography (37). Toll is a cytoplasmic leucine rich repeat protein which mediates protein–protein interactions and this protein has 60% homology to a four leucine-rich repeat of p34 ribosomal binding protein (37). Binding of NMT to such ribosomal binding proteins could direct the localization of NMT to the site of protein synthesis independent of poly-lysine regions.

The significance of a phosphorylation-dependent interaction between NMT and a tyrosine kinase is not known at present. However, it is tempting to speculate that phosphorylated NMT may be binding to Src family tyrosine kinases upon phosphorylation and this binding may have a regulatory role for the tyrosine kinases. c-Src is regulated through the cytoplasmic CSK (c-Src kinase) which phosphorylates a tyrosine residue near the C-terminus, which then binds to its own SH2 group and leads to the inactivation of Src (38). Binding of phosphorylated NMT to the c-Src SH2 group could bypass this regulation perhaps resulting in the constitutive activation of c-Src. The SH2 domain is a non-catalytic domain of Src family tyrosine kinases and other proteins that participate in intermolecular and intramolecular interactions of tyrosine phosphorylated proteins (39). It has also been shown that the accessibility of the c-Src-SH2 domain for binding to cellular proteins *in vivo* is increased during mitosis (40).

We have observed NMT activities in membrane fractions of various tissues (41, 42). Src family members are localized to the plasma membrane after they are myristoylated by NMT (1). Perhaps the presence of NMT in these fractions could be a result of NMT translocation from the cytoplasm by binding to Src family tyrosine kinases, once the NMT is phosphorylated.

It will be important now to assess whether the phosphorylation occurs *in vivo*. Certainly, the phosphorylation of NMT and site-directed mutagenesis studies to map out the essential tyrosine residue(s) responsible for phosphorylation and interaction with Src family tyrosine kinases are suggestive that they could be physiological. Our observations lead to several intriguing questions. Does the binding of phosphorylated NMT to Src family members have a regulatory role for either NMT or the tyrosine kinase *in vivo?* Myristoylation is required for Src family members to function in signal transduction pathways. Our studies indicate that cross talk exists between NMT and their protein substrates; myristoylation of protein substrates and phosphorylation of NMT by N-myristoylated protein substrates. The cross-talk between NMT, tyrosine kinases and phosphatases are probably determined by their subcellular localization and by the physiological state of the cell.

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REFERENCES

1. Resh, M. D. (1994) Myristylation and palmitylation of Src family members: The fats of the matter. *Cell* **76,** 411–413.
2. Bhatnagar, R. S., and Gordon, J. I. (1997) Understanding covalent modifications of proteins by lipids: Where cell biology and biophysics mingle. *Trends Cell. Biol.* **7,** 14–18.
3. Resh, M. D. (1999) Fatty acylation of proteins: New insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta* **1451,** 1–16.
4. Rajala, R. V., Datla, R. S., Moyana, T. N., Kakkar, R., Carlsen, S. A., and Sharma, R. K. (2000) *N*-Myristoyltransferase. *Mol. Cell. Biochem.* **204,** 135.
5. Wilcox, C., Hu, J. S., and Olson, E. N. (1987) Acylation of proteins with myristic acid occurs cotranslationally. *Science* **238,** 1275–1278.
6. Deichaite, I., Casson, L. P., Ling, H. P., and Resh, M. D. (1988) In vitro synthesis of pp60v-src: Myristylation in a cell-free system. *Mol. Cell. Biol.* **8,** 4295–4301.

