Journal of Cellular Biochemistry 92:573–578 (2004)

N-Myristoyltransferase Inhibitor Protein Is Homologous to Heat Shock Cognate Protein 70

Ponniah Selvakumar,[1] Ashakumary Lakshmikuttyamma,[1] Mohammed Khysar Pasha,[1] Martin J. King,[1] Douglas J.H. Olson,[2] Sumiko Mori,[3] Andrew R.S. Ross,[2] Kiyoshi Hayashi,[3] Jonathan R. Dimmock,[4] and Rajendra K. Sharma[1]*

[1]Department of Pathology, College of Medicine and Research Unit, Saskatchewan Cancer Agency, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 4H4, Canada
[2]NRC Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, S7N 0W9, Canada
[3]National Food Research Institute, 2-1-2 Kannondai, Tsukuba 305, Japan
[4]College of Pharmacy and Nutrition, University of Saskatchewan, 110 Science Place, Saskatoon, Saskatchewan, S7N 5C9, Canada

Abstract Many of viral and eukaryotic proteins are required for signal transduction and regulatory functions which undergo a lipid modification by the enzyme *N*-myristoyltransferase (NMT). In this study, we demonstrated that heat shock cognate protein 70 (HSC70) is homologous to NMT inhibitor protein (NIP71), which was discovered in our laboratory, based on MALDI-TOF mass spectrometric analysis. The purified bovine cytosolic HSC70 and particulate NIP71 produced a dose-dependent inhibition of human NMT having half maximal inhibitions of 235 and 230 nM, respectively. Further, Western blot analysis revealed that the purified particulate NIP71 and cytosolic HSC70 cross-reacted with both anti-NIP71 and anti-HSC70 antibodies. The results we obtained imply that molecular chaperones could be involved in the regulation of NMT in normal and cancerous cells. Further studies directed to revealing the role of HSC70 in the regulation of NMT may lead to the development of gene based therapies of colon cancer. J. Cell. Biochem. 92: 573–578, 2004.

Key words: myristoylation; *N*-myristoyltransferase; molecular chaperone; heat shock cognate protein 70; colon cancer therapy

N-myristoylation involves the co-translational addition of a fatty acyl moiety to the amino terminus of many eukaryotic cellular proteins and this modification is catalyzed by *N*-myristoyltransferase (NMT) and is recognized to be a widespread and functionally important modification of proteins [Resh, 1999; Farazi et al., 2001; Selvakumar et al., 2002]. Several viruses have been shown to require myristoylation of structural and non-structural proteins for viral assembly and replication. Activated proto-oncogenes in colon cancer include mutant *c-ras*, over expressed *c-myc*, amplified *c-myc*, *c-myb*, or *neu*, rearranged *trk*, and an activated *c-src* gene product, $pp60^{c\text{-}src}$ [Boutin, 1997]. *N*-myristoylation of the Pr55gag precursor encoded by HIV-1 inhibits its proteolytic processing and blocks viral replication [Bryant et al., 1991]. Furthermore, some myristoylated proteins are involved in the pathogenesis of cancer. For example, myristoylated $pp50^{c\text{-}src}$ and $pp62^{c\text{-}yes}$ protein kinase activities are significantly elevated in primary colorectal adenocarcinoma as well as in their corresponding cell lines relative to those of normal cells [Park et al., 1993].

It has been previously established that myristoylation of the *c-src* oncogene product may be very important for tumorigenicity of *c-src* gene expressed cells. The myristoyl blocking

Grant sponsor: Canadian Institutes of Health Research, Canada; Grant number: MOP-36484.

*Correspondence to: Dr. Rajendra K. Sharma, Department of Pathology, College of Medicine, and Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, University of Saskatchewan, 20 Campus Drive, Saskatoon, SK, S7N 4H4, Canada.
E-mail: rsharma@scf.sk.ca

