Hrs Is Associated with STAM, a Signal-transducing Adaptor Molecule

ITS SUPPRESSIVE EFFECT ON CYTOKINE-INDUCED CELL GROWTH*

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We previously reported a new type of signal-transducing adaptor molecule, STAM, which was shown to be involved in cytokine-mediated intracellular signal transduction. In this study, we molecularly cloned a 110-kDa phosphotyrosine protein inducible by stimulation with interleukin 2 (IL-2). The 110-kDa molecule was found to be a human counterpart of mouse Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and to be associated with STAM. Tyrosine phosphorylation of Hrs is induced rapidly after stimulation with IL-2 and granulocyte-macrophage colony-stimulating factor as well as hepatocyte growth factor. The mutual association sites of Hrs and STAM include highly conserved coiled-coil sequences, suggesting that their association is mediated by the coiled-coil structures. Exogenous introduction of the wild-type Hrs significantly suppressed DNA synthesis upon stimulation with IL-2 and granulocyte-macrophage colony-stimulating factor, while the Hrs mutant deleted of the STAM-binding site lost such suppressive ability. These results suggest that Hrs counteracts the STAM function which is critical for cell growth signaling mediated by the cytokines.

Activation of tyrosine kinases is an initial biochemical event in intracellular signal transduction from cytokine receptors after their bindings with ligands. Although most of the cytokine receptors do not contain any consensus motif of tyrosine kinase, several families of tyrosine kinases, such as the Src family tyrosine kinases, Jak family tyrosine kinases, Syk/ZAP70 family tyrosine kinases, and other family tyrosine kinases (Fes and Tec), are known to be associated with the cytoplasmic domains of cytokine receptors (1). Upon activation of the tyrosine kinases, cytokine receptor subunits are phosphorylated on tyrosine residues, which results in association of the receptor subunits with signal transducers and activators of transcription (Stats) via interaction between the phosphorylated tyrosine residues of receptor subunits and the Src homology 2 (SH2) domains of Stats, and subsequently the Stats are tyrosine-phosphorylated by the Jak family tyrosine kinases to be activated as transcription factors (2, 3). For example, interleukin-2 (IL-2) induces activation of Lck (Fyn or Lyn), Syk, and Jak1, all of which are associated with the IL-2 receptor β chain, and Jak3, which is associated with the IL-2 receptor γc chain, together with activation of other signaling molecules such as phosphatidylinositol 3-kinase and Shc/Grb2/Sos/Ras/Raf1/mitogen-activated protein kinase cascade (4). Stat5 associated with the IL-2 receptor β chain is activated by Jak3 but not Jak1 upon IL-2 stimulation (5–7). IL-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) also seems to activate Stat5 through Jak2 (8, 9). Activation of Stat5 has been shown to be involved in signaling for DNA synthesis mediated by IL-2 and IL-3 in certain cell lines (10, 11). Jak3 and Jak2 are also known to be essential for induction of c-myc and c-fos upon stimulation with IL-2 and GM-CSF, respectively (12, 13). The target genes for Stat5, such as OSM (oncostatin M), c-fos, pim-1, Id-1, and CIS (cytokine inducible SH2-containing protein) have been defined (11, 14), but there is no evidence for involvement of Stat5 in c-myc induction. Therefore, we suspected that signaling molecule(s) other than Stat5 would be involved in the signaling pathway immediately downstream of the Jak5 for induction of c-myc.

To search for such molecule(s), we have investigated cellular phosphotyrosine proteins induced by IL-2 stimulation, and detected several phosphotyrosine molecules distinct in molecular masses. Among them, the 75-kDa molecule was identified as the β chain of IL-2 receptor, and a cDNA encoding the 70-kDa molecule was cloned and identified as a novel signal-transducing adaptor molecule, which we named STAM, containing an SH3 domain and a tyrosine cluster region including an immunoreceptor tyrosine-based activation motif (ITAM) (15). We also succeeded in determination of a partial amino acid sequence of the 110-kDa phosphotyrosine molecule induced by IL-2 stimulation, and here demonstrated that the 110-kDa molecule is a human counterpart of mouse Hrs. Hrs containing

* The abbreviations used are: Stat, signal transducer and activator of transcription; SH, Src homology; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; ITAM, immunoreceptor tyrosine-based activation motif; HGF, hepatocyte growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; NTPase, nucleotide triphosphatase; PBL, peripheral blood leukocytes; PHA, phytohemagglutinin; mAb, monoclonal antibody; HA, hemagglutinin; FISH, fluorescence in situ hybridization; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCS, fetal calf serum; bp, base pair(s); kb, kilobase pair(s).

