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Association of MTG8 (ETO/CDR), a Leukemia-related Protein, with Serine/Threonine Protein Kinases and Heat Shock Protein HSP90 in Human Hematopoietic Cell Lines

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A proto-oncogene, *MTG8* (*ETO*/*CDR*), is disrupted in the t(8;21) translocation associated with acute myeloid leukemia, and the gene product, MTG8, is a phosphoprotein capable of cell transformation in concert with v-H-ras. To obtain insight into functional regulation of MTG8 by phosphorylation, we studied protein kinases that interact with, and phosphorylate, MTG8 *in vitro*. Recombinant MTG8 protein was first found to be associated with two serine/threonine protein kinases in cell extracts from both HEL cells and a leukemic cell line carrying t(8;21). A cytoplasmic protein kinase of 61 kDa (MTG8N-kinase) phosphorylated the amino-terminal of MTG8, and another of 52 kDa (MTG8C-kinase) phosphorylated the carboxyl-terminal domain. In addition, we demonstrated that heat shock protein 90 (HSP90) specifically binds to the amino-terminal domain of MTG8 *in vitro* and *in vivo*. Thus, our results shed new light on post-translational regulation of MTG8, perturbation of which, in AML1-MTG8 protein, probably contributes to leukemogenesis.

Key words: MTG8N-kinase — MTG8C-kinase — Leukemogenesis

The proto-oncogene, *MTG8* (also called *ETO* or *CDR*), is disrupted and fused with the *AML1* gene in the t(8;21) chromosomal translocation, which is frequently found in acute myeloid leukemia of M2 subtype.[1–5] The *AML1-MTG8* fused transcript contains exons 1 to 5 of *AML1* and most of the coding region of *MTG8*.[5,6] Recent investigations have shown that AML1-MTG8 protein acts as a dominant inhibitor,[7–10] or as an activator[11,12] in gene expression regulated by AML1 and C/EBP-α *in vitro*, and that MTG8 sequences are essential for dominant repression or activation by AML1-MTG8.[9–12] The deduced amino acid sequence of the *MTG8* gene product has some characteristic features, such as proline-, serine-, threonine-(PST) rich regions, putative zinc-finger motifs, and a region homologous to *Drosophila* TAF110 protein.[5,13] The *MTG8* gene is highly expressed in adult mouse brain,[5] and may function in the neural system of mammals. In addition, the *MTG8* transcript is expressed in various hematopoietic tumor cell lines, especially those of lymphoid origin,[14] and in $CD34^+$ enriched peripheral blood cells.[15] All of these results suggest that MTG8 protein plays a significant role in both leukemogenesis and normal hematopoiesis. Recently, we reported that overexpression of the *MTG8* gene in Bhas 42 cells (BALB/3T3 cells transfected with v-H-*ras* gene) induced tumorigenicity; it did not have this effect in untransfected BALB/3T3 cells, which suggests that MTG8 acts as an oncogene with the activated ras-signaling cascade.[16] In addition, since we found, in agreement with Erickson *et al*.,[15] that MTG8 is a putative phosphoprotein and is localized in nuclei of human erythroleukemia (HEL) cells,[15] we tried to identify protein kinases that interact with MTG8 in leukemic cell lines. We also found heat shock protein 90 (HSP90) as a major associated protein of MTG8 *in vitro* and *in vivo*. HSP90 is a well conserved protein family that is abundant in animal cells.[17] This paper reports that MTG8 is associated with protein kinases and HSP90 in the cellular signaling pathway.

MATERIALS AND METHODS

Plasmids The MTG8-expression plasmids were prepared as follows. For *in vitro* translation of full-length MTG8 protein tagged with S-peptide (S-MTG8), pCITE/MTG8: a polymerase chain reaction (PCR) product encoding MTG8a[5] amino acids 1–578 (5′ primer; 5′-GATGCCTGATCGTACTGAGAAGCA and 3′ primer; 5′-CTAGCGAGGGGTTGTCTCTATGGT) was inserted into the pCITE-3C(+)-T vector (Novagen, Madison, WI). For expression in *E. coli*, pS-MTG8: the S-MTG8 coding region (*Nde*I-*Xho*I fragment) of pCITE/MTG8 was subcloned into pET-31b(+) vector (Novagen). For glutathione S-transferase (GST) fusion proteins, pGST-MTG8N and

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pGST-MTG8C: the *Rsa*I fragment of *MTG8a* cDNA (amino acids 4–183) and a PCR fragment encoding amino acids 447–578 (5′ primer; 5′-AGAGACTCGAGAATTC-TAGAGCCCACGACATGATCACAAC and 3′ primer, 5′-GGACTGAATTCCTAGCGAGGGGTTGTCTCTA) were inserted into the pGEX-3X vector (Amersham Pharmacia Biotech, Little Chalfaut, UK).

