Published online November 10, 2005

Nucleic Acids Research, 2005, Vol. 33, No. 19 ***6393–6404***
doi:10.1093/nar/gki940

T:G mismatch-specific thymine-DNA glycosylase (TDG) as a coregulator of transcription interacts with SRC1 family members through a novel tyrosine repeat motif

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Received August 30, 2005; Revised and Accepted October 17, 2005

ABSTRACT

Gene activation involves protein complexes with diverse enzymatic activities, some of which are involved in chromatin modification. We have shown previously that the base excision repair enzyme thymine DNA glycosylase (TDG) acts as a potent coactivator for estrogen receptor-α. To further understand how TDG acts in this context, we studied its interaction with known coactivators of nuclear receptors. We find that TDG interacts *in vitro* and *in vivo* with the p160 coactivator SRC1, with the interaction being mediated by a previously undescribed motif encoding four equally spaced tyrosine residues in TDG, each tyrosine being separated by three amino acids. This is found to interact with two motifs in SRC1 also containing tyrosine residues separated by three amino acids. Site-directed mutagenesis shows that the tyrosines encoded in these motifs are critical for the interaction. The related p160 protein TIF2 does not interact with TDG and has the altered sequence, F-X-X-X-Y, at the equivalent positions relative to SRC1. Substitution of the phenylalanines to tyrosines is sufficient to bring about interaction of TIF2 with TDG. These findings highlight a new protein–protein interaction motif based on Y-X-X-X-Y and provide new insight into the interaction of diverse proteins in coactivator complexes.

INTRODUCTION

Estrogens play a critical role in reproductive physiology, are important in other diverse processes and have been implicated in breast and endometrial cancers, as well as cardiovascular disease, osteoporosis and in Alzheimer's disease (1–3). Estrogen actions are mediated by two ligand-dependent transcription factors, estrogen receptors α (ERα) and β (ERβ). These receptors belong to the nuclear receptor (NR) superfamily, which includes high affinity receptors for the steroid hormones, vitamin D3, thyroid hormone and retinoic acid. The so-called 'orphan receptors' include receptors that bind with low affinity to dietary lipids such as fatty acids, oxysterols, bile acids and xenobiotics, and a large number of receptors for which no ligand has been identified to date (4). NR share a common modular structure, with a core DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). Upon binding estrogen, ERα and β stimulate gene expression by binding as homo- or hetero-dimers to estrogen response elements (ERE) in promoters of estrogen-regulated genes (5). Two activation domains, AF1 and AF2, mediate transcription activation. AF1 activity is regulated by phosphorylation (2,6), AF2 is integral to the LBD and requires estrogen-binding for its activity. The LBD is comprised of conserved α-helical

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Simak Ali and Lakjaya Buluwela would like to dedicate this work to the memory of their colleague and friend Dr David Vigushin.

sequences (7). Agonist-binding induces conformational changes that orient the C-terminal AF2 helix, helix 12, to create a binding pocket to which coactivators of transcription can be recruited. Anti-estrogens are known to prevent coactivator binding to the ERα LBD, by reorienting helix 12, such that helix 12 lies over and blocks the binding pocket (8,9).

Several coactivator proteins have been implicated in estrogen action and include the distinct, but related, p160 proteins, SRC1/N-CoA1, TIF2/GRIP1 and AIB1/pCIP/ACTR/RAC3/NCoA-3 (10–12). These coactivators interact with the LBD of agonist-bound receptors through α-helical motifs, which include a sequence with the consensus LXXLL (13–15). These so-called NR boxes orient within the hydrophobic pocket containing helix 12, held by a charge clamp composed of conserved residues in helices 3, 4, 5 and 12 (9,16–19). The p160 coactivators recruit other proteins required for transcription activation, including CBP/p300 and the associated factor P/CAF, as well as CARM1 and PRMT1 (20–26). CBP/p300 and P/CAF possess intrinsic histone acetyltransferase activities. CARM1 and PRMT1 are methyltransferases that methylate arginine 17 of histone H3 and arginine 3 of histone H4, respectively (25,27). These modifications facilitate gene expression by transcription factors by chromatin remodeling and/or recruitment of additional factors.

Recent studies have shown that DNA repair-associated enzymes can stimulate transcription factor activity. The basal transcription factor TFIIH, required for nucleotide excision repair (NER), also regulates the activity of a number of transcription factors including retinoic acid receptors α and γ (28,29), ERα (30) and the androgen receptor (AR) (31). BRCA1, which has been implicated in double-strand break repair, represses ERα and stimulates AR activity (32–35), whilst the modified O^6-methylgluanine-DNA methyltransferase involved in the reversal of DNA alkylation damage can also repress ERα activity (36) and 3-methyladenine DNA glycosylase inhibited transactivation by ERα (37). The AP endonuclease Ref-1/APE1, required for the repair of abasic DNA damage, was found to activate c-jun and p53 (38–40). Thymine DNA glycosylase (TDG), which excises damaged cytosine and 5-methylcytosine bases opposite G (41), represses the activity of the homeodomain containing transcription factor TTF-1 (42), and potentiates the activities of retinoic acid receptor α and retinoid X receptor α (43,44).

More recently, we have shown that TDG associates with and stimulates the activity of ERα, acting as a transcriptional coactivator (43). The only known enzymatic activity of TDG is its DNA glycosylase activity and, in our previous work, we showed that coactivation by TDG does not require this activity. Rather, it is likely that TDG acts as a coactivator by interacting with other co-regulators of gene expression. To explore this possibility, we have investigated the interaction of TDG with coactivators of ERα. We show that TDG interacts with SRC1 and this interaction is mediated by a novel tyrosine-containing motif present in both proteins. Together, these studies identify a new protein–protein interaction motif, which features in the association of proteins in coactivator complexes.

MATERIALS AND METHODS

Plasmids and peptides

All expression plasmids and the reporter genes have been described (26,43,45). Additional constructs were derived from these and verified by sequencing. Details are available on request. The peptides were synthesized, high-performance liquid chromatography (HPLC) purified and verified by mass-spectrometric analysis by the ABC, Imperial College London.

Protein expression, purification and glutathione *S*-transferase (GST)-based interaction assay

In vitro transcription/translations were performed using TNT rabbit reticulocyte lysates (Promega, UK), in the presence of ^{35}S-labelled methionine. GST proteins were induced and lysates prepared as described previously (46). For pulldowns, GST fusion proteins were purified by affinity chromatography on glutathione-agarose beads and retained as a 50% slurry in 20 mM HEPES (pH 7.6), 100 mM KCl, 1 mM EDTA, 1 mM DTT and 20% glycerol, supplemented with protease inhibitors. A total of 100 μl volumes of glutathione-agarose bead slurry loaded with 10 μg of GST fusion proteins were then used directly in binding assays with 10 μl radiolabelled *in vitro* translation reactions and 890 μl of low-salt buffer [50 mM HEPES (pH 7.6), 250 mM NaCl, 0.5% NP-40, 5 mM EDTA, 0.1% BSA, 0.5 mM DTT, 0.005% SDS and protease inhibitors]. Following 1 h incubation at room temperature, the beads were washed twice with low-salt buffer and twice with high-salt buffer (low-salt buffer containing 1 M NaCl). Samples were boiled for 10 min in 80 μl of Laemmli buffer and fractionated by SDS–PAGE. Gels were dried and autoradiographed.