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a double zinc-finger motif was reported previously to be a phosphotyrosine protein induced by stimulation with heparan-
cyte growth factor (HGF), epidermal growth factor (EGF), and
platelet-derived growth factor (PDGF), and localized in the
cytoplasm (16). Very recently, Hrs-2, a rat homolog of Hrs,
which is different in the C terminus, has been reported to have
nucleotide triphosphatase (NTPase) activity and be associated
with SNAP-25, possibly modulating vesicular transport (17).
However, the biological significance of Hrs has not yet been
elucidated. On the other hand, we have recently demonstrated
that STAM is directly associated with and phosphorylated
by Jak3 and Jak2 upon stimulation with IL-2 and GM-CSF,
respectively, and involved in signaling for DNA synthesis and
c-myc induction mediated by IL-2 and GM-CSF (18). The pres-
cent study documents that Hrs is associated with STAM and
implicated in regulation of intracellular signal transduction
mediated by IL-2 and GM-CSF.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines used were a human T cell line, MOLTβ,
which is a MOLT-4 transfectant clone expressing the exogenous β and
endogenous γ c chain expression plasmids, respectively (19); a mouse IL-2 receptor
pre-B cell line, BAF-B03 (20); a human GM-CSF-responsive cell line,
TF-1 (21); a SV40-transformed monkey kidney cell line, COS7; human
B cell lines, Raji and Dauidi; a human T cell line, Jurkat; a human
monocytic cell line, THP-1; a human eosinophilic cell line, Eol-3; a
human myelogenic cell line, KU812; and a human GM-CSF-dependent
cell line, M-TAT (22). TF-1 and M-TAT were maintained in RPMI 1640
medium supplemented with 10% FCS and recombinant GM-CSF. BAF-
B03 was maintained in RPMI 1640 medium supplemented with 10% FCS,
20% conditioned medium derived from culture supernatant of
BAF-B03 was maintained in RPMI 1640 medium supplemented with 10% FCS, 20% conditioned medium derived from culture supernatant of
WEHI-3 cell line (as a source of IL-3) and 50 µg 2-mercaptoethanol.
COST was maintained in Dulbecco’s modified Eagle’s medium supple-
mented with 10% FCS. Other cell lines were maintained in RPMI 1640
medium supplemented with 10% FCS. Peripheric blood leukocytes (PBL)
from a healthy donor were treated with 1.0% phytohemagglutinin
(PHA) (Difco) for 2 days and maintained in RPMI 1640 medium
supplemented with 10% FCS and 1 mM IL-2.

Plasmids—pSBR5 and pSRG1 are human IL-2 receptor β and γ c
chain expression plasmids, respectively (19, 23), and hGMRα and
hGMRβ are human GM-CSF receptor α and β c chain expression plas-
mids, respectively (24, 25). pEML is a β-galactosidase expression
plasmid.

Cytokines and Antibodies—Human recombinant cytokines used here
were IL-2 (obtained from Ajinomoto Co. Ltd., Japan) and GM-CSF
(Hoechst Japan). Monoclonal antibodies (mAbs) used were TUS-1
(IgG1) specific for STAM (15), 12CA5 (IgG2b) specific for influenza virus
hemagglutinin (HA) epitope (Boehringer Mannheim), 401G (IgG2b
(Upstate Biotechnology Inc.) and P1D11 (Amersham Life Science))
were subcloned into pCXN2 expression vector (28).

Immunoprecipitation and Immunoblotting—Immunoprecipitation
was carried out as described elsewhere (5). In brief, cells were lysed
in Nonidet P-40 cell extraction buffer (1% Nonidet P-40, 25 mM Tris-HCl,
ph 7.5, 140 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluo-
ride, 20 µg/ml aprotinin, 1 mM Na3VO4), and immunoprecipitated with
indicated antibodies. The immunoprecipitates were separated by
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
and then transferred to polyvinylidene difluoride filters (Millipore).
After blocking with 5% bovine serum albumin in phosphate-buffered
saline containing 0.1% Tween 20 or Tris-buffered saline containing
0.1% Tween 20 was performed with anti-FLAG antibody. The
filters were incubated with indicated antibodies, fol-
lowed by incubation with anti-mouse IgG coupled with horseradish
peroxidase and visualized by using the enhanced chemiluminescence
(ECL) detection system (Amersham Life Science).

