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Team Work in Protein Processing

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MetAP substrates and their physiological roles have remained elusive. In this issue of *Chemistry & Biology*, Hu and colleagues [1] employ a small molecule approach to study the impact of MetAP inhibition on the molecular regulation and cellular functions of the proto-oncogene *c*-Src.

All mammalian cells express two catalytically active isoforms of a class of metallopeptidases, methionine aminopeptidases type 1 (MetAP-1) and type 2 (MetAP-2). These enzymes cotranslationally remove the initiator methionine from nascent polypeptide chains, the most common protein modification that is involved in the regulation of important cellular processes, including protein targeting to specific cellular compartments and cell proliferation. Not surprisingly, studies of a wide range of biological systems in which the MetAPs were manipulated, using chemical, genetic, or combined approaches have yielded results that have reinforced the notion of an essential role for these enzymes, ultimately for cell growth and survival [2–4]. These studies have also shown a significant degree of conserved functional redundancy, yet distinct substrate specificities between MetAP-1 and MetAP-2 [5, 6]. Contradicting the concept of functional redundancy shared by these two enzymes are observations that the proliferation of endothelial cells and a few other cell types is exquisitely sensitive to inhibition of either MetAP isoform [5, 7, 8]. Moreover, the recent analysis of conditional MetAP-2 knockout mice has revealed an essential role for this enzyme in vertebrate development, further highlighting the critical role of individual MetAPs in the regulation of cellular processes [4].

These dramatic changes that are set in motion as a consequence of MetAP inhibition demand explanations that extend beyond the phenotypic description of affected cells. Specifically, the molecular targets and the underlying downstream effector mechanism(s) involved in the control of these cellular responses need to be identified. However, the identification of physiological substrates processed by MetAPs and the decoding of their links to specific cellular responses have remained largely elusive and constitutes the "black box" of MetAP biology. This "black box" holds the key to a number of fundamental questions. The answers to those questions could trigger a new interest and stepped-up efforts in basic MetAP research and importantly, could have significant therapeutic implications for the discovery of novel clinical targets.

In this issue of *Chemistry & Biology*, Hu and colleagues [1] take an important first step in this direction. The authors employ a clever combination of in vitro and in vivo experiments to demonstrate conclusively that the proto-oncogene *c*-Src is a common substrate of MetAP-1 and MetAP-2 and that the simultaneous inhibition of both MetAPs provokes serious "predicted" consequences. An essential prerequisite for *c*-Src translocation to the plasma membrane and for its activity as a tyrosine kinase is its myristoylation at the penultimate glycine residue by the enzyme N-myristoyltransferase (Figure 1) [9]. This cotranslational modification in turn is dependent on the prior removal of the N-terminal methionine from *c*-Src by MetAP peptidase activity. Here, the authors advance a hypothesis that is built on prior findings from an investigation of the global pattern of N-myristoylated proteins in endothelial cells following the selective inhibition of MetAP-2 in these cells by TNP-470, a small-molecule MetAP-2 inhibitor of the fumagillin class [5]. With the single exception of endothelial nitric oxide synthase (eNOS), no differences in N-myristoylation were observed, suggesting that MetAP-1 activity compensated for the loss of MetAP-2 activity in these cells. Nevertheless, differentially myristoylated eNOS offered a first view into the world of myristoylated proteins directly affected by MetAP activity. Moreover, these results suggested that more targeted and innovative approaches would be necessary to gain further insights into the myristoylation of physiologically relevant MetAP substrates and their link to cellular processes. Such strategy is successfully implemented in Hu's current study.

The authors first examined c-Src myristoylation levels following the inhibition of either MetAP-1 or MetAP-2, or after simultaneous inhibition of both enzymes. In these experiments, several chemically and mechanistically diverse MetAP inhibitors were used, including bengamide A, a nonspecific, reversible inhibitor of MetAP-1 and MetAP-2, IV-43, a reversible MetAP-1 inhibitor, and TNP-470, a selective, covalent MetAP-2 inhibitor. Bengamide A treatment of HEK293 cells engineered to transiently overexpress a tagged version of *c*-Src resulted in the retention of its initiator methionine, while neither IV-43 nor TNP-470 treatment protected *c*-Src from N-terminal processing by MetAP peptidase activity. These results confirmed the existence of functional redundancy of MetAP-1 and MetAP-2 and that *c*-Src is a common in vivo substrate of these enzymes. The authors then correctly predicted that the retention of the *c*-Src initiator methionine in bengamide A treated cells would result in a decrease of N-terminal *c*-Src myristoylation and conversely, that treatment with either IV-43 or TNP-470 would