Reporter gene assays

COS-1 cells were maintained in DMEM, supplemented with 5% fetal calf serum (FCS). Cells were seeded onto 9 cm dishes and transiently transfected with 4 μg of β-galactosidase and chloramphenicol acetyl transferase (CAT) reporter genes and 0.5 μg of expression plasmids, using the calcium phosphate co-precipitation technique. Cells were harvested after a further 24 h and reporter gene activities were assayed as described (47). For experiments involving ERα, the cells were seeded in DMEM lacking phenol red and containing 5% dextran-coated charcoal-stripped FCS. E2 (10 nM) was added as appropriate.

Immunoprecipitations and immunoblotting

COS-1 cells were plated in 9 cm dishes in DMEM supplemented with 5% FCS 16 to 24 h before transfection and, transfected with 4 μg of HA-TDG and/or HA-SRC1 expression plasmids using Lipofectamine 2000 (Invitrogen, UK). Following transfection (48 h), the cells were lysed in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris–HCl (pH7.5)] containing protease inhibitors. Lysates (2 mg) were immunoprecipitated using an anti-TDG mouse monoclonal antibody, which has been described previously (48), or using a rabbit polyclonal SRC1 antiserum [a gift of Dr H. Hurst, Barts and the

London School of Medicine and Dentistry (49)]. Control immunoprecipitations were carried out using rabbit IgG (Sigma-Aldrich, UK). Immunoprecipitates were resolved by SDS–PAGE and immunoblotted using horseradish-peroxidase (HRP)-labelled HA antibody (Sigma).

Chromatin Immunoprecipitation (ChIP)

Estrogen (10 nM) was added to MCF7 cells maintained in phenol red-free DMEM supplemented with 5% dextran-coated charcoal stripped FCS, for 30 min before cell fixation and harvesting. ChIP and re-ChIP assays were performed essentially as described by Shang *et al*. (50), using the TDG mouse monoclonal antibody and SRC1 rabbit polyclonal antisera described above, or using the ERα mouse monoclonal B10 (51). Control ChIP/re-ChIP were carried out using rabbit IgG.

For ChIP/re-ChIP experiments an initial ChIP immunoprecipitate was generated. This and the corresponding supernatant were each divided into three equal amounts. For re-ChIP, each of these was subjected to ChIP using antibodies against two other proteins. The final re-ChIP products were analysed by PCR against the ERE encoding region of the pS2 gene promoter using primers with the sequences 5′-GCCATCTCTCACT-ATGAATCACTTCTGC-3′ and 5′-GGCAGGCTCTGTTTG-CTTAAAGAGCG-3′, as described (50).

Yeast 2-hybrid analysis of TDG: SRC1 interaction

The *Saccharomyces cerevisiae* reporter L40a strain (MATa, trp1, his3, leu2, ade2, LYS::(LexAop)4-HIS3, URA3::(LexAop)8-LacZ), expressing LexA-SRC1(989–1240) (45), was transformed with VP16-fused mutants of mouse TDG as described previously (44). β-galactosidase activities were determined as described (45).

RESULTS

TDG interacts with SRC1 *in vivo*

We have shown previously that TDG acts as an ERα coactivator (43). Previous studies have also demonstrated TDG association with CBP/p300 (52). In confirming the latter findings we also investigated whether TDG interacts with other nuclear receptor coactivators. In order to test this possibility, COS-1 cells were co-transfected with HA-tagged TDG and HA-SRC1a. Immunoprecipitation of whole cell lysates with antibodies to TDG resulted in the co-precipitation of SRC1a, indicating that TDG interacts with SRC1a *in vivo* (Figure 1A). Immunoprecipitation using antibodies to SRC1 co-precipitated TDG, whilst immunoprecipitation with rabbit IgG did not (Figure 1B).

Interaction between endogenous ERα, TDG and SRC1 was further investigated using ChIP by performing serial immunoprecipitations in which soluble chromatin prepared from E2-treated MCF7 cells was divided in three. One aliquot was immunoprecipitated with TDG antibodies, followed by release of the immune complexes, which were further divided in three and re-immunoprecipitated with ERα and SRC1 antibodies, whilst DNA was prepared from the third portion. The second aliquot was immunoprecipitated with SRC1 antibodies, followed by re-immunoprecipitation (re-IP) with ERα and TDG antibodies. Re-IP were similarly performed using the supernatants from the primary immunoprecipitations, again following division into two aliquots. TDG and SRC1 antibodies immunoprecipitated the pS2 promoter following E2-treatment, as described previously (43) (Figure 1B). However, re-IP using supernatants did not immunoprecipitate the pS2 promoter, whereas re-IP of the eluted primary immunoprecipitates from the TDG IP using ERα or SRC1 antibodies did immunoprecipitate the pS2 promoter. Similarly, performing re-IP following primary immunoprecipitation for SRC1

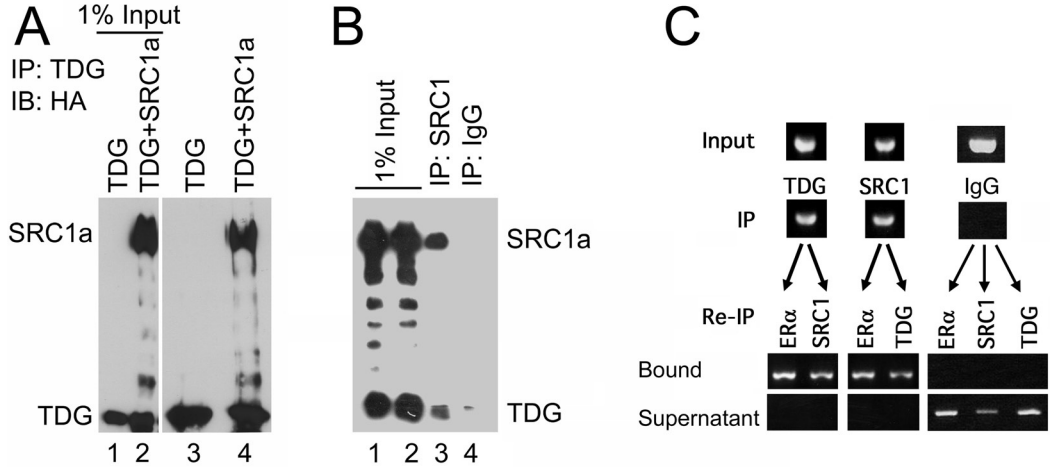

Figure 1. SRC1 and TDG interact *in vivo*. (**A** and **B**) Lysates prepared from COS-1 cells transfected with HA-tagged TDG and/or HA-SRC1a were immunoprecipitated with anti-TDG (A), anti-SRC1 (B) or rabbit IgG (B). Immunoprecipitates were resolved by SDS–PAGE and immunoblotted using HA antibodies. (**C**) MCF-7 cells were stimulated with 100 nM E2 for 30 min before fixation, harvesting and aliquoting in three. One aliquot was immunoprecipitated with antibodies to TDG (IP). The other two aliquots were immunoprecipitated with antibodies against TDG, release of immune complexes and re-IP with antibodies against ERα and SRC1 (Re-IP Bound). The supernatants from the primary IPs were also re-immunoprecipitated (Re-IP Supernatant). A similar procedure was followed whereby primary IP performed with antibodies to SRC1 were re-immunoprecipitated, using antibodies against ERα and TDG. Primary IPs using mouse immunoglobulins, followed by re-IP with antibodies to ERα, TDG or SRC1, using supernatants or pellets was carried out as a control. The bound immunoprecipitated DNA was amplified by PCR (25 cycles) using primers for the pS2 gene directed against a region of the gene encompassing the ERE.