Isolation of cDNA Clone Coding for pp110 (Human Hrs)—Based on
a partial amino acid sequence (GQTVHDEVANK) of pp110, a pair of
primers, 3′F (5′-GGAGCTCTCAAGACAGCATGTCGTC-3′) and 5′R (5′-
TCAGATGTAGGCTGCAGCTACATCTG-3′), were chemically syn-
thetized. To isolate a cDNA coding for pp110, we prepared polyclonal
RNA from MOLTβ, and synthesized first-strand cDNA from the
poly(A)+ RNA as a template and AR primer with the First-Strand DNA
synthesis kit (Pharmacia, Sweden). The synthesized cDNA were
amplified by PCR method with A3F and AR primers, and the first
strands cDNA as templates. To obtain a full-length cDNA, we
amplified DNA fragments were digested with KpnI and BglII
and then ligated with pKU-Hrs vector at the
BglII site cloned into pKU-Hygro expression vector at the
XhoI and XbaI sites, and pKU-Hrs was obtained as a cDNA
plasmid for pp110. pKU-HdC1 and pKU-Hdc2 are expression plasmids for
the C-terminal truncation mutants of Hrs, named Hrs-dC1 and
Hrs-dC2, respectively, which were deleted up to nucleotides 1786 and
1429 from pp110 with exonuclease III and mung bean nuclease,
respectively, and then a multiple-terminator linker (pGCTAGCT
TTAGTCTAGTACACTCTACTAGC) was inserted. pKU-Hdm is an
expression plasmid for the mutant deleted from Ser432 to Met573 of Hrs,
named Hrs-dM, of pKU-Hdm, we amplified Hrs DNA as PCR method with two pairs of primers, 5′-TGGAAGACAGTTGGAGGTCC-3′ and 5′-TAAAAGTACCGAGTCATTGGTGATC-3′, or
5′-AAAAGTACCGAGCCCGCCGGTGCGTGCT-3′ and 5′-GGAGGTGTGGCGGGAGTTCCTT-3′, respectively, and with pKU-Hrs as a template.
Amplified DNA fragments were digested with KpnI and the resultant
fragments were ligated to each other and then digested with BgII and
XbaI. The digested fragment was ligated to pKU-Hrs vector after re-
moval of the BgII/XbaI fragment. The wild-type and mutants of Hrs
were subcloned into pCXN2 expression vector (28).

pKU-Hrs-HA is an expression plasmid for the HA epitope (YPDVPDY
DYASO)-tagged Hrs (Hrs-HA). For preparation of pKU-Hrs-HA, we
amplified Hrs DNA by PCR method with two pairs of primers, 5′-AT-
ATCGAGCCGGCGCGCTGCGTTTT-3′ and 5′-ATAATCCGGAAACTC
ATAGATCATCCCAGGCGACCTGCG-3′, or 5′-ATGTTCCGAGTT
TTGCTAGTACGAGGGCGAGGTGACCT-3′, respectively, and with pKU-Hrs as a template. Amplified DNA fragments were digested with BglII and
then digested with XhoI and BgII.Digested fragments were ligated to
pKU-Hrs vector after removal of the XhoI-BgII fragment. pKU-HrsHA expresses Hrs-HA, which consists of the HA-epitope linked
to Gly2 at the N terminus of Hrs. For HA-tagging to all the Hrs
mutants, pKU-HdC1, pKU-Hdc2, and pKU-Hdm were digested with
BglII and XbaI, and the resultant fragments were ligated into pKU-
Hrs-HA vector at the BgII and XbaI sites. These expression vectors
were named pKU-Hdc11HA, pKU-Hdc21HA, pKU-Hdc31HA, and pKU-
Hdm-HA, respectively.

All the constructed plasmids were sequenced as performed in an Applied Bio-
systems model 373A DNA sequencer.

