

# A putative nuclear receptor coactivator (TMF/ARA160) associates with hbrm/hSNF2 $\alpha$ and BRG-1/hSNF2 $\beta$ and localizes in the Golgi apparatus

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**Abstract** An ATP-dependent chromatin remodeling factor, SNF/SWI complex, acts as a coactivator for numerous transcriptional factors. One of the best-documented examples is nuclear receptors, although the molecular mechanism for this coactivation has not been sufficiently elucidated. Here we show that hbrm/hSNF2 $\alpha$  and BRG-1/hSNF2 $\beta$ , the ATPase subunits of the human SNF/SWI complexes, specifically associate *in vitro* and *in vivo* with TATA element modulatory factor (TMF)/ARA160, which has been described as a binding protein to and coactivator for the androgen receptor. This interaction requires highly conserved N-terminal regions of hbrm/hSNF2 $\alpha$  and BRG-1/hSNF2 $\beta$  and a C-terminal region of TMF/ARA160. Immunofluorescence and Western blot studies revealed that the TMF isoforms differentially localize in the Golgi apparatus and the nucleus. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Transcription; SWI/SNF; Nuclear receptor; Cofactor; Golgi apparatus

## 1. Introduction

The SNF/SWI proteins have been genetically identified as gene-specific transcriptional factors ([1–3], reviewed in [4–5]). The subunits are highly conserved in eukaryotes, from yeast to mammals, ([1–3,6–17], reviewed in [18,19]) and have been found to form multisubunit complexes [20–24]. These complexes remodel the chromatin of target genes in an ATP-dependent manner [20,25] through interaction with transcription factors and thereby facilitate the following processes (e.g. factor assembly) of transcriptional initiation [26,27]. It has been shown that the SNF/SWI components interact directly with various types of transcription factors: Rb [28], EKLF [29], c-myc [30], AP1 [31] and mSin3A [32], as well as nuclear receptors [33], which are best characterized. Transcriptional activation by the glucocorticoid receptor (GR) and the estrogen

receptor (ER) is enhanced by ectopic expression of ATPase subunits of the SNF/SWI complexes: hbrm/hSNF2 $\alpha$  and BRG-1/hSNF2 $\beta$  [6–8] (for simplicity, mostly referred to hereafter as hSNF2 $\alpha$  and hSNF2 $\beta$ ). Nucleosome remodeling and activation of cell-free transcription of a chromatin template by the SNF/SWI complexes are enhanced by GR in a binding element-dependent manner [34,35]. There are reports that nuclear receptors directly interact with the SNF/SWI subunits in a ligand-enhanced manner: GR directly interacts with SNF2/SWI2 [36] and BAF250/p250 (one of the largest subunits of human SNF/SWI complexes) [14]; ER also directly interacts with hbrm/hSNF2 $\alpha$  [37]. It is not clear, however, whether these direct interactions are determinants of *in vivo* activation and common to other nuclear receptors. Thus mechanisms by which the SNF/SWI complexes or components act to enhance nuclear receptor-dependent transcriptional activation still remain to be sufficiently understood.

In an effort to identify proteins that interact with hSNF2 $\alpha$  and hSNF2 $\beta$ , we used the yeast two-hybrid screening method and isolated a cDNA whose product was originally isolated as an HIV-1 TATA element modulatory factor (TMF) [38] and has also been characterized as a coactivator (ARA160) for the androgen receptor (AR) [39]. We showed that this protein (TMF/ARA160, referred to as TMF in most cases) specifically interacts with hSNF2 $\alpha/\beta$  as well as with the SNF/SWI complexes, both *in vitro* and *in vivo*, and that short and mutually identically positioned N-terminal regions of hSNF2 $\alpha$  and hSNF2 $\beta$  as well as a C-terminal region of TMF are sufficient for the interaction. We also showed that TMF localizes in the Golgi apparatus at high density and that the 120 kDa form of TMF is also present in the nucleus, which may lead to the finding of new events regulating the SNF/SWI complexes.

