Possible Involvement of a Novel STAM-associated Molecule “AMSH” in Intracellular Signal Transduction Mediated by Cytokines*

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STAM containing an SH3 (Src homology 3) domain and an immunoreceptor tyrosine-based activation motif was previously revealed to be implicated in signaling pathways immediately downstream of Jak2 and Jak3 tyrosine kinases associated with cytokine receptors. We molecularly cloned a novel molecule interacting with the SH3 domain of STAM, which was named AMSH (associated molecule with the SH3 domain of STAM). AMSH contains a putative bipartite nuclear localization signal and a homologous region of a c-Jun activation domain-binding protein 1 (JAB1) subdomain in addition to a binding site for the SH3 domain of STAM. AMSH mutant deleted of the C-terminal half conferred dominant negative effects on signaling for DNA synthesis and c-myc induction mediated by interleukin 2 and granulocyte macrophage-colony-stimulating factor. These results suggest that AMSH plays a critical role in the cytokine-mediated intracellular signal transduction downstream of the Jak2/Jak3-STAM complex.

Cytokines are soluble ligands inducing growth, differentiation, survival, and activation of target cells through their interaction with specific receptors on cell surfaces. The Jak family tyrosine kinases such as Jak1, Jak2, Jak3, and Tyk2 are associated with a variety of cytokine receptors to transduce downstream signalings from the receptors (1). Signal transducers and activators for transcription (Stats)1 are known to be activated by the Jaks upon stimulation with cytokotines, which play crucial roles in cellular responses (2). The γc chain originally identified as the third component of IL-2 receptor is commonly used as a receptor subunit for IL-2, IL-4, IL-7, IL-9, and IL-15, and its cytoplasmic domain is associated with Jak3, of which activation on IL-2 stimulation leads to activation of Stat3 and Stat5 and induction of cell growth and expression of early responsive genes such as c-myc, c-jun, and c-fos (3, 4). Several target genes for Stat5 have been defined, such as osm, c-fos, pim-1, Id-1, and cis (5–8); however, those did not include the c-myc proto-oncogene, and IL-3-mediated c-myc induction was not affected by the dominant negative Stat5 (9). Furthermore, accumulating evidence suggests that activation of Stat5 is not directly involved in the induction of DNA synthesis in response to IL-2 (10–12). On the other hand, activation of Stat3 was recently demonstrated to be essential for cell cycle progression and c-myc induction mediated by IL-6 (13, 14). Although Stat3 was also shown to bind the c-myc E2F site upon stimulation with IL-2, and to be involved in induction of the IL-2 receptor α chain expression, Stat3 is not essential for cell growth signaling mediated by IL-2 (15). Hence, little is known about additional signaling molecules possibly associated with Jak3, which may play critical roles in intracellular cell growth signaling mediated by IL-2.

During the search for signaling molecules involved in the IL-2-induced signaling pathway downstream of Jak3, at least two molecules were revealed to be associated with Jak3, STAM, a signal-transducing adaptor molecule (16) and Pyk2, a member of the focal adhesion kinase family (17). The kinase-negative Pyk2 showed a suppressive effect on IL-2-mediated cell proliferation, suggesting a possible involvement of Pyk2 in the IL-2-mediated cell growth signaling (17). On the other hand, we demonstrated that STAM contains an SH3 domain and an ITAM region and directly interacts with Jak2 as well as Jak3 through its ITAM region (18). Since the SH3 deletion mutant of STAM conserving the association site of the Jak3s acts as a dominant negative in signaling for cell growth, and the wild type STAM but not the STAM mutant enhances c-myc induction mediated by IL-2 and GM-CSF, we suspect that a certain critical signaling molecule interacts with the SH3 domain of STAM to transduce downstream signals from the Jak3/STAM complex. The present study demonstrates that a novel molecule named AMSH is associated with the SH3 domain of STAM and plays an important role in signal transduction for cell growth and c-myc induction mediated by IL-2 and GM-CSF.