Northern Blot Analysis—Northern blot analyses were performed as
described previously (29). In brief, poly(A)+ RNA derived from various
human cell lines was purified with guaninium isothiocyanate extraction
and oligo(dT) column (Oligotex™) (Takara Shuzo Co.), and Multi-
ple Tissue Northern blot containing poly(A)+ RNA preparations derived
from various human tissues were purchased (CLONTECH). 2 µg of
poly(A)+ RNA derived from each cell lines were electrophoresed on 1%
agarose gel containing formaldehyde, and transferred to GeneScreen
membrane (NEN Life Science Products). A 2.9-kb fragment of Hrs
cDNA and 0.5-kb fragment of glyceraldehyde-3-phosphate dehydrogen-
ase (GAPDH) cDNA were used as probes for hybridization after labeling with
[32P]dCTP. Radioactivity was measured with a Bio-Image Ana-
lyzer BAS 1500 (Fuji Film).

Chromosomal Mapping—The chromosomal location of human Hrs
gene was determined by fluorescence in situ hybridization (FISH).
RESULTS

Molecular Cloning of Human Hrs—An IL-2-induced phosphorysine protein, pp110, detected previously (15), was purified from immunoprecipitates of MOLT\(/2 cell lysates with anti-phosphotyrosine mAb, 4G10, 4G10, and PY20 (Fig. 3). We investigated expression of Hrs mRNA in various human tissues and cell types. Hrs mRNA was detected as a 3.0-kb band in all the tissues and cell types tested, including spleen, lymph node, thymus, appendix, PBL, bone marrow, fetal liver, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2A, T and B lymphoid cell lines (MOLT\(/2, Jurkat, Daudi, and Raji), non-lymphoid hematopoietic cell lines, and their schematic structures are shown in Fig. 1A. The N terminus containing 180 amino acid residues showed 99% homology among them, and the regions containing the double zinc-finger motif, proline-rich region, proline- and glutamine-rich regions, putative coiled-coil sequences, and nucleotide-binding sites, and their schematic structures are shown in Fig. 1A. The N terminus containing 180 amino acid residues showed 99% homology among them, and the regions containing the double zinc-finger motif, proline-rich region, proline- and glutamine-rich regions, putative coiled-coil sequence were also highly homologous. However, the C-terminal 147 amino acid residues of Hrs-2 were not included in human and mouse Hrs. The chromosomal location of human Hrs gene was determined by FISH with Hrs cDNA probes. The human Hrs gene was mapped on chromosome 17q25 (Fig. 1B).

We investigated expression of Hrs mRNA in various human tissues and cell types. Hrs mRNA was detected as a 3.0-kb band in all the tissues and cell types tested, including spleen, lymph node, thymus, appendix, PBL, bone marrow, fetal liver, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2A). T and B lymphoid cell lines (MOLT\(/2, Jurkat, Daudi, and Raji), non-lymphoid hematopoietic cell lines (THP-1, TF-1, EoL-3, KU812, K562, and M-TAT), and PHA-treated PBL (PHA-PBL) (Fig. 2B). These results suggest that human Hrs is ubiquitously expressed in a variety of human tissues and cell lines.

Cytokine-induced Tyrosine Phosphorylation of Hrs—Since human Hrs was originally detected as an IL-2-induced phosphorysine protein, and mouse Hrs has been shown to be tyrosine-phosphorylated by stimulation with HGF, EGF, and PDGF, we examined tyrosine phosphorylation of Hrs upon stimulation with IL-2. MOLT\(/2 cells were lysed before and after IL-2 stimulation. Their lysates were immunoprecipitated with anti-Hrs antibody and then immunoblotted with anti-phosphotyrosine mAbs, 4G10 and PY20 (Fig. 3A). Tyrosine-phosphorylated Hrs was detected at a 110-kDa molecular mass in the cell lysates after IL-2 stimulation but not before IL-2 stimulation. Immunoblotting with anti-Hrs mAb indicated comparable expressions of Hrs before and after IL-2 stimulation. Similarly, GM-CSF stimulation of TF-1 cells also induced tyrosine phosphorylation of Hrs (data not shown). These results indicate that tyrosine phosphorylation of Hrs is induced by stimulation with IL-2 and GM-CSF, as well as HGF, EGF, and PDGF. In addition to the 110-kDa Hrs, phosphotyrosine molecules with 120-kDa and 70-kDa molecular masses were detected in the Hrs immunoprecipitate, suggesting that the 120-kDa and 70-kDa molecules may be coimmunoprecipitated with Hrs. The kinetic study of IL-2-induced tyrosine phosphorylation of Hrs was done with MOLT\(/2 cells (Fig. 3B). Tyrosine-phosphorylated Hrs became detectable within 3 min of IL-2 stimulation, and maximized at 5 min.