***In vitro* translation and expression of GST fusion proteins** The ^{35}S-labeled S-MTG8 protein was produced with a TNT T7 Coupled Reticulocyte Lysates System (Promega, Madison, WI) using [^{35}S]methionine (Amersham Pharmacia Biotech). S-MTG8, GST-fused MTG8N-terminal region (GST-MTG8N) and GST-fused MTG8C-terminal region (GST-MTG8C) proteins were produced in *E. coli* in the presence of isopropyl-β-thiogalactoside (IPTG) (Wako Pure Chemicals, Osaka), and purified on S-protein agarose (Novagen) or glutathione (GSH)-Sepharose beads (Amersham Pharmacia Biotech), respectively.

Preparation of anti-MTG8 antibody Anti-MTG8 polyclonal antibodies were raised in rabbits injected with purified GST-MTG8 (amino acids 87–189) and with GST-MTG8N. The sera were affinity-purified by using the antigens, absorbed with anti-GST antibodies, and then purified by Mono-Q column chromatography.

Cells and preparation of cell extracts HEL and HL60 (human promyelocytic leukemia) cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Myeloid leukemia cell lines carrying t(8;21), Kasumi-1 and SKNO-1 were obtained from Dr. N. Kamada (Hiroshima University, Hiroshima) and Dr. T. Nakagawa (Hyogo Medical Center for Adult Diseases, Akashi), respectively.[18, 19] Kasumi-1 and SKNO-1 cell lines were grown in IMDM medium supplemented with 20% FCS and 75 μM thioglycerine, and RPMI 1640 medium supplemented with 10% FCS and GM-CSF (10 ng/ml, supplied by Schering-Plough Research Institute, Kenilworth, NJ), respectively. For ^{32}P-labeling, HEL cells were preincubated with phosphate-free RPMI medium containing 10% FCS, and then labeled with [^{32}P]orthophosphate (3.7 MBq/ml) for 3 h, followed by incubation with or without 100 n*M* okadaic acid for another 2 h. Cell extracts were prepared in lysis buffer (25 m*M* Tris-Cl, pH 7.4, 1.5 m*M* $MgCl_2$, 10 m*M* NaF, 0.2 m*M* sodium vanadate, 0.5% NP-40, 250 m*M* NaCl, 1 m*M* phenylmethanesulfonyl fluoride (PMSF), 8 μM aprotinin, 50 μM leupeptin), followed by centrifugation to remove cell debris. For preparation of cytoplasmic and nuclear fractions, HEL cells were lysed in lysis buffer containing 140 m*M* NaCl, and separated nuclei. The supernatant was centrifuged at 50,000 rpm for 60 min at 4°C, resulting in cytoplasmic fraction. Nuclei were sonicated in buffer containing 20 m*M* N-2-hydroxyethylpiperazine-N′-3-propanesulfonic acid (HEPES), pH 7.4, 10 m*M* NaF, 0.2 m*M* sodium vanadate, 0.5% NP-40, 125 m*M* NaCl, 1 m*M* PMSF, 8 μM aprotinin, and 50 μM leupeptin, followed by centrifugation to remove debris, affording the nuclear fraction.

Immunoblot analysis Cell extracts and *in vitro*-translated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Laboratories), and subjected to western blotting using anti-MTG8 antibody and anti-HSP90 antibody (provided by Dr. I. Yahara, Tokyo Metropolitan Institute of Medical Science, Tokyo). Antigen-antibody complex was visualized with the Hyperfilm-ECL system (Amersham Pharmacia Biotech).

Immunoprecipitation Aliquots of cell lysates, which were precleared with normal rabbit serum, were incubated at 4°C with anti-MTG8 antibody, followed by addition of protein A-Sepharose beads to precipitate immune complexes.

***In vitro* binding and protein kinase assays** The *in vitro* assays were performed as described by Hibi *et al.*[20] with some modifications. Cell extracts, cytoplasmic and nuclear fractions were each mixed for 2 h at 4°C with 40 μl of solution containing 5 μg of S-MTG8-linked S-protein agarose, GST-MTG8N-linked GSH-Sepharose, or GST-MTG8C-linked GSH-Sepharose beads. The beads were collected, washed extensively and used for protein kinase activity assay.

Phosphoamino acid analysis Phosphorylated S-MTG8 and GST-fusion proteins were separated by SDS-PAGE, and transferred to PVDF membrane. ^{32}P-Labeled protein bands were excised, hydrolysed in 6 *N* HCl for 2 h, and analyzed by two-dimensional thin-layer cellulose chromatography, according to Kamps and Sefton.[21] Standard phosphoamino acids (Sigma, St. Louis, MO) were visualized by using ninhydrin solution.