**MATERIALS AND METHODS**

Gene Cloning—A Agt11 phage cDNA library was constructed with cDNAs derived from human peripheral blood leukocytes (PBLs) stimulated with phytohemagglutinin and recombinant human IL-2. An Escherichia coli strain Y1090R* was incubated with the phages, mixed with top agarose, and plated onto the LB plates and incubated at 42 °C until small plaques just become visible. Then the nitrocellulose membranes pretreated with isopropyl-β-d-thiogalactoside was overlaid onto the
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Plates were incubated at 37 °C for 4 h to allow the transfer of proteins produced. The transferred membranes were immersed in the blocking solution at 4 °C and then incubated overnight at 4 °C with glutathione S-transferase (GST) fusion protein containing the SH3 domain of STAM. The membranes were washed three times for 5 min each in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and then incubated with anti-GST antibody for 1 h at 4 °C. After washing in TBST, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG for 1 h and then immersed into the color development solution with 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml)/nitro blue tetrazolium chloride (0.3 mg/ml) for 10 min. In this Western screening, the GST protein was used as a negative control. One positive clone (clone 11) containing a 1.1-kbp insert was obtained from 5 × 10⁶ independent phage clones. To obtain a full-length cDNA clone, a ZAPII cDNA library from activated human PBLS was screened with the 1.1-kbp fragment used as a probe. Among the overlapping clones obtained, the longest cDNA clone (K1) was sequenced for both strands and determined to be the full-length cDNA clone.

Cells and Antibodies—Cell lines used were MOLT4 (a human T cell leukemic cell line stably transfected with the IL-2 receptor β chain), BAF-B03 (an IL-3-dependent murine pro-B cell line), and COS7 (a simian fibroblastoid cell line). Antibodies used were anti-STAM mAb (TUS1, IgG1 (16), anti-FLAG mAb, M2 (Eastman Kodak Co.), and anti-B19 parvovirus mAb (PAR3, IgG1) as a control mAb, and horseradish peroxidase-labeled anti-rabbit IgG (Stratagene). An anti-AMSH mAb (TUAM1, IgG1) was prepared by immunization of mice with a GST fusion protein containing 56 amino acid residues (Lys125 to Lys180) of human AMSH.

Northern Blotting—A mixture of total RNA containing poly(A)+ RNA preparations derived from various human tissues was purchased (CLONTECH). A 1.9-kbp fragment from the AMSH clone K1 and a 2.0-kbp fragment of human β actin were used as probes. The probes were labeled with random primers, [α-32P]dCTP (Amersham Pharmacia Biotech), and Bca-BEST labeling kit (Takara Shuzo, Kyoto). Hybridization was performed for 20 h at 42 °C in 50% formamide, 5× Denhardt’s solution, 5× SSPE, 0.1% SDS, 20 μg/ml Tris-HCl (pH 7.5), and 200 μg/ml sonicated and denatured salmon sperm DNA. The membranes were washed in 0.2× SSC and 0.1% SDS three times at 65 °C. Radioactivity was analyzed with a bio-image analyzer MacBas1500 (Fuji Film).

Plasmids—pGST-STAMSH3 is a prokaryotic expression vector for the GST-SH3 fusion protein. The gene fragment coding for the SH3 domain (Gly122 to Lys169) of STAM was amplified by polymerase chain reaction and inserted into pGEX-2T bacterial expression vector in frame. The gene fragment coding for the GST-SH3 fusion protein. pCMV-AMSH was constructed by cloning the cDNA fragment from AMSH in frame into the GST fusion protein containing 56 amino acid residues (Lys125 to Lys180) of human AMSH.

