Tumour-specific chromosomal rearrangements are known to create chimaeric products with the ability to generate many human cancers. hTAF\textsubscript{II}68-TEC (where hTAF\textsubscript{II}68 is human TATA-binding protein-associated factor II 68 and TEC is translocated in extraskeletal chondrosarcoma) is such a fusion product, resulting from a t(9;17) chromosomal translocation found in extraskeletal myxoid chondrosarcomas, where the hTAF\textsubscript{II}68 NTD (N-terminal domain) is fused to TEC protein. To identify proteins that control hTAF\textsubscript{II}68-TEC function, we used affinity chromatography on immobilized hTAF\textsubscript{II}68 (NTD) and MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS and isolated a novel hTAF\textsubscript{II}68-TEC-interacting protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase). GAPDH is a glycolytic enzyme that is also involved in the early steps of apoptosis, nuclear tRNA export, DNA replication, and transcription. hTAF\textsubscript{II}68-TEC and GAPDH were co-immunoprecipitated from cell extracts, and glutathione S-transferase pull-down assays revealed that the C-terminus of hTAF\textsubscript{II}68 (NTD) was required for interaction with GAPDH. In addition, three independent regions of GAPDH (amino acids 1–66, 67–160 and 160–248) were involved in binding to hTAF\textsubscript{II}68 (NTD). hTAF\textsubscript{II}68-TEC-dependent transcription was enhanced by GAPDH, but not by a GAPDH mutant defective in hTAF\textsubscript{II}68-TEC binding. Moreover, a fusion of GAPDH with the GAL4 DNA-binding domain increased the promoter activity of a reporter containing GAL4 DNA-binding sites, demonstrating the presence of a transactivation domain(s) in GAPDH. The results of the present study suggest that the transactivation potential of the hTAF\textsubscript{II}68-TEC oncogene product is positively modulated by GAPDH.

Key words: chromosome translocation, co-activator, extraskeletal myxoid chondrosarcoma, hTAF\textsubscript{II}68-TEC, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), protein–protein interaction, transactivation.
hTAFI68 (NTD) is believed to act as a transactivation domain for hTAFI68-TEC oncoprotein. To search for binding partners that control hTAFI68-TEC function in vivo, we performed GST (glutathione S-transferase) pull-downs in HEK (human embryonic kidney)-293T cell lysates, followed by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS, and identified GAPDH as a binding partner. We confirmed the interaction between hTAFI68-TEC and GAPDH in vitro using bacterially expressed fusion proteins and in vivo using immunoprecipitation and Western blot analysis. In transient transfection assays, the transcriptional activity of hTAFI68-TEC was stimulated by GAPDH, but not by a GAPDH mutant defective in hTAFI68-TEC binding. In addition, fusion of GAPDH to the GAL4 DNA-binding domain produced a chimera capable of transactivating a reporter gene containing GAL4-binding sites, indicating that GAPDH is a potential transcriptional co-activator. These data demonstrate that hTAFI68-TEC-mediated transcriptional activation is positively regulated by GAPDH.

MATERIALS AND METHODS

Materials and general methods

Restriction endonucleases, polynucleotide kinase, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. Plasmid DNA preparation, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformation and SDS/PAGE of proteins were carried out using standard methods [52]. PCR amplification products were sequenced by the chain termination method, using double-stranded DNA templates, to ensure the absence of mutations.

Isolation of hTAFI68-TEC complexes and MS

For affinity purification of hTAFI68-TEC-associated proteins, a GST–hTAFI68 (NTD) was constructed as described below. Pull-downs were carried out in binding buffer [50 mM Tris/Cl (pH 8.0), 1% Nonidet P40, 2 mM EDTA, 150 mM NaCl, 1 mM DTT (dithiothreitol) and 1× Complete Protease Inhibitor Cocktail (Roche Diagnostics)] as described previously [53], using HEK-293T lysates. Beads were collected by brief centrifugation (12,000 g for 10 s at 4°C) and washed four times with wash buffer (same composition as the binding buffer minus the 1× Complete Protease Inhibitor Cocktail). Bound proteins were eluted with SDS/PAGE sample buffer and subjected to SDS/PAGE. Gels were stained with Coomassie Blue for MALDI-TOF MS [54].

Plasmid construction

The construction of GST–hTAFI68 (NTD) [55] and GST–EWS (NTD) has been previously described [56]. For Tag2A/Flag-hTAFI68-TEC, plasmid pSKII/hTAFI68-TEC was digested with BamHI and cloned into the same site of pCMV-Tag2A (Invitrogen). Details on the construction of GST–TLC (NTD), pcDNA3/GST and pcDNA3/GST–hTAFI68 (NTD) have been given previously [55,56].