## 2. Materials and methods

### 2.1. Yeast two-hybrid screening and assays

Plasmids pGBT9 and pGADGH (Clontech) were used to express the bait and prey proteins, respectively. A cDNA fragment encoding an N-terminal 1243 amino-acid portion of hSNF2 $\beta$  was inserted into pGBT9 to express a fusion protein with the Gal4 DNA binding domain (GAL4BD). This plasmid was used as bait to screen a HeLa cDNA-pGADGH library (Clontech) in the HF7c yeast indicator cells. Clones positive for the His<sup>+</sup> phenotype were selected in the presence of 5 mM 3-aminotriazole and then assayed for  $\beta$ -galactosidase activity as described [40]. Interaction was also quantitated with indicator strains, SFY526 (Clontech) and Y187 [41] (kindly provided by S. Elledge, Baylor College of Medicine).

### 2.2. Protein preparations

Fusion proteins with glutathione S-transferase (GST) were expressed by employing pGEX5x (Amersham-Pharmacia) in an *Esche-*

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*richia coli* strain BL21(DE3)LysS (Novagen). For in vitro transcription-translation, template RNAs were prepared by transcription of pBluescript SK derivatives containing an hSNF2 $\alpha$  or hSNF2 $\beta$  cDNA fragment (truncated with *Bgl*II or *Bam*HI, respectively) with T7 RNA polymerase in the presence of mGpppG and labeled protein was synthesized with rabbit reticulocyte lysate (Promega) in the presence of [<sup>35</sup>S]methionine. For size fractionation, the HeLa nuclear extract [42] was fractionated stepwise on DEAE-Sephacrose (Amersham-Pharmacia) with 0.5 M KCl after a 0.1 M KCl wash in column buffer (20 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 0.05% NP-40) and then further fractionated on a molecular sieving agarose matrix Bio Gel A-5m (Bio-Rad) in column buffer containing 200 mM KCl. To prepare cytoplasmic and nuclear fractions of RL95-2, cell pellet was suspended in the hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.5% NP-40). After centrifugation in microtube for 5 min at 5000 rpm, the supernatant was isolated as cytoplasmic fraction and the nuclear pellet was additionally treated with the lysis buffer once or twice. The resulting nuclear pellets as well as the cytoplasm were directly suspended in sodium dodecyl sulfate (SDS) sample buffer, sonicated and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.3. Pull-down assays

Pull-down assay with GST fusion proteins was performed as described [15]. Briefly, an *E. coli* extract containing an expressed recombinant protein was incubated with glutathione-Sephacrose (Amersham-Pharmacia), washed extensively and used for the binding reaction with <sup>35</sup>S-labeled protein or HeLa nuclear extract. After being washed with a buffer containing 0.3 M NaCl, bound fractions were directly heated for 5 min at 95°C in SDS sample buffer and separated by (SDS-PAGE). Gels were either analyzed by autoradiography after treatment with the fluorography reagent (Amplify, Amersham-Pharmacia) or processed by Western blotting analysis.

### 2.4. Two-hybrid assay in mammalian cells

The GAL4BD-hSNF2 plasmids were constructed by inserting the full-length hSNF2 $\alpha$  or hSNF2 $\beta$  cDNA into pECE, yielding pECE-hSNF2 $\alpha$  or pECE-hSNF2 $\beta$  [43]. The VP16-TMF plasmid was constructed by inserting both the VP16 activation domain sequence and a TMF C-terminal cDNA fragment (amino acids (aa) 699–1093) into the pSV vector (designated pSVVP16-TMFC) [8]. The plasmids were transfected by the standard calcium phosphate coprecipitation method with the reporter plasmid G6(–119)HIVLTRATAR [43] in Cos-1 cells, and the interaction activity was measured by a standard CAT assay with <sup>14</sup>C-chloramphenicol (Amersham-Pharmacia) and by silica gel thin layer chromatography.

### 2.5. Antibody preparations and Western blot analyses

Rabbit antisera were raised against hSNF2 $\alpha$  (aa 1374–1484) or TMF (aa 831–1093) recombinant protein containing a histidine-tag. The anti-hSNF2 $\alpha$  antiserum (N3C2) recognizes both hSNF2 $\alpha$  and hSNF2 $\beta$  proteins in Western blotting. For immunostaining, antisera were affinity purified with antigen-conjugated Sepharose as described [15]. A monoclonal antibody against the 58 kDa Golgi protein was purchased (clone 58K-9, Sigma). Western blotting was carried out under standard conditions with Hybond-ECL membranes and ECL detection reagents (Amersham-Pharmacia).

