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Human spot 14 protein interacts physically and functionally with the thyroid receptor

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Abstract

Spot 14 (S14) is a small acidic protein with no sequence similarity to other mammalian gene products. Its biochemical function is elusive. Recent studies have shown that, in some cancers, human S14 (hS14) localizes to the nucleus and is amplified, suggesting that it plays a role in the regulation of lipogenic enzymes during tumorigenesis. In this study, we purified untagged hS14 protein and then demonstrated, using various biochemical methods, including analytic ultracentrifugation, that hS14 might form a homodimer. We also found several lines of evidence to suggest physical and functional interactions between hS14 and the thyroid hormone receptor (TR). The ubiquitous expression of hS14 in various cell lines and its cell-type-dependent functions demonstrated in this study suggest that it acts as a positive or negative cofactor of the TR to regulate malic enzyme gene expression. These findings provide a molecular rationale for the role of hS14 in TR-dependent transcriptional activation of the expression of specific genes.

Keywords: Spot 14; Dimerization; Thyroid receptor; Malic enzyme

Spot 14 (S14) is a small (about 17 kDa) acidic (pI 4.65) protein with no sequence similarity to other functional motifs. It initially came to attention in 1982 through its marked and rapid induction by thyroid hormone [1]. The multifaceted regulation and nuclear localization of S14 prompted the hypothesis that it functions in the tissue-specific control of metabolism in response to changing dietary and hormonal milieux [2–4]. Cunningham et al. found that it exists as a homodimer and coimmunoprecipitates with a 36-kDa protein from rat liver to form the tripartite S14–S14–p36 complex [5].

The human S14 (hS14) gene product is abundant in tissues active in long-chain fatty acid synthesis, including lactating mammary glands. The gene is located on the 11q13 cancer amplicon, where the cyclin D1 mammary oncogene occurs [6–9]. The lipogenic tumor phenotype is characterized by high rates of fatty acid synthesis, elevated content of lipogenic enzymes in tumors, such as fatty acid synthase, acetyl-CoA carboxylase, and malic enzyme, and a dependence on lipogenesis for tumor cell growth [10,11]. Malic enzyme is a member of a group of adaptive enzymes involved in the *de novo* synthesis of long-chain fatty acids. Several lines of evidence indicate that thyroid hormone plays important roles in mediating the changes in malic enzyme activity caused by nutritional manipulation [12–14].

Thyroid hormone receptors (TRs), located in the cell nuclei, mediate the action of thyroid hormone by the positive and negative regulation of thyroid-hormone-responsive genes [15]. Triiodothyronine (T3) binds to nuclear TRs, which bind to the target DNA sequence (thyroid-hormone-responsive element [TRE]), and both induces the

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transcription of some genes and inhibits the transcription of other genes. Recent studies have indicated that the transcriptional activity of TR depends on the type of TRE located in the promoter regions of T3 target genes and on a host of corepressors and coactivators [16,17]. The binding of T3 leads to the release of corepressors from TR and the recruitment of coactivators, which leads to gene activation.

Until now, the biochemical function of the hS14 protein has not been well understood. To clarify its mechanism of action, we undertook studies to define the interactions of hS14 with TR and other factors that affect the malic enzyme promoter. Using purified untagged hS14 protein, we found that hS14 protein exists as a homodimer in solution and is ubiquitously expressed in the mammalian cell lines tested. We also observed that the effect of the hS14 protein–TR complex on the human malic enzyme promoter depends, at least in part, on stimulation by T3 and the type of cell line. Taking these data together, we suggest that hS14 functions as a TR coactivator on the malic enzyme promoter element.