RESULTS

Molecular Cloning and Expression of AMSH—To isolate cDNA clones encoding a protein associated with the SH3 domain of STAM, GST fusion protein containing the SH3 domain of STAM was used as a probe for far Western screening of a λgt11 cDNA library derived from human-activated PBLS. As described under “Materials and Methods,” a full-length cDNA clone was identified, which encodes a protein consisting of 424 amino acids with a predicted molecular mass of 48.1 kDa and named AMSH (associated molecule with the SH3 domain of STAM). The deduced amino acid sequence of AMSH indicated the presence of at least three characteristic regions (Fig. 1A): 1) two Pro-Xaa-Xaa-Pro (PXXP) motifs known as possible binding sites for the SH3 domain, located at Pro195-Pro198 (P1) and
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Pro$^{227}$–Pro$^{311}$ (P2); 2) a JAB1 subdomain homologous (JSH) region between Gln$^{123}$ and Pro$^{239}$, defined to be highly homologous to one of the three subdomains of c-Jun activation domain-binding protein 1 (JAB1) (25) and yeast Pad1 (26) (Fig. 1B); and 3) a putative bipartite nuclear localization signal (NLS) (Lys$^{112}$–Lys$^{277}$) homologous to the NLS of human p53 (27) (Fig. 1C).

The chromosomal location of human AMSH gene was determined by FISH with AMSH cDNA probes. The human AMSH gene was mapped onto chromosome 2p12–13 (data not shown).

Expression of AMSH gene was examined for various human tissues by Northern blot analyses. A single 2.1-kbp mRNA species related to AMSH was seen in all the human tissues tested, including spleen, lymph node, thymus, appendix, PBLs, bone marrow, fetal liver, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2). In addition to the 2.1-kbp major band, two minor bands of approximately 4.0 and 1.6 kbp in size were also detected in the human tissues.

These results indicate that AMSH is ubiquitously expressed in various human tissues.

**Association between AMSH and STAM**—We assessed association between STAM and AMSH in a human T cell line, MOLTb, expressing the functional IL-2 receptor. Lysates of IL-2-stimulated and unstimulated MOLTb cells were treated with a chemical cross-linker, DSP, and then subjected to immunoprecipitation with anti-AMSH mAb (TUAM1), anti-STAM mAb (TUS1), or control PAR3 mAb. The immunoprecipitates were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted (IB) with anti-STAM mAb. After being stripped of the antibody, the membrane was reblotted with anti-AMSH mAb (lower panel).

Fig. 2. **AMSH mRNA expression in various human tissues.** A multiple tissue Northern blot containing 2 μg of poly(A)$^+$ RNA prepared from various human tissues was purchased (CLONTECH). The blot was first hybridized with the AMSH probe and then rehybridized with the β-actin probe.

Since AMSH was isolated as a protein interacting with the SH3 domain of STAM in the far Western screening, we next confirmed whether the SH3 domain of STAM is the binding site for AMSH in co-immunoprecipitation studies using various STAM mutants. The FLAG-tagged wild type AMSH was co-transfected into COS7 cells with the wild type STAM and three mutants of STAM, namely DSH3 deleted of the SH3 domain, DIT deleted of the ITAM, and DY2 deleted of the tyrosine cluster region (Fig. 4A). Their lysates were immunoprecipitated with anti-FLAG mAb (M2), and the immunoprecipitates were then immunoblotted with anti-STAM mAb (TUS1).

AMSH co-immunoprecipitated the wild type STAM, DIT, and DY2 but not DSH3 (Fig. 4B, upper panel). The expression levels of the wild type STAM, STAM mutants, and AMSH were assessed in the transfectant cells (Fig. 4B, middle and bottom panels), confirming that DSH3 was expressed equally well with the other STAMs, and AMSH expression was constant. These results indicate that the SH3 domain of STAM indispensably contributes to the binding with AMSH.

We also attempted to define the binding site of AMSH for STAM. Expression vectors were prepared for the FLAG-tagged wild type AMSH and five AMSH mutants such as DBS2 deleted of a PX motif (Pro$^{227}$–Pro$^{231}$), DBS3 deleted of two PX motifs (Pro$^{195}$–Val$^{199}$), DC2 deleted of the C-terminal half (Asp$^{224}$–Arg$^{234}$) containing JSH, DJS deleted of the JSH region (Gln$^{223}$–Pro$^{239}$), and DNL deleted of the NLS region (Lys$^{112}$–Lys$^{277}$) (Fig. 4C).