demonstrated that ERα and TDG were co-immunoprecipitated with SRC1. Control ChIP/re-ChIP were also performed, using isotype-matched mouse immunoglobulins for the first IP. On re-IP, pS2 was immunoprecipitated from the supernatants and not from the eluted primary immunoprecipitates. This shows that TDG and SRC1 are recruited concomitantly by ERα-binding to the promoters of estrogen-responsive genes, and therefore indicate that TDG and SRC1 associate *in vivo*, or at least that they are present in the same complex at promoters of estrogen-responsive genes.

Interaction of TDG with SRC1 is mediated by a novel tyrosine repeat-containing protein–protein interaction motif

We next attempted to determine whether SRC1 and TDG interact directly *in vitro*. GST-binding assays using non-overlapping regions of SRC1 fused to GST (Figure 2A) showed that TDG interacts with a region of SRC1 encoded within amino acids 989–1240 of SRC1 (Figure 2B). Absence of TDG-binding to GST-SRC1(1107–1399) suggested that amino acids 1107–1240 are critical for SRC1 interaction with TDG, although amino acids 989–1106 also appear to be important, since TDG-binding to GST-SRC1(349–1106) was lower than that observed with GST-SRC1(989–1240) (Figure 2C). Further, in a mammalian 2-hybrid assay using the Gal4 DNA-binding domain fused to overlapping regions of SRC1, significant interaction of VP16 fused to TDG was only observed with amino acids 989–1240 (Figure 2D), in agreement with the results of the *in vitro* GST-binding assays.

The sequences in TDG required for interaction with SRC1 were also determined with a yeast 2-hybrid assay, using a LexA fusion with SRC1 amino acids 989–1240, for interaction with VP16 fused to overlapping portions of mouse TDG (Figure 3A). These deletions showed that amino acids 32–307 of mouse TDG are insufficient for interaction with SRC1, whereas TDG(32–346) was able to interact, indicating

