Regulation of N-Myristoyltransferase by the Calpain and Caspase Systems

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N-myristoyltransferase (NMT) is an essential eukaryotic enzyme which catalyzes the transfer of the myristoyl group to the terminal glycine residue of a number of proteins including those involved in signal transduction and apoptotic pathways. In higher eukaryotes, two isoforms of NMT have been identified (NMT1 and NMT2) which share about 76% amino acid sequence identity in humans. Protein-protein interactions of NMTs reveal that m-calpain interacts with NMT1 whereas caspase-3 interacts with NMT2. These findings reveal differential interactions of both isoforms of NMT with various signaling molecules. This minireview provides an overview of the regulation of N-myristoyltransferase by calpain and caspase systems.

Keywords: Myristoylation, N-myristoyltransferase, Calpain, Caspase, Cancer

Introduction

Post-translational modification has long been recognized as a way in which the properties of proteins may be subtly altered after the synthesis of the polypeptide chain is complete. Amongst the moieties most commonly encountered covalently attached to proteins are oligosaccharides, phosphates, acetyl and formyl groups as well as nucleosides. Their addition to polypeptides occurs during nascent chain biosynthesis as well as later as the protein becomes integrated into cellular metabolic activity. In many cases, it is clear that a defined sequence of amino acids is the primary determining factor in controlling such modifications. These modifications have been a source of study for many years. Recently, it has become apparent that the binding of lipids to proteins is also widespread and is certainly of great importance. How these modifications affect cellular functions is known in only a few cases.

Lipid modifications

Lipid modifications of proteins constitute one of the most common post-translational modifications in eukaryotic cells¹. The process is sequence-specific and involves several enzymatic steps. These modifications generally occur either at or near the amino terminus or carboxy terminus of the protein. There are four broad types of lipidic modifications which have been classified according to the identities of the attached lipid. These modifications include: myristoylation, palmitoylation, prenylation and glycosylphosphatidylinositol.

Myristoylation of proteins and myristoyl CoA: protein N-myristoyltransferase

Recently, there has been a major emphasis to understand the role of protein myristoylation. Protein myristoylation is an irreversible lipidic modification that generally refers to the covalent attachment of myristate, a 14 carbon saturated fatty acid to the N-terminal glycine residue of a number of eukaryotic proteins²-⁴. Protein myristoylation is catalyzed by the enzyme myristoylCoA: protein N-myristoyltransferase (NMT), which is ubiquitously distributed among eukaryotes, including humans and often exists as isoforms in vivo⁵-¹⁶. The enzyme is very specific for the transfer of myristate in vivo². Across mammalian species, NMT exists in two major isoforms generally termed NMT1 and NMT2, both of which are highly conserved¹³. NMT1 and NMT2 have an overall sequence identity of 76-77% with most divergence being at their N termini¹³. While NMT2 appears as a single 65 kDa protein, NMT1 exists as four distinct isoforms ranging from 49 to 68 kDa in size¹³. The smaller isoform of 416 amino acids is catalytically active and there is no functional requirement for the

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longer isoform\textsuperscript{17,18}. The N-terminus in yeast NMT is shown to be located close to the myristoyl-CoA binding site as well as the peptide-binding site, suggesting that this region plays an important role in the coordinated control of the catalytic activity of NMT\textsuperscript{19}. The N-terminal extensions of the human NMTs also play a role in targeting the enzyme to ribosomes\textsuperscript{20}.

N-Myristoyltransferase in cancer

Several proteins involved in signaling processes are myristoylated, including those which regulate cell proliferation and growth. Following our observations that NMT activity and expression is unregulated during the progression of colorectal cancer\textsuperscript{21}, NMT has been proposed as a potential therapeutic target\textsuperscript{22,23,24}. We have observed significantly higher NMT activity in rat colonic tumors\textsuperscript{25} and a several fold increase in NMT activity in polyps and stage B1 tumors compared to normal colonic mucosa, implying that NMT could be used as a diagnostic/prognostic tool for colorectal cancer\textsuperscript{21,23,24,25}. Elevated NMT activity has also been observed in human adenocarcinoma\textsuperscript{21,23,24,25}, gall bladder cancer\textsuperscript{25} and various other carcinomas\textsuperscript{26,27,28,29,30,31}. Clegg et al\textsuperscript{28} reported that the proliferative capacity of mammary epithelial cells correlates with NMT activity.

We have reported for first time a higher specific expression of NMT2 in human colorectal tumor tissues, compared to normal tissues and high expression also in polyps\textsuperscript{32,33}. We have also demonstrated a higher expression of NMT1 and NMT2 in HCCLs\textsuperscript{29,31}. These results suggest that the NMT2 gene is up-regulated during molecular events that take place during the malignant formation of colon tissues. A higher expression of NMT2 is reported in rat hepatoma cells by dioxin toxicity and the inducible level of NMT2 is a direct consequence of Ah receptor activation\textsuperscript{32}. siRNA-mediated NMT knockdown shows that of the two human NMT isoforms, silencing NMT1 strongly inhibits tumor growth in a mouse model of mammary adenocarcinoma, mainly through loss of c-Src activation and its target FAK\textsuperscript{33}. Futhermore, it has been observed that NMT1 and NMT2 have only partially overlapping functions and NMT1 is critical for tumor cell proliferation, suggesting that isoform-specific inhibitors might be developed as potential anti-cancer agents\textsuperscript{34}.