Received 5 November 2003; Accepted 6 February 2004
DOI 10.1002/jcb.20085

compounds depressed colony formation, cell proliferation, and specific localization to the plasma membrane of $pp60^{c\text{-}src}$ [Shoji et al., 1990]. Therefore, it is possible that the increased synthesis of $pp60^{c\text{-}src}$ in colon cancer requires increased levels of *N*-myristoyl-dependent targeting of newly synthesized $pp60^{c\text{-}src}$ to the cytoskeleton. To support this suggestion, we reported for the first time that NMT was more active in various colonic epithelial neoplasms than in normal colonic tissue and this elevation of NMT activity was present in all tumors examined, including colonic polyps in rats [Magnuson et al., 1995]. Increased NMT activity was also observed in some human colonic tumors and was predominantly cytosolic [Magnuson et al., 1995]. Furthermore, in the human colorectal tumors there was increased immunohistochemical staining for NMT compared to normal mucosa and the staining appeared to be cytoplasmic [Raju et al., 1997]. An endogenous NMT inhibitor protein (NIP71) was discovered from bovine brain [King and Sharma, 1993] and inhibited NMT activity in rat colonic tumors [Magnuson et al., 1995]. We have also demonstrated increased NMT mRNA levels in certain well-differentiated adenocarcinomas [Rajala et al., 2000a]. NMT and $pp60^{src}$ mRNA levels were generally elevated in a subset of human colon cancer cell lines [Rajala et al., 2000a]. In addition, high levels of NIP71 in low-expressing $pp60^{src}$ cell lines and low levels of NIP71 in high-expressing $pp60^{src}$ cell lines were observed [Rajala et al., 2000a]. In this study, we demonstrate that heat shock cognate protein 70 (HSC70) contains NMT inhibitory activity.

MATERIALS AND METHODS

Materials

[^{3}H]Myristic acid (39.3 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). *Pseudomonas* acyl CoA synthetase, coenzyme A, benzamidine, phenylmethanesulfonyl fluoride (PMSF), leupeptin, ATP-agarose were obtained from Sigma-Aldrich Canada (Oakville, ON). Anti-HSC70 was obtained from Affinity BioReagents (Golden, CO). The peptide based on the NH_2-terminal sequence of the type II catalytic subunit of cAMP-dependent protein kinase (GNAAAAKKRR) was obtained from Research Genetics (Huntsville, Alabama). Centricon 10 was obtained from Millipore (Bedford, MA). The expression and purification of recombinant human NMT were undertaken as described elsewhere [Raju et al., 1996]. The production and purification of the polyclonal antibody of NIP71 has been described previously [Rajala et al., 2000a].

Purification of HSC70

HSC70 was purified from bovine brain according to a modified two-step purification protocol described previously [Welch and Feramisco, 1985]. Fresh bovine brains were obtained from a local slaughterhouse and transferred to the laboratory in packed ice. Brains were homogenized in 2 vol. of buffer A (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 20 μg/ml soybean trypsin inhibitor, 0.2 mM PMSF) in a Waring industrial blender (2×15 s). The homogenate was adjusted to pH 7.4 and then centrifuged at 8,000*g* for 25 min at 4°C. The supernatant was then centrifuged at 100,000*g* for 60 min at 4°C. The supernatant obtained represented the soluble (cytosolic) fraction of the cell. The pellet obtained was resuspended in buffer A. This represented the particulate (membranous) fraction of the cell. The cytosolic fraction was applied to a DEAE-Sepharose CL-6B column (1.6×6 cm^2) which was pre-equilibrated with buffer A. After application, the column was washed with buffer A, followed by elution of HSC70 with buffer A containing 1,000 mM NaCl. Further, the 1,000 mM fraction was dialyzed against buffer A and applied to a Affi-Gel blue column (0.6×3 cm^2) which was previously equilibrated with buffer A. After sample applications, the column was washed with buffer A contained 400 mM NaCl. The protein eluted with buffer A containing 1,000 mM NaCl. The eluted protein fraction was dialyzed against buffer A.

The dialyzed fraction was heat treated at 100°C for 5 min and the clear supernatant was applied to an ATP-agarose affinity column (0.6×3 cm^2) which was previously equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl) at 4°C. After sample applications, the column was washed with buffer B containing 2 M NaCl and HSC70 was eluted with buffer B containing 10% glycerol, 25 mM ATP and the resulting HSC70 containing fractions were pooled and dialyzed. The dialyzed sample was concentrated using Centricon-10 (Millipore) centrifugal concentra-

tors at 5,000*g* for 30 min. The purity of HSC70 was analyzed by SDS–PAGE and Western blot.

Purification of NIP71

The delipidated protein containing NIP71 was prepared by the procedure of King and Sharma [1993] and was further purified to apparent homogeneity using ATP-agarose affinity chromatography as described in "Materials." Most of the NIP71 protein was bound to the ATP-agarose affinity column. The recovery of the NIP71 activity in this step was more than 90%.

NMT and Inhibition Assay

NMT activity and its inhibition were assayed as described earlier [King and Sharma, 1991, 1993].