Purification of MTG8N- and MTG8C-protein kinases For large-scale preparation of cytoplasmic extracts, Kasumi-1 cells (approximately 1×10^9 cells) were used. The cytoplasmic fraction was passed through a 2-ml GST-bound GSH-Sepharose column, and then loaded onto 2 ml of GST-MTG8N beads or GST-MTG8C beads. The proteins binding to each column were eluted with binding buffer containing 1 *M* NaCl. Eluted proteins were dialyzed extensively against 20 m*M* Tris-Cl, pH 7.6, 1 m*M* dithiothreitol (DTT), 10 m*M* NaF, 0.5 m*M* PMSF, 25 m*M* NaCl and concentrated with sucrose, followed by in-gel protein kinase assay or ATP-Sepharose column chromatography.

ATP-Sepharose column chromatography Half of the eluate from the GST-MTG8N column was applied to an ATP-Sepharose column.[22] After extensive washing with binding buffer (25 m*M* Tris-Cl, pH 7.4, 60 m*M* $MgCl_2$, 10 m*M* NaF, 1 m*M* DTT, 1 m*M* PMSF), the proteins were eluted with 1, 2, 3, 4, 5 and 10 m*M* ATP. Each eluate was

subjected to in-gel protein kinase assay or SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. The main protein band in the 2 m*M* ATP eluate was transferred to PVDF membrane, and microsequenced by Takara Biomedicals, Otsu.

In-gel protein kinase assay In-gel protein kinase assay was performed according to Kameshita and Fujisawa.[23] The 1 *M* NaCl eluate from the GST-MTG8N affinity column and the fractions of ATP-Sepharose column eluted with 1 m*M*, 2 m*M*, and 3 m*M* ATP were subjected to 10% SDS-PAGE containing myelin basic protein (MBP) (0.16 mg/ml) in the resolving phase. For the eluate from the GST-MTG8C column, the GST-MTG8C protein (0.21 mg/ml) was dissolved in the resolving phase. After electrophoresis, the gel was equilibrated in protein kinase buffer for 1 h. Reactions were carried out for 1 h in 10 ml of protein kinase buffer containing [γ-^{32}P]ATP (1.85 MBq) and 50 μM cold ATP at 30°C. The gel was washed with 100 ml solution containing 5% trichloroacetic acid and 1% sodium pyrophosphate at 25°C, followed by bioimage analysis.

RESULTS

Immunochemical detection of MTG8 and AML1-MTG8 proteins The full-length MTG8 protein with S-peptide (S-MTG8) was obtained by *in vitro* translation in rabbit reticulocyte lysate containing [^{35}S]methionine. The ^{35}S-labeled S-MTG8 protein migrated at 80 kDa on SDS-PAGE, and was recognized by western blotting with anti-MTG8 antibody (Fig. 1A). To detect cellular MTG8 and AML1-MTG8 proteins in leukemic cells, western blot analysis of cell lysates was performed (Fig. 1B). HEL cell lysates contained a single 76 kDa protein, and SKNO-1 cell lysates contained 2 protein bands with a broad signal of 100 to 107 kDa and 76 kDa, whereas the HL60 cell lysates did not contain any protein which cross-reacted with the antibody (Fig. 1B). Preimmune serum did not react with either 76 kDa or 100–107 kDa proteins (data not shown). These results indicated that the 76 kDa protein is MTG8, and the 100–107 kDa proteins are AML1-MTG8. The presence of AML1-MTG8 protein with various molecular weights is probably due to an alternative first exon of the *AML1* gene, which produces 2 types of AML1 proteins, AML1b and AML1c.[6] HL60 cells did not express either MTG8 or AML1-MTG8. Our recombinant MTG8 protein that was originally produced as S-MTG8 in *E. coli* followed by removal of its S-tag by thrombin digestion, migrated at 64 kDa on SDS-PAGE, which was consistent with its molecular weight 64,392 (data not shown). Thus, the retardation of cellular MTG8 on SDS-PAGE suggests post-translational modification, probably phosphorylation. We next studied whether MTG8 protein is phosphorylated in HEL cells. As shown in Fig. 1C, the anti-MTG8 antibody immunoprecipitated ^{32}P-labeled MTG8 protein with the same molecular weight of 76 kDa, whereas no phosphoprotein was precipitated by nonimmune serum (NRS). Furthermore, treatment of HEL cells with 100 n*M* okadaic acid during ^{32}P-labeling resulted in hyperphosphorylation of MTG8 protein. Thus, MTG8 was confirmed to be a phosphoprotein[15] whose phosphorylation is enhanced by incubation with okadaic acid, an inhibitor of protein phosphatases 1 and 2A.[24]