7. da Silva, A. M., and Klein, C. (1990) A rapid posttranslational myristylation of a 68-kD protein in *D. discoideum*. *J. Cell. Biol.* **111,** 401–407.
8. Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R. T., Katagiri, F., and Dangl, J. L. (2000) Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae. Cell* **101,** 353.
9. Zha, J., Weiler, S., Oh, K. J., Wei, M. C., and Korsmeyer, S. J. (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science* **290,** 1761–1765.
10. Krueger, J. G., Garber, E. A., Goldberg, A. R., and Hanafusa, H. (1982) Changes in amino-terminal sequences of pp60src lead to decreased membrane association and decreased in vivo tumorigenicity. *Cell* **28,** 889–896.
11. Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V., and Rosen, N. (1987) Activation of pp60c-src protein kinase activity in human colon carcinoma. *Proc. Natl. Acad. Sci. USA* **84,** 2251–2255.
12. Cartwright, C. A., Meisler, A. I., and Eckhart, W. (1990) Activation of the pp60c-src protein kinase is an early event in colonic carcinogenesis. *Proc. Natl. Acad. Sci. USA* **87,** 558–562.
13. Shoji, S., Kurosawa, T., Inoue, H., Funakoshi, T., and Kubota, Y. (1990) Human cellular src gene product: Identification of the myristoylated pp60c-src and blockage of its myristoyl acylation with N-fatty acyl compounds resulted in the suppression of colony formation. *Biochem. Biophys. Res. Commun.* **173,** 894–901.
14. Magnuson, B. A., Raju, R. V., Moyana, T. N., and Sharma, R. K. (1995) Increased *N*-myristoyltransferase activity observed in rat and human colonic tumors. *J. Natl. Cancer Inst.* **87,** 1630–1635.
15. Raju, R. V., Moyana, T. N., and Sharma, R. K. (1997) *N*-Myristoyltransferase overexpression in human colorectal adenocarcinomas. *Exp. Cell. Res.* **235,** 145–154.
16. Sharma, R. K., Taylor, W. A., and Wang, J. H. (1983) Use of calmodulin affinity chromatography for purification of specific calmodulin-dependent enzymes. *Methods Enzymol.* **102,** 210–219.
17. Cheng, H. C., Nishio, H., Hatase, O., Ralph, S., and Wang, J. H. (1992) A synthetic peptide derived from p34cdc2 is a specific and efficient substrate of src-family tyrosine kinases. *J. Biol. Chem.* **267,** 9248–9256.
18. Cheng, H. C., Litwin, C. M., Hwang, D. M., and Wang, J. H. (1991) Structural basis of specific and efficient phosphorylation of peptides derived from p34cdc2 by a pp60src-related protein tyrosine kinase. *J. Biol. Chem.* **266,** 17919–17925.
19. Raju, R. V., Datla, R. S., and Sharma, R. K. (1996) Overexpression of human N-myristoyltransferase utilizing a T7 polymerase gene expression system. *Protein Expression Purif.* **7,** 431–437.
20. Raju, R. V., Datla, R. S., and Sharma, R. K. (1996) Expression of human *N*-myristoyltransferase in *Escherichia coli.* Comparison with *N*-myristoyltransferases expressed in different tissues. *Mol. Cell. Biochem.* **155,** 69–76.
21. Raju, R. V., Datla, R. S., Warrington, R. C., and Sharma, R. K. (1998) Effects of L-histidine and its structural analogues on human *N*-myristoyltransferase activity and importance of EEVEH amino acid sequence for enzyme activity. *Biochemistry* **37,** 14928–14936.
22. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.
23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
24. King, M. J., and Sharma, R. K. (1991) *N*-Myristoyltransferase assay using phosphocellulose paper binding. *Anal. Biochem.* **199,** 149–153.
25. Raju, R. V., and Sharma, R. K. (1999) Preparation and assay of myristoyl-CoA:protein *N*-myristoyltransferase. *Methods Mol. Biol.* **116,** 193–211.
26. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294,** 1351–1362.
27. Rudnick, D. A., Johnson, R. L., and Gordon, J. I. (1992) Studies of the catalytic activities and substrate specificities of *Saccharomyces cerevisiae* myristoyl-coenzyme A: Protein *N*-myristoyltransferase deletion mutants and human/yeast Nmt chimerase in *Escherichia coli* and *S. cerevisiae. J. Biol. Chem.* **267,** 23852–23861.
28. Lodge, J. K., Johnson, R. L., Weinberg, R. A., and Gordon, J. I. (1994) Comparison of myristoyl-CoA:protein *N*-myristoyltransferases from three pathogenic fungi: *Cryptococcus neoformans, Histoplasma capsulatum,* and *Candida albicans. J. Biol. Chem.* **269,** 2996–3009.
29. McIlhinney, R. A., Patel, P. B., and McGlone, K. (1994) Characterization of a polyhistidine-tagged form of human myristoyl-CoA: Protein *N*-myristoyltransferase produced in *Escherichia coli. Eur. J. Biochem.* **222,** 137–146.
30. Raju, R. V., Anderson, J. W., Datla, R. S., and Sharma, R. K. (1997) Molecular cloning and biochemical characterization of bovine spleen myristoyl CoA:protein *N*-myristoyltransferase. *Arch. Biochem. Biophys.* **348,** 134–142.
31. Qi, Q., Rajala, R. V., Anderson, W., Jiang, C., Rozwadowski, K., Selvaraj, G., Sharma, R., and Datla, R. (2000) Molecular cloning, genomic organization, and biochemical characterization of myristoyl-CoA:protein *N*-myristoyltransferase from *Arabidopsis thaliana. J. Biol. Chem.* **275,** 9673–9683.
32. Glover, C. J., Hartman, K. D., and Felsted, R. L. (1997) Human *N*-myristoyltransferase amino-terminal domain involved in targeting the enzyme to the ribosomal subcellular fraction. *J. Biol. Chem.* **272,** 28680–28689.
33. Giang, D. K., and Cravatt, B. F. (1998) A second mammalian *N*-myristoyltransferase. *J. Biol. Chem.* **273,** 6595–6598.
34. Arfin, S. M., Kendall, R. L., Hall, L., Weaver, L. H., Stewart, A. E., Matthews, B. W., and Bradshaw, R. A. (1995) Eukaryotic methionyl aminopeptidases: Two classes of cobalt-dependent enzymes. *Proc. Natl. Acad. Sci. USA* **92,** 7714–7718.
35. Pathak, V. K., Nielsen, P. J., Trachsel, H., and Hershey, J. W. (1988) Structure of the beta subunit of translational initiation factor elF-2. *Cell* **54,** 633–639.
36. Donahue, T. F., Cigan, A. M., Pabich, E. K., and Valavicius, B. C. (1988) Mutations at a Zn(II) finger motif in the yeast elF-2 beta gene alter ribosomal start-site selection during the scanning process. *Cell* **54,** 621–632.
37. Ntwasa, M., Egerton, M., and Gay, N. J. (1997) Sequence and expression of *Drosophila* myristoyl-CoA:protein *N*-myristoyl transferase: Evidence for proteolytic processing and membrane localisation. *J. Cell. Sci.* **110,** 149–156.
38. Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287,** 121–149.
39. Anderson, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F., and Pawson, T. (1990) Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. *Science* **250,** 979–982.
40. Bagrodia, S., Laudano, A. P., and Shalloway, D. (1994) Accessibility of the c-Src SH2-domain for binding is increased during mitosis. *J. Biol. Chem.* **269,** 10247–10251.
41. Rajala, R. V., and Sharma, R. K. (1995) Myristoyl CoA:protein *N*-myristoyltransferase: Subcellular localization, activation and kinetic behavior in the presence of organic solvents. *Biochem. Biophys. Res. Commun.* **208,** 617–623.
42. Magnuson, B. A., Raju, R. V., and Sharma, R. K. (1996) Distribution of myristoyl-CoA:protein *N*-myristoyl transferase activity in rabbit intestine. *Biochim. Biophys. Acta* **1300,** 119–124.