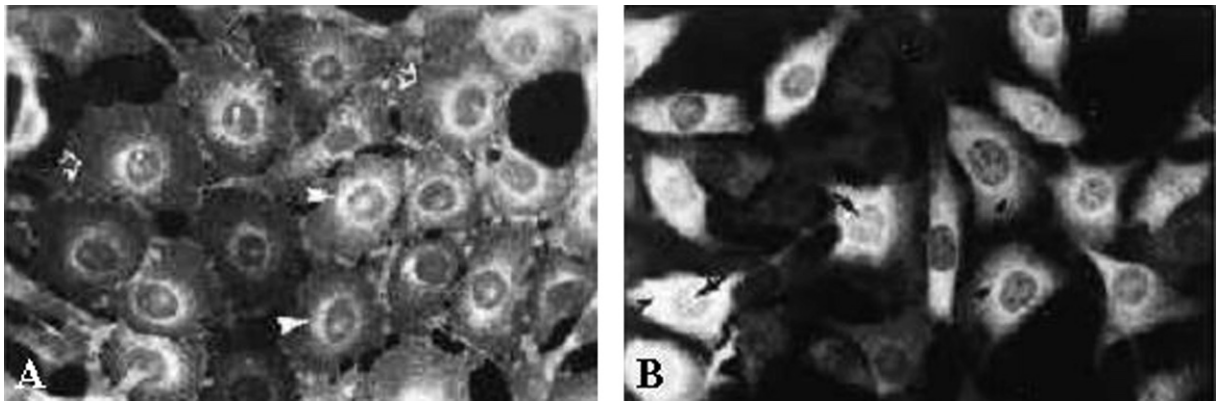

Figure 1. c-Src Myristoylation Determines its Cellular Localization
(A) In NIH-3T3 cells which overexpress wild-type c-Src, c-Src detected by immunostaining is associated with the plasma membrane (open arrows) and perinuclear structures (arrowheads). (B) In contrast, the overexpression of a myristoylation-deficient c-Src mutant shows nuclear localization (black arrows) and diffuse cytoplasmic distribution (arrowheads). Reproduced with permission of the Company of Biologists.

only moderately affect this cotranslational modification. Hu et al. [1] went on to demonstrate that the altered myristoylation of *c*-Src in bengamide A treated cells significantly impacted the subcellular localization of the kinase. The association of *c*-Src with the plasma membrane is a critical step for the ability of the *c*-Src to initiate signaling pathways upon activation by a broad spectrum of membrane-bound receptors. Bengamide A treatment of HeLa cells resulted in a marked redistribution of *c*-Src from the plasma membrane to the cytosolic compartment and the nucleus, a phenomenon not observed in IV-43 or TNP-470 treated cells.

The dramatic changes in the cellular localization of *c*-Src observed after bengamide A treatment prompted the authors to investigate whether this redistribution would also affect its tyrosine kinase activity. Tyrosine kinase activity of plasma membrane-associated *c*-Src is dependent on sequential dephosphorylation (tyrosine-530) by a protein tyrosine phosphatase and *c*-Src autophosphorylation of tyrosine residue 419. The finding that *c*-Src immunoprecipitated from bengamide A treated cells had significantly lower tyrosine kinase activity than that from control cells, combined with results that treatment with either IV-53 or TNP-470 had no detectable effects, further supported the notion that *c*-Src is a common substrate of MetAP-1 and MetAP-2. Moreover, the authors provide evidence that the decreased tyrosine kinase activity of *c*-Src in bengamide A treated cells is molecularly linked to elevated levels of tyrosine-530 phosphorylation and decreased levels of tyrosine-419 phosphorylation, but failed to observe similar regulatory mechanisms in IV-43 and TNP-470 treated cells, consistent with the fact that *c*-Src tyrosine kinase activity was not affected in these cells. The proto-oncogene c-Src is known to play a pivotal role in cell cycle progression from G_2 into mitosis. Here, the authors show that bengamide A induced MetAP inhibition in HeLa cells resulted in a significant cell cycle delay, but not cell cycle arrest, at the G_2/M phase transition. These results support a direct link between MetAP inhibition, deregulation *c*-Src, and the observed cell cycle effects.