Detection of protein kinases for MTG8 To identify pro-

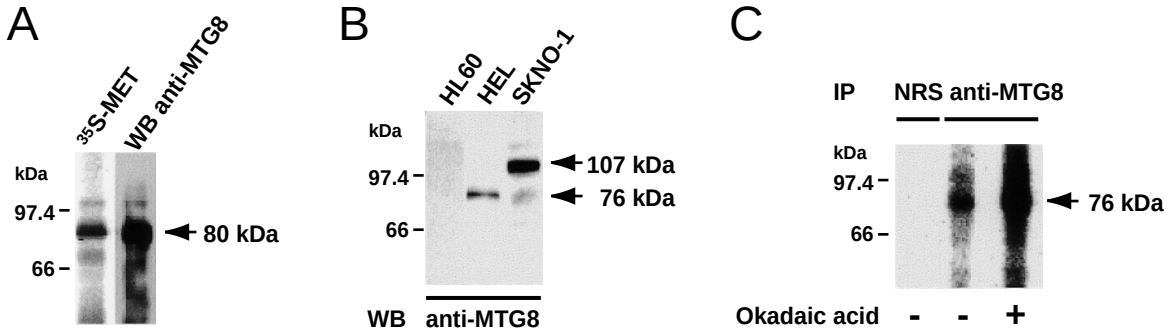

Fig. 1. Detection of MTG8 and AML1-MTG8 proteins using anti-MTG8 antibody. (A) [^{35}S]Methionine-labeled S-MTG8 protein translated in rabbit reticulocyte lysate was separated by SDS-PAGE. ^{35}S-Autoradiogram of SDS-gel (left), and western blotting (WB) with anti-MTG8 antibody of the same gel (right). (B) Detection of cellular MTG8 and AML1-MTG8 proteins by western blotting with anti-MTG8 antibody. Cell lysates (20 μg protein in each lane) from HL60 cells (left), HEL cells (middle) and SKNO-1 cells (right) were subjected to SDS-PAGE. Molecular weight markers are indicated in kDa on the left. (C) Phosphorylation of MTG8 in the cells. HEL cells were labeled with [^{32}P]orthophosphate for 3 h in phosphate-free medium, followed by 2 h incubation with or without okadaic acid (100 n*M*). Proteins were immunoprecipitated from the cell lysate with nonimmune serum (NRS, left), or with anti-MTG8 antibody (middle and right). After SDS-PAGE, radioactive phosphoproteins were visualized with a bioimage analyzer.