Materials and methods

Plasmids. We constructed pSG5.HA vectors encoding hS14 fragments (amino acids 1–146, 1–114, 1–77, 78–146) by inserting an *Eco*RI–*Xho*I fragment of the appropriate PCR-amplified hS14 into the *Eco*RI and *Xho*I sites of the pSG5.HA vector. Vectors encoding the Gal4 DNA-binding domain (Gal4DBD) or the Gal4 activation domain (Gal4AD) fused with various hS14 fragments were constructed by inserting *Eco*RI–*Xho*I hS14 fragments cut from the respective pSG5.HA.hS14 into the *Eco*RI and *Sal*I sites of the pM or pVP16 vector (Clontech, CA, USA), a vector used for the expression of Gal4DBD or Gal4AD fusion proteins from a constitutive SV40 early promoter. Reporter genes for the human malic enzyme promoter were constructed with the appropriate PCR-amplified fragments cloned into the pGL3-basic-Luc vector (Promega, WI, USA). The reporter constructs GK1–LUC and MMTV(TRE)–LUC and the expression from pCMX.hTRβ1 of human TRβ1 have been described previously [18,19].

A bacterial expression vector for intein fused with full-length hS14 (codons 1–146) was constructed by inserting an *Nde*I–*Sap*I fragment of the appropriate PCR-amplified hS14 into the *Nde*I and *Sap*I sites of the pTYB1 vector (New England BioLabs, MA, USA). Site-directed mutagenesis was used to construct pTYB1 vectors encoding intein fused to various hS14 fragments in which cysteine residues 12, 67, or both were mutated to alanine. Bacterial expression vectors encoding GST fused with various hS14 fragments were constructed by inserting *Eco*RI–*Xho*I hS14 fragments cut from the respective pSG5.HA.hS14 vectors into the *Eco*RI and *Xho*I sites of the pGEX 4T1 expression vector (GE HealthCare, USA). GST–$GRIP1_{563–1121}$ has been described previously [20].

Analytical ultracentrifugation. Sedimentation velocity was performed in a Beckman-Coulter XL-A analytical ultracentrifuge with an An50Ti rotor at 20 °C and 42,000 rpm in 12-mm double-sector *Epon* charcoal-filled centerpieces. The UV absorption of the cells at 280 nm was scanned in a continuous mode with time interval of 8 min and a step size of 0.003 cm. The partial specific volume of the protein, solvent density, and viscosity were calculated by the Sednterp program (http://www.jphilo.mailway.com/). All samples were visually checked for clarity after ultracentrifugation to make sure that there was no indication of precipitation due to unfolding of the protein. Multiple scans at different time points were analyzed with the SedFit program [21].

Protein–protein interaction assays. For GST pulldown assays, [^{35}S]-labelled proteins (TR or various hS14 proteins) were produced using the TNT T7-coupled reticulocyte lysate system (Promega). The bound radioactive proteins are then eluted and analyzed by SDS–PAGE as previously described [22].

Cell culture and transient transfection assays. HEK293, COS7, HepG2, MCF-7, and HeLa cells were grown in DMEM supplemented with 10% charcoal/dextran-treated fetal bovine serum. The cells in each well (24-well plate) were transfected with jetPEI (PolyPlus-transfection, Illkirch, France) according to the manufacturer's protocol; total DNA was adjusted to 1.0 μg by addition of the empty vector pSG5.HA. Luciferase assays were performed with the Promega Luciferase Assay kit, and the measurement is expressed numerically as relative light units (RLU). Luciferase activities are shown as the mean and deviation from the mean of two transfected sets. The results shown are representative of at least three independent experiments.

Immunoblots analysis. Cell lysates were prepared in RIPA buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100) at 4 °C and were separated by SDS–PAGE, transferred onto a polyvinylidine difluoride membrane (Immobilon-P, Millipore, MA, USA) and detected by using antibodies against hS14 (Bioman, Taiwan, Republic of China) and loading control protein α-tubulin and HuR (B-7 and 3A2, Santa Cruz, USA).

Results

Human S14 is a dimeric protein

To determine the association state of hS14 in solution, we constructed and purified untagged hS14 using the IMPACT (intein-mediated purification with an affinity chitin-binding tag) system. Purified untagged hS14 was collected as the self-cleavable chitin-tagged hS14 fusion protein. The purification profile was subjected to SDS–PAGE analysis and one purified hS14 protein band corresponding to a molecular weigh of about 16 kDa was observed (Fig. 1A, lane 4). The purified hS14 protein had an estimated molecular weight of about 36 kDa in the 8–25% gradient gel on native PAGE and two major cross-linked bands were corresponded to molecular weights of about 16 kDa (monomer) and 36 kDa (dimer) on 15% SDS–PAGE, at 16 °C (data not shown). The association states of the purified hS14 were confirmed by analytical ultracentrifugation analysis (Fig. 1B). Analysis of the sedimentation data with SEDFIT software resulted in high-quality data fits with randomly distributed residuals and root mean square deviations below 0.1%. The calculated sedimentation coefficient revealed a well-resolved peak corresponding to a homodimer of hS14. hS14 was predicted from its amino acid composition to have a molecular weight of about 17.5 kDa, and in solution to have a molecular weight of 34.5 kDa (Fig. 1B). Therefore, hS14 is a homodimeric protein.

