The XPB subunit of repair/transcription factor TFIIH directly interacts with SUG1, a subunit of the 26S proteasome and putative transcription factor

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ABSTRACT

Mutations in the basal transcription initiation/DNA repair factor TFIIH are responsible for three human disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The non-repair features of CS and TTD are thought to be due to a partial inactivation of the transcription function of the complex. To search for proteins whose interaction with TFIIH subunits is disturbed by mutations in patients we used the yeast two-hybrid system and report the isolation of a novel XPB interacting protein, SUG1. The interaction was validated in vivo and in vitro in the following manner. (i) SUG1 interacts with XPB but not with the other core TFIIH subunits in the two-hybrid assay. (ii) Physical interaction is observed in a baculovirus co-expression system. (iii) In fibroblasts under non-overexpression conditions a portion of SUG1 is bound to the TFIIH holocomplex as deduced from co-purification, immunopurification and nickel-chelate affinity chromatography using functional tagged TFIIH. Furthermore, overexpression of SUG1 in normal fibroblasts induced arrest of transcription and a chromatin collapse in vivo. Interestingly, the interaction was diminished with a mutant form of XPB, thus providing a potential link with the clinical features of XP-B patients.

INTRODUCTION

Transcription of protein encoding genes in higher eukaryotes by RNA polymerase II (Pol II) is a multi-step process involving a preinitiation complex containing several basal transcription factors, including TFIIH (for review see 1). An essential step is the conversion of a closed to an open initiation complex by local melting of the transcription start site probably due to the DNA-unwinding activity of the XPB and XPD subunits of TFIIH (2–5). Purification of TFIIH to homogeneity demonstrated that it is a multisubunit protein complex that contains a minimum of nine proteins (6). TFIIH also has kinase activity specific for the large subunit of RNA polymerase II (1). Biochemical evidence in both yeast and mammalian cells has shown TFIIH to be part of the RNA polymerase II holocomplex. Included in this megadalton complex with all basal transcription factors except TFIID are the SRB proteins, transcriptional activators and mediators and the chromatin remodelling SWI/SNF factors (7–13).

The p89 and p80 subunits of TFIIH were found to be identical to the XPB and XPD helicases involved in nucleotide excision repair (NER) (3,4,14–17). The NER pathway removes a variety of structurally unrelated DNA lesions in a multi-step pathway (18). The consequences of inborn errors in NER are highlighted by the phenotype of XP (XP), an autosomal recessive condition displaying sun (UV) sensitivity, photophobia and predisposition to skin cancer (19). Two other distinct excision repair disorders have been recognized: Cockayne syndrome (CS) a neurodevelopmental, photo-sensitive condition and trichothiodystrophy (TTD) which resembles CS but shows, in addition, brittle hair and nails (20,21). The TTD syndromes are genetically heterogeneous and comprise at least 10 complementation groups: seven in XP (XP-A to XP-G), five in CS, part of which overlap with XP complementation groups (CS-A, CS-B, XP-B, XP-D and XP-G) and three in TTD (TTD-A, and two XP groups; XP-B and XP-D) (18 and references therein). Strikingly, the proteins involved in XP-B, XP-D and TTD-A, i.e., all complementation groups with TTD features and most of the combined XP/CS groups, appear to be part of TFIIH (22). The dual function of TFIIH in repair and basal

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transcription and the specific link between mutations in this complex and the heterogeneous clinical features of CS and TTD, prompted the idea that at least part of the CS and TTD symptoms arise from (viable) defects in the transcription function of the complex. Thus, different mutations in the XPB and XPD helicases differentially affect the transcription and repair function of the complex giving rise to either XP, or XP/CS or TTD manifestations (22–24). In the case of XP-B patient XP11BE molecular analysis revealed a splice mutation in the XPB gene leading to a frameshift altering the last 41 amino acids of the encoded protein. Cells of this XP/Cs patient are almost completely deficient in NER (14). The enzymatic activity of TFIIH isolated from XP11BE lymphoblast cells is reduced in both the XPB-derived 3′→5′ helicase and in the in vitro basal transcription activity (25). Perhaps these effects are in part exerted by impaired protein–protein interactions. To identify proteins whose binding to XPB protein is diminished by the XP11BE mutation, we utilized the yeast two-hybrid system (26). With XPB as a bait we identified SUG1 as a protein specifically interacting with TFIIH and whose affinity is diminished by the XP11BE mutation. SUG1 has previously been identified as a part of the 26S proteasome complex (27 and references therein) and part of the RNA polymerase II holocomplex (7).