### 2.6. Cell culture, transfection, immunofluorescence and green fluorescent protein (GFP)-fusion protein localization assays

HeLa, Cos-1 and endometrial carcinoma RL95-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HeLa cells were synchronized at G2-M phase by treating them with 2.5 mM thymidine (at 0 h), released from the thymidine block at 24 h, further treated with 0.1  $\mu$ g/ml nocodazole (Sigma) at 36 h and used at 48 h. For immunofluorescence analysis, cells were fixed with 3.7% paraformaldehyde/phosphate-buffered saline (PBS) and permeabilized with 1% Triton X-100/PBS. After washing with PBS, the cells were incubated with affinity-purified antibody and then with FITC-conjugated anti-rabbit IgG antibody. For the GFP analysis, the cells were transfected with a pEGFP-C1 derivative plasmid containing the full-length TMF cDNA, fixed with 3.7% paraformaldehyde/PBS at 48 h after transfection, washed with PBS, and examined with a fluorescent microscope.

## 3. Results

### 3.1. TMF/ARA160 is an hSNF2-binding protein

To identify hSNF2-binding proteins, we have used a yeast two-hybrid screening method with an N-terminal aa region of the hSNF2 $\beta$  protein [8] as bait and a HeLa cDNA library as prey. From a total of 10<sup>6</sup> cDNAs in the library, one of the primary positive clones exhibited high  $\beta$ -galactosidase activities in combination with a prey plasmid expressing hSNF2 $\alpha$  or hSNF2 $\beta$  but not with any of several negative control prey plasmids. This cDNA encodes a C-terminal region (395 aa) of a protein that has been reported as a specific DNA-binding protein of the HIV-1 TATA element (TMF) [38] and also as a cofactor protein of AR (ARA160) [39] (Fig. 1). This protein attracted our interest, because the SNF/SWI complexes have been implicated in nuclear receptor-dependent transcriptional activation.

To further investigate this interaction, we performed two experiments: a pull-down assay with in vitro translated proteins and a mammalian two-hybrid assay. In vitro translated <sup>35</sup>S-labeled hSNF2 $\alpha$  and hSNF2 $\beta$  proteins (N-terminal 633 and 837 aa, respectively) were tested for their interaction with GST-fused TMF bound on Sepharose resin. As a result, GST-TMF but not GST alone efficiently trapped these proteins (Fig. 2A). We next tested the interactions of these two proteins by a mammalian two-hybrid assay in which transcription activity of the reporter plasmid was measured by a CAT assay. Transcription activities were strongly enhanced with the combinations of GAL4BD-hSNF2 $\alpha$  or GAL4BD-hSNF2 $\beta$  and VP16-TMF but not with each partner alone (Fig. 2B), indicating that the interaction clearly occurred with specificity in vivo in yeast.

### 3.2. TMF associates with human SNF/SWI complexes in vitro and in vivo

The hSNF2 $\alpha$  and hSNF2 $\beta$  proteins are components of multiprotein complexes with different compositions and also interact with numerous factors [15,28–32]. It is therefore possible that some of other SNF/SWI subunits or interacting proteins sterically exclude the interaction. To study the interaction between TMF and the SNF/SWI complexes, a pull-down assay with GST-fusion proteins and HeLa nuclear extract was carried out. GST-TMF, but neither GST nor GST-USF, efficiently bound the hSNF2 proteins (Fig. 3A). We next analyzed the chromatographic behavior of native TMF with a gel filtration column. Previously, the SNF/SWI complexes were reported to be fractionated at an approximate molecular mass of 2 MDa [7]; this finding appears to be reproduced in this study (Fig. 3B). A Western blot analysis of the chromato-

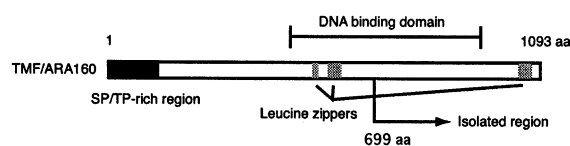


Fig. 1. Isolation of an hSNF2 $\beta$  (BRG-1)-binding protein by yeast two-hybrid screening. A cDNA encoding a protein that interacts with the amino-terminal region (1–1243 aa) of hSNF2 $\beta$  was isolated from the 10<sup>6</sup> cDNA clones of a HeLa cDNA expression library and identified as a C-terminal region (aa residues 699–1093, indicated by an arrow) of TMF/ARA160. The domain structure of TMF/ARA160 is shown.