GSTM-fusion plasmids GST–hTAFI68 (1–72) and GST–hTAFI68 (29–108) have been previously described [55]. For GST-hTAFI68 (106–159), plasmid pGEX4T-1-hTAFI68 (NTD) was amplified by PCR using primers 5′-hTAFI68-106 (5′-GA-TCGAATTCTGACTTGCAACAAAGAT-3′, EcoRI site underlined) and 3′-hTAFI68-159 (5′-GATCCCTCGAGTCTTGTGTGGTGGCTG-3′, Xhol site underlined), digested with EcoRI and Xhol, and cloned into the same sites of pGEX (4T-1).

The six histidine-containing fusion plasmids (His6)-GAPDH, (His6)-GAPDH (1–66), (His6)-GAPDH (67–160), (His6)-GAPDH (160–248) and (His6)-GAPDH (217–335) were generated using the following steps. (i) For (His6)-GAPDH, plasmid pcDNA3/GAPDH was digested with BamHI and EcoRI and cloned into the same sites of pTag3B (Stratagene). This in turn was digested with ScaI and KpnI and cloned into the same sites of pTRC-HisB (Invitrogen). (ii) For (His6)-GAPDH (1–66), pTRC-HisB/GAPDH was digested with HindIII and self-ligated with DNA ligase. (iii) For (His6)-GAPDH (67–160), GAPDH (67–160) was amplified from pcDNA3/GAPDH by PCR using primers 5′-GAPDH-67 (5′-GATCCTGCTCAAGCTTTGCATCAATGGAG-3′, BamHI site underlined) and 3′-GAPDH-160 (5′-GATCCGAGTCCCCGTTGAGGAGGCACT-3′, EcoRI site underlined), digested with BamHI and EcoRI, and cloned into the same sites of pTRC-HisA. (iv) For (His6)-GAPDH (160–248), GAPDH (160–335) was amplified from pcDNA3/GAPDH by PCR using primers 5′-GAPDH-161 (5′-GATCCTGCTCAAGCTTTGCATCCATGAC-3′, BamHI site underlined) and 3′-GAPDH-335 (5′-GATCGGAATTCCTTTGCTGAC-3′, EcoRI site underlined), digested with BamHI and EcoRI, and cloned into the same sites of pTRC-HisA. (v) For (His6)-GAPDH (217–335), GAPDH (217–335) was amplified from pcDNA3/GAPDH by PCR using primers 5′-GAPDH-217 (5′-GATCGGATCCCTTTGCTGAC-3′, BamHI site underlined) and 3′-GAPDH-335 (5′-GATCGGAATTCCTTTGCTGAC-3′, EcoRI site underlined), digested with BamHI and EcoRI, and cloned into the same sites of pTRC-HisA. (vi) For (His6)-GAPDH (217–335), GAPDH (217–335) was amplified from pcDNA3/GAPDH by PCR using primers 5′-GAPDH-217 (5′-GATCGGATCCCTTTGCTGAC-3′, BamHI site underlined) and 3′-GAPDH-335 (5′-GATCGGAATTCCTTTGCTGAC-3′, EcoRI site underlined), digested with BamHI and EcoRI, and cloned into the same sites of pTRC-HisA.

The reporter plasmid pB1aLuc has been previously described [55]. For Tag3B/GAPDH, pGEX (4T-1)/GAPDH was digested with BamHI and Xhol and cloned into the same sites of Tag3B. For Tag3B/GAPDH (217–335), pTRC-HisA/GAPDH (217–335) was digested with BamHI and EcoRI and cloned into the same sites of Tag3B.

Subcellular localization of GAPDH

Subcellular fractions were prepared essentially as described by Dignam [57]. Nuclear and cytoplasmic fractions were boiled in 2% SDS for 10 min and the insoluble material was removed by centrifugation in a microcentrifuge at 12,000 g for 10 min at 4°C. Equivalent amounts of each supernatant on a cell basis were fractionated by SDS/PAGE, transferred to PVDF membranes and probed with the indicated antibodies. Immunocytochemical analyses were performed as previously described [55]. Briefly, HeLa, COS-7 and C28/I2 human juvenile costal chondrocyte cells (provided by Dr M. Goldring, Hospital for Special Surgery, Cornell Medical College in New York City, NY, U.S.A.) were plated on glass coverslips and transfected with PVDF membranes and probed with the indicated antibodies. Immunocytochemical analyses were performed as previously described [55]. Briefly, HeLa, COS-7 and C28/I2 human juvenile costal chondrocyte cells (provided by Dr M. Goldring, Hospital for Special Surgery, Cornell Medical College in New York City, NY, U.S.A.) were plated on glass coverslips and transfected with pTag2A/Flag-hTAFI68-TEC plasmid. After transfection (48 h), the cells were washed in PBS and fixed for 10 min at −20°C with acetone/methanol (1:1, v/v). To detect hTAFI68-TEC and GAPDH, we used primary antibodies for Flag-tagged hTAFI68-TEC antibody (M2, Sigma) and GAPDH (V-18; Santa Cruz Biotechnology) respectively. The subcellular distribution of hTAFI68-TEC or GAPDH was examined using a confocal laser scanning microscope (LSMS Pascal, Carl Zeiss Co).