SDS–PAGE and Western Blot Analysis

Proteins were separated on 10% SDS–PAGE according to the procedure described by Laemmli [1970]. The protein expression was determined by immunoblotting method of Towbin et al. [1979].

Mass Spectrometric Analysis

The purified HSC70 from bovine brain cytosol and particulate was cut from the SDS–PAGE gel using a spot cutter (Protean, Bio-Rad, Hercules, CA) and placed in a 96-well microtitre plate (Corning costar MTP, Corning, NY). The excised gel pieces were then processed using a robotic digest station (MassPrep, Micromass, Manchester, UK), following a standard protocol that included de-staining, reduction with dithiothreitol, alkylation with iodoacetamide, and digestion with modified trypsin (sequencing grade, Promega, Madison WI). The resulting peptides were extracted from the gel pieces, and 0.75 μl of each extract combined with an equal volume of alpha cyano-4-hydroxycinnamic acid (5 mg/ml) in 75.0/24.9/0.1 v/v/v acetonitrile:water:TFA on a MALDI target plate. The sample spots were air-dried and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA.) operating in the positive ion and reflectron modes. Spectra were acquired by combining and averaging 400 scans using the instrument software (Data Explorer v.4.0, Applied Biosystems). The resulting spectra were de-isotoped and internally calibrated using the autolytic trypsin fragment 108–115 (m/z 842.5100), where present. If this peptide was not observed, the sample was close externally calibrated using des-Arg Bradykinin (m/z 904.4681) and ACTH 18-39 (m/z 2465.1989). The mono-isotopic peptide masses were then submitted to Protein Prospector (http://prospector.ucsf.edu/) and searched against the NCBI database using the MS-Fit program.

Other Methods

Protein concentration was measured by the method of Bradford [1976] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Mass spectrometry technology offers a powerful and sensitive tool to study co- and/or post-translational proteins in the cell. In the present study, we have demonstrated that NIP71 is 43% homologous to HSC70. Mass spectrometric analysis of a trypsin digest of NIP71 analysis showed an identical homology with HSC70 based upon the peptides detected (Fig. 1). To further support the mass spectrometry results, we carried out the potential inhibition of human NMT by purified bovine brain particulate NIP71 and bovine brain cytosolic HSC70. The purified particulate NIP71 inhibited human NMT in a dose-dependent manner with half maximal inhibition at 230 nM (Fig. 2A). Similarly, the purified cytosolic HSC70 produced a dose-dependent inhibition on human NMT with the half maximal inhibition at 235 nM (Fig. 2B). To further explore and validate NIP71 is similar-like protein to HSC70, we performed a Western blot analysis using a specific polyclonal antibody against NIP71 and monoclonal antibody against HSC70. The purified particulate NIP71 and cytosolic HSC70 cross reacted with both anti-NIP71 (Fig. 3A) and anti-HSC70 (Fig. 3B) antibodies suggesting that the particulate NIP71 and cytosolic HSC70 have common antigenic epitopes.

HSC70 is a constitutively expressed member of the HSP70 protein family that has been implicated in many processes including the folding of newly synthesized polypeptides, translocation of proteins across the endoplasmic reticulum, stabilizing proteins under stress conditions, and antigen presentation [Bukau and Horwich, 1998]. The classification of various

A

MSKGPAVGIDLGTTYSCVGVFQHGK**VEIIANDQGNRTTPSYVAFTDTER**LIGDA
AKNQVAMNPTNTVFDAKRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPKVQV
EYKGETKSFYPEEVSSMVLTK**MKEIAEAYLGKTVTNAVVTVPAYFNDSQR**QA
TK**DAGTIAGLNVLR**IINEPTAAAIAYGLDKKVGAERNVLIFDLGGGTFDVSILTIE
DGIFEVK**STAGDTHLGGEDFDNR**MVNHFIAEFKRKHKKDISENKRAVRRLRTA
CERAKRTLSSSTQASIEIDSLYEGIDFYTSITR**ARFEELNADLFR**GTLDPVEKALR
DAKLDK**SQIHDIVLVGGSTR**IPKIQK**LLQDFFNGK**ELNKSINPDEAVAYGAAVQ
AAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYS
DNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILN
VSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSKNS
LKSYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIINWLDKNQTAEKEEFEHQ
QKELEKVCNPIITKLYQSAGGMPGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD

B

MSKGPAVGIDLGTTYSCVGVFQHGK**VEIIANDQGNRTTPSYVAFTDTER**LIGDA
AKNQVAMNPTNTVFDAKRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPKVQV
EYKGETKSFYPEEVSSMVLTK**MKEIAEAYLGKTVTNAVVTVPAYFNDSQR**QA
TK**DAGTIAGLNVLR**IINEPTAAAIAYGLDKKVGAERNVLIFDLGGGTFDVSILTIE
DGIFEVK**STAGDTHLGGEDFDNR**MVNHFIAEFKRKHKKDISENKRAVRRLRTA
CERAKRTLSSSTQASIEIDSLYEGIDFYTSITR**ARFEELNADLFR**GTLDPVEKALR
DAKLDK**SQIHDIVLVGGSTR**IPKIQK**LLQDFFNGK**ELNKSINPDEAVAYGAAVQ
AAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYS
DNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILN
VSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSKNS
LKSYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIINWLDKNQTAEKEEFEHQ
QKELEKVCNPIITKLYQSAGGMPGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD

Fig. 1. Protein identification by mass spectrometry. Molecular masses determined by MALDI-TOF MS for each peptide fragment were matched to the corresponding protein sequence in the NCBI database, as described in the ''Materials and Methods.'' Peptide sequences are shown in bold letters for both bovine brain particulate NIP71 (**A**) and cytosolic HSC70 (**B**).

HSP(s) in families is based on the related function, size and the cellular compartment in which they reside. HSP70 multi-gene family consists of at least four members: *HSP70*, *HSC70*, *Grp 78* (BiP), and mitochondrial HSP75 (*mt HSP75*). HSP70, HSC70 are found in the cytosol and nucleus, mt HSP75 in the matrix of mitochondria and Grp 78 in endoplasmic reticulum [Bhattacharyya et al., 1995]. In our present study, the HSC70 purified from particulate may be the nuclear form of HSC70. However, both cytosolic and nuclear HSC70 inhibited human NMT at nanomolar concentration. The function of HSC70 in various types of cancer and its association with key molecules of the cell cycle control system is not clear yet. In breast carcinomas, mutation was observed in the NH_2-terminal ATPase domain of HSC70 [Bakkenist et al., 1999]. The mutations identified may affect the binding and hydrolysis of ATP and/or the binding of the two cofactors, p48 and BAG-1, BAG is an anti-apoptotic protein capable of interacting with and enhancing the activity of Bcl-2 [Sturzbecher et al., 1987]. Several studies have found the constitutively expressed HSC70, and heat-induced HSP70 or HSP90 to be associated with mutant p53 but not with wild-type p53 protein [Sturzbecher et al., 1987; Blagosklonny et al., 1996; Ehrhart et al., 1998]. Mutations in the *p53* gene are among the most common genetic disorders in human cancer, including those of breast, colon, lung, and liver origin [Zylicz et al., 2001]. Increased expression of NMT in p53 mutant cases suggesting that wild-type p53 may have a negative regulatory effect on NMT gene expression [Rajala et al., 2000b]. Similarly, high expression of NMT in human colon cancer [Raju et al.,