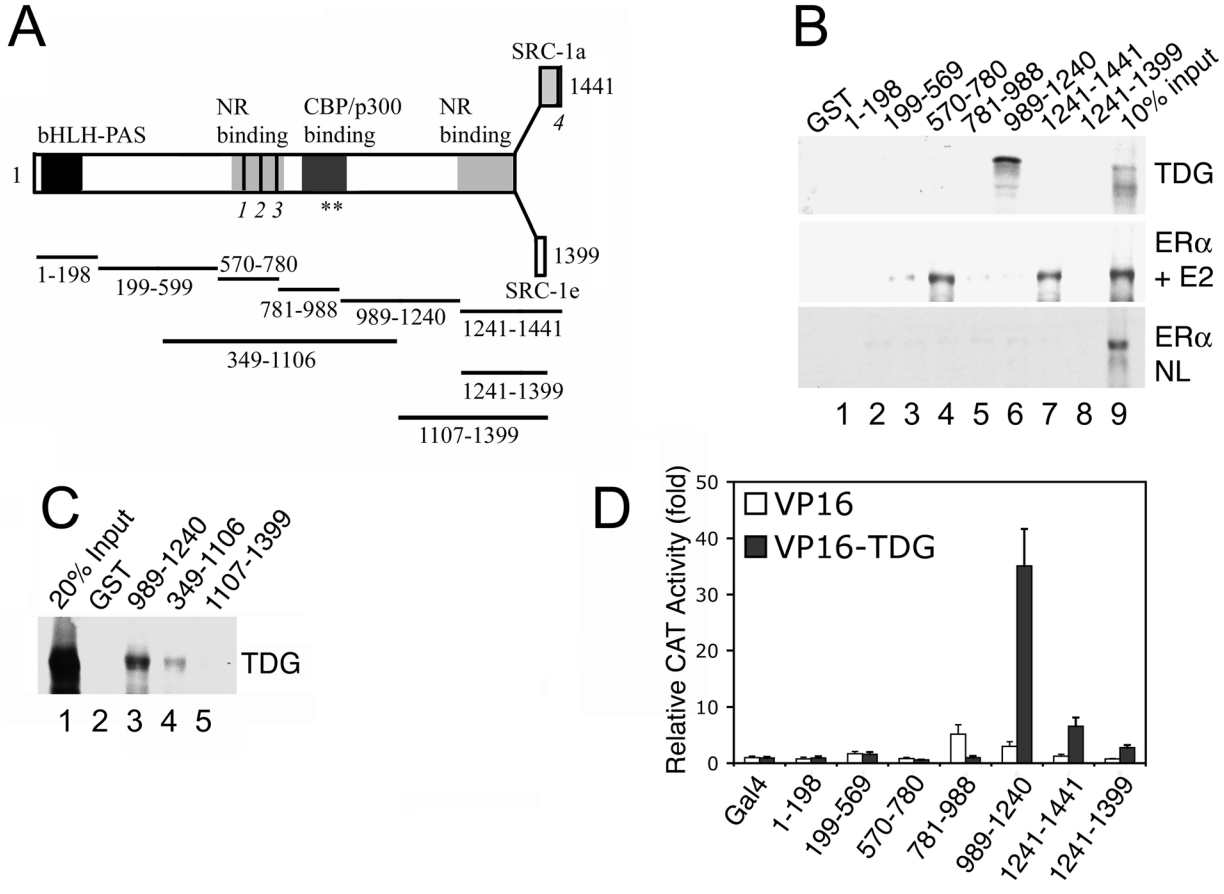

Figure 2. Mapping the region of SRC1 required for interaction with TDG. (**A**) Schematic representation of human SRC1 isoforms and the regions that were fused to GST and to the Gal4 DBD. Numbers refer to amino acids. Indicated are the bHLH-PAS homology region, the binding regions for NR and the functional LXXLL motifs (numbered 1–4). The fusion proteins (1241–1444) and (1241–1399) contain amino acid sequences unique to the SRC1a and SRC1e proteins, respectively (26). (**B**) GST-binding assays were carried out by incubation of ^{35}S-labelled TDG or ERα with GST (lane 1) or GST–SRC1 fusion proteins encoding amino acids 1–198 (lane 2), 199–569 (lane 3), 570–780 (lane 4), 781–988 (lane 5), 989–1240 (lane 6) or 1241–1441 (lane 7) of SRC1a, or with amino acids 1241–1399 of SRC1e (Lane 8), as depicted in (A). In the case of ERα, binding assays were carried out in the absence of ligand or with E2 (100 nM). The input lanes represent 10% of the total volume of the *in vitro* translation reaction used in the binding assay. (**C**) GST-binding assays were carried out as above by incubation of ^{35}S-labelled TDG with GST (lane 2) or GST–SRC1 fusions encoding amino acids 989–1240 (lane 3), 349–1106 (lane 4) or 1107–1399 (lane 5) of SRC1e, as depicted in (A). The input lanes represent 20% of the total volume of the *in vitro* translation reaction used in the binding assay. (**D**) Regions of SRC1 fused to the DBD of the yeast transcription factor Gal4 (amino acids 1-147) were tested for their ability to simulate a Gal4 reporter in COS-1 cells, following cotransfection with VP16 or VP16 fused to TDG. Fold activity indicates the activity of the Gal4 fusions over the empty Gal4 plasmid contransfected with the VP16 plasmid.

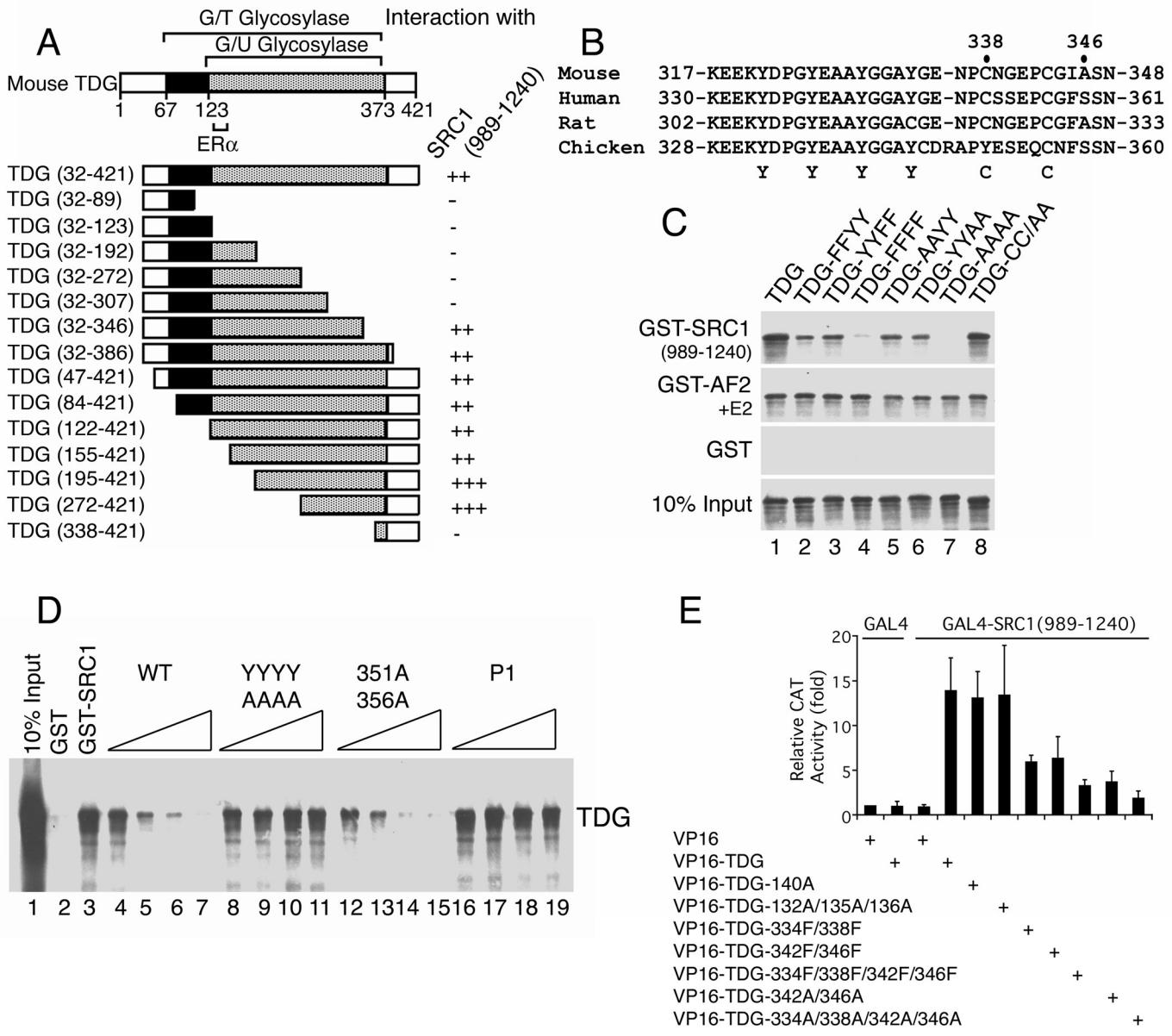

Figure 3. Association of TDG with SRC1 is mediated by a tyrosine repeat-containing motif in TDG. (**A**) Fusions of VP16 with regions of mouse TDG were expressed in yeast, together with LexA-SRC1 (989–1240) and the activity of an integrated LexA-regulated β-galactosidase gene was determined. Activities are shown relative to the activity observed with mouse TDG (32–421). Also indicated are the regions in TDG required for the DNA glycosylase activity and the region required for TDG interaction with ERα. (**B**) Amino acid sequence of mouse TDG amino acids 317–348 is shown, aligned to TDG from other species. The numbers above the sequences refer to mouse TDG. Alignments were generated using dbClustal (81). (**C**) Binding assays were done following incubation of ^{35}S-labelled human TDG or TDG mutants with GST, GST-SRC1(989–1240) or with GST fusion with the ERα LBD/AF2 (GST-AF2). E2 (100 nM) was present in the case of GST-AF2. The input lanes represent 10% of the total volume of the *in vitro* translation reaction used in the binding assay. (**D**) Binding assays were done in the presence of 1 μM (lanes 4, 8, 12, 16), 5 μM (lanes 5, 9, 13, 17), 10 μM (lanes 6, 10, 14, 18) or 20 μM (lanes 7, 11, 15, 19) of peptides corresponding to amino acids 330–346 of human TDG. A peptide corresponding to wild-type TDG, or containing the substitutions shown, were used in the competitions. P1, a peptide corresponding to amino acids 115–146 of human TDG served as a further control. (**E**) COS-1 cells transfected with Gal4-SRC1(989–1240) and VP16-TDG or mutants, were tested for their ability to stimulate a Gal4 reporter in COS-1 cells. Fold activity refers to activity over the empty Gal4 plasmid co-transfected with the VP16 plasmid.

that amino acids 307–346 are critical for the interaction. Deletions lacking N-terminal sequences showed that residues 272–421 were sufficient, whereas amino acids 338–421 did not interact. Together, these data are indicative of a SRC1-interacting region in residues 307–338 of mouse TDG.