Purification of six histidine-tagged GAPDH mutant proteins

Recombinant six histidine-tagged GAPDH proteins were purified as described in [53]. The elution profiles of GAPDH mutant
Functional regulation of hTAFII68-TEC by GAPDH

Figure 1 Identification of GAPDH as an hTAFII68-TEC-associated protein

(A) SDS/PAGE of hTAFII68 (NTD) complexes isolated from HEK-293T cells. Total HEK-293T cell lysates were incubated with either GST or a GST-fusion protein containing the NTD of hTAFII68 (indicated above the panel). Complexes were resolved by SDS/PAGE (15 % gels) and stained with Coomassie Blue. Molecular mass markers are shown on the left; they are derived from pre-stained protein standards (broad range; New England Biolabs). The band investigated in this analysis is indicated by the arrow to the right. Lane 1, molecular mass marker; lane 2, GST; lane 3, GST plus cell lysate; lane 4, GST–hTAFII68 (NTD); lane 5, GST–hTAFII68 (NTD) plus cell lysate. (B) Peptide sequences of GAPDH identified by MALDI-TOF analysis. The GAPDH peptides matched by MALDI-TOF are shown with amino acid numbers displayed at both ends. (C) Association of hTAFII68 (NTD) with GAPDH. HEK-293T cell lysate was incubated with either GST–hTAFII68 (NTD) or GST. After affinity-selection, the pellets from GST pull-down assays were analysed by SDS/PAGE (15 % gels), and bound GAPDH was detected with anti-GAPDH antibody (MAB374; Chemicon) and chemiluminescence (Perkin Elmer Life Science). The positions of the molecular mass markers are indicated on the left-hand side, and the GAPDH band is indicated by the arrow on the right-hand side. Lane 1, GST; lane 2, GST plus cell lysate; lane 3, GST–hTAFII68 (NTD); lane 4, GST–hTAFII68 (NTD) plus cell lysate. IB, immunoblotting; Ab, antibody.

proteins were monitored by Western blot analysis using the monoclonal Xpress antibody (Invitrogen).

GST pull-down assays, co-affinity precipitations and co-immunoprecipitations

GST pull-down assays were performed as described previously [53]. Co-affinity precipitations and co-immunoprecipitation assays were also performed as described in [53,56] and bound proteins were detected by immunoblotting [58].

Cell culture, electroporation and reporter assays

HeLa, COS-7 or HEK-293T cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % heat-inactivated fetal calf serum (HyClone), penicillin and streptomycin. Cells were transiently transfected with plasmids by electroporation using the Gene Pulser II RF module system according to the manufacturer’s instructions (Bio-Rad). Luciferase assays were performed using the Promega Luciferase Assay System or Dual-luciferase Assay System (Promega). Renilla luciferase activities were used to normalize transfection efficiencies.

RESULTS

Identification of GAPDH as an hTAFII68-TEC-interacting protein

To isolate potential regulators of the hTAFII68-TEC oncoprotein, we used MS to identify proteins associating with it. Bacterially expressed GST or GST–hTAFII68 NTD-fusion proteins immobilized on glutathione–Sepharose beads were mixed with HEK-293T cell extracts. After extensive washing, bound proteins were eluted with sample buffer and analysed by SDS/PAGE and Coomassie Blue staining. Two proteins, of ~34 kDa (denoted by an arrow) and ~24 kDa (denoted by an asterisk), were visible on the Coomassie Blue-stained gel (Figure 1A). The 24 kDa species was present in eluates from both the GST and GST–hTAFII68 (NTD) affinity matrices (Figure 1A, lanes 1 and 3) and was not observed if the HEK-293T cell extract was omitted from the GST or GST–hTAFII68 (NTD) beads (Figure 1A, lanes 2 and 4). This suggested that the 24 kDa protein species was not specifically retained by hTAFII68 (NTD), and this species was not further characterized. The 34 kDa protein species was observed in eluates from the GST–hTAFII68 (NTD) beads (Figure 1A, lane 3), not from the column that had GST coupled to it and had been exposed to HEK-293T cell extracts (Figure 1A, lane 4), and not
Figure 2 Binding of GAPDH to hTAFII68 (NTD) in vitro