MATERIALS AND METHODS

Cells and viruses

*Spodoptera frugiperda* clones 9 or 21 (S′/9 or S′/21) and the baculovirus transfer vector pVL1392 were purchased from PharMingen. Baculoviruses were propagated in insect cells at 28°C in Hinks insect medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The human fibroblast cell line C5RO was grown in F10/DMEM medium containing 10% FCS and antibiotics. In the case of spinner cultures, the SV40-immortalized 

28 PharMingen. Baculoviruses were propagated in insect cells at

2604 medium supplemented with 20 mM glutamine, 10% FCS and antibiotics.

Expression of the recombinant XPB and mSUG1 in insect cells

The *Bam*H1 fragment containing the entire coding region of XPB was inserted into pVL1392, yielding pVLXBP. The pVL-ERCC3 virus containing the entire XPB coding sequence with an eighth-codon addition, including the codons for six histidines, at the 5′ terminus of the ORF was described in ref. 28. A Xho–*Bam*H1 fragment comprising the coding region of mSUG1 was inserted into pAcSGHisA, yielding pAcSMUG1. An ~1.4 kb *Bam*H1 mSUG1 cDNA fragment tagged with the Hemagglutinin epitope (HA) of the influenza virus was ligated in the *Bam*H1 digested pVL1393 vector, yielding pVLMSUGHA.

Insect cells were co-transfected with a mixture of linearized AcNPV DNA (BaculoGold™) and transvector DNA as described according to the manufacturer’s protocol. Expression of recombinant His-tagged mSUG1, HA-tagged mSUG1, XPB, His-tagged XPB infected cells was determined by analyzing the cell extracts with immunoblotting.

For immunoprecipitations, monolayer insect cells (6-well plates) were (co)infected with recombinant virus at a multiplicity of infection (M.O.I.) of ~5 p.f.u. per cell. At 3 days post-infection cells were dislodged by pipetting and centrifuged at 2000 g for 3 min. Subsequently, they were washed twice with phosphate-buffered saline (PBS) and then resuspended in 1 ml E1A immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.1% NP-40), including 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 µg/ml trypsin inhibitor and 1 µg leupeptin and pepstatin (referred as protease inhibitors).

For nickel-chelate affinity chromatography whole cell extract (WCE) was dialyzed against NiTA binding buffer (20 mM Tris pH 7.9, 500 mM NaCl, 5 mM imidazole, 5 mM DTT and protease inhibitors) prior to application onto an equilibrated NiTA column. The column was washed extensively with wash buffer (binding buffer with 60 mM imidazole) and then eluted into 0.5 ml column fractions with 100 and 500 mM imidazole containing binding buffer.