Examination of the corresponding amino acid sequence revealed that this region of low amino acid complexity is distinguished by the presence of four equally spaced tyrosine residues (334-YDPGYEAAYGGAY-346 in human TDG), which is highly conserved in vertebrate TDG proteins (Figure 3B). Note, however, that this region maps C-terminal to the DNA glycosylase domain and does not appear to be required for the enzymatic activity of TDG (48,53). We generated TDG mutants in which two or all four tyrosine residues in this region were substituted by phenylalanine or by alanine. Binding of human TDG to GST-SRC1(989–1240)

was reduced significantly if the first two or the third and fourth tyrosines were substituted by phenylalanine or alanine residues. Substitution of all four tyrosine residues prevented interaction with SRC1 (Figure 3C). Two cysteine residues in mammalian TDG follow the equally spaced tyrosine residues, whilst the N-terminal-most of these cysteine residues is replaced by a tyrosine in chicken TDG, which might mean that the cysteine residues extend the SRC1-interacting region of TDG. However, substitution of the cysteine residues by alanines had no effect on the interaction of human TDG with SRC1. Interaction of TDG with the LBD/AF2 of ERα is mediated by a sequence motif around amino acids 132–136 of TDG (43). Mutation of the tyrosine residues in TDG did not inhibit the interaction of ERα AF2 with TDG, suggesting that these substitutions do not grossly affect the structure of TDG. This possibility was further explored using peptides corresponding to the tyrosine motif region. A peptide corresponding to human TDG amino acids 330–364 competed for TDG-binding to SRC1 (Figure 3D), whereas substitution of the tyrosine residues prevented competition. A peptide in which the conserved cysteine residues were substituted by alanines (351A/356A) blocked interaction of SRC1 and TDG. Finally, peptide (P1), corresponding to amino acids 115–146 of human TDG, which contain the motif required for TDG association with ERα, also failed to compete.

The interaction between TDG and SRC1 was confirmed using the mammalian 2-hybrid assay (Figure 3E). Substitution of the first two or the third and fourth tyrosine residues by phenylalanine significantly reduced the interaction, and substitution of all four tyrosine residues completely prevented the interaction of TDG with SRC1. Substitutions in the ERα-interacting motif (VP16-TDG-132A/135A/136A) did not prevent the interaction. Asparagine 140 is absolutely required for the DNA glycosylase activity of TDG (54). Substitution of this residue by alanine also did not inhibit the interaction of TDG and SRC1. Collectively, these data indicate that the interaction of TDG with SRC1 is mediated by a novel tyrosine-containing interaction motif located within amino acids 334–346 of human TDG.

Interaction of TDG with SRC1 is mediated by a novel tyrosine motif in TDG and a similar motif in SRC1

Intriguingly, examination of the sequence of SRC1 encoded within amino acids 989–1240 highlighted the presence of two Y-X-X-X-Y motifs separated by about 170 amino acids in SRC1 (Figure 4A). The region around these tyrosine-containing motifs is conserved in the two related genes, AIB1 and TIF2. The Y-X-X-X-Y motifs are also conserved in AIB1, but are altered in TIF2, where the first tyrosine (Y1 and Y3) in each of these motifs is substituted by phenylalanine in TIF2 (Figure 4A). In agreement with the potential importance of these tyrosine residues for interaction with TDG, SRC1 and AIB1 interacted with GST-TDG in GST-binding assays, whereas TIF2 did not interact (Figure 4B).

In order to determine whether these tyrosine residues are required for interaction of SRC1 with TDG, Y1, Y3 or Y1/Y3 were mutated to phenylalanines, to reflect the difference observed in TIF2 compared with AIB1 and SRC1. We investigated whether these tyrosine residues are involved in mediating the interaction of SRC1 with the tyrosine-containing motif in TDG. Substitution of Y1, Y3 or Y1/Y3 by phenylalanine significantly reduced interaction of GST–SRC1(989–1240) with TDG (Figure 4C). Substitution of Y1 and Y3 also prevented interaction of SRC1 with TDG in a yeast 2-hybrid assay (Figure 4D). Interestingly, in the yeast 2-hybrid assay Y3 appeared to be more important for the interaction with TDG than Y1. In agreement with these results, SRC1e bound GST-TDG, but substitution of Y1 by phenylalanine reduced binding, whereas substitution of Y3 or Y1/Y3 prevented the interaction of SRC1 with TDG (Figure 4E), suggesting that the C-terminal Y-X-X-X-Y motif may be more important for interaction with TDG than the N-terminal motif.

The importance of the Y-X-X-X-Y motif for interaction of p160 coactivators with TDG was confirmed by substitution of the phenylalanine residues at positions equivalent to Y1 and Y3 in SRC1 and AIB1 by tyrosine in TIF2. Substitution of the phenylalanine at position 1, 3 or 1/3 allowed TIF2 interaction with TDG (Figure 4E). As observed for SRC1, the C-terminal motif appeared to be more important for interaction with TDG than the N-terminal motif. Together, these data indicate that the interaction of TDG with members of the p160 family of nuclear receptor coactivators is mediated by a motif containing tyrosine residues separated by three amino acids in TDG with a motif in SRC1 also containing tyrosine residues spaced by three amino acids.

Cooperativity between SRC1 and TDG is mediated by the tyrosine repeat motif

SRC1 and TDG are coactivators for ERα. In order to determine whether the novel tyrosine-containing motifs that mediate SRC1/TDG interaction have functional significance *in vivo*, TDG and SRC1 were cotransfected into COS-1 cells, together with ERα and an estrogen-responsive CAT reporter gene. As has been described previously, both TDG and SRC1 stimulated ERα activity in the presence of estrogen (E2) (Figure 5A). Interestingly, co-transfection of TDG with SRC1 significantly stimulated ERα in the absence of ligand (Figure 5A), suggesting that TDG and SRC1 can cooperate. The coactivation by TDG and SRC1, individually or together, required the integrity of helix 12 in the ERα LBD, since substitution of the key leucine residues in helix 12, at amino acid positions 539 and 540 prevented transcriptional stimulation by TDG and SRC1. In the absence of ligand, there was little stimulation of ERα activity by the other two members of the p160 family. However, co-transfection of AIB1 with TDG resulted in stimulation of ERα activity, whereas cotransfection of TIF2 with TDG did not (Figure 5B).

The importance of the tyrosine motif in the cooperativity between SRC1 and TDG was demonstrated by the fact that substitution of Y1 and Y3 of SRC1 prevented stimulation of ERα activity by TDG and SRC1 (Figure 5C). Similarly, a TDG mutant where all of the tyrosine residues were replaced by alanines did not cooperate with SRC1. Finally, whilst TIF2/TDG did not stimulate ERα activity, TIF2[YY...YY] was able to cooperate with TDG to stimulate ERα activity (Figure 5D). Taken together, these data provide *in vivo* evidence for the interaction between TDG and p160 coactivators being mediated by a novel protein–protein interaction motif involving tyrosine residues separated by three amino acids in one protein, with a similar motif in a second protein.