(A) Specific association of hTAFII68 (NTD) with GAPDH. GST-fusion proteins containing the NTDs of hTAFII68, EWS or TLS were incubated with cell lysates. An aliquot of the inputs (20%) and the pellets from the various pull-downs were analysed on SDS/PAGE (15% gels) and bound GAPDH protein was detected using an anti-GAPDH antibody (MAB374). The identities of the GST-fusion proteins are indicated above the panel. The positions of the molecular mass markers are indicated on the left-hand side. GAPDH is indicated by an arrow on the right-hand side. Three independent experiments were performed, all of which gave similar results. Lane 1, 20% input; lane 2, GST alone; lane 3, GST–hTAFII68 (NTD); lane 4, GST–EWS (NTD); lane 5, GST–TLS (NTD). IB, immunoblotting; Ab, antibody.

(B) Quantitation of the GST-fusion proteins used in the GST pull-down assays. The GST-fusion proteins utilized in the pull-down assays were fractionated by SDS/PAGE (15% gels) and visualized by Coomassie Blue staining. Three independent experiments were performed, all of which gave similar results. Lane 1, GST alone; lane 2, GST–hTAFII68 (NTD); lane 3, GST–EWS (NTD); lane 4, GST–TLS (NTD).

in eluates from GST–hTAFII68 (NTD) beads that had not been exposed to HEK-293T cell extracts (Figure 1A, lane 4). These results indicated that the ~ 34 kDa protein species was specifically retained by and eluted from the GST–hTAFII68 (NTD) affinity matrix. The band was extracted from the gel matrix by trypsin digestion and analysed by MALDI-TOF MS, revealing peptides derived from GAPDH (Figure 1B). Binding to GAPDH was confirmed by Western blot analysis using a monoclonal anti-GAPDH antibody (Figure 1C).

hTAFII68-TEC interacts with GAPDH in vitro and in vivo

The in vitro interaction between hTAFII68-TEC and GAPDH was further investigated using GST-fusion proteins containing the NTDs of hTAFII68 and its homologues, such as EWS and TLS. As shown in Figure 2(A), approximately 30% of input GAPDH protein was retained on hTAFII68 (NTD)-conjugated Sepharose beads. However, neither EWS (NTD) nor TLS (NTD) was able to bind GAPDH (Figure 2A, lanes 4 and 5) suggesting that GAPDH binds differentially to the NTDs of TET (hTAFII68, EWS and TLS) family members. This provides a molecular criterion for distinguishing between them. As GAPDH did not bind to GST alone (Figure 2A, lane 2), the interaction was considered to be specific. The amounts of GST-fusion proteins utilized in this assay were fractionated on SDS/PAGE (15% gels) and visualized by Coomassie Blue staining which revealed that similar amounts of protein had been used in the pull-down assay (Figure 2B).

To determine whether the interaction between hTAFII68 (NTD) and GAPDH occurred in vivo, we performed co-affinity precipitations following transfection of COS-7 cells with an expression vector driving the synthesis of hTAFII68 (NTD). For these assays, mammalian expression vectors containing the NTD of hTAFII68 fused to GST [pcDNA3/GST–hTAFII68 (NTD)] and the GST domain alone (pcDNA3/GST) were transfected into COS-7 cells. The cells were lysed 48 h after transfection and the fusions were incubated with glutathione beads. Immunoblotting of the eluates with an anti-GAPDH antibody revealed that endogenous GAPDH was co-precipitated with GST–hTAFII68 (NTD), but not with

Figure 3 Association of hTAFII68-TEC with GAPDH in vivo

(A) Co-affinity purification of hTAFII68 (NTD) with GAPDH from COS-7 cells. After transfection (48 h) of COS-7 cells with 10 µg of either pcDNA3/GST or pcDNA3/GST–hTAFII68 (NTD), cell extracts were prepared as described in the Materials and methods section and affinity-precipitated with glutathione–Sepharose beads. After fractionation by SDS/PAGE (15% gels), the proteins were analysed by Western blot with an anti-GAPDH antibody (MAB374). The identities of the transfected DNAs are indicated above the panel. The positions of the molecular mass markers are indicated on the left-hand side and the position of GAPDH is indicated by the arrow on the right-hand side. Three independent experiments were performed, all of which gave similar results. AP, affinity precipitation; IB, immunoblotting; Ab, antibody.