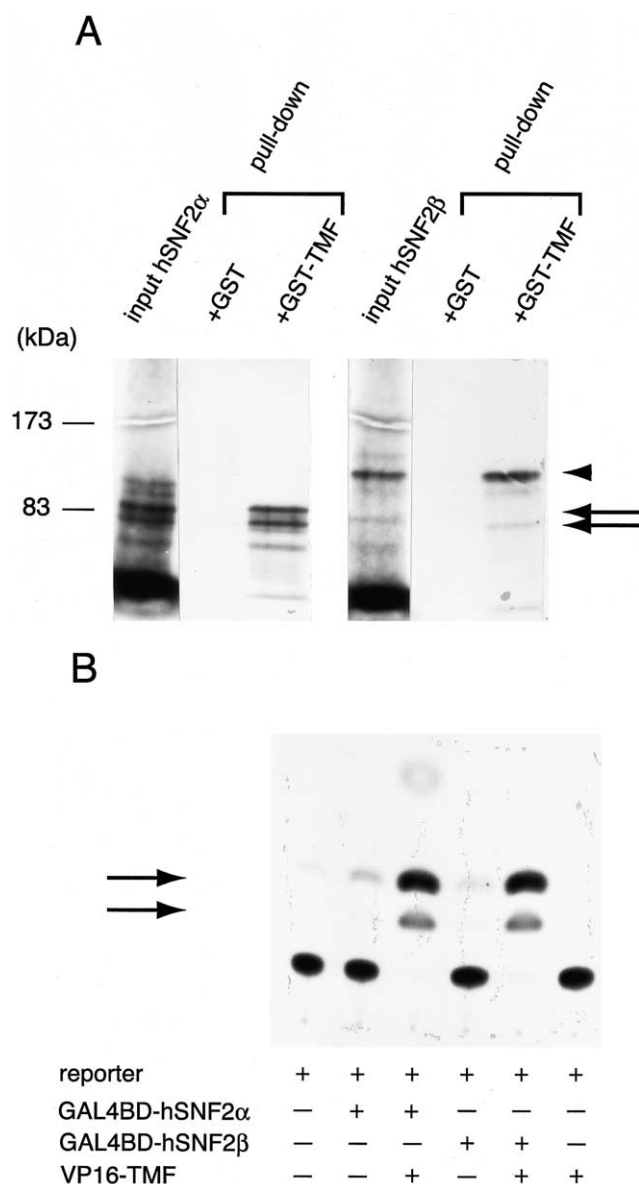


Fig. 2. Interaction between hSNF2 $\alpha/\beta$  and TMF in vivo and in vitro. **A**: Pull-down assay. In vitro translated [ $^{35}$ S]-labeled hSNF2 $\alpha$  and hSNF2 $\beta$  proteins were incubated with glutathione-Sepharose bearing GST or GST-TMF and washed with a buffer containing 0.3 M NaCl. The bound fraction as well as the same amount of each input was separated by SDS-PAGE and analyzed by fluorography. The hSNF2 $\alpha$  and hSNF2 $\beta$  bands, indicated by an arrow and arrow-head, appear somewhat larger than their respective theoretical molecular masses of 70 and 94 kDa. **B**: Mammalian two-hybrid interaction assay. A plasmid (8.0  $\mu$ g) expressing GAL4BD-hSNF2 $\alpha$  or GAL4BD-hSNF2 $\beta$  was transfected with pSVVP16-TMF (4.0  $\mu$ g) in the presence of a reporter plasmid G6(–119)HIVLTRATAR (2.0  $\mu$ g) as denoted. Acetylated forms of [ $^{14}$ C]chloramphenicol are indicated by arrows. The same amounts of plain vector plasmids were included in the blank (–) transfection samples.