cDNA library screening and yeast transactivation assays

Construction of the 14.5 day-old CD-1 mouse embryo cDNA library in the yeast AAD fusion vector pPC96 was described elsewhere (29). The starting plasmid for construction of GAL4(DB) fusion vectors was pPC97. Generation of the GAL4 fusion vector that was used as bait to screen the cDNA library is constructed as follows. A 2.4 kb *Sma*I–*Nsi*I fragment containing the human XPB cDNA (14) lacking 30 amino acids at the N-terminus, was ligated in the *Sma*I site of pPC97 (29), yielding pPC97E3. XPB cDNA plasmids containing the C-terminal truncations C21 and C42 have been described previously (30) and were subcloned into the GAL4 fusion vector pPC97 as described above. Similar constructs were generated fusing the other TFIIH core components with the GAL4 DNA-binding domain, including XBP–XP11BE (aa 31–742), XPD–XP11BE (aa 75–760), XPD–XP11BE (aa 24–548), p44 (aa 37–395), p52 (aa 1–462) and p34 (aa 22–303). Note that in all cases GAL4 fusion constructs have been tested for expression levels in the yeast strain BJ5459 (ura3-52, trpl-1, lys2-801, leu2Δ1, his3Δ200, pep4::HIS3 prb1Δ1, 6R, can1, GAL) by immunoblotting using the antibodies against GAL4 DBD region (MAb 5C1, kindly provided by R. Bernards, Amsterdam) or the corresponding TFIIH subunit. The XPB bait in the pPC97 plasmid was introduced by LiAc transformation into the *Saccharomyces cerevisiae* Y190 reporter strain (MATa, leu2-3, 112, ura3-52, trpl-901, his3Δ200, ade2-101, gln3::GAL1-1,3-ADH1-LacZ, ELY GAL-HIS3 cyf– (31). Approximately 1.5 × 106 yeast transformants were selected on 40 9-cm plates of supplemented synthetic dextrose medium including 25 mM 1,2,4 triazol-3-ylamin 3-amino-1,2,4,-triazole lacking tryptophan, leucine and histidine. After 4–5 days, 39 transformants of which GAL4 activity had been reconstituted were assayed for β-galactosidase activity by transferring the resulting colonies directly to Hybond (Amersham) filters which were then treated with 5-bromo-4-chloro-3-indolyl-[β]-galactoside (1 mg/ml) as described earlier (29). β-Galactosidase assays on individual yeast transformants were determined as described earlier (32) except that the substrate was 4-methylumbelliferyl-β-D-galactopyranoside (MU-βGal) (0.66 mM, final concentration) assayed in 30 µl, 50 mM phosphate buffer pH 7.5, and incubation was at 37°C for 1 h. The reaction was stopped by addition of 200 µl sodiumcarbonate buffer, pH 10.7, and fluorescence was measured at 448 nm (ext. 365 nm) in a fluorometer.

**Plasmids**

The ∼1.4 kb msUG1 cDNA was subcloned into the eukaryotic expression vector pCDNA3 vector (Invitrogen), yielding pCMSUG1. Oligonucleotides p209 (5′-CCGATCCAAGATGG-
CGCTTGATGGG) and p210 (5′-CCGGCATCTCCAGCTAGC- 
GTAATCTGCACAATGTGGAATCTTTGATGGATTTG-3′) as 5′ and 3′ PCR primers containing a BamHI restriction site were used to construct the HA epitope-tagged SUG1 with an HA epitope added at the C-terminus (amino acid sequence YYDYVP- 
DYAS). The amplified DNA was purified and digested with BamHI and subsequently cloned into the pCDNA3 vector, yielding pCMSUG1HA and sequenced by the dideoxy-chain termination method. Oligonucleotides p123 (5′-CGCGGAAATTCACCA- 
TGGGACAGCCTATCATCATACATACAGCAGC- 
GGCCTGGTGACGGCGCCAGGCTAATGGGCAAAAGAG- 
ACCGG) and p90 (5′-CCCCGATCTCGACTAGCCTAATCTGG- 
AACATCGATGGGATTTCTCCTAAAGCGTTTGAAG) were used as 5′ and 3′ PCR primers to construct a XBP cDNA including six histidine codons (preceded by a thrombin cleavage site) at the N-terminus and an HA epitope at the C-terminus. The PCR fragment was sequenced to ensure correctness, digested with EcoRI and BamHI, and ligated into the modified eukaryotic expression vector pSG5 containing the SV40 early promoter, yielding pSHE3HA. Microinjection and DNA transfection of the tagged XBP construct corrects UDS and UV resistance [Winkler et al., manuscript submitted]. The eukaryotic mSUG1 cDNA tagged with HA was transfected into HeLa TK− cells using electroporation. The anti-HA immuno- and nickel-affinity purification of TFIIH complexes was performed as described earlier (37). RNA synthesis was determined by a 1 h pulse labeling with [3H]uridine (10 μCi/ml; s.a.: 50 Ci/mmol). DNA at a concentration of 0.1 μg/ml was microinjected into one of the nuclei of polykaryons. Cells were assayed 24 h after microinjection.