(B) Co-immunoprecipitation of hTAFII68-TEC and GAPDH in vivo. After transfection (48 h) of HeLa cells with 10 µg of either Tag2A or Tag2A/Flag-hTAFII68-TEC, cell extracts were immunoprecipitated with an anti-Flag antibody (M2), resolved by SDS/PAGE (15% gels), and probed with an anti-GAPDH antibody (MAB374). The identities of the transfected DNAs are indicated above the panel. The positions of the molecular mass markers are indicated on the left-hand side, and the positions of IgG and GAPDH are indicated by arrows on the right-hand side. Three independent experiments were performed, all of which gave similar results. IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.
Mapping the hTAFII68 (NTD) region that interacts with GAPDH

**Figure 4** Schematic representation of the GST–hTAFII68 (NTD) fusion proteins and their ability to bind to GAPDH. Numbers refer to amino acid residues, and binding ability is indicated by + or −. (A) Strong binding of GAPDH to GST–hTAFII68 (106–159). Recombinant GST–hTAFII68 (NTD) deletion mutants were incubated with HEK-293T cell lysates. Following GST pull-down assays, the bound proteins were eluted with SDS loading buffer and analysed by Western blotting with an anti-GAPDH antibody. The positions of molecular mass markers and of GAPDH are indicated. Three independent experiments were performed, all of which gave similar results. Lane 1, 20 % input; lane 2, GST alone; lane 3, GST–hTAFII68 (NTD); lane 4, GST–hTAFII68 (1–72); lane 5, GST–hTAFII68 (29–105); lane 6, GST–hTAFII68 (106–159). IB, immunoblotting; Ab, antibody. (C) Coomassie Blue staining of the GST–hTAFII68 deletions. The amounts of GST-fusion proteins utilized in these assays were fractionated on SDS/PAGE (15 % gels) and visualized by Coomassie Blue staining. Lane 1, GST alone; lane 2, GST–hTAFII68 (NTD); lane 3, GST–hTAFII68 (1–72); lane 4, GST–hTAFII68 (29–105); lane 5, GST–hTAFII68 (106–159).

As shown in Figure 4(B), GAPDH strongly bound to GST–hTAFII68 (NTD) (Figure 4B, lane 3) and GST–hTAFII68 (106–159) (Figure 4B, lane 6). However, the GST-fusions with hTAFII68 (1–72) (Figure 4B, lane 4) and hTAFII68 (29–105) (Figure 4B, lane 5) failed to bind, indicating that amino acids 106–159 of the hTAFII68 (NTD) is necessary and sufficient for the interaction with GAPDH. The amounts of GST-fusion proteins utilized in this assay were fractionated on SDS/PAGE (15 % gels) and visualized by Coomassie Blue staining which revealed that similar amounts of protein had been used in the pull-down assay (Figure 4C).

GAPDH contains at least three independent hTAFII68 (NTD)-interacting motifs

To delineate the regions of GAPDH responsible for the interaction with hTAFII68 (NTD), GAPDH (1–66), GAPDH (67–160), GAPDH (160–248) or GAPDH (217–335) were expressed as six histidine proteins in *Escherichia coli* and purified by Ni²⁺-NTA (Ni²⁺-nitrilotriacetate)–agarose resin (Figure 5A). In GST pull-down assays with GST or the GST–hTAFII68 (NTD) fusion...
protein, deletions of GAPDH containing amino acids 1–66, 67–160 and 160–248 interacted strongly with hTAFII68 (NTD), whereas GAPDH (217–335) interacted only weakly (Figure 5B). These results suggested that at least three sites (amino acids 1–66, 67–160 and 160–248) of GAPDH can bind to hTAFII68 (NTD) independently. The amounts of GST-fusion proteins utilized in this assay were fractionated on SDS/PAGE (15 % gels) and visualized by Coomassie Blue staining, which revealed that similar amounts of protein had been used in the pull-down assay (Figure 2B).

hTAFII68-TEC co-localizes with a nuclear form of GAPDH

Since hTAFII68-TEC is a nuclear protein [55], we wanted to establish the subcellular location of endogenous GAPDH. HeLa (Figure 6A, left panels) and COS-7 (Figure 6A, right panels) cells were lysed, and cytosolic and nuclear protein fractions were prepared and subjected to SDS/PAGE and Western blot analysis. As shown in Figure 6(A), GAPDH was present in both the cytoplasm and nucleus in HeLa and COS-7 cells. As a further control for the quality of the fractionation procedure, a cytoplasmic protein, actin [59], was detected only in the cytoplasmic fraction (Figure 6A, lower panel).