Physical Association between Hrs and STAM—Since we previously found that STAM has a 70-kDa molecular mass and is tyrosine-phosphorylated upon stimulation with a wide variety of cytokines including IL-2, GM-CSF, EGF and PDGF, we considered the possibility that the 70-kDa phosphotyrosine molecule detected in the immunoprecipitate of Hrs is STAM. To examine this possibility, we performed coimmunoprecipitation assays between Hrs and STAM with MOLT\(/2 cells. They were

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treated or untreated with IL-2, and their lysates were immunoprecipitated with rabbit anti-Hrs antibody, preimmune serum, or anti-STAM mAb. The immunoprecipitates were separated by SDS-PAGE, and then immunoblotted with anti-STAM mAb or anti-Hrs mAb, respectively. Irrespective of IL-2 stimulation, Hrs was apparently coimmunoprecipitated with STAM, or vice versa (Fig. 4). These results indicate that Hrs is physically associated with STAM without IL-2 stimulation.

To gain insight into the association site of Hrs with STAM, we further carried out coimmunoprecipitation between various deletion mutants of Hrs and STAM. Firstly, we prepared HA-tagged Hrs, Hrs-dC1, Hrs-dC2, and Hrs-dM, which are the wild-type Hrs, and three mutants of Hrs deleted of the C terminus up to Gln571, of the C terminus up to Gln452, and of the portion between Ser432 and Met573, respectively, as shown in Fig. 5A. COS7 cells were transiently transfected with the wild-type STAM and the wild-type Hrs or Hrs mutants, their lysates were immunoprecipitated with anti-STAM mAb, and then immunoblotted with anti-HA mAb or anti-STAM mAb. STAM was coimmunoprecipitated with Hrs-dC1 as well as the wild-type Hrs, but not with Hrs-dC2 and Hrs-dM (Fig. 5B, upper blot). Conversely, the cell lysates were immunoprecipitated with anti-HA mAb, and immunoblotted with anti-STAM mAb or anti-HA mAb. The wild-type Hrs and Hrs-dC1 but not Hrs-dC2 and Hrs-dM mutants was coimmunoprecipitated with STAM (Fig. 5C, upper blot). The expression levels of the wild-type mutants of Hrs and STAM were confirmed to be comparable among transfections (Fig. 5, B, C, and E, lower blots). These results suggest that the portion between Gln552 and Leu570 of Hrs includes the binding site for STAM. Next, we examined the association of STAM mutants with the wild-type Hrs. DSH3, DIT, and DY2 mutants of STAM are deleted of the SH3 domain, ITAM region and the tyrosine cluster region, respectively (Fig. 5D). COS7 cells were transiently transfected with the wild-type STAM, DSH3, DIT, and DY2 together with HA-tagged wild-type Hrs, their lysates were immunoprecipitated with anti-STAM mAb, and then immunoblotted with anti-phosphotyrosine mAbs (upper blot), or anti-Hrs mAb, lmos-I (lower panel). A, MOLTβ cells deprived of serum fo r 6 h were incubated with (+) or without (−) 10 nM of IL-2 in serum-free medium for 10 min. Their lysates were immunoprecipitated with rabbit anti-Hrs antibody. The immunoprecipitates were separated by SDS-PAGE, and then immunoblotted with anti-phosphotyrosine mAbs (upper blot), or anti-Hrs mAb, lmos-I (lower panel). B, MOLTβ cells deprived of serum were incubated with (+) or without (−) IL-2 in serum-free medium for the indicated times. Their lysates were immunoprecipitated with rabbit anti-Hrs antibody, and then immunoblotted with anti-phosphotyrosine mAbs.