Could the myristoylation-targeted strategy employed so effectively by Hu and coworkers [1] be copied to become a standard approach to successfully investigate the link between MetAPs and their substrates and thereby support the analysis of their cellular functions? While estimates for N-terminal processing by MetAPs range from 60%–70% of all cellular proteins, similar estimates for myristoylated proteins place this number close to 100, thus highlighting a potentially promising direction for future MetAP research [10]. In the current report, Hu and colleagues make an important contribution to the field by starting to unlock the "black box."

Beyond fundamental research, this strategy could have significant implications for the discovery of novel therapeutic targets. In the clinic, MetAP-2 has a long history as a cancer target of pharmacological inhibitors of the fumagillin class, and the therapeutic promise linked to MetAP-2 inhibition has now been extended to a broad array of disease states involving hyperproliferative cells and angiogenesis [11]. Thus, it seems reasonable to envision that the discovery of novel MetAP substrates or downstream effectors in signaling pathways governed by MetAPs will also translate into the discovery of a new generation of therapeutic targets. On a more cautious note, however, no *c*-Src inhibitor has yet found application in the clinic, despite *c*-Src being one of the most extensively studied protein kinases with well-established roles in tumorigenesis and metastasis.

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Tying the Knot: Making of Lasso Peptides

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In this issue of *Chemistry & Biology*, Duquesne and colleagues [1] describe the biosynthetic machinery of microcin J25, an antibacterial peptide that adopts an exceptional three-dimensional structure resembling a knot. The dedicated enzymes, McjB and McjC, were identified and employed to produce this modification in vitro.

The problem of increasing antibacterial resistance, that modern medicine faces today, has resulted in quite a number of studies that deal with natural antibacterial products and their biosynthesis. Microcins are defined as small antibacterial peptides that are produced by members of the Enterobacteriaceae. The designation "microcin" was coined to set off the low molecular weight antibacterial peptides (<10 kDa) against the large protein bacteriocins (e.g., the colicins) that are also produced by these bacteria. All microcins are ribosomally synthesized and they are often posttranslationally modified. The microcin group comprises peptides that differ widely with respect to their structure and mode of action. For example, microcin B17, a peptide that contains thiazole and oxazole rings, targets the DNA gyrase, microcin C7, which carries a C-terminal adenosine monophosphate, inhibits protein biosynthesis [2], and microcin E492 permeabilizes the cytoplasmic membrane in its unmodified as well as in its modified form [3].

Microcin J25 is characterized by a very unusual and fascinating structure: a so-called "lasso peptide" [4]. Lasso peptides are short peptides (16–21 amino acids) that contain an N-terminal ring structure that is closed by an amide bond between the N-terminal amino acid and the side chain carboxyl group of a glutamic or aspartic amino acid residue in position 8 or 9. The C-terminal tail is threaded through this ring structure and forms a loop or noose, which, in the case of microcin J25, is stabilized by bulky aromatic residues in the C terminus (Phe19 and Tyr20) that anchor the loop above and below the ring (Figure 1). The resulting backbone structure resembles a lasso and is very stable, thus, even after cleavage of the loop by proteases, the C-terminal part will stay anchored in the ring [5].

Several other examples of lasso peptides have been described so far. Although their modes of action are very diverse, the majority of these peptides bind to proteins. For example, the group includes MS-271, an inhibitor of the calmodulin-activated myosin light chain kinase [6], anantin, a peptide binding to the atrial natriuretic factor (ANF) [7], and two closely related peptides, NP-06 and RP 71955, which are active against human immunodeficiency virus 1 [8, 9]. The lariatins A and B [10] and microcin J25 are the only lasso peptides with antibacterial activity. Microcin J25 is produced by and is active against *Escherichia coli* strains. Furthermore, it inhibits growth of some *Salmonella* serovars. It passes the outer membrane, taking advantage of the FhuA iron siderophore transporter [11], and is taken up into the cytoplasm via other membrane proteins (Smb, TonB, ExbD and ExbB). Intracellularly it acts as an inhibitor of the RNA polymerase and stimulates the production of superoxide [12].

The main producers of lasso peptides characterized so far are members of the actinobacteria and belong to the genera *Streptomyces*, *Microbiospora*, and *Rhodococcus*. In order to synthesize a lasso peptide, like microcin J25, the prepeptide has to be folded in the correct manner, with the N terminus embracing the C terminus. Subsequently, the leader peptide cleavage and the ring formation need to take place. The enzymes that catalyze these maturation steps were unknown until now. For the first time, Duquesne and coworkers [1]