To confirm the hTAFII68-TEC–GAPDH interaction in vivo (Figure 3), we also performed immunofluorescence staining of the transfected cells. To do this we constructed a pTag2A/Flag-hTAFII68-TEC construct that expressed Flag-tagged hTAFII68-TEC protein. In control HeLa cells not expressing hTAFII68-TEC, the majority of GAPDH protein was localized to the cytoplasm [Figure 6B(a), arrowheads]. A significant amount of GAPDH was also detected in the nucleus, as previously reported [16,47,50]. However, in HeLa cells expressing hTAFII68-TEC, the amount of nuclear GAPDH was as high as that of cytoplasmic GAPDH, suggesting that hTAFII68-TEC promotes translocation of GAPDH to the nucleus [Figure 6B(a), arrows]. Consistent with a previous report [55], hTAFII68-TEC protein localized to the nucleus in HeLa cells [Figure 6B(b)]. The overlay images indicated that hTAFII68-TEC and GAPDH in the nucleoplasm partially overlapped [Figure 6B(c)]. Similar results were obtained in COS-7 and C28/I2 human chondrocyte cells [Figure 6B(d–i)]. Therefore we conclude that hTAFII68-TEC and a nuclear form of GAPDH co-localize or are in close proximity, and that the formation of hTAFII68-TEC–GAPDH complexes results in a redistribution of GAPDH.

GAPDH modulates hTAFII68-TEC-dependent transactivation

To investigate the consequences of the hTAFII68-TEC–GAPDH interaction, we measured the effect of GAPDH expression on transcriptional activation by hTAFII68-TEC. This was performed in HeLa cells by examining gene expression from a luciferase-based reporter plasmid transfected along with Tag2A/hTAFII68-TEC, with or without co-transfection of Tag3B/GAPDH. The structures of the reporter plasmid and the expression vectors used in this study are shown schematically in Figure 7(A). The introduction of hTAFII68-TEC into HeLa cells activated transcription (up to ~ 60-fold) from p(B1a)8-Luc, a reporter plasmid containing the TATA minimal promoter with eight NBREs (nerve growth factor inducible factor B response elements) [60] driving synthesis of Renilla luciferase (Figure 7B, open bars).
Figure 7 GAPDH enhances hTAFII68-TEC-mediated transcription

(A) Schematic representation of the reporter and expression plasmids used in the present study. The p(B1a)8-Luc reporter plasmid contains eight copies of NBRE upstream of a basal promoter-luciferase gene construct. The eight copies of hTAFII68-TEC recognition sites are indicated by solid bars, the TATA box is represented by an open box, and the luciferase gene is indicated by a solid box. The expression vectors driving the production of hTAFII68-TEC, GAPDH or GAPDH (217–335) are also shown. The positions of the first and last amino acids are indicated below each construct. CMV, cytomegalovirus promoter. (B) Stimulation of hTAFII68-TEC-mediated transactivation by GAPDH. Aliquots of hTAFII68-TEC (bars 1–4) were co-transfected into HeLa cells with 2 µg of empty vector (open bars) or GAPDH expression plasmid (black bars). After 48 h the cells were harvested and luciferase assays performed. The experiments were repeated three times, and the averages of two independent experiments are presented and error bars are shown. (C) A GAPDH (217–335) mutant lacking the hTAFII68-TEC-interacting domain does not stimulate hTAFII68-TEC-mediated transcriptional activation. HeLa cells were transfected with 1 µg of p(B1a)8-Luc reporter plasmid, 5 µg of hTAFII68-TEC, and 5 µg of GAPDH (217–335) or empty vector, and luciferase activity was assayed. Relative transcriptional activation values were calculated with hTAFII68-TEC taken as 100%. The experiments were repeated three times, and the averages of two independent experiments are presented with error bars.

Interestingly, GAPDH augmented hTAFII68-TEC-mediated transactivation up to ∼210-fold (Figure 7B, solid bars). These results indicated that GAPDH can stimulate hTAFII68-TEC-mediated activation.

To demonstrate that binding between GAPDH and hTAFII68-TEC is required for the observed effects on transcriptional activation, we constructed a GAPDH deletion mutant lacking the hTAFII68-TEC-binding domain, called pTag3B/GAPDH (217–335). This deletion mutant did not activate hTAFII68-TEC-dependent transactivation (Figure 7C), indicating that GAPDH modulated hTAFII68-TEC-mediated transactivation by physical association with hTAFII68-TEC.

GAPDH is a potential transcriptional co-activator

To assess how GAPDH activates hTAFII68-TEC-mediated transcription, it was fused to the GAL4 DNA-binding domain, generating GAL4–GAPDH (Figure 8A). The pG5 luc reporter contains five GAL4 DNA-binding sites upstream of the TATA box and was used as a reporter. An expression vector driving the synthesis of only the GAL4 DNA-binding domain, pGAL4, had no effect on the levels of luciferase produced from pG5 luc when transfected into COS-7 cells (Figure 8B, bar 1). In contrast, pGAL4–GAPDH activated luciferase production up to 70-fold (Figure 8B, bars 2–4), indicating that GAPDH contained a transactivation domain(s). These results demonstrate that GAPDH can act as a co-activator of transcription if it is recruited to the vicinity of the promoter.