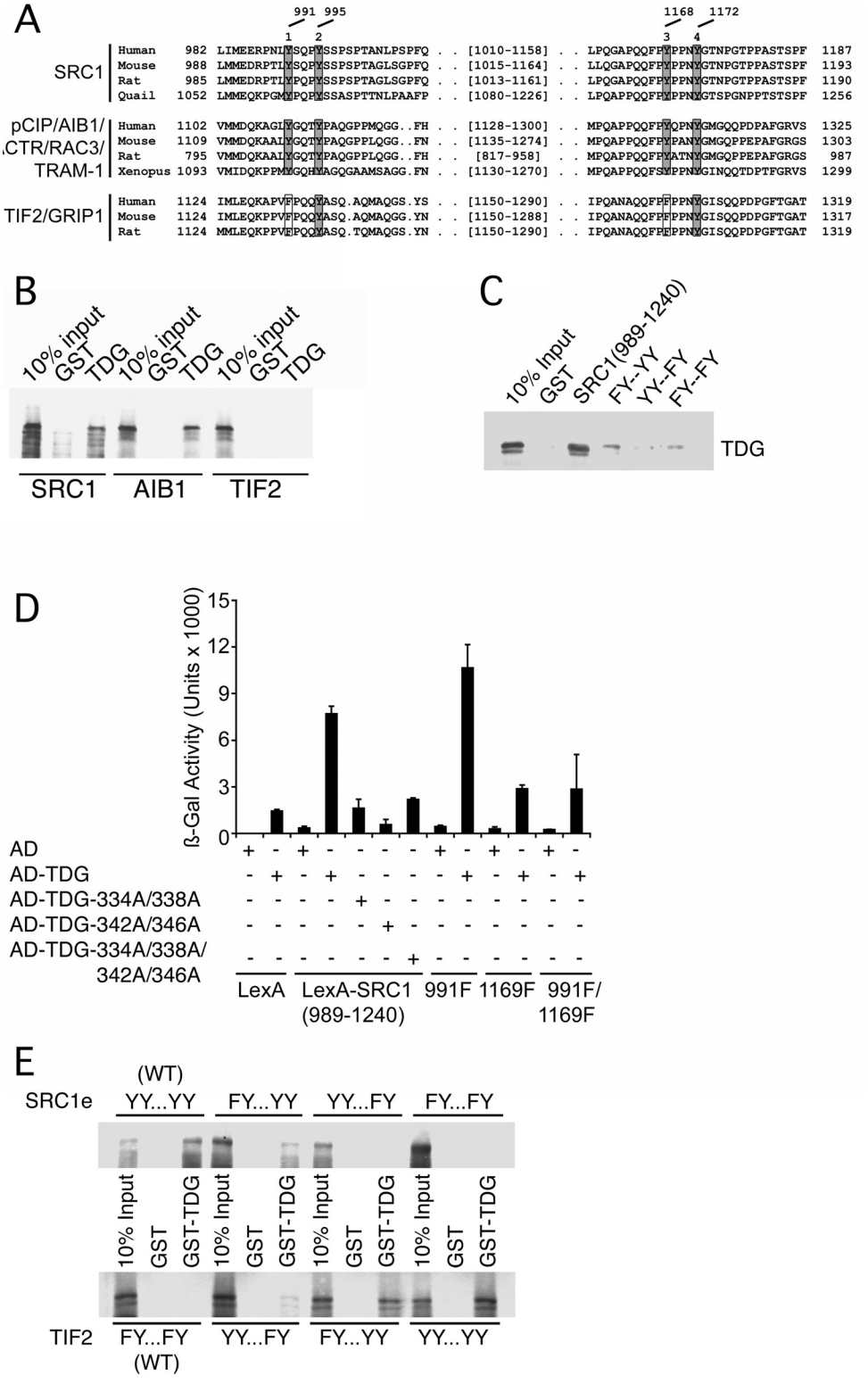

Figure 4. Y-X-X-X-Y motifs in SRC1 mediate its interaction with TDG. (**A**) SRC1, AIB1 and TIF2 were aligned using dbClustal (81). The various other names of these coactivators are listed. The conserved tyrosine residues are highlighted and are numbered 1 to 4. The amino acid numbering above the sequences refers to human SRC1. (**B**) GST-binding assays were done with ^{35}S-labelled SRC1e, AIB1 or TIF2, using GST or GST-TDG. The input lanes represent 10% of the total volume of the *in vitro* translation reaction used in the binding assay. (**C**) TDG-binding assays were done using GST-SRC1(989–1240) or mutants in which tyrosine 1, 3 or 1 and 3 were substituted by phenylalanine (F). The input lanes represent 10% of the total volume of the *in vitro* translation reaction used in the binding assay. (**D**) TDG fusions with the Gal4 activation domain (Gal AD-TDG), were expressed in yeast, together with LexA-SRC1(989–1240) and the activity of an integrated LexA-regulated β-galactosidase gene was determined. Activities are displayed as β-galactosidase units. The activities obtained for three independent yeast colonies are shown. (**E**) GST pulldown assays were performed using SRC1e and SRC1e in which tyrosine residues 1, 3 or 1/3 were substituted by phenylalanine. Binding assays were also performed using TIF2 or TIF2 in which phenylalanine residues were substituted by tyrosines.