The SH3 domain of ITAM region and the tyrosine cluster region, respectively (Fig. 5D). COS7 cells were transiently transfected with the wild-type STAM, DSH3, DIT, and DY2 together with HA-tagged wild-type Hrs, their lysates were immunoprecipitated with anti-STAM mAb, and then immunoblotted with anti-HA mAb. Hrs was coimmunoprecipitated equally well with the wild-type STAM and DSH3 mutant STAM, but weakly with DY2 mutant STAM, while DIT mutant STAM was not coimmunoprecipitated with Hrs (Fig. 5E). These results indicated that the ITAM region, in particular, the amino acid position between Glu356 and Leu370, of STAM includes the association site for Hrs. The SH3 domain of STAM is not involved in the association with Hrs.

Hrs-2, a rat homolog of Hrs, was recently reported to include two putative coiled-coil sequences in the amino acid position between Gln450 and Asp477, and between Met515 and Arg544 (17), which correspond to the STAM-binding site of Hrs. The SH3 domain of STAM is not involved in the association with Hrs. The amino acid sequence of Hrs was hence searched for a coiled-coil sequence with COILS 2.1 program (31). A highly conserved coiled-coil sequence (p = 0.99) was detected at the amino acid position between Gln552 and Asp570, and between Met515 and Arg544 (17), which correspond to the STAM-binding site of Hrs. These results suggest that the portion between Gln552 and Leu570 of Hrs includes the binding site for STAM. Next, we examined the association of STAM mutants with the wild-type Hrs. DSH3, DIT, and DY2 mutants of STAM are deleted of the
coiled-coil sequence. The coiled-coil sequence of Hrs was deleted in Hrs-dM mutant, which lost STAM binding activity. The amino acid sequence of STAM was also searched for a coiled-coil sequence in a similar manner. STAM also contained a putative coiled-coil sequence (p = 0.96) at the amino acid position between Ile and Glu (Fig. 6B). Most of the coiled-coil sequence of STAM was included in the ITAM region at the amino acid position between Ser and Gin, of which the deletion mutant, DIT, lacked Hrs binding activity. DY2 mutant of STAM carrying a weak Hrs binding activity is deleted of a C-terminal half from Tyr, including a small region consisting of 7 amino acid residues (from Tyr to Glu) of the total 28 amino acid residues of the coiled-coil sequence of STAM. These results suggest a possible involvement of the coiled-coil structures of Hrs and STAM in their association.

Effects of Hrs on DNA Synthesis Mediated by IL-2 and GM-CSF—Since STAM is involved in signaling for DNA synthesis mediated by IL-2 and GM-CSF, we investigated the effect of Hrs on DNA synthesis mediated by IL-2 and GM-CSF. BAF-B03 cells without starvation for IL-3 and serum were transiently transfected with the wild-type Hrs or Hrs-dM mutant, together with human IL-2 receptor β and γ genes or human GM-CSF receptor α and β genes. They were then stimulated with human IL-2 or GM-CSF, respectively, and assayed for [3H]thymidine incorporation. Transfection with the wild-type Hrs resulted in 76% and 73% inhibition of [3H]thymidine incorporation induced by IL-2 and GM-CSF, respectively, in comparison with the control, whereas Hrs-dM mutant, which has no binding site for STAM, induced little if any inhibition of [3H]thymidine incorporation upon stimulation with IL-2 and GM-CSF (Fig. 7, A and B). The suppression of [3H]thymidine uptake mediated by IL-2 and GM-CSF was dose-dependent on the wild-type Hrs plasmids (Fig. 7, C and D). These results suggest that Hrs has the ability to inhibit signaling for DNA synthesis mediated by IL-2 and GM-CSF, and that Hrs-STAM association is a prerequisite for such activity.

DISCUSSION

Mouse Hrs was molecularly identified as a phosphotyrosine protein induced by stimulation with HGF, EGF, and PDGF, but its biological significance has not yet been elucidated (16). We here demonstrated that human Hrs is implicated in regulation of intracellular signal transduction mediated by cytokines through interaction with STAM, which we have already shown to be associated with Jak3 and Jak2 tyrosine kinases, and involved in signaling for cell growth and c-Myc induction mediated by IL-2 and GM-CSF (15, 18). Human Hrs was shown to have structural characteristics similar to mouse Hrs, such as a double zinc-finger motif, a proline-rich region, and a proline- and glutamine-rich region, and found to be rapidly tyrosine-phosphorylated upon stimulation with cytokines such as IL-2 and GM-CSF with similar kinetics to stimulation with HGF (16). These observations suggest that Hrs is possibly involved in signaling induced by a wide variety of cytokines and growth factors, which well correlates with its ubiquitous expression among various human tissues and cell types. Although receptors for HGF, EGF, and PDGF carry tyrosine kinases in themselves (32), and receptors for IL-2 and GM-CSF are associated with non-receptor type tyrosine kinases such as the Jak family and Src family (1), tyrosine kinase(s) catalyzing Hrs phosphorylation has not yet been detected. Since STAM directly associates with Jak3 and Jak2 (18), it is possible that Hrs is phosphorylated by Jak3 and Jak2 upon stimulation with IL-2 and GM-CSF, respectively.