DISCUSSION

Aberrant expression or structural alteration of transcription factors is often a critical event in tumour development, and the characterization of specific chromosome abnormalities associated with human tumours has led to the discovery of new mechanisms involved in tumorigenic transformation. EMC is a malignant soft tissue tumour that occurs primarily in the extremities [61]. A portion of EMCs harbours a characteristic translocation t(9;17)(q22;q11) involving the hTAFII68 gene at 17q11 and the TEC gene at 9q22 [4,5]. This chimaeric hTAFII68-TEC encodes a potent transcriptional activator (S. Kim and J. Kim, unpublished work).

The results reported in the present study begin to reveal the regulation of the hTAFII68-TEC oncogene. Using affinity chromatography and MALDI-TOF MS, we showed that GAPDH interacts with hTAFII68-TEC and we confirmed this interaction using GST pull-down assays in vitro (Figure 2) and by co-immunoprecipitation experiments on extracts of cultured cells (Figure 3). Although it is unclear whether the interaction is direct, residues 106–159 of the hTAFII68 N-terminal domain were found to be required for the association with GAPDH (Figure 4).

GAPDH is an essential glycolytic enzyme in all prokaryotic and eukaryotic organisms, converting glyceraldehyde 3-phosphate into 1,3-diphosphoglycerate. However, it has a number of other seemingly independent activities, including a uracil DNA-glycosylase function, RNA binding, modulation of apoptosis,
Our finding of the presence of some GAPDH in the nucleus after cells are treated with genotoxic drugs suggests that GAPDH harbours at least three independent binding sites for hTAFII68 (NTD) (Figure 6), consistent with other reports [16,66]. Since hTAFII68-TEC is nuclear [55], the interaction between hTAFII68-TEC and GAPDH may occur within the nucleoplasm. Interestingly, this interaction also appears to lead to the translocation of a fraction of GAPDH from the cytoplasm to the nucleus (Figure 6B). Using GST pull-down assays, we showed that the C-terminal part of hTAFII68 (NTD) harbours a binding site for GAPDH, and it is interesting that amino acids 106–159 of hTAFII68 (NTD) are required for the interaction with GAPDH, because we have shown that amino acids 75–159 are involved in the interaction with v-Src [55]. It is therefore possible that the C-terminal part of hTAFII68 (NTD) is specialized to participate in protein–protein interactions. Using recombinant proteins in GST pull-down assays, we showed that GAPDH harbours at least three independent binding sites for hTAFII68 (NTD) (Figure 5), suggesting that the association of GAPDH with hTAFII68 (NTD) can be mediated by three different domains of a single molecule or that the stoichiometry of the interaction is greater than one. Further experiments are required to distinguish these possibilities.

We showed that GAPDH can modulate hTAFII68-TEC-mediated transactivation (Figure 7). However, although GAPDH has intrinsic transactivation activity (Figure 8), the precise mechanism underlying this effect is unclear. One possibility is that GAPDH acts as a bridging factor linking an hTAFII68-TEC molecule to components of the transcription initiation machinery. Consistent with this speculation, GAPDH was recently identified as a cofactor of the Oct-1 coactivator complex, OCA-S (Oct-1 CoActivator in S-phase) [47], which is essential for S-phase-dependent histone H2B transcription; using stimulation of Oct-1 transcription in an in vitro assay, OCA-S was purified from a HeLa cell nuclear extract and found to contain seven proteins: nm23-H1, nm23-H2, uracil-DNA glycosylase, lactate dehydrogenase, Hsp70, Sti1 and GAPDH. The last also associates with PML in an interaction that is dependent on the presence of RNA [50], and the possible significance of this GAPDH–PML interaction was indicated by a recent study which revealed an association of PML nuclear bodies with transcriptionally active genomic regions [51].

In conclusion, the findings of the present study provide new evidence that hTAFII68-TEC function can be modulated by GAPDH. Unlike EWS-TEC which can transform CFK2 chondrogenic cells [67], the transforming activity of hTAFII68-TEC has yet to be assessed. Thus it would be of interest to determine whether GAPDH can collaborate with hTAFII68-TEC to transform cells into EMC tumours.

This research was supported by a Korea Research Foundation Grant funded by the Korean Government (M0EHRD, Basic Research Promotion Fund: R14-2002-012-01002-0) of the KOSEF. S. K. and J. L. were recipients of Seoul Science Fellowships and of a research fellowship BK21 from the Ministry of Education and Human Resources Development.