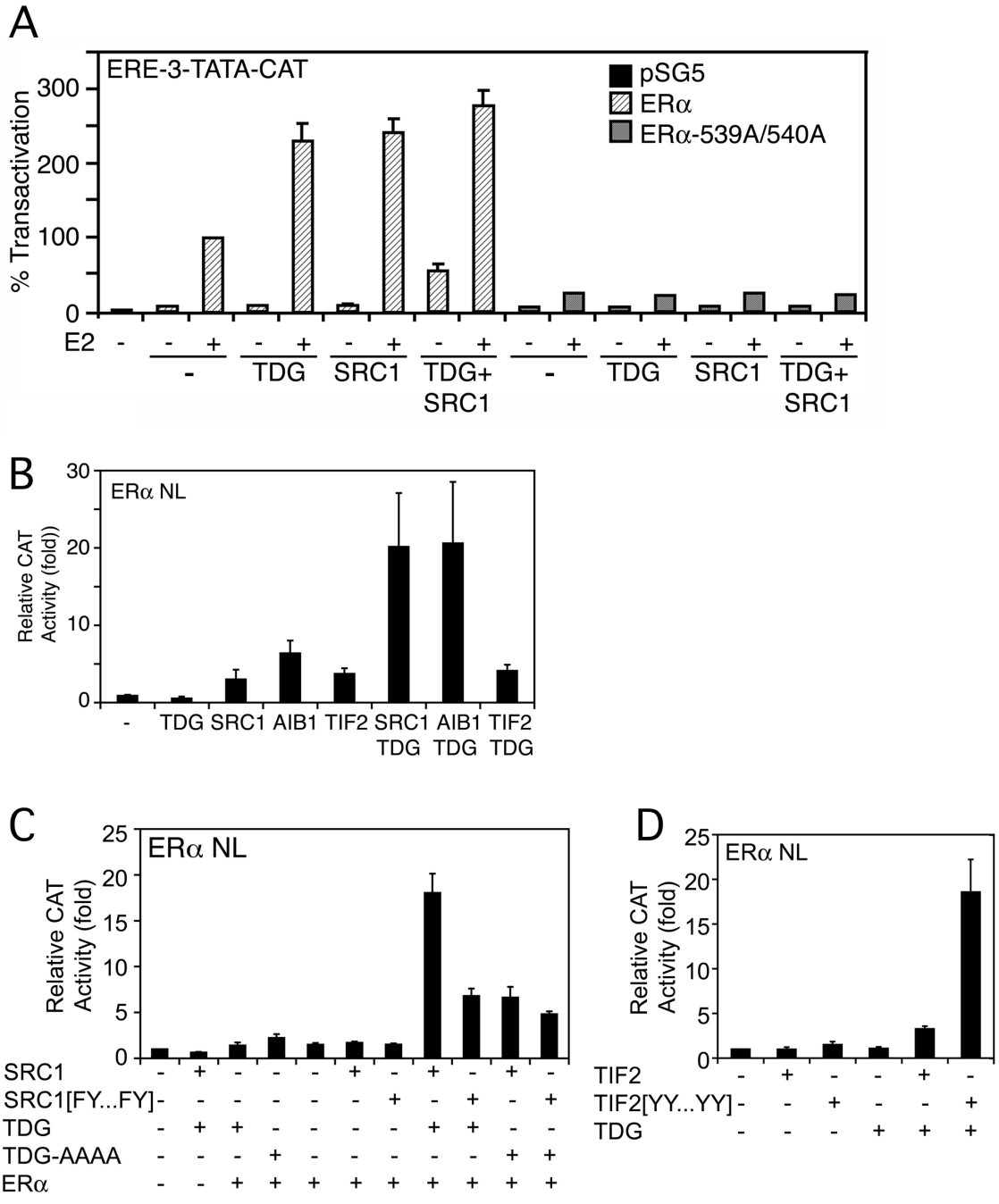

Figure 5. TDG cooperates with SRC1 to stimulate ERα activity in the absence of ligand. (**A**) COS-1 cells were transfected with an estrogen-regulated reporter gene and the expression plasmid pSG5, ERα or ERα with substitution of leucine residues at amino acids 539/540. TDG and/or SRC1a were co-transfected, as appropriate. The results of three independent experiments are shown. Transcription activation by ERα in the presence of E2 was taken as 100%. All other activities are shown relative to this. (**B**) COS-1 cells grown in DMEM, lacking phenol red and supplemented with 5% dextran-coated charcoal-stripped FCS, were transfected with a ERE-3-TATA-CAT, together with ERα. TDG, SRC1, AIB1 and TIF2 were transfected as shown. The results of three independent experiments are shown. Activities are shown relative to the activity observed for ERα alone. (**C** and **D**) COS-1 cells grown in estrogen depletion conditions were transfected with ERE-3-TATA-CAT, together with ERα. TDG or TDG-AAAA was co-transfected with SRC1, SRC1 mutated in each of the tyrosine motifs, TIF2 or TIF2 in which phenylalanines were replaced by tyrosines. The results of three independent experiments are shown. Fold activation is shown relative to the activity observed for ERα alone (−).

DISCUSSION

It is now clear that nuclear receptors interact with proteins involved in DNA repair, such interactions leading to transcriptional repression or activation. In the case of transcription activation, interaction with TFIIH suggests that phosphorylation of ERα can be an important event in this activation and leads to increased responsiveness to low levels of estrogen, thereby potentiating the estrogen response by ERα (30). Recently, we have shown that TDG interacts with ERα to stimulate its activity (43). We have now demonstrated that TDG also interacts with the NR coactivators SRC1

and AIB1 to stimulate their recruitment by ERα. To our knowledge, this is the first demonstration of a direct interaction between SRC1 and AIB1 with a DNA damage recognition and repair protein, and is suggestive of an as yet unknown functional relationship between certain transcriptional activation and DNA bases excision repair processes.

Interaction between TDG and SRC1 is mediated by a novel tyrosine containing motif

Co-immunoprecipitation and ChIP assays were used to show that TDG interacts with SRC1 *in vivo*. Deletion mapping in yeast demonstrated that the interaction between SRC1 and TDG required a highly conserved region of TDG that was distinguished by the presence of four equally spaced tyrosine residues. Substitution of the tyrosines prevented association with SRC1 *in vitro* and *in vivo*, whereas substitution of two highly conserved cysteines in the same region did not prevent SRC1-binding. Although this region is highly conserved in TDG from different vertebrate species it is not present in other DNA glycosylases. Moreover, this region is not required for DNA glycosylase activity, although the lysine at position 330, just N-terminal to the tyrosine repeat, is a target for SUMO modification, which modulates TDG activity by reducing its DNA substrate and abasic binding site affinity (48). Substitution of these tyrosine residues in TDG inhibited interaction with SRC1 *in vivo* and *in vitro* and prevented cooperative stimulation of ERα activity by SRC1 and TDG.

Amino acids 989–1240 in SRC1 mediated its interaction with TDG. Alignment of this region with AIB1 and TIF2 revealed the presence in human SRC1 of two regions with the sequence 991-Y-S-Q-P-Y-995 and 1168-Y-P-P-N-Y-1172. There were no other Y-X-X-X-Y or F-X-X-X-Y motifs in the human SRC1 amino acid sequence. Alignment of SRC1 with AIB1 and TIF2 showed that the two motifs were strictly conserved between SRC1 and AIB1 from different species. In TIF2, however, these motifs had the sequences 1133-F-P-Q-Q-Y-1137 and 1300-F-P-P-N-Y-1304, both having a phenylalanine in place of the tyrosines present in SRC1 and AIB1. Substitutions in each of the two Y-X-X-X-Y motifs in SRC1 inhibited *in vitro* and *in vivo* interaction with TDG. Moreover, simply by replacing the phenylalanines by tyrosines it was possible to obtain interaction of TIF2 with TDG and to allow ERα activation in the absence of ligand. Of the two Y-X-X-X-Y motifs in SRC1 the C-terminal one appeared to be more important. This was confirmed in TIF2, where substitution of phenylalanine at position 3 by tyrosine enabled more efficient binding to TDG than replacement of the phenylalanine at position 1. These findings indicate that a single Y-X-X-X-Y motif may be sufficient for interaction with a second such motif in a different protein. Alternatively, multiple motifs may be required but if so then their spacing appears to demonstrate considerable flexibility, as in the case of SRC1 and AIB1, where in contrast to TDG, the two Y-X-X-X-Y motifs are separated by ~170 amino acids. Certainly, the data presented here indicates that both motifs in SRC1 are required for maximal interaction with TDG. Few clues are provided as to the additional sequence requirements in these motifs by examination of the TDG and p160 sequences due to the high sequence conservation of these proteins across species. Careful mutagenesis around this region should help to define sequence requirements more precisely. Nevertheless, our data clearly demonstrate the importance of the tyrosine residues for interaction between TDG and SRC1 and define a protein–protein interaction motif involving tyrosine residues separated by three amino acids that has not been described previously.