graphic fractions with anti-hSNF2 $\alpha/\beta$  and anti-TMF antisera revealed that hSNF2 $\alpha/\beta$  and TMF were exactly cofractionated at the same position of an approximate molecular mass of 1–2 MDa (Fig. 3B). A lesser fraction of TMF migrated at an isolated position of a lower molecular mass (approximately 600 kDa). This result indicates that most (apparently more than 50%) of the TMF protein associates with (at

least certain forms of) the SNF/SWI complexes in the nuclear extract, albeit TMF is not a stoichiometric component.

### 3.3. An N-terminal domain of hSNF2 $\alpha$ and hSNF2 $\beta$ , and a C-terminal domain of TMF are required for the interaction

To determine which regions are required for the interaction by the yeast two-hybrid assay, a set of deletion mutants were constructed for hSNF2 $\alpha$ , hSNF2 $\beta$ , and TMF. Interaction was tested in the yeast indicator strains SFY526 and Y187 with combinational transformation of one of the full-length constructs and one of the deletion constructs of the partner proteins. Consequently, short interaction regions have been identified: the N-terminal regions of hSNF2 $\alpha$  (aa 338–585) and hSNF2 $\beta$  (aa 359–601) as well as a C-terminal region of TMF (aa 835–1093) (Fig. 4A,B). The regions of hSNF2 $\alpha$  and hSNF2 $\beta$  locate in an identical position (almost completely overlapped in homology alignment) and have high identity (87.6%) with each other, suggesting that the regions have a very similar interaction domain. The interaction domain of TMF contains a potential leucine zipper [38].

### 3.4. Subcellular localization of TMF

Localization and activities of the SNF/SWI complexes and their components are dynamic. For example, hbrm/hSNF2 $\alpha$  and BRG-1/hSNF2 $\beta$  are phosphorylated and excluded from the condensed chromosome during mitosis [44] coincident with the inactivation of the remodeling activity [45]. The BAF complex (SNF/SWI complexes) also becomes associated with chromatin and the nuclear matrix by PIP2 treatment [16]. We therefore investigated the subcellular localization of TMF in HeLa cells by immunofluorescence analysis with an affinity-purified anti-TMF antibody. Unexpectedly, we found that it was mainly localized in the Golgi apparatus (Fig. 5A). This pattern is identical to that seen with anti-mannosidase II antiserum (kindly provided by Dr. K. Moremen, University of Georgia; data not shown), which is a typical Golgi-localizing protein [46]. In HeLa cells treated with nocodazole after a thymidine block (i.e. synchronized in G<sub>2</sub>-M phase), fluorescence was observed in the fragmented bodies of the Golgi apparatus (Fig. 5B,C). At these stages, nuclei appear to be more densely stained than they are in interphase, whereas the nucleoli are excluded from staining.

To confirm the TMF localization, we utilized an EGFP (enhanced GFP) fusion strategy. When pEGFP-TMF was transfected into Cos-1 cells, the fluorescence was again detected in the Golgi apparatus with moderate staining of the cytoplasm (Fig. 5D). This higher background could be due to proteolysis of EGFP-TMF with no Golgi-localization signal or to mislocalization of the overexpressed protein, which was shown for a Golgi-localizing protein, phospholipase D [47].

To further investigate whether TMF is present in the nucleus, cytoplasmic and nuclear fractions of RL95–2 cells were prepared by a hypotonic-NP40 lysis method and analyzed by Western blotting. As a result, a substantial amount (estimated as 20–30%) of a shorter form (120 kDa), but not the full length form (160 kDa), of TMF was detected in the fractions from the extensively washed nuclei (Fig. 5E, upper panel). In contrast, a control 58 kDa Golgi protein (48) was exclusively detected in the cytoplasmic fraction (Fig. 5E, lower panel). It is thus likely that at least significant amount of the 120 kDa TMF protein is indeed present in the nucleus.