REFERENCES


Figure 8 Transactivation by a GAL4–GAPDH fusion protein

(A) Schematic representation of the reporter plasmid and expression vectors. The pG5 luc reporter plasmid contains five GAL4-binding sites upstream of the luciferase gene. The GAL4 recognition sites are indicated with solid bars, the TATA box is represented by a shaded box, and the luciferase gene by a solid box. The expression vectors driving the production of GAL4 or GAL4–GAPDH are also presented. The positions of the first and last amino acids of the GAPDH construct are indicated below the construct. (B) Transactivation by GAPDH. COS-7 cells were co-transfected with 0.1 µg of GAL4–GAPDH construct are indicated below the construct. (Figure 7). This effect was dose-dependent and required the region of GAPDH that interacts with hTAFII68-TEC. It seems possible that GAPDH also interacts with other transcriptional regulators.

Because of its abundance, GAPDH is often regarded as a stable marker protein in contrast with the dynamic changes of other proteins. However, GAPDH expression is also dynamic, being affected by hypoxic stress [62]. It also accumulates in the nucleus after cells are treated with genotoxic drugs such as mercaptopurine or thioguanine [16,63]. Because hTAFII68-TEC is a transcriptional activator and its activity is modulated by GAPDH (Figure 7), it would be interesting to see whether exposure to hypoxic stress or genotoxic drugs modulates its activity. In fact, treatment with 6-mercaptopurine stimulates the transcriptional activity of the orphan nuclear hormone receptor NOR-1 (the rat homologue of the human TEC receptor) and Nurrl [64,65], but little is currently known of these effects. Our finding of the presence of some GAPDH in the nucleus (Figure 6) is consistent with other reports [16,66]. Since hTAFII68-TEC is nuclear [55], the interaction between hTAFII68-TEC and GAPDH may occur within the nucleoplasm. Interestingly, this interaction also appears to lead to the translocation of a fraction of GAPDH from the cytoplasm to the nucleus (Figure 6B). Using GST pull-down assays, we showed that the C-terminal part of hTAFII68 (NTD) harbours a binding site for GAPDH, and it is interesting that amino acids 106–159 of hTAFII68 (NTD) are required for the interaction with GAPDH, because we have shown that amino acids 75–159 are involved in the interaction with v-Src [55]. It is therefore possible that the C-terminal part of hTAFII68 (NTD) is specialized to participate in protein–protein interactions. Using recombinant proteins in GST pull-down assays, we showed that GAPDH harbours at least three independent binding sites for hTAFII68 (NTD) (Figure 5), suggesting that the association of GAPDH with hTAFII68 (NTD) can be mediated by three different domains of a single molecule or that the stoichiometry of the interaction is greater than one. Further experiments are required to distinguish these possibilities.

We showed that GAPDH can modulate hTAFII68-TEC-mediated transactivation (Figure 7). However, although GAPDH has intrinsic transactivation activity (Figure 8), the precise mechanism underlying this effect is unclear. One possibility is that GAPDH acts as a bridging factor linking an hTAFII68-TEC molecule to components of the transcription initiation machinery. Consistent with this speculation, GAPDH was recently identified as a cofactor of the Oct-1 coactivator complex, OCA-S (Oct-1 CoActivator in S-phase) [47], which is essential for S-phase-dependent histone H2B transcription; using stimulation of Oct-1 transcription in an in vitro assay, OCA-S was purified from a HeLa cell nuclear extract and found to contain seven proteins: nm23-H1, nm23-H2, uracil-DNA glycosylase, lactate dehydrogenase, Hsp70, Sti1 and GAPDH. The last also associates with PML in an interaction that is dependent on the presence of RNA [50], and the possible significance of this GAPDH–PML interaction was indicated by a recent study which revealed an association of PML nuclear bodies with transcriptionally active genomic regions [51].

In conclusion, the findings of the present study provide new evidence that hTAFII68-TEC function can be modulated by GAPDH. Unlike EWS-TEC which can transform CFK2 chondrogenic cells [67], the transforming activity of hTAFII68-TEC has yet to be assessed. Thus it would be of interest to determine whether GAPDH can collaborate with hTAFII68-TEC to transform cells into EMC tumours.

This research was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund: R14-2002-012-01002-0) of the KOSEF. S. K. and J. L. were recipients of Seoul Science Fellowships and of a research fellowship BK21 from the Ministry of Education and Human Resources Development.

REFERENCES

59 Geetha, T., Kenchappa, R. S., Wooten, M. W. and Carter, B. D. (2005) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. EMBO J. 24, 3859–3868

Received 25 August 2006/9 January 2007; accepted 15 February 2007
Published as BJ Immediate Publication 15 February 2007, doi:10.1042/BJ20061297