**Immunological methods and antibodies**

Anti-XBP MAb (1B3) and anti-p62 MAb (3C9) are described earlier (3,38). MAb 2SU was raised against the N-terminal region of mSUG1 (aa 1–149) (39). Mab 3SU was raised against the C-terminal region of mSUG1 (40). MAB-CTmut was raised against the 43 C-terminal amino acids of the mutant XBP (XP11BE): QAGISALWHHEFYWGRHVCVPLAEGQAQTC- 
PALQAL as described in 25). Monoclonal antibody 12CA5 was raised against influenza hemagglutinin peptide HA1 (75–110) (41). In some immunoprecipitation experiments antibodies were crosslinked to protein A–Sepharose.

Immunoprecipitation was performed at 4°C by addition of 40–100 μl of protein A/G–Sepharose (10%). The immunoprecipitated proteins were separated by 11% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore) or nitrocellulose in 25 mM Tris–HCl–192 mM glycine buffer (pH 8.3) containing 20% methanol. Antigen-bound antibodies were detected with a horseradish peroxidase- 
linked Goat anti-mouse IgG and an enhanced chemiluminescence detection system. For immunoprecipitation/immunoblot analysis, a horseradish peroxidase-linked anti-mouse IgG (κ) (Southern Biotechnology Associates Inc.) was used as secondary antibody.

**RESULTS**

**Isolation and characterization of mSUG1**

The wild-type human XBP (aa 31–742) was fused to the GAL4 DNA-binding domain (DBD) (aa 1–47) and used as bait in a two-hybrid screen for interacting proteins encoded by a library of cDNAs (mouse) and fused to the GAL4 activation domain (AD) (29). Yeast transformants (1.5 × 10⁶) were selected by plating on synthetic dextrose medium lacking tryptophan, leucine and histidine to select for those transformants that could express a 
GAL1-HIS3 gene which is regulated by GAL4 binding sites. His⁺ 
transformants were subsequently screened for the expression of a second reporter gene, GALI-LacZ. Among the 39 His⁺ clones, 
one yeast clone was positive for LacZ expression. DNA sequence 
analysis revealed an open reading frame of 406 amino acids fused in frame with the GAL4 activation domain. The encoded protein appeared identical to the mouse protein, mSUG1 (39), a functional homolog of the yeast transcription factor/mediator 
SUG1 (42,43) and to p45 of the P A700 regulatory subunit of the 
26S proteasome (44). The mSUG1 protein is also 99.3% identical (three amino acids difference; Fig. 1 and Discussion) to the 
human Trip1 protein (45). SUG1 contains a putative ATP-binding 
site (46,47) (the ‘A’ and ‘B’ sites of NTP- binding domains, 
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Figure 1. Sequence analysis of mSUG1 cDNA. mSUG1 is 100% identical to the human p45 protein component of the PA700 regulatory complex of the human 26 proteasome (44). Comparison of the predicted amino acid sequence of mSUG1, yeast SUG1 (39,42), MSS1, a positive modulator of HIV Tat-mediated transactivation (52) and the yeast homolog of MSS1, CIM5 (54), TBP1 (55) and the yeast homolog of TBP1 (YTA1) (56). Sequence identity is presented in black boxes, whereas similar residues (A, S, T, P and G; D, E, N and Q; R, K and H; I, L, V and M; F, Y and W) are given in grey boxes. Three amino acids differ between mSUG1 and the human published TRIP1 protein (45) (amino acid residues 266 S, 272 Q and 300 which are respectively D, T and I in SUG1 and p45, but S, Q and M in the Trip1 sequence). The putative functional domains are indicated. The fact that the aspartic acid (D), the threonine (T) at positions 266 and 272 are completely conserved in the AAA-family members from yeast to human and isoleucine (I) at position 300 is conserved between yeast and human SUG1 suggests that they are invariant and that the differences with the Trip1 protein might represent sequencing errors.
TFIIH components were fused to the GAL4 DNA-binding domain (aa 1–406) was fused to the GAL4 activation domain (AD) and other core subunits of TFIIH. For these experiments, mSUG1 (aa 2278–2319) was used, which like XPB, is ubiquitously expressed (data not shown).