Hrs was revealed to bind to STAM, suggesting a possible biological significance of Hrs in cytokine-mediated signal transduction. The association between Hrs and STAM exists in cells even before ligand stimulation, because IL-2 stimulation did not affect their coimmunoprecipitation. The STAM-association site of Hrs was identified to locate in the portion between Glu and Leu, which includes a highly conserved coiled-coil sequence. Furthermore, using DIT mutant STAM deleted of the ITAM region, the Hrs-binding site of STAM was determined to locate in the ITAM region, which also contains most of a highly conserved coiled-coil sequence. These results suggest the possibility that Hrs forms a complex with STAM through interaction between their coiled-coil structures. The weaker association of DY2 mutant STAM with Hrs than the wild-type STAM may be explained by deletion of a small part (seven amino acids) of the coiled-coil sequence of STAM. So far, the ITAM region of STAM is also known to be involved in association with Jak3 and Jak2 (18). However, DY2 mutant STAM, retaining Hrs binding activity, completely lost the ability for association with the Jaks. Furthermore, the Jaks do not contain any highly conserved coiled-coil sequence (data not shown). These observations suggest that the Hrs-binding site of STAM does not completely overlap the Jak-binding site of STAM, and the coiled-coil sequence of the ITAM region may not be involved in the interaction with the Jaks.

It has been demonstrated that native intrinsic STAM is involved in signaling for DNA synthesis mediated by IL-2 and GM-CSF, since the STAM mutant deleted of the SH3 domain functions as a dominant negative effect on such signal transduction (18). In the present study, exogenous introduction of Hrs associated with STAM induced suppression of DNA synthesis mediated by IL-2 and GM-CSF. Hrs-dM mutant lacking the STAM-binding site, however, restored DNA synthesis mediated by the cytokines, suggesting that the interaction of Hrs with STAM may result in negative regulation of DNA synthesis induced by the cytokines. These observations further suggest that STAM is associated with signaling molecules, which either positively or negatively regulate DNA synthesis mediated by IL-2 and GM-CSF. Hrs is thought to be a signaling molecule negatively regulating DNA synthesis mediated by the cytokines. In contrast, molecule(s) associated with the SH3 domain of STAM may contribute to the positive effect, because the SH3
domain of STAM is essentially involved in signaling for DNA synthesis mediated by IL-2 and GM-CSF (18). Since stimulation of cells with IL-2 and GM-CSF induced DNA synthesis even in cells expressing endogenous Hrs, the negative effect of Hrs on DNA synthesis may not be dominant in signaling pathways from the cytokine receptors. It is also interesting to examine whether Hrs affects the signaling for c-myc induction, where STAM is implicated. Such study to define the functional significance of Hrs in intracellular signal transduction is currently in progress.

Together with the evidence for the physical association between Hrs and STAM, the apparent effects of exogenous Hrs on DNA synthesis mediated by the cytokines suggest the implication of endogenous Hrs in the cytokine-mediated signaling pathways. Very recently, an Hrs-homologous rat protein, Hrs-2, has been molecularly cloned and demonstrated to have NTPase activity with four nucleotide-binding sites in itself (17). NTPase activity of Hrs remains to be tested. However, since Hrs was also shown to have three nucleotide-binding sites, it is also predicted to have NTPase activity. Little is known about