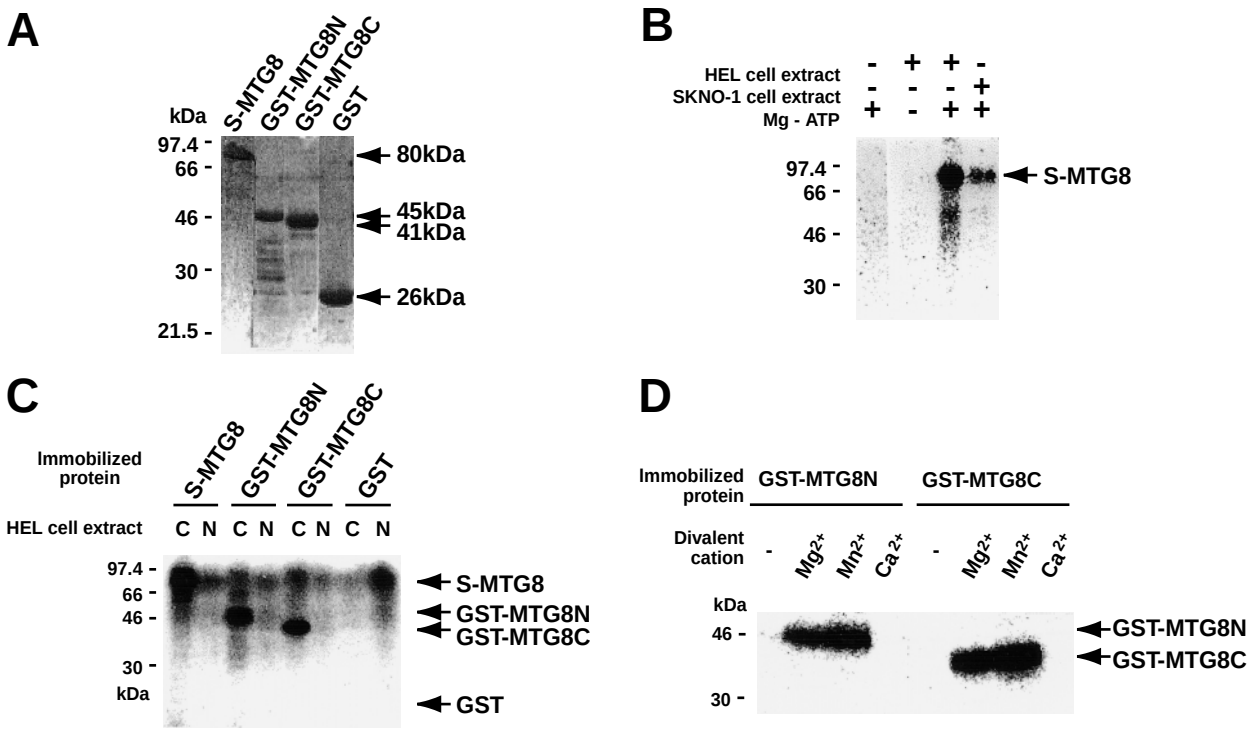

Fig. 2. Association of protein kinase with MTG8 *in vitro.* (A) CBB-stained SDS-gel of purified recombinant proteins: S-MTG8 (80 kDa), GST (26 kDa), GST-MTG8N (45 kDa), and GST-MTG8C (41 kDa). (B) Immobilized S-MTG8 on S-protein agarose was incubated without cell extract, with HEL cell extract or with SKNO-1 cell extract, and then subjected to *in vitro* protein kinase assay, with or without Mg-ATP, as described in "Materials and Methods." Phosphorylation of S-MTG8 was analyzed in terms of ^{32}P-radioactivity, after separation of the protein kinase reaction mixture on SDS-PAGE. (C) S-MTG8 beads as in B, and GST-MTG8N, GST-MTG8C, and GST bound to GSH-Sepharose were incubated with cytoplasmic (C) or nuclear (N) fractions of HEL cells. After the protein kinase reaction, phosphorylation of the immobilized proteins was analyzed. (D) Immobilized GST-MTG8N and GST-MTG8C were incubated with the cytoplasmic fraction of HEL cells. The protein kinase reactions were performed without divalent cation (–), with Mg^{2+}, with Mn^{2+}, and with Ca^{2+}.

tein kinases that bind and phosphorylate MTG8 protein, pull-down assay was performed using immobilized recombinant MTG8 proteins, such as S peptide-tagged full-length MTG8 (S-MTG8), GST-fused MTG8N-terminal region (GST-MTG8N) and GST-fused MTG8C-terminal region (GST-MTG8C) (Fig. 2A). Fig. 2B shows that S-MTG8 was phosphorylated in an Mg^{2+}- and ATP-dependent reaction with HEL cell extract, whereas it was not phosphorylated when it was incubated without Mg^{2+} and ATP. The SKNO-1 cell extract also showed phosphorylation of MTG8. Thus, both HEL and SKNO-1 cells contain protein kinases that phosphorylate MTG8 *in vitro*.

To analyze the cellular localization and properties of protein kinases in HEL cells, cytoplasmic and nuclear fractions of HEL cells were both incubated with S-MTG8, GST-MTG8N, and GST-MTG8C beads. S-MTG8, GST-MTG8N and GST-MTG8C proteins were all phosphorylated by protein kinase contained in the cytoplasmic fraction of HEL cells; little activity was found in the nuclear fraction, and GST protein was not phosphorylated by either the cytoplasmic or nuclear fraction (Fig. 2C). Thus, the N-terminal and C-terminal regions of MTG8 are able to bind to protein kinase in cytoplasmic fraction and be phosphorylated. To characterize the protein kinase, the divalent cation requirement for catalytic activity was analyzed: Phosphorylation of both N- and C-terminal domains required Mg^{2+} or Mn^{2+}, but not Ca^{2+} (Fig. 2D).

Amino acids phosphorylated by MTG8N- and MTG8C-protein kinases We next examined which amino acids were phosphorylated by protein kinases associated with MTG8. As shown in Fig. 3, S-MTG8 was phosphorylated on serine (76%), and to a lesser extent on threonine (24%); GST-MTG8N was phosphorylated on serine and threonine almost equally (50%:50%); and GST-MTG8C was phosphorylated mainly on threonine (62%). This suggests that various protein kinases are involved in phosphorylation of MTG8. Since no tyrosine of MTG8 was phosphorylated by the associated protein

kinases, we assume that the associated protein kinases are of the serine/threonine type.