The wild type STAM was expressed in COS7 cells, and their lysates were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-STAM mAb. STAM was co-immunoprecipitated with the wild type AMSH, DJS, DNL, and DC2 but not with DBS2 and DBS3 (Fig. 4D, upper panel). Expression levels of DBS2 and DBS3 were not significantly different from those of the other AMSHs (Fig. 4D, lower panel).

These results indicate that the PX motif (Pro$^{227}$–Pro$^{231}$) of AMSH is a major binding site for the SH3 domain of STAM.

**Involvement of AMSH in Signaling for DNA Synthesis Mediated by IL-2 and GM-CSF**—Since the SH3 deletion mutant of STAM was previously reported to have a dominant negative effect on signaling for DNA synthesis mediated by IL-2 and GM-CSF (18), we assumed that the binding of AMSH to the SH3 domain of STAM is also involved in a similar signal transduction pathway. To investigate this possibility, we ex-
amined the effects of the wild type and various mutants of AMSH on DNA synthesis mediated by IL-2 and GM-CSF in IL-3-dependent BAF-B03 cells, transiently reconstituted with receptors for IL-2 or GM-CSF. Initially, BAF-B03 cells were transfected with the wild type AMSH or its mutants along with human IL-2 receptor β and γc chains and stimulated with human IL-2. The cells transfected with the wild type AMSH and its mutants of either DJS, DNL, or DBS2 exhibited significant increases of [3H]thymidine incorporation, comparable with the control vector transfecnt cells. In sharp contrast, the transfecnt cells with DC2 mutant AMSH resulted in 58% inhibition of [3H]thymidine uptake as compared with those with the control vector (Fig. 5A). The inhibition of IL-2-induced [3H]thymidine uptake was dependent on the DC2 plasmid dose (Fig. 5B). Similar results were obtained with the GM-CSF stimulation system. BAF-B03 cells were transfected with the wild type AMSH, AMSH mutants, and control vector along with human GM-CSF receptor α and βc chains and stimulated with human GM-CSF. Only the transfecnt with DC2 mutant showed 55% inhibition of [3H]thymidine uptake mediated by GM-CSF as compared with the control vector (Fig. 5C). The inhibition of the GM-CSF-induced [3H]thymidine uptake was also observed in the plasmid dose-dependent manner (Fig. 5D). These results indicate that exogenous expression of DC2 mutant of AMSH induces appreciable inhibition of DNA synthesis mediated by IL-2 and GM-CSF, suggesting a dominant negative effect of DC2 mutant on intrinsic AMSH, which is involved in the cell growth signal transduction from receptors for IL-2 and GM-CSF.

Involvement of AMSH in c-myc Induction Mediated by IL-2 and GM-CSF—STAM was reportedly shown to be involved in c-myc induction in response to IL-2 and GM-CSF (18). Hence, we examined whether AMSH also contributes to signaling for c-myc induction mediated by IL-2 and GM-CSF. BAF-B03 cells were transfected with the wild type AMSH, its mutants, or the control vector together with the human IL-2 receptor β and γc chains and the c-myc promoter-driven luciferase reporter gene. They were then stimulated with IL-2 and assayed for c-myc induction by luciferase activity. Transfectant cells with the wild type AMSH, DJS, DNL, and DBS2 exhibited significant increases of c-myc induction upon IL-2 stimulation, similar to those achieved by the control vector (Fig. 6A). However, with the DC2 mutant, c-myc induction was suppressed to less than half the activity observed with the control vector (Fig. 6A). The suppression of IL-2-mediated c-myc induction was dependent on the DC2 plasmid dose (Fig. 6B). Similar results were obtained with the GM-CSF stimulation system. BAF-B03 cells were transfected with the wild type AMSH, AMSH mutants, or the control vector, together with the human GM-CSF receptor.