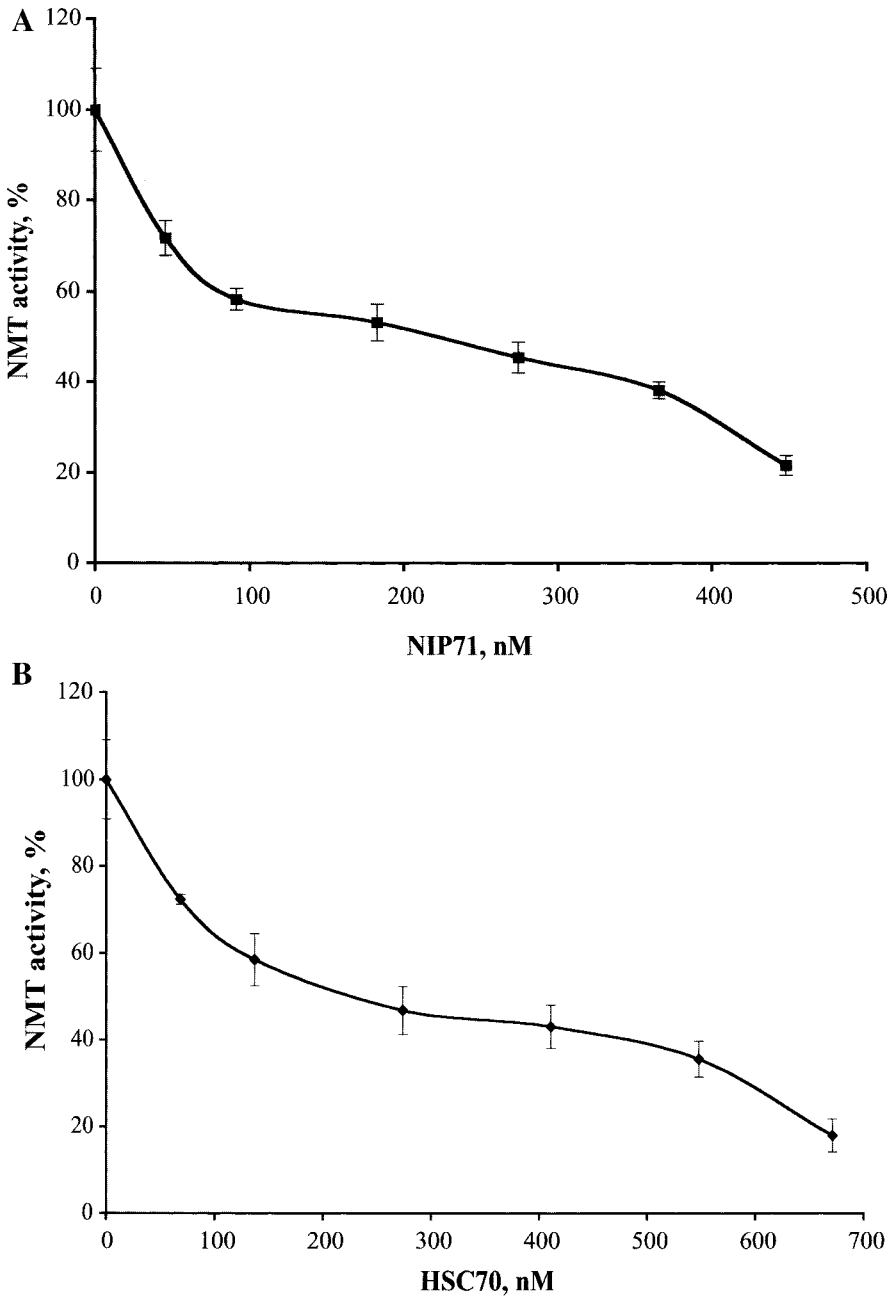

Fig. 2. Inhibition of human *N*-myristoyltransferase (NMT) by bovine brain particulate NIP71 and cytosolic HSC70. The recombinant human NMT (0.2 μg/assay) was incubated with various concentrations of purified (**A**) particulate NIP71 and (**B**) cytosolic HSC70 using cAMP-dependent protein kinase derived peptide as a substrate described under the "Materials and Methods." Representative data from three independent experiments are shown, with ± SD from three determinations.

1997] as well as human gallbladder carcinoma [Rajala et al., 2000b] was observed.

In our present study, we demonstrated that the protein NIP71, a potential inhibitor of NMT is homologous to HSC70. We observed that HSC70 inhibited human NMT activity at nanomolar concentration level and may have a role in the regulation of NMT, and the molecular chaperons could be involved in the down regulation of oncoproteins. Hence, HSC70 could be used as an anticancer therapeutic target. Further studies of this work would aid in the

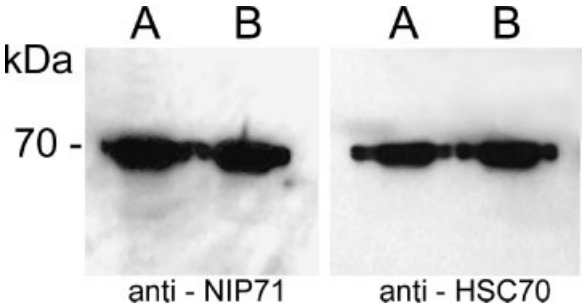

Fig. 3. Western blot analysis of bovine brain particulate NIP71 and cytosolic HSC70. Equal amounts (5 μg) of proteins (cytosolic HSC70, **lane A** and particulate NIP71, **lane B**) were probed with antibody against NIP71 (1:1,000 dilution) and HSC70 (1:1,000 dilution) as described under the ''Materials and Methods.''

interpretation of the present results. Site-directed mutagenesis and siRNA studies will reveal the role of HSC70 in the regulation of NMT which may lead to the development of a gene based therapy of colon cancer.