Identification of protein kinases for GST-MTG8N and GST-MTG8C To further characterize the protein kinases, cytoplasmic fraction of Kasumi-1 cells was applied to a GST-MTG8N column and a GST-MTG8C column. The eluate with 1 *M* NaCl contained one protein kinase specific for GST-MTG8N and one specific for GST-MTG8C (data not shown). We also found that the protein kinase for MTG8N efficiently phosphorylated exogenous substrates, such as histone H1 and MBP (data not shown). Thus, MBP was used as a substrate for MTG8N-protein kinase. As shown in Fig. 4A, MTG8N-protein kinase was detected as a protein of 61 kDa (MTG8N-kinase) that phosphorylated MBP, and MTG8C-protein kinase was detected as a protein of 52 kDa (MTG8C-kinase) that phosphorylated GST-MTG8C. To determine the ATP-binding ability of each protein kinase, both fractions eluted from GST-MTG8N and GST-MTG8C columns were applied to γ-phosphate-linked ATP-Sepharose columns. MTG8N-kinase was eluted from the column with 2 m*M* ATP as confirmed by in-gel protein kinase assay (Fig. 4B, left). MTG8C-kinase was not recovered from the ATP-Sepharose column, possibly due to its low quantity. When S-MTG8 was used as a bait in pull-down assay, 61 kDa MTG8N-kinase was detected by in-gel-kinase assay, whereas 52 kDa MTG8C-kinase was not (data not shown). These data strongly suggest that the whole molecule of MTG8 can be phosphorylated by at least two different protein kinases in cells.

Association of HSP90 with MTG8 *in vitro* and *in vivo* When eluates from the ATP-Sepharose column were subjected to SDS-PAGE, the major component in the 2 m*M* ATP eluate was a 90 kDa protein (p90) detected by CBB staining of the gel (Fig. 4B, right), whereas the MTG8N-protein kinase band (61 kDa) was not visible. Judging from the stoichiometry of the protein contents, the association of p90 with GST-MTG8N was apparently different

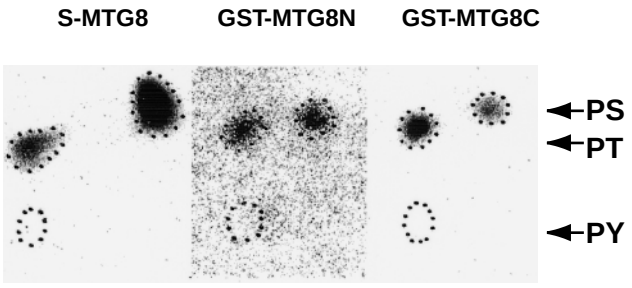

Fig. 3. Phosphoamino acid analysis of MTG8 proteins phosphorylated *in vitro*. Phosphorylated S-MTG8 (left panel), GST-MTG8N (middle panel), and GST-MTG8C (right panel) bands as in Fig. 2 were cut out from PVDF membranes, hydrolyzed with 6 *N* HCl, and then subjected to two-dimensional thin-layer chromatography. The locations of unlabeled marker phosphoamino acids on the thin-layer plates were determined by ninhydrin staining and are indicated by the dotted circles. The relative migration positions of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) are indicated on the right.