C-terminal region and two conserved cysteine residues of hS14 are not essential for its homodimerization

Work from Kinlaw's laboratory using yeast two-hybrid analysis demonstrated that rat S14 forms a homodimer primarily via its C-terminal hydrophobic heptad repeat (zipper) [5]. We also examined the homodimer domain of hS14 using mammalian two-hybrid and GST pulldown analyses. The transcriptional activation of the luciferase reporter gene in the mammalian (or yeast) two-hybrid system indicates a protein–protein interaction. Because this

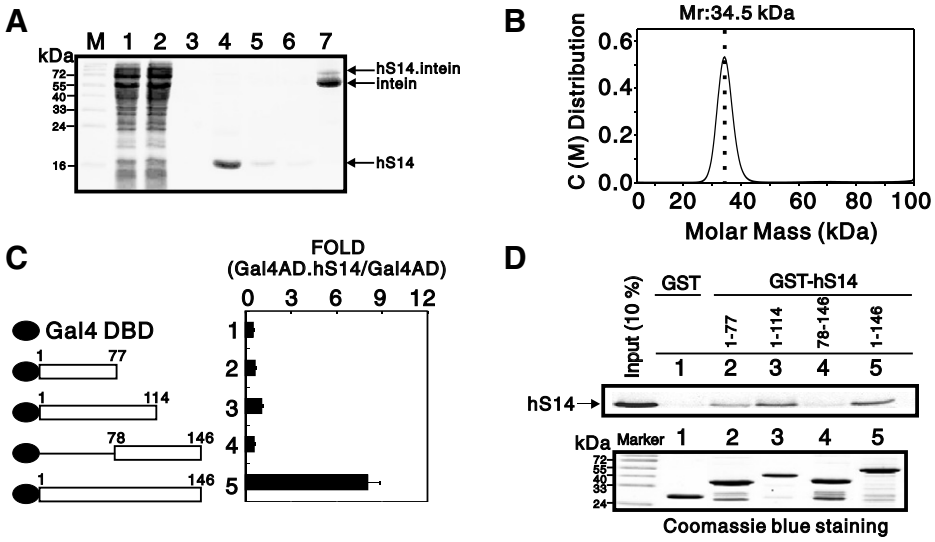

Fig. 1. Purified hS14 protein is identified as a homodimer through it N-terminal region. (A) The purification profile of the IMPACT system was obtained with 15% SDS–PAGE. Lane 1: crude fraction; lane 2: flow-through fraction; lane 3: washed fraction; lanes 4–6: DTT-cleaved hS14 protein elution fractions; lane 7: chitin resin. (B) Analytical ultracentrifugation was performed as described in Materials and methods. The C(M) distribution of the dimer of hS14 protein at a protein concentration of 0.5 mg/mL was analyzed by sedimentation velocity. (C) HeLa cells were transfected with 0.25 μg of the GK1–LUC reporter, 0.35 μg of Gal4DBD, or the various Gal4DBD.hS14 fragments indicated, and 0.35 μg of Gal4AD or full-length Gal4AD.hS14 protein. The luciferase activities of the transfected cell extracts were determined. Activation fold by Gal4AD.hS14 indicates the values that were calculated from the ratio of Gal4AD.hS14 to Gal4AD vector alone. The data are the means of three experiments (means ± SD, $n = 3$). (D) Full-length hS14 proteins were translated *in vitro* and incubated with various bead-bound GST–hS14 fusion proteins or GST alone. The bound proteins were eluted, separated by SDS–PAGE, and visualized by autoradiography (top panel). Eluted GST and GST–hS14 fusion proteins were subjected to SDS–PAGE and stained with Coomassie blue (bottom panel). The results shown are representative of three independent experiments.