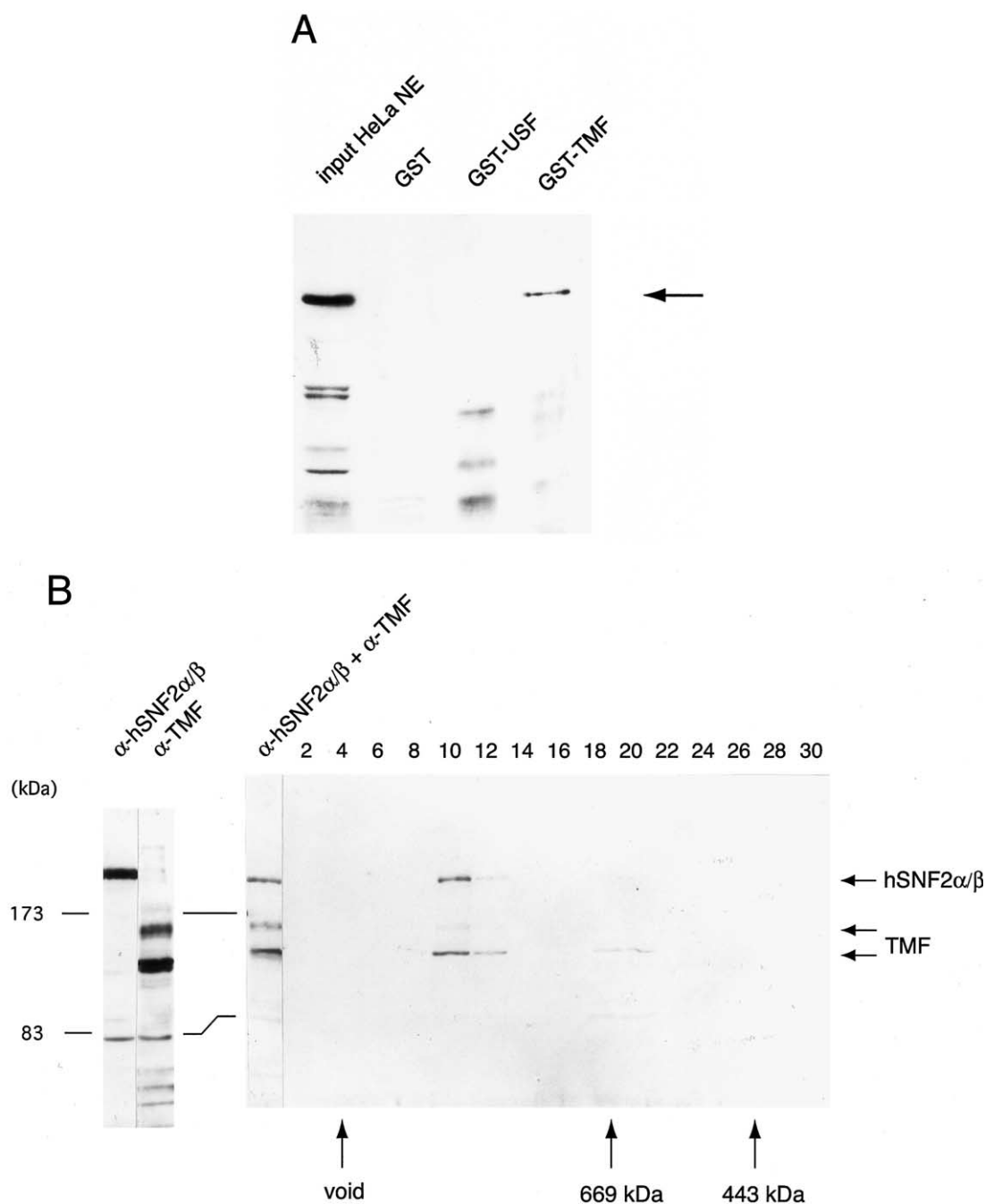


Fig. 3. Association of TMF with human SNF/SWI complexes. A: pull-down assay. HeLa nuclear extract (NE) was incubated with GST, GST-USF, or GST-TMF. After washing, the bound fractions were analyzed by 5% PAGE and Western blotting with anti-hSNF2 antiserum. A 190 kDa hSNF2α/β band is indicated by an arrow. B: Cofractionation of TMF with human SNF/SWI complexes. A 0.3 M KCl DEAE-Sepharose eluate was fractionated by gel filtration chromatography. Fractions 2–30 were analyzed as for (A) with a mixture of anti-hSNF2α/β and anti-TMF antisera. Western blotting patterns for HeLa NE with anti-hSNF2, anti-TMF, or their mixture, are included in the left three lanes. Specific bands are indicated by arrows. The peak positions of 669 and 443 kDa marker proteins are fractions 19 and 27, respectively.