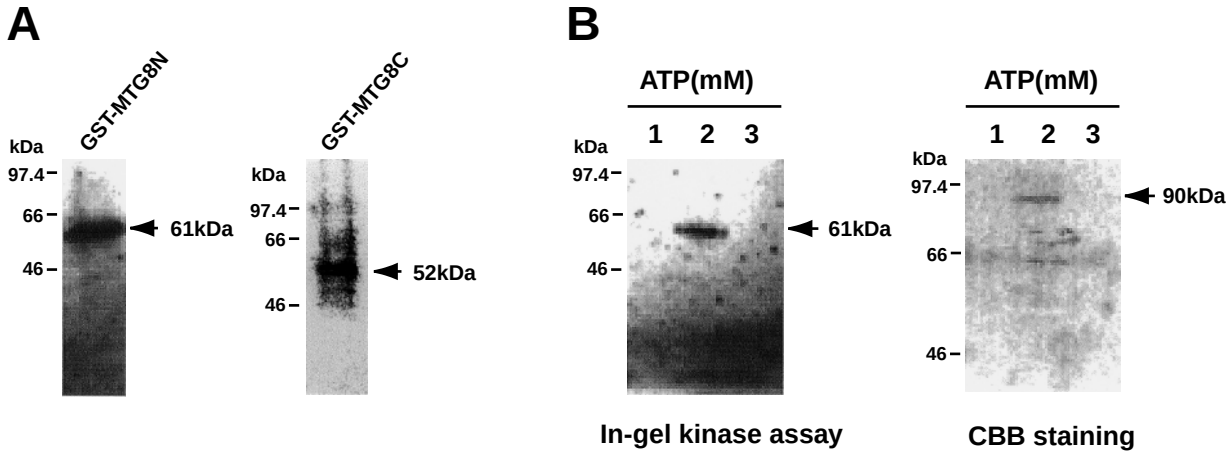

Fig. 4. In-gel protein kinase assay of MTG8 protein kinases. (A) Fractions eluted with 1 *M* NaCl from either GST-MTG8N or GST-MTG8C columns were subjected to SDS-PAGE containing MBP and GST-MTG8C respectively in the resolving phase, and subjected to protein kinase assay in gel. (B) ATP-Sepharose column chromatography of the eluate from the GST-MTG8N column. The eluates from the ATP-Sepharose column were fractionated on SDS-PAGE containing MBP in the resolving phase, followed by in-gel protein kinase assay. CBB staining of SDS-gel of GST-MTG8N shows proteins eluted from the ATP-Sepharose column by 1 m*M*, 2 m*M*, and 3 m*M* ATP.

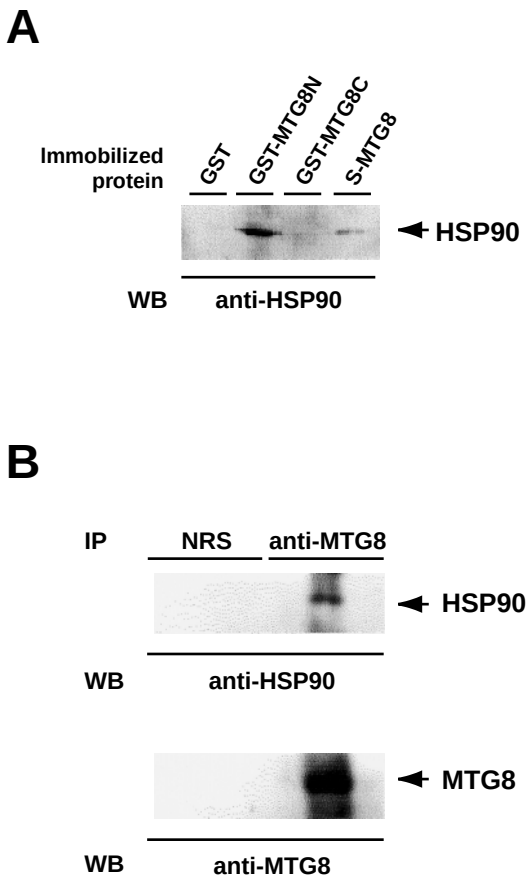

Fig. 5. Association of MTG8 with HSP90. (A) Western blotting of recombinant MTG8 proteins in HEL cytoplasmic fraction with anti-HSP90 antibody. (B) Immunocomplex of HSP90 and MTG8 from HEL cell lysates. NRS designates normal rabbit serum. Immunoprecipitants (IP) were fractionated by 10% SDS-PAGE, followed by immunoblotting with anti-HSP90 antibody (upper panel) and with anti-MTG8 antibody (lower panel).

from that with MTG8N-kinase. The p90 was then blotted onto PVDF membrane to conduct protein microsequencing. The amino-terminal sequence of p90 was N-PEEVX-HGEXEVETFA-C (X is an unidentified amino acid), which displayed high homology with the amino-terminal portion of human HSP90β.[25] Since the ATP-binding nature of HSP90 was recently confirmed by crystallography,[26] we concluded that p90 is HSP90β. Next, we examined whether MTG8N protein binds significantly to HSP90 *in vitro* by western blotting. HSP90 was recovered with both GST-MTG8N and S-MTG8, but not with GST-MTG8C or GST (Fig. 5A). Thus, HSP90 binds to the N-terminal domain of MTG8 *in vitro*. The association of HSP90 with MTG8 was confirmed by immunoprecipitation using HEL cell lysates. The anti-MTG8 antibody precipitated HSP90 along with endogenous MTG8, while NRS did not precipitate either HSP90 or MTG8 (Fig. 5B). Immunoprecipitation with anti-HSP90 antibody was not performed. S-MTG8 translated in rabbit reticulocyte lysates was also found to bind to HSP90 (data not shown). Thus, endogenous MTG8 is shown to form a stable complex with HSP90 in HEL cells.