The TDG tyrosine repeat motif is present in histone acetyl transferases

Searching protein databases with Y-X-X-X-Y or Y-X-X-X-Y-X-X-X-Y was not possible due to the large number of possible hits. However, a search of the SwissProt database for matches with the sequence Y-X-X-X-Y-X-X-X-Y-X-X-X-Y as found in TDG identified 174 matches when searching all species, with 17 of these matches being human proteins (Swiss Institute of Bioinformatics myhits search for patterns in proteins, http://myhits.isb-sib.ch/cgi-bin/pattern_search) and including TDG. Many of the genes identified on this basis encode transcription regulatory proteins. In particular, one of the identified proteins is human GCN5, the prototype histone acetyltransferase for the superfamily of acetyltransferses known as GCN5-related *N*-acetyltransfereases (GNATs) (55,56). Previous alignment of the GCN5 HAT domains shows that the tyrosine repeat motif and a second similar motif are present in the related HAT domain of P/CAF, but not in other *N*-acetyltransferases (57–60). Structural studies of the GCN5 and P/CAF HAT domains have shown that the regions encoding the tyrosine containing motifs are solvent accessible and likely to be involved in histone substrate recognition, with one of the motif tyrosines also contacting acetyl coenzyme A cofactor. Based on our findings, it is interesting to speculate that these regions may be involved in protein–protein interactions with non-histone proteins, to facilitate their acetylation and/or for regulation of the HAT activity. Additionally, yeast Gcn5 and human P/CAF are present in coactivator complexes required for ERα activity in yeast and in human cells, respectively (61,62); thus interaction with TDG could be involved in regulating the recruitment of Gcn5 and P/CAF-containing coactivator complexes by nuclear receptors, in a manner similar to that which we have described here.

DNA repair is likely to be affected by chromatin structure and to be regulated by chromatin remodeling complexes. Certainly, hyperacetylated nucleosomes facilitate enhanced recognition of DNA lesions and their repair (63). Histone H3 acetylation levels increase following UV-irradiation in mammalian cells and the HAT activity of the mammalian GCN5-containing TFTC complex is greater on nucleosomes containing UV-irradiated DNA (64). Given that the TDG tyrosine repeat motif is found in very few human proteins and the possible requirement of chromatin remodeling for DNA repair by TDG, it would be interesting to determine whether with GCN5 or P/CAF might be involved in facilitating DNA repair by TDG through interactions mediated by the tyrosine repeat motif. Certainly, the GCN5-containing STAGA complex can associate with the UV-damage-DNA-binding protein DDB1 (65). Moreover, TDG associates with CBP/p300 and is a substrate for acetylation by CBP, its acetylation regulating recruitment of the APE1 endonuclease (52). It is therefore, not

unreasonable to suppose that GCN5 also acetylates TDG and thereby regulates DNA repair by TDG.

The significance of TDG interaction with transcription factors and transcriptional coactivators

In vertebrate genomes the dinucleotide CpG is often found to specify modifications leading to 5′ methylation of the cytosine. This is an important feature of gene regulation, but can be corrupted by spontaneous deamination to thymine and this deamination may contribute to as much as 30% of all germline mutations, despite comprising only about 1% of the genome (66–68). In this context TDG is responsible for initiating correction of the T:G mismatches that arise at sites of 5-methylcytosine deamination, but also for the repair of U:G mismatches resulting from deamination of cytosines. Our studies have highlighted the importance of another aspect of TDG activity, namely interaction with transcription factors such as ERα, leading to the recruitment of coactivators and the subsequent activation of transcription. Such interactions may be a general feature of transcriptional regulation and raise the possibility that TDG would localize to gene promoters. This in turn would allow TDG to interrogate and maintain local DNA sequence integrity, including the CpG islands found in the promoters of many genes. Such a function would have great significance, as CpG islands are associated with about one-half of all mammalian genes (69) and may be a major feature in maintaining the stability and functionality of mammalian genomes. Transcription factors such as ERα could play an important role in DNA repair by 'sensing' DNA damage at transcriptionally important regions of the genome through their ability to recruit DNA repair enzymes such as TDG. Conversely, the involvement of TDG as a transcriptional coactivator may form part of a signal for DNA integrity at gene promoters.

It is now well recognized that DNA methylation is an important feature of vertebrate gene regulation, where methylation of CpG dinucleotide sequences in and around gene promoters is often associated with gene silencing and is a key epigenetic regulator of gene expression (70,71). However, it is also clear that reversal of this methylation state is necessary for gene activation to occur. In this situation, the exact nature of the demethylation mechanism is unclear, although it has been suggested that such demethylation could occur through the activity of base excision and repair mechanisms. In this respect, it has been shown previously that a DNA glycosylase, named 5-methylcytosine DNA glycosylase (5-MCDG) copurifies with a DNA demethylation activity in chicken embryos and liver, mouse myoblasts and in HeLa cells (72–75). Further, it is clear that 5-MCDG is TDG and that, in an experimental model of myoblast differentiation featuring genome-wide demethylation, antisense-RNA to TDG/5-MCDG inhibits the demethylation, whilst its overexpression correlates with demethylation of a reporter gene in a human embryonic kidney cell line (76,77). Further, ERα has been shown to promote the demethylase activity of 5-MCDG *in vitro* (78) and longstanding observations show that estrogen treatment results in the demethylation of chicken vitellogenin genes in the liver (79,80). Therefore, TDG could act to promote and/or help to maintain the demethylated status of CpG dinucleotides in promoters of estrogen-responsive genes through a process requiring chromatin remodeling/modification, by facilitating the recruitment of transcriptional coactivator complexes through direct interaction with p160 coactivators SRC1 and AIB1, as well as the reported interaction with CBP/p300 (52). Clearly, a role for complexes containing TDG and coactivators in the DNA demethylation and chromatin remodeling associated with gene activation requires further investigation.

ACKNOWLEDGEMENTS

We are grateful to Drs C. Bevan, B. Katzenellenbogen, P. Meltzer and M. Stallcup for generous gifts of plasmids. Our thanks also go to Dr P. Freemont for his help and advice in analyzing the significance of the tyrosine repeat motif. We thank R. Striesow and I. Moss of the Advanced Biotechnology Centre, Imperial College London, for preparing the peptides. This work was funded by grants from Cancer Research UK, the Wellcome Trust and Association for International Cancer Research. Funding to pay the Open Access publication charges for this article was provided by Cancer Research UK.

Conflict of interest statement. None declared.

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