The two-hybrid assay was used to assess the ability of mSUG1 to bind to the wild-type and XP11BE mutant XPB protein and the other core subunits of TFIIH. For these experiments, mSUG1 (aa 1–406) was fused to the GAL4 activation domain (AD) and TFIIH components were fused to the GAL4 DNA-binding domain (DBD). β-galactosidase reporter activity indicates that only the wild-type XPB protein was able to interact with mSUG1 (Fig. 2A, first lane). No significant increase in reporter activity was observed when the AD protein fusion bearing mSUG1 was co-expressed with the DBD fusion of XPB (Fig. 2A, last lane), or p62, p52, p44, p34 and a vector control were paired with mSUG1 (data not shown). Interestingly, the XP11BE mutant interacts with mSUG1, but generated a transcript. These data suggest that the C-terminal 41 amino acid nonsense part of the mutant XPB protein interferes with mSUG1 binding. To localize the region(s) required for the interaction with SUG1, truncation mutants of XPB, lacking the carboxy 21 and 42 amino acids residues (CA21 and CA42, respectively) were fused to the GAL4–DBD domain and assayed for functional interaction. All the fusion proteins were expressed at similar levels, as judged by immunoblot analysis (Fig. 2B) ruling out the possibility that reporter activity is reduced due to different expression levels of the DBD-fusion proteins. Both C-terminal truncated fusion proteins resulted in a decrease of interaction with mSUG1 compared to wild-type XPB. These data indicate that XPB C-terminus, though important, is not indispensable for functional interaction. It appears that the C-terminal frame-shifted nonsense sequence may interfere in a semi-dominant fashion with mSUG1 interaction, since the binding with the XP11BE mutant protein is even more affected.

**Figure 2.** Functional interaction between mSUG1 and XPB in yeast cells. (A) Plasmids expressing wild-type XPB and wild-type or (deletion) mutants of XPB fused to GAL4-DBD were introduced into the yeast reporter strain Y190 together with the GAL4-AD-mSUG1 fusion construct. Transformants were grown in liquid media. Extracts (each bar is the average of four individual yeast colonies, each extract was measured in triplicate) were prepared and assayed for β-galactosidase activity, which is expressed in arbitrary units. (B) Equivalent amounts of total yeast cell extracts were analyzed for the presence of the DBD-fusion proteins (DBD–XPD fusion not shown) by immunoblot analysis using antibodies 1B3 [recognizing the wild-type and mutant XPB protein, upper panel, and CTmut, lower panel]. The upper bands (the wild-type XPB fusion protein is ∼105 kDa) correspond to the different full length fusion proteins.

A 1.4 kb transcript of SUG1 was found by Northern blot analysis in all tested mouse tissues, including thymus, spleen, lung, brain, testis, ovary, muscle, heart, kidney and liver. The levels of expression in all tissues tested indicated that mSUG1, like XPB, is ubiquitously expressed (data not shown).

**Specificity of mSUG1 and XPB binding**

The two-hybrid assay was used to assess the ability of mSUG1 to bind to the wild-type and XP11BE mutant XPB protein and the other core subunits of TFIIH. For these experiments, mSUG1 (aa 1–406) was fused to the GAL4 activation domain (AD) and TFIIH components were fused to the GAL4 DNA-binding domain (DBD). β-galactosidase reporter activity indicates that only the wild-type XPB protein was able to interact with mSUG1 (Fig. 2A, first lane). No significant increase in reporter activity was observed when the AD protein fusion bearing mSUG1 was co-expressed with the DBD fusion of XPB (Fig. 2A, last lane), or p62, p52, p44, p34 and a vector control were paired with mSUG1 (data not shown). Interestingly, the XP11BE mutant interacts with mSUG1, but generated a transcript. These data suggest that the C-terminal 41 amino acid nonsense part of the mutant XPB protein interferes with mSUG1 binding. To localize the region(s) required for the interaction with SUG1, truncation mutants of XPB, lacking the carboxy 21 and 42 amino acids residues (CA21 and CA42, respectively) were fused to the GAL4–DBD domain and assayed for functional interaction. All the fusion proteins were expressed at similar levels, as judged by immunoblot analysis (Fig. 2B) ruling out the possibility that reporter activity is reduced due to different expression levels of the DBD-fusion proteins. Both C-terminal truncated fusion proteins resulted in a decrease of interaction with mSUG1 compared to wild-type XPB. These data indicate that XPB C-terminus, though important, is not indispensable for functional interaction. It appears that the C-terminal frame-shifted nonsense sequence may interfere in a semi-dominant fashion with mSUG1 interaction, since the binding with the XP11BE mutant protein is even more affected.