Elevated NMT activity during carcinogenesis may be due to the higher demand for myristoylation of various proteins/oncoproteins (src, ras etc.), which are overexpressed and activated during tumorogenesis. Among several proteins in the intestine that are myristoylated, tyrosine kinases of the src family are the most studied. The levels of myristoylated tyrosine kinases (pp60\textsuperscript{-src} and pp60\textsuperscript{-yes}) are reported to be several fold higher in colonic pre-neoplastic lesions and neoplasms, compared with normal colon cells\textsuperscript{34-37}. Differential expression of pp60\textsuperscript{-src} has been observed in colonic tumor-derived cell lines\textsuperscript{34,38} in and colonic polyps prone to developing cancer\textsuperscript{38,39}. We also have observed that in colon cancer cell lines, the elevated expression of NMT correlates with high levels of c-Src\textsuperscript{30}. Higher levels of cytoskeletal-associated pp60\textsuperscript{-src} protein tyrosine kinase activity have been observed in intestinal crypt cells along with higher expression of pp60\textsuperscript{-yes} in the normal intestinal epithelium\textsuperscript{40,41}. Studies have revealed that pp60\textsuperscript{-src} is overexpressed in human colon carcinoma and it has enhanced kinase activity in progressive stages and metastases of human colorectal cancer\textsuperscript{34,35}. In colonic cell lines, blockage of pp60\textsuperscript{-src} N-myristoylation results in depressed colony formation and reduced proliferation\textsuperscript{42}. Recently, it is shown that src kinase activity is positively regulated by myristoylation and the non-myristoylated c-Src exhibits reduced kinase activity\textsuperscript{43}.

NMT as a therapeutic target

NMT is essential for the survival and growth in a number of organisms and thus has been a candidate drug target for many human pathogens, including Candida albicans, C. neoformans, L. major, T. brucei and Plasmodium falciparum\textsuperscript{44-53}. It has also been studied as an anti-HIV molecule\textsuperscript{54}. Given its role in cancer, NMT also represents both a valuable clinical marker and therapeutic target for anticancer drugs\textsuperscript{23,24,27,33,55}. A large number of moieties of diverse structures (generally grouped into four structural classes: analogs of myristate and myristoyl-CoA, myristoylpeptide derivatives, histidine analogs and other synthetic compounds) have been shown to inhibit NMT activity\textsuperscript{56}. We have identified heat shock cognate protein 70 as a regulator of NMT activity both \textit{in vitro} and \textit{in vivo}\textsuperscript{57,58,59}. We have also discovered enolase (a glycolytic enzyme)\textsuperscript{59} and \textit{E. coli} 10 kDa protein as potent inhibitors of human NMT\textsuperscript{60}.

N-Myristoyltransferase has been extensively reviewed in several articles\textsuperscript{2,26,27,56}. This mini review provides an overview of the regulation of N-myristoyltransferase by calpain and caspase systems.
Interaction of NMT1 and NMT2 with proteases

Recently, the importance of calpains, a family of Ca\(^{2+}\)-dependent cysteine proteases has been implicated in tumorigenesis and various aspects of cell physiology, including apoptosis, cell migration and cell proliferation\(^{61-64}\). The calpain and ubiquitin-proteasome pathways function as the major proteolytic systems responsible for the degradation of various proteins\(^{65,66}\). Numerous lines of evidence demonstrate that calpains are involved in oncocytic cell death in a variety of models\(^{66}\). Calpains cause limited proteolysis of substrates, resulting in the alteration of substrate activity\(^{61}\). PEST sequences are hydrophilic stretches of amino acids greater than or equal to 12 residues in length which are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues. PEST sequences are believed to be putative intramolecular signals for rapid proteolytic degradation. The sequence of both NMT isoforms contains a higher percentage of proline, glutamic acid, serine and threonine which are believed to be a signal for the rapid proteolytic degradation by m-calpain. Our earlier study has suggested that bovine cardiac NMT1 activity is completely abolished by m-calpain \(^{67}\). Degradation of NMT1 by m-calpain is inhibited by the calpain inhibitor calpastatin. Numerous lines of evidence demonstrate that calpains are involved in oncocytic cell death in a variety of models\(^{61,66}\). Earlier, we have reported that the activity and protein expression of m-calpain is significantly higher in colorectal adenocarcinomas than in normal samples\(^{68}\).