#### 4. Discussion

A number of coactivators for nuclear receptors, which include the SNF/SWI complexes, have been reported and their actions are likely to involve multiple steps that depend on cooperative physical interactions (reviewed in [49–51]). We have searched for factors that interact with hbrm/hSNF2α and/or BRG-1/hSNF2β and, consequently, have identified TMF/ARA160 by yeast two-hybrid screening. TMF/

ARA160 has been reported as a putative coactivator for AR. It was observed to bind to AR in a ligand (DHT)-dependent manner in vitro (by pull-down assay) and in vivo (by mammalian two-hybrid assay). Furthermore, its enhancement of transcription from the MMTV promoter-driven reporter is dependent on AR, GR, or PR (progesterone receptor) and their cognate ligand. Molecular mechanisms that control the actions of this putative coactivator, however, have not been elucidated.

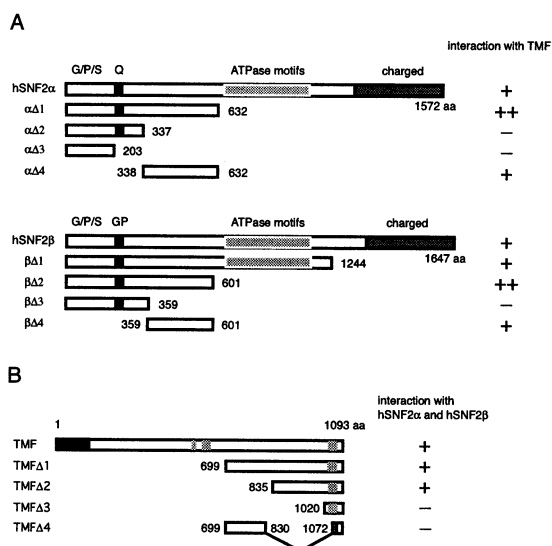


Fig. 4. Interaction regions of hSNF2 $\alpha/\beta$  and TMF. Regions required for the interaction were determined by the yeast two-hybrid assay with sets of deletion constructs. Interaction activities were measured by  $\beta$ -galactosidase assays (—, negative; +, positive; ++, strongly positive). Interaction regions of hSNF2 $\alpha$  and hSNF2 $\beta$  (A), and TMF (B) are shown. Minimal regions reside in aa residues 338–585 of hSNF2 $\alpha$ , 359–601 of hSNF2 $\beta$  and 835–1093 of TMF. The glutamine-rich and glycine/proline-rich regions (black), ATPase motifs (light gray), and charged regions (dark gray) are depicted.

We believe that the interaction between hSNF2 $\alpha/\beta$  and TMF is physiologically relevant for several reasons. First, TMF associates not only with the hbrm/hSNF2 $\alpha$  and BRG-1/hSNF2 $\beta$  proteins but also with the SNF/SWI complexes in vitro and in vivo; second, the binding activities we obtained seem to be sufficiently strong; third, the regions of hSNF2 $\alpha/\beta$  required for the interaction mapped to an identical position with a mutually high identity (87.6%); and fourth, a substantial amount of TMF was detected in the nuclear fractions. An important question would be raised as to whether only some special forms of the SNF/SWI complexes (or the PBAF complex) or their derivatives containing interacting factors can associate with TMF. This remains to be addressed. Although it is possible that TMF was solubilized from the Golgi apparatus and contaminated during the nuclear extract preparation, we believe from our immunofluorescence study that at least some fraction of TMF is present in the nucleus especially during mitotic phase. Thus, on the premise that TMF-(ARA160) is a coactivator for nuclear receptors such as AR, it is tempting to suppose that TMF mediates actions of the SNF/SWI complexes on the nuclear receptors and participates in the sequential and highly cooperative actions of the numerous nuclear-receptor coactivators [52]. Alternatively, TMF could confer totally different functions on the SNF/SWI complexes. The limited information so far available on TMF has precluded us from speculating on other possibilities at present.