**FIG. 5.** Schematic structures of Hrs mutants and STAM mutants, and coimmunoprecipitation between Hrs and STAM. A, schematic structures of the wild-type and three mutants of Hrs are indicated: a zinc-finger motif (zinc), a proline-rich region (Pro), a proline- and glutamine-rich region (Pro/Gln), and a putative coiled-coil structure (coil). Hrs-dC1 is deleted of the C-terminal 207 amino acid residues, Hrs-dC2 is deleted of the C-terminal 326 amino acid residues, and Hrs-dM is deleted of the portion between Ser322 and Met373. These mutants and the wild-type Hrs were tagged with HA. COS7 cells were transiently transfected with 10 μg of expression plasmids for the wild-type and three mutants of Hrs, together with 10 μg of expression plasmids for the wild-type STAM by electroporation. The cells were incubated for 24 h; their lysates were immunoprecipitated with anti-STAM mAb, TUS-1, and anti-HA mAb, and then immunoblotted with anti-HA mAb (B, upper blot) or TUS-1 (B, lower blot), and with TUS-1 (C, upper blot) or anti-HA mAb (C, lower blot). D, schematic structures of the wild-type and three mutants of STAM are indicated: a Src homology 3 domain (SH3), an immunoreceptor tyrosine-based activation motif (ITAM), and tyrosine residues (Y). DSH3 mutant lacks the SH3 domain, DIT mutant is deleted of the ITAM region, and DY2 mutant is deleted of the tyrosine cluster region. E, COS7 cells were transiently transfected with 10 μg of expression plasmids for the wild-type and three mutants of STAM, together with 10 μg of expression plasmids for the HA-tagged wild-type Hrs. After a 24-h incubation of cells, their lysates were immunoprecipitated with anti-STAM mAb, TUS-1, and then immunoblotted with anti-HA mAb (upper blot) or TUS-1 (lower blot).

**FIG. 6.** Prediction of coiled-coil structures in Hrs and STAM. The amino acid sequences of human Hrs and STAM were searched for coiled-coil sequence with COILS 2.1 program using MTIDK matrix in scanning windows of 28 amino acid residues as described previously (31). A, Hrs contains a putative coiled-coil sequence at the amino acid position between Leu350 and Arg397, with a potential \( p = 0.99 \) to form a coiled-coil structure. B, STAM also contains a putative coiled-coil sequence at the amino acid position between Ile350 and Glu377, with a potential \( p = 0.96 \) to form a coiled-coil structure.
mediated by the cytokines. Moreover, it is still obscure the exact relationship between genes coding for Hrs and Hrs-2, because a single mRNA band but not two distinct mRNA bands was detected in all the tissues and cell types tested with Hrs probes, which are highly homologous to Hrs-2.

Hrs together with STAM, both of which are tyrosine-phosphorylated upon stimulation with a variety of cytokines and growth factors, may contribute to the general understanding of signal-transducing pathways from receptors for such ligands.

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FIG. 7. Effect of Hrs on [3H]thymidine incorporation of BAF-B03 cells in response to IL-2 and GM-CSF. BAF-B03 cells without starvation for IL-3 and serum were transfected with 20 μg of the wild-type Hrs, Hrs-DM, and pCXB control plasmids, together with human IL-2 receptor β and γc plasmids (A), or together with human GM-CSF receptor α and βc plasmids (C), in addition to pENL, by electroporation. BAF-B03 cells without starvation for IL-3 and serum were also transfected with the indicated doses of the wild-type Hrs plasmids and pCXB control, together with human IL-2 receptor β and γc plasmids (B), or human GM-CSF receptor α and βc plasmids (D), in addition to pENL, by electroporation. Subsequently, the cells were cultured with (+) or without (−) IL-2 (A and B) and GM-CSF (C and D) and assayed for [3H]thymidine incorporation. The values shown are means ± S.E. of triplicate determinants. Results represent one of three comparable experiments. Transfection efficiencies were assessed by β-galactosidase activities of transfected samples, and they were comparable among transfections with Hrs plasmids used.

any relationship between molecules carrying NTPase activity and regulation of signaling for gene expression and DNA synthesis. Hrs-2 was shown to bind to SNAP-25, which is considered to modulate vesicular transport, and in fact, recombinant Hrs-2 inhibited calcium-triggered noradrenaline release from PC12 cells (17). Interaction, however, between Hrs and SNAP-25 is still unknown, but since expression of SNAP-25 is specific for nerve tissues (33), SNAP-25 may not be implicated in the Hrs-induced regulation of signaling for DNA synthesis.