ACKNOWLEDGMENTS

Ponniah Selvakumar is a recipient of Post Doctoral Fellowship from the Saskatchewan Health Research Foundation, Saskatoon, Canada.

REFERENCES

Bakkenist CJ, Koreth J, Williams CS, Hunt NC, McGee JO. 1999. Heat shock cognate 70 mutations in sporadic breast carcinoma. Cancer Res 59:4219–4221.

Bhattacharyya T, Karnezis AN, Murphy SP, Hoang T, Freeman BC, Phillips B, Morimoto RI. 1995. Cloning and subcellular localization of human mitochondrial hsp70. J Bio Chem 270:1705–1710.

Blagosklonny MV, Toretsky J, Bohen S, Neckers L. 1996. Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90. Proc Natl Acad Sci USA 93:8379–8383.

Boutin JA. 1997. Myristoylation. Cell Signal 9:15–35.

Bradford MM. 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.

Bryant ML, Ratner L, Duronio R, Kishore NS, Devadas B, Adams SP, Gordon JI. 1991. Incorporation of 12-methoxydodecanoate into the human immunodeficiency virus 1 gag polyprotein precursor inhibits its proteolytic processing and virus production in a chronically infected human lymphoid cell line. Proc Natl Acad Sci USA 88: 2055–2059.

Bukau B, Horwich AL. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366.

Ehrhart JC, Duthum A, Ullrich S, Appella E, May P. 1998. Specific interaction between a subset of the p53 protein family and heat shock proteins hsp72/hsc73 in a human osteosarcoma cell line. Oncogene 3:595–603.

Farazi TA, Waksman G, Gordon JI. 2001. The biology and enzymology of protein *N*-myristoylation. J Biol Chem 276:39501–39504.

King MJ, Sharma RK. 1991 *N*-myristoyl transferase assay using phosphocellulose paper binding. Anal Biochem 199:149–153.

King MJ, Sharma RK. 1993. Identification, purification and characterization of a membrane-associated *N*-myristoyl-transferase inhibitor protein from bovine brain. Biochem J 291:635–639.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Magnuson BA, Raju RV, Moyana TN, Sharma RK. 1995. Increased *N*-myristoyltransferase activity observed in rat and human colonic tumors. J Natl Cancer Inst 87: 1630–1635.

Park J, Meisler AI, Cartwright CA. 1993. c-Yes tyrosine kinase activity in human colon carcinoma. Oncogene 8: 2627–2635.

Rajala RV, Dehm S, Bi X, Bonham K, Sharma RK. 2000a. Expression of *N*-myristoyltransferase inhibitor protein and its relationship to c-Src levels in human colon cancer cell lines. Biochem Biophys Res Commun 273:1116–1120.

Rajala RV, Radhi JM, Kakkar R, Datla RS, Sharma RK. 2000b. Increased expression of *N*-myristoyltransferase in gallbladder carcinomas. Cancer 88:1992–1999.

Raju RV, Moyana TN, Sharma RK. 1996. Overexpression of human *N*-myristoyltransferase utilizing a T7 polymerase gene expression system. Protein Expr Purif 7: 431–437.

Raju RV, Moyana TN, Sharma RK. 1997. *N*-Myristoyl-transferase overexpression in human colorectal adenocarcinomas. Exp Cell Res 235:145–154.

Resh MD. 1999. Fatty acylation of proteins: New insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1451:1–16.

Selvakumar P, Pasha MK, Ashakumary L, Dimmock JR, Sharma RK. 2002. Myristoyl-CoA:protein *N*-myristoyl-transferase: A novel molecular approach for cancer therapy. Int J Mol Med 10:493–500.

Shoji S, Kurosawa T, Inoue H, Funakoshi T, Kubota Y. 1990. Human cellular src gene product: Identification of the myristoylated $pp60^{c\text{-}src}$ 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.

Sturzbecher HW, Chumakov P, Welch WJ, Jenkins JR. 1987. Mutant p53 proteins bind hsp 72/73 cellular heat shock-related proteins in SV40-transformed monkey cells. Oncogene 1:201–211.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Welch WJ, Feramisco JR. 1985. Disruption of the three cytoskeletal networks in mammalian cells does not affect transcription, translation, or protein translocation changes induced by heat shock. Mol Cell Biol 5:1571–1581.

Zylicz M, King FW, Wawrzynow A. 2001. Hsp70 interactions with the p53 tumour suppressor protein. EMBO J 20:4634–4638.