assay is performed in mammalian cells, mammalian proteins are more likely to retain their native conformations, and the results are more likely to represent biologically significant interactions. Our results demonstrate that the full-length hS14 in the mammalian two-hybrid analysis (Fig. 1C) and the full-length or N-terminal hS14 (amino acids 1–77 or 1–114) in the GST pulldown analysis (Fig. 1D) were able to form dimeric proteins. In contrast, the C-terminal region of hS14 (amino acids 78–146) failed to form a dimer in both analyses. Furthermore, single-, double-, or triple-point mutations on hS14 C-terminal hydrophobic residues remained its homodermic form in the ultracentrifugation analysis (data not shown).

Two conserved cysteine residues (positions 12 and 67 in human, mouse, and rat), which potentially form a disulfide bond, were substituted with alanine to determine whether disulfide cross-linking is involved in the dimerization of hS14. All these mutant proteins (C12A, C67A, and C12/67A) and wild-type hS14 were purified to homogeneity and evaluated by native gel electrophoresis, cross-linking with glutaraldehyde, and analytical ultracentrifugation (Fig. 2 and data not shown). All observations implied that the two conserved cysteine residues are not required for hS14 dimerization in solution.

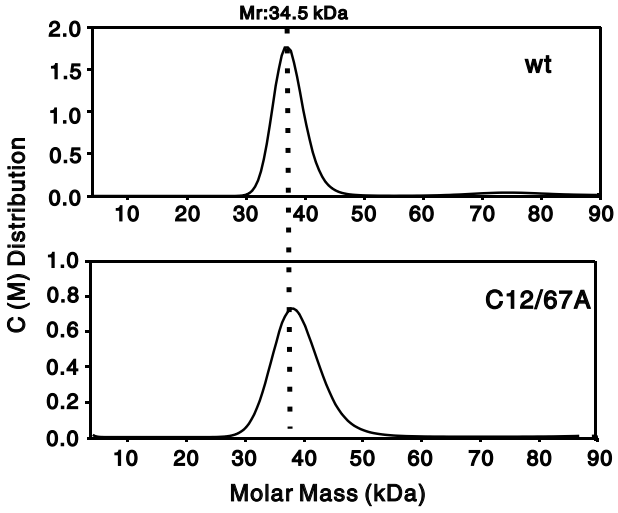

Fig. 2. Two conserved cysteine residues are not required for hS14 protein self-association. Analytical ultracentrifugation analysis was performed as described in Materials and methods and the legend of Fig. 1. The results shown are representative of three independent experiments.

Ubiquitous hS14 expression is not regulated by T3 stimulation

Recent studies have demonstrated that, as with the lipogenic enzymes, rat hepatic S14 mRNA is induced by T3 and other factors [4,23,24]. A specific antibody directed against hS14 was made using the purified full-length untagged hS14 protein as antigen. The hS14 antibody specificity was verified by the competition of purified hS14 protein and it was also able to recognize GFP- or Gal4- fused with hS14 protein (data not shown). hS14 was detected in hepatic cells (such as HepG2 cells), and in kidney cells (HEK 293 and COS7 cells), mammary gland cells (MCF-7 cells), and cervical carcinoma cells (HeLa cells)

using this new hS14 antibody (Fig. 3, lane 1). No apparent T3 stimulation of hS14 protein expression was observed by western blot analysis (Fig. 3, compare lanes 1 and 2).

Functional role of hS14 in the effect of T3-activated TR on malic enzyme promoter activity

Immunohistochemistry showed the nuclear localization of hS14, suggesting its possible function in the modulation of gene expression [25,26]. Therefore, we examined the potential relationship between hS14 and the T3-binding protein, TR. From the GST pulldown analysis, comparison with the GST protein negative control suggests that hS14 interacts physically with TR (Fig. 4A, compare lanes 1–6). Unlike the T3-dependent binding protein, GRIP1, the physical interaction between hS14 and TR is independent of T3 stimulation (Fig. 4A, compare lanes 3–8). A strong correlation between the regulation of rat S14 gene expression and malic enzyme activity has been established in rat liver tissue treated with T3 and dietary carbohydrate [27,28]. However, no apparent T3 stimulation of human malic enzyme promoter activity was observed in several cell lines, including HEK 293, HeLa, and COS7 cells (data not shown). The physical interaction between hS14 and TR (Fig. 4A) suggests that hS14 might be a cofactor for TR-dependent transcriptional activation.