Fig. 4. Schematic structures of STAM mutants and AMSH mutants and co-immunoprecipitation between AMSH and STAM. A, schematic structures of the wild type and mutants of STAM. DSH3, DIT, and DY2 mutants of STAM are deleted of the SH3 domain (Ala217 to Ala266), ITAM (Glu356 to Leu387), and tyrosine cluster region (Tyr 371 to Leu387), respectively. B, COS7 cells were transiently transfected with 10 µg of expression plasmids for the wild type and three mutants of STAM, together with 20 µg of expression plasmids for FLAG-tagged AMSH. After cultivation for 28 h, the cell lysates were immunoprecipitated with anti-FLAG mAb (M2) (upper panel) or anti-STAM mAb (TUS1) (bottom panel) and then immunoblotted with anti-STAM mAb (upper and bottom panels) or anti-FLAG mAb (lower panel). C, schematic structures of the wild type and mutants of AMSH. DBS2, DBS3, DC2, DJS, and DNL mutants of AMSH are deleted of the P2 region (Pro227 to Pro231), the P1 to P2 region (Pro195 to Val233), a C-terminal half-region (Asp234 to Arg264), and the NLS region (Lys112 to Lys127), respectively. D, COS7 cells were transiently transfected with 10 µg of expression plasmids for the wild type and five mutants of AMSH together with 10 µg of wild type STAM expression plasmid. After cultivation for 28 h, the cell lysates were immunoprecipitated (IP) with anti-FLAG mAb and then immunoblotted (IB) with anti-STAM mAb (upper panel) or anti-FLAG mAb (lower panel).
and c-myc promoter-driven luciferase reporter gene. After stimulation with GM-CSF, they were assayed for c-myc induction by luciferase activity. The c-myc induction was significantly suppressed by transfection of the DC2 mutant but not of the wild type AMSH, other AMSH mutants, and the control vector (Fig. 6C). The suppression of GM-CSF-mediated c-myc induction was dependent on the DC2 plasmid dose (Fig. 6D). These results indicate that the DC2 mutant of AMSH also acts as a dominant negative in c-myc induction mediated by IL-2 and GM-CSF, suggesting the involvement of AMSH in signal transduction for c-myc induction mediated by these cytokines. On the other hand, we detected marginal effects of the wild type AMSH and its mutants on c-fos promoter-driven luciferase activities mediated by IL-2 and GM-CSF (data not shown), suggesting non-involvement of AMSH in signaling for c-fos induction.

**DISCUSSION**

The present study documented the existence of a novel molecule named AMSH, which is associated with STAM and is involved in the signal transduction mediated by IL-2 and GM-CSF. We previously demonstrated that STAM containing the SH3 domain and ITAM region is associated with both Jak2 and Jak3 tyrosine kinases via its ITAM region irrespective of cytokine stimulation (18). Jak2 and Jak3 are known to be associated with the βc chain of GM-CSF receptor and γc chain of IL-2 receptor, respectively, and to be indispensable for intracellular signal transduction mediated by GM-CSF and IL-2, respectively (28–33). The SH3 deletion mutant of STAM acts as a dominant negative in cell growth signal transduction mediated by IL-2 and GM-CSF, indicating the possible existence of an interacting molecule with the SH3 domain of STAM, which plays a critical role in the downstream signal transduction of the Jak2/Jak3-STAM complex (18). Upon investigation of a signaling molecule associated with the SH3 domain of STAM, we hereby successfully cloned a cDNA encoding AMSH. AMSH contains two regions with the ProXXPro motif known to be a putative binding site for the SH3 domain. It is present in many signaling molecules including kinases and adaptor molecules and appears to be responsible for intermolecular interaction (34, 35). Co-immunoprecipitation with various deletion mutants of AMSH and STAM demonstrated that the PXXP region (Pro227–Pro231) of AMSH is a binding site for the SH3 domain of STAM, which plays a critical role in the downstream signal transduction of the Jak2/Jak3-STAM complex (18). Upon investigation of a signaling molecule associated with the SH3 domain of STAM, we hereby successfully cloned a cDNA encoding AMSH. AMSH contains two regions with the PXXP motif known to be a putative binding site for the SH3 domain of STAM. AMSH also contains a putative bipartite NLS. However, since nuclear localization of AMSH has not been observed even after stimulation of cells with cytokines (data not shown), the functional significance of AMSH NLS is still unknown. Another unique sequence, JSH, is present in AMSH, which is homologous to one of the three subdomains of c-Jun activation...
domain-binding protein 1 (JAB1). The JSH sequence of AMSH is conserved among human JAB1 and yeast Pad1. Although JAB1 and Pad1 are known to be co-activators for transcription of AP-1 target genes through interaction with c-Jun or Jun D, we have no evidence for association of AMSH with c-Jun or Jun D in co-immunoprecipitation or electrophoretic mobility shift assays (data not shown). Hence, the biological significance of the JSH region of AMSH is still unknown.