Unique observations are that TMF localizes in the Golgi apparatus and only the shorter (presumable processed) form of TMF is also present in the nucleus. There are several prece-

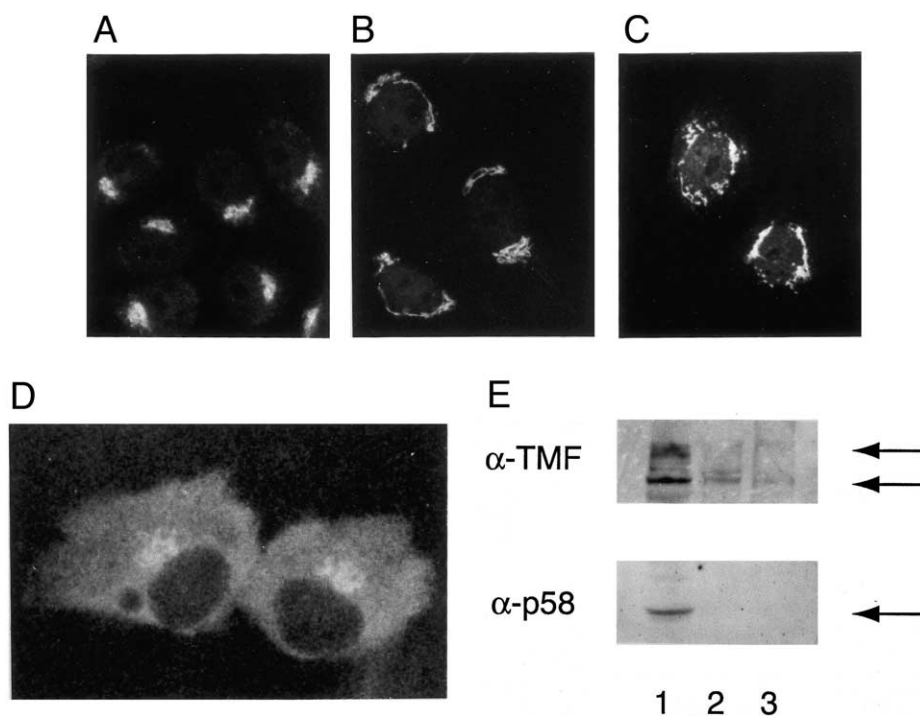


Fig. 5. Immunofluorescence analysis of TMF protein. HeLa cells were either asynchronous (A) or synchronized by treatment with nocodazole after releasing them from thymidine block (B and C). Cells were fixed and permeabilized as described in Section 2 and incubated with affinity-purified anti-TMF/ARA160 antibody and then with FITC-conjugated anti-rabbit IgG antibody. FITC immunofluorescence images of the Golgi apparatus show that fragmentation in (C) is further advanced than that in (B). D: Cos-1 cells were transfected with the pEGFP-TMF plasmid and EGFP fluorescence is shown. E: Cytoplasmic (lane 1) and nuclear fractions (lanes 2 and 3) of RL95-2 cells were analyzed by Western blotting with anti-TMF antiserum (upper panel) and monoclonal antibody to the Golgi 58 kDa protein [48] (lower panel). Nuclear extracts were prepared from nuclei washed two (lane 2) or three times (lane 3). The arrows indicate specific bands.

dents, such as for phospholipase D1 and cyclin B2 [47,53], for proteins that exhibit dual localization in the Golgi apparatus and nucleus. The nuclear localization of TMF appears to be enhanced in the G<sub>2</sub>-M phase (Fig. 5B,C), suggesting that TMF has a preference for being present in the nucleus, unless otherwise trapped. In this context it is quite interesting to note that PLD1 is dissociated from the Golgi apparatus upon stimulation with 1-BtOH, which diminishes PIP2 synthesis, possibly because PLD1-binding to the Golgi membrane is weakened [47]. We therefore imagine that TMF may be translocated as a result of biological changes or stimulations; this possibility is currently being investigated.

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