Therefore, we transiently transfected human TRβ1 into HEK 293 cells, and stimulated them with 200 nM T3 for 48 h. We found that the malic enzyme promoter activity was increased by T3-activated TR and the stimulation was undetectable when the promoter was truncated from positions –202 to –55, suggesting that at least one TRE occurs in this region (data not shown). Next, we examined the role of the TRE in this promoter using TR and/or hS14 protein transiently transfected into HEK 293 cells. There was no T3 induction of the malic enzyme promoter in HEK 293 cells (Fig. 4B, histograms 1). In the absence of T3 stimulation, we observed a negative effect on the TRE by exogenous TR protein in the HEK 293 cell line (Fig. 4B, top panel, compare histograms 1 and 2, closed columns). The negative effect of TR changed to a positive effect when the promoter lacked a TRE (Fig. 4B, top panel, compare histograms 1 and 2, open columns). In contrast,

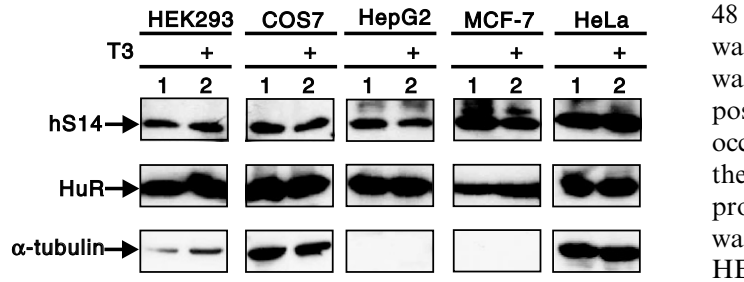

Fig. 3. The expression of hS14 protein is ubiquitous and is not affected by T3 stimulation in various cell lines. Various indicated cell lines were grown in charcoal-treated Dulbecco's modified Eagle's medium (DMEM) with or without 200 nM T3 treatment for 12 h. These fractions were subjected to western blot analysis with antibodies directed against hS14, α-tubulin, or HuR. α-tubulin and HuR are loading control proteins. The results shown are representative of three independent experiments.