AMSH was revealed to be implicated in signal transduction mediated by IL-2 and GM-CSF. An AMSH mutant, DC2, deleted of the C-terminal half including the JSH region, possessed an apparent suppressive activity for DNA synthesis and c-myc induction mediated by IL-2 and GM-CSF. Although the JSH region is included in the C-terminal half, it seems unnecessary for such molecule association, because the DJ5 mutant deleted of the JSH region did not show any dominant negative effect in the signaling. Accumulating evidence suggests a critical role of c-myc gene activation in cytokine-induced cell cycle progression from G1 to S phase (36). Despite extensive study in cytokine-mediated intracellular signal transduction, little is known about the signaling pathway for c-myc induction and cell cycle progression except for the Stat3-mediated pathway. Stat3 was recently demonstrated to be involved in signaling for the G1 to S cell cycle transition and c-myc induction through its binding to the c-myc E2F site in response to IL-6 (13, 14). The binding of Stat3 to the c-myc E2F site was also detectable upon stimulation with IL-2 (14), suggesting a possible involvement of Stat3 in IL-2-mediated c-myc induction. However, we have evidence that the c-myc E2F site of c-myc gene is not essential for full induction of c-myc in response to IL-2. Furthermore, the recent study using the Stat3 knock-out mice demonstrated an essential involvement of Stat3 in the IL-2 receptor α chain expression mediated by IL-2 but nonessential for IL-2-induced cell growth signaling (15). Although another member of the Stat family, Stat5, is activated by stimulation with various cytokines including IL-2, IL-3, and GM-CSF (10, 37–41), Stat5 is reportedly dispensable for the c-myc induction mediated by IL-3 (9). In concert with these observations, Stat5 activations mediated by IL-2 and GM-CSF were not affected by overexpression of the wild type AMSH and its mutants (data not shown), suggesting AMSH is nonessential for Stat5 activation.

Our present study demonstrated that the STAM-AMSH complex plays a critical role in signaling for c-myc induction and cell cycle progression mediated by IL-2 and GM-CSF in the downstream pathway of Jak3 and Jak2, which may be distinct from the pathway for Stat5 activation.

STAM interacting with AMSH binds to Jak2 and Jak3 (18), which are also associated with the βc and γc chains of the cytokine receptors, respectively (29, 30, 33), and all the interactions between AMSH and STAM, STAM and the Jaks, and the Jaks and the cytokine receptors are independent of cytokine stimulation. These observations suggest a possible formation of the tetrameric complex composed of the cytokine receptors, Jaks, STAM, and AMSH in vivo. However, since AMSH is co-immunoprecipitable with STAM, only when the cells are treated with the chemical cross-linker DSP, the binding affinity between AMSH and STAM may be too low for detection of the tetrameric complex.

AMSH was found to be expressed ubiquitously among various human tissues including thymus, spleen, brain, testis, liver, kidney, and lung, which is well correlated with the expression pattern of STAM (16). Since STAM is known to be tyrosine-phosphorylated upon stimulation with a variety of cytokines and growth factors such as IL-2, IL-4, IL-7, GM-CSF, IL-3, platelet-derived growth factor, and epidermal growth factor (16), we suspect a possible involvement of the STAM-AMSH complex in signaling pathways downstream of multiple cytokine and growth factor receptors.

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