**mSUG1 interacts with XPB in vitro**

To confirm the interaction detected in the two-hybrid system with a biochemical assay, XPB and His-tagged mSUG1 were co-expressed in insect cells using the baculovirus system. Crude extracts infected with either XPB alone or in combination with His-tagged mSUG1 baculoviruses were loaded onto a nickel-chelate affinity column. After washing and elution with 100 and 500 mM imidazole, immunoblot analysis indicated that XPB is retained onto the affinity column only in the presence of His-tagged mSUG1, whereas XPB overexpressed alone mainly flowed through the column [Fig. 3A, compare the test column (XPB/mSUG1 lysate) with the control column (XPB lysate, lanes 4–11)]. It is worth noting that XPB-infected cell extracts do not contain any immunoreactive SUG1 (Fig. 3A, control column, lane 1). To further demonstrate the specificity of the interaction between XPB and SUG1, the 500 mM imidazole eluted fraction 2 (Fig. 3A, lane 10) was immunoprecipitated with a MAb-XPB antibody (1B3). Even after extensive washing with buffer containing 500 mM KCl, mSUG1 still remained associated with XPB (Fig. 3B, lane 3), whereas neither XPB nor SUG1 were retained on the control beads cross-linked to Mab GST (lane 5).

Since XPB interaction could also have been mediated through the His-tag we used an alternative approach (Fig. 4). Extract from insect cells co-infected with viruses expressing XPB and Hemagglutinin (HA)-tagged mSUG1 were immunoprecipitated with a MAb-XPB (1B3) or an anti MAb-SUG1 (2SU). Immunoprecipitates were analyzed on immunoblots with a MAb-HA antibody (12CA5) (Fig. 4A) and MAb-XPB (1B3) (Fig. 4B). Several control infections confirm that the interactions were specific. The MAb-SUG1 (2SU) did not co-immunoprecipitate XPB when only XPB virus was used for infection (Fig. 4A and B) showing that exogenous SUG1 was required. XPB antibodies did not precipitate mSUG1 when infected alone (Fig. 4A and B). Based on Coomassie-stained gels of extracts of S9/21-infected cells we showed that HA-tagged SUG1 is expressed to a higher extent than His-tagged XPB which, in part, may explain the difference in immunoblot signals. Related to this is the observation that SUG1 can form homodimers in vitro and in vivo (our own unpublished results). These results, combined with the two-hybrid and the
Figure 3. Purification of a SUG1–XPB complex from insect cells. Sf9 cells were infected with XPB or co-infected with XPB and mSUG1-His baculoviruses (pVLXPB and pAcSMUG1, respectively) as described in Materials and Methods. Equal amounts of XPB/mSUH1-His and XPB lysates were loaded onto a nickel-chelated affinity test column and control column respectively and extensively washed with binding buffer containing 60 mM imidazole. The bound proteins were then sequentially eluted with 100 and 500 mM imidazole. (A) The load (lane 1), the flow through (FT) (lane 2), the wash (lane 3) and the eluted fractions (lanes 4–11) were analyzed by immunoblotting with MAb against XPB (1B3) and SUG1 (2SU). (B) The 500 mM imidazole eluted fraction 2 [(A) lane 10 of the test column] was immunoprecipitated with either XPB MAb (1B3) or, as negative control, GST MAb crosslinked to protein A–Sepharose. The load (lane 1), the flow through (FT, lanes 2 and 4) as well as the proteins bound to control MAb (lane 5) and to XPB MAb (lane 3) were analyzed by immunoblotting and probed with MAbS against SUG1 (2SU) and XPB (1B3).