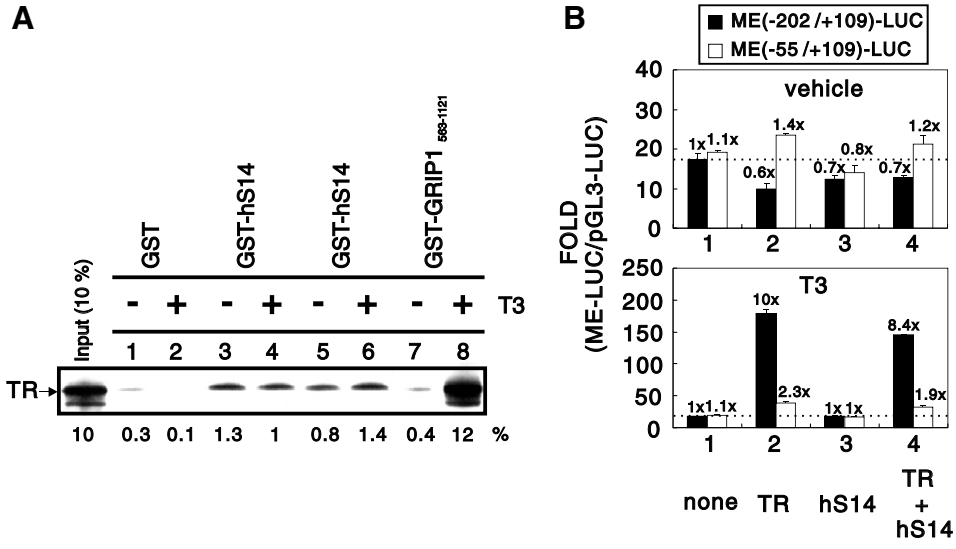

Fig. 4. Human S14 protein physically and functionally interacts with TR. (A) TRs were translated *in vitro* and incubated with bead-bound GST-full-length hS14 fusion protein or GST alone in the absence and presence of 100 nM T3. Bound proteins were eluted, separated by SDS–PAGE, and visualized by autoradiography. GST–GRIP1$_{563–1121}$ fusion protein was the ligand-dependent TR-binding control and GST protein alone was the negative TR-binding control for the GST pulldown assay. The percentage of protein bound, as determined by phosphorimager analysis, is shown below each lane. (B) HEK 293 cells were transfected with 0.25 μg of the pGL3–LUC, pGL3–ME(−202/+109)–LUC, or pGL3-ME(−55/+109)-LUC reporter, and either 0.04 μg of TR, 0.3 μg of pSG5.HA.hS14, or both. Where indicated, the transfected cultures were grown in 200 nM T3 or alcohol (vehicle) for 48 h. The luciferase activity of the transfected cell extracts was determined and the fold induction of activity was compared with that of pGL3–ME(−202/+109)–LUC to pGL3–LUC (closed) or pGL3–ME(−55/+109)–LUC to pGL3–LUC (open). The data are the means of three experiments (means ± SD, $n = 3$).

TR enhanced the activity of the malic enzyme promoter 17-fold through the TRE region when the cells were activated with T3 (Fig. 4B, bottom panel, compare histograms 1 and 2, closed columns). Exogenous hS14 had either no effect or a small effect on these malic enzyme promoters, whether or not T3 was added (Fig. 4B, compare histograms 1 and 3). The effect of hS14 protein on TR functions depended on T3 stimulation. The null or negative effect of unliganded TR on the promoter was mitigated by exogenous hS14. The enhancement of malic enzyme promoter activity by liganded TR was suppressed by exogenous hS14 (Fig. 4B, compare histograms 2 and 4, closed columns). The specificities of TR and T3 were reconfirmed using a malic enzyme promoter lacking a TRE (Fig. 4B, compare histograms 2 and 4, open columns).

Discussion

In this study, the purified untagged hS14 protein prepared by the IMPACT system allowed us to demonstrate, using analytical ultracentrifugation, that hS14 self-associates as a 34.5-kDa homodimer (Figs. 1 and 2). Our findings further suggest that full-length (amino acids 1–146) hS14 *in vivo* or, at least, its N-terminal region (amino acids 1–114) is necessary to form the homodimer *in vitro* (Fig. 1D). The only other relevant study, by Cunningham et al., suggested that the homodimerization of rat S14 depends on a C-terminal zipper domain. However, their yeast two-hybrid results seemingly provide evidence that any deleted sequences in the N- or C-terminal region of rat S14 results in the loss of homodimerization [5]. Until now, there have been insufficient studies to define the homodimerization domain of human or rat S14 protein.

Although the biochemical mechanism of its action is unknown, it is clear that the S14 protein in general acts to transduce hormone- and nutrient-related signals to genes involved in lipid metabolism [2,24,28]. Its localization in the hepatocyte nucleus also suggests that hS14 plays a role in the regulation of lipogenic enzymes [23,28]. We observed no similar induction of rat hepatic S14 protein [29] by T3 or T3-activated TR in the study of hS14 protein (data not shown), but our findings demonstrate that might physically and functionally interact with TR *in vitro* (Fig. 4). The involvement of hS14 in TR-dependent transcriptional activation might open a new avenue in the study of the effects of T3 on hS14 or other S14-interacting protein(s). Its nuclear localization also suggests that hS14 might act as a transcription cofactor in the regulation of specific genes, such as the TR cofactor studied here.

Our study provides solid biochemical evidence for hS14 homodimerization. However, we must determine how hS14 undergoes homodimerization, including the dimerization interface or interacting residues and the functional role of the homodimeric form *in vivo*. The precise identification of residues critical for hS14 homodimerization will facilitate the design of soluble molecules that prevent its assembly. X-ray crystallography should allow us to identify the surfaces of hS14 that allow it to interact with TR and other proteins.

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