DISCUSSION

We first demonstrated in this study that two different protein kinases, which are present in cytoplasm of leukemic cells, associate with MTG8 and phosphorylate MTG8 *in vitro*. Protein kinase specific for the MTG8N-terminal region is a 61 kDa protein that phosphorylates serine and threonine almost equally. Protein kinase specific for the MTG8C-terminal region is a 52 kDa protein that dominantly phosphorylates threonine residues (62%). Full-length MTG8 is phosphorylated on serine and threonine in the ratio of 3:1 upon *in vitro* incubation with cytoplasmic fraction of HEL cells. The central region of MTG8 may be phosphorylated by different protein kinases from MTG8N- and MTG8C-kinases. These two protein kinases seem to be previously unknown protein kinases based on their molecular weights. It is important to understand how both MTG8N and MTG8C are phosphorylated in HEL cells or SKNO-1 cells. From this point of view, the ratio of *in vivo*-phosphorylated serine/threonine residues in MTG8, including MTG8N, MTG8C, and endogenous MTG8, is being investigated in this laboratory (to be published elsewhere). The ratio of phosphoamino acids in full-length MTG8 was compatible with the ratio of *in vivo*-phosphorylated MTG8 in Kasumi-1 cells.[15] Thus, we think that HEL cell cytoplasmic fraction contains protein kinases that phosphorylate MTG8 specifically. It is possible that MTG8 phosphorylation has the function of regulating subcellular localization of MTG8, because MTG8, though it lacks a typical nuclear localization signal, is located dominantly in the nucleus. The difference in the phosphorylation of nuclear and cytoplasmic MTG8 is very likely significant. The C-terminal region of MTG8 (aa 447–578) contains Zn-finger like motifs reported as an MYND domain,[27] consisting of two CXXC and two C/HXXXC, along with a PST domain. We have not yet found any specific DNA binding activity of MTG8 but we have found protein binding activity.[28] Recently, the MYND domain has been reported as being responsible for the AML1-MTG8 function to suppress MDR1 transcription.[29] Therefore, phosphorylation in the MYND domain could be important in the regulation of MTG8 function through protein-protein interactions.

It is important to note that the N-terminal region of MTG8 is stably associated with HSP90 *in vitro* and *in vivo*, because HSP90 acts as specific chaperone for a wide range of client proteins involved in signal transduction,

cell cycle regulation, and hormone responsiveness, as well as stress response.[17, 30] Geldanamycin is reported to inhibit HSP90-mediated conformational maturation/refolding reaction, resulting in the degradation of some HSP90 client proteins.[30–35] However, the amount of MTG8 in HEL cells was not reduced after 24 h treatment with geldanamycin (data not shown). Therefore, MTG8 is unlikely to be protected from protein degradation by HSP90 in cells. Rather, MTG8 may be conformationally activated after the association of HSP90, to be transported to the nucleus. At present, we cannot exclude the possibility of the existence of adapter molecule(s) that link MTG8 and HSP90. Screening for such cellular proteins that interact directly with MTG8 is under way in this laboratory using a yeast two-hybrid system to clarify the nature of the MTG8-HSP90 complex.

We have previously reported that overexpression of MTG8 caused morphological changes of cells and tumorigenicity in nude mice in concert with v-H-ras in Bhas 42 cells (BALB/3T3 cells carrying v-H-*ras* gene), whereas no significant changes were observed in BALB/3T3 cells overexpressing MTG8 (without v-H-*ras* gene) or in Bhas 42 cells with MTG8 at the basal level. One interpretation of this result is that excess MTG8 activated the ras-signaling cascade, resulting in induction of malignant transformation.[16] Our data suggest the significance of the biochemical pathway mediated through protein-protein interaction of MTG8 in hematopoietic cells and the HSP90-mediated signal transduction. HSP90 has also been shown to be critical for signaling via MAP kinase, since Raf and Mek components of the MAP kinase signaling system exist in native complexes with HSP90.[30] Thus, the association of MTG8 with serine/threonine protein kinases and HSP90 allows us to hypothesize that MTG8 is a signaling mediator functioning downstream of MAP kinase in nuclei.

Recently, two new genes closely related to MTG8, MTGR1 and MTG16, have been identified.[36, 37] The protein sequence of MTGR1 is 61% homologous to MTG8, and that of MTG16 is 75% homologous, and both are expressed in a broader range of tissues than MTG8. Since our antibody against MTG8 reacts with NHR1 (nervy homology region 1), which is a strongly conserved region of *Drosophila* Nervy,[36, 37] the antibody probably cross-reacts with other proteins of the MTG8 family. In fact, the antibody reacted with an 85 kDa protein in K562 cells, though K562 cells did not express MTG8 mRNA (our unpublished results). MTGR1 forms a heterodimer with AML1-MTG8 through the NHR2 domain of the central region,[36] and MTG16 is a new fusion partner of AML1 similar to MTG8 in t(16;21) chromosome translocation in secondary leukemia.[37] The evidence presented in this paper, specifically the new association of MTG8 with protein kinases and HSP90, should be helpful in understanding the role of MTG8 family proteins in leukemogenesis.

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