In an study on the interaction of both forms of NMT with m-calpain and caspase-3 in human colorectal normal and adenocarcinoma tissues and in HT29 colon cancer cells by immunoprecipitation analysis, we have observed the interaction of NMT1 with m-calpain in normal, tumor and HT29 samples (Fig. 1A, lanes 1-3). Furthermore, immunoprecipitation analysis of m-calpain using a NMT2 antibody has revealed no interaction between NMT2 and m-calpain (Fig. 1A, lanes 4-6). The data reveal that m-calpain could interact with NMT1, but not with NMT2. These results suggest that both forms of NMT may differentially regulate cellular signalling. However, cross-talk between the calpain and caspase proteolytic systems has complicated efforts to determine their distinct roles in apoptotic cell death. Calpastatin overexpression has been observed to decrease calpain activation, increase caspase-3-like activity and accelerate the appearance of apoptotic nuclear morphology\(^{69}\).

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<tr>
<th>IP: Ab-NMT1</th>
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<tr>
<td>A 1 2 3</td>
<td>4 5 6</td>
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<td>WB: Ab-m-Calpain</td>
<td>WB: Ab-Caspase-3</td>
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Fig. 1—Interaction between NMTs (NMT1 and NMT2) and proteases (m-calpain and caspase-3) by immunoprecipitation analysis in human colon cancer [Lanes 1 and 4, human normal colorectal sample; lanes 2 and 5, human tumor sample; and lanes 3 and 6, HT29 sample. For details see Selvakumar et al (29)]

The involvement of calpains and caspases in pathological conditions are unclear. Besides, calpain-mediated degradation of calpastatin during apoptosis, caspases can also degrade calpastatin\(^{70}\). It may be possible that caspase-3 might indirectly activate calpain via calpastatin degradation. To study the protein-protein interaction of NMTs with caspase-3, we have examined the immunoprecipitation analysis of caspase-3 with NMT1 and NMT2 antibodies using human normal, tumor and HT29 cells (Fig. 1B). We have observed that NMT2 interacts with caspase-3 in normal and cancerous samples (Fig. 1B, lanes 4-6), whereas NMT1 does not interact with caspase-3 (Fig. 1B, lanes 1-3). These data reveal that NMTs may be involved in the calpain/caspase-mediated pathway during the development of cancer. It is worth noting that the there are difference in the interactions of two forms of NMT with m-calpain and caspase-3. NMT1 is able to interact with m-calpain, but not the NMT2, whereas NMT2 could interact with caspase-3, but not m-calpain. It is plausible that a differential regulation exists for NMT1 and NMT2 by m-calpain and caspase-3.

Interaction of NMTs between p53 and Bcl2

Mutations in the p53 gene are among the most common genetic disorders in human cancer, including those originating in the breast, colon, lung and liver\(^{71}\). Increased expression of NMT in p53 mutant cases suggests that wild-type p53 may have a negative regulatory effect on NMT gene expression\(^{25}\). It is critical to identify the pathways responsible for the activation and suppression of p53 activity in cancerous cells. Immunoprecipitation analysis of p53 with NMT1 and NMT2 antibodies has revealed the interaction of p53 with NMT1 and NMT2 in human normal, tumor and HT29 (Fig. 2A). Interaction of p53 with NMT1 is more intense in human colorectal adenocarcinoma than in normal mucosa (Fig. 2A, lane 1 vs 2). Similarly, interaction of p53 with NMT1
in HT29 cells is more significant (Fig. 2A, lane 1 vs 3). We have also observed the interactions of p53 with NMT2 in human and normal tumor tissues extracts and in HT29 cells. While no change in the intensity of the interaction between NMT2 and p53 is observed across normal and cancerous tissues, a slightly higher interaction occurs in HT29 cells (Fig. 2A, lane 4-6). These data suggest that NMTs may be involved in the p53 pathway during cancer development.

Bcl2 overexpression leading to inhibition of cell death signaling has been observed as a relatively early event in colorectal cancer development. Several studies of colorectal adenocarcinomas have detected the expression of the Bcl2 protein using immunohistochemistry. We have examined the interaction of Bcl2 with NMT1 and NMT2 by immunoprecipitation analysis in human normal, tumor and HT29 cells (Fig. 2B) and have found that NMT1 fails to interact with Bcl2 in any of the samples tested (Fig. 2B, lanes 1-3), whereas Bcl2 interacts with NMT2 (Fig. 2B, lanes 4-6). These data suggest that Bcl2 associates with NMT2, but not with NMT1.

Despite the massive amount of knowledge that has accumulated about p53, there is still much to learn about its role in tumor suppression. Tumors with increased expression of NMT and p53 are associated with poor clinical outcomes, as evidenced by their mean survival time. A strong interaction has been shown between NMT1 and p53 in colon cancer tissues and in HT29 cells. However, the interaction of NMT2 with p53 is similar in cancerous and normal mucosa. It is critical to identify the pathways responsible for suppression of apoptosis by NMTs in colon cancer. Our ongoing investigations on the specific role of NMT1 and NMT2 by RNAi studies on myristoylation of proteins and their involvement in the regulation of apoptosis may shed light on these events in future.

References
71 Fearon ER & Vogelstein B (1990) *Cell* 61, 759-767
77 Flohil CC, Janssen PA & Bosman FT (1996) *J Pathol* 178, 393-397