His-tag affinity pull-downs, indicate a specific and direct interaction between SUG1 and XPB in vivo.

Characterization of the hSUG1 complex

The direct and specific interaction between XPB and mSUG1 by the two-hybrid assay in yeast and the co-expression studies in insect cells do not prove that this interaction is of physiological significance. For instance, SUG1 may interact with an XPB domain not available when the latter is in a complex with TFIIH. Therefore, TFIIH from fractionated HeLa WCE (Fig. 5A) was tested for the presence of hSUG1 using MAb-SUG1 (2SU). Notably, SUG1 was detected in the Heparin 5PW fractions 11 and 12, identical to the elution pattern of TFIIH (Fig. 5A). A physical association between hSUG1 and XPB was demonstrated by immunoprecipitations of Heparin 5PW fraction 12 using MAbs against SUG1 (C- and N-terminal 2SU and 3SU, respectively).

Both 2SU and 3SU specifically and efficiently depleted the fraction of hSUG1 (Fig. 5B, lanes 1–3). Moreover, in support of the two-hybrid and co-infection experiments, XPB, although not quantitatively depleted, also eluted from the SUG1 immunoprecipitations (Fig. 5B). To verify the specificity of the interaction the inverse immunoprecipitation was performed using MAb against XPB. Immunoprecipitates were washed with increasing salt concentrations prior to elution (Fig. 5C). Even after treatment with 1 M KCl salt, hSUG1 remained bound to the MAb XPB complex. However, immunoreactivity of SUG1 could not be found in the most pure (HAP) fraction of TFIIH (HAP is the final column of TFIIH purification), suggesting that SUG1 may dissociate during the preceding phenyl column.

To confirm the association of SUG1 with TFIIH in HeLa cells, we developed an alternative method for purification of TFIIH under more physiological conditions. A His6 and HA epitope was...
Figure 5. Co-purification by conventional chromatography of hSUG1 with XPB from a HeLa extract. (A) Immunoblot analysis of whole cell extract (WCE), peak TFIIH containing fractions (Heparin Ultrogel 0.40, DEAE 0.2., Sulfoethylpropyl 0.45) and fractions eluting from a Heparin-SPW from 0–0.9 M (NH4)2SO4 gradient indicate that hSUG1 co-elutes with XPB in Heparin SPW fractions 10–14. Samples (25 µl) were subjected to SDS–PAGE. Immunoblots were probed with MAbs against SUG1 and XPB. (B) Immunoprecipitations of hSUG1 from Heparin SPW fraction 12 with the C-terminal-specific anti SUG1 MAb, 3SU or N-terminal-specific anti SUG1 MAb 2SU bound to protein G–Sepharose, specifically precipitate XPB (lanes 5 and 6). No non-specific binding of hSUG1 and XPB proteins to the resin was detected (lane 4). Moreover, the flow through of the 3SU and 2SU immunoprecipitations are both depleted of hSUG1 in comparison to the resin mock depletion (lanes 1, 2 and 3). For each immunoprecipitation, Heparin SPW fraction 12 (1 ml) flow through from protein G–Sepharose (100 µl) was incubated at 4°C for 2 h with 10 µl of the respective MAb (3SU or 2SU) bound to the protein G–Sepharose (100 µl) or with protein G–Sepharose alone (RESIN). The flow through was collected and the beads washed with 30 bed volumes of 500 mM KCl, and the bound proteins were released from the resin by addition of Laemmli load buffer (100 µl). Samples (25 µl of flow through, 50 µl of supernatant) were analyzed by immunoblotting using MAbs against SUG1 and XPB proteins to the resin was detected (lane 4). The above experiments combined with its presence in the RNA polymerase II holocomplex (7,42,43,50), suggest that SUG1 may become very densely stained. Concomitantly, transcription chromatin material clumped into a small area and the cytoplasm became very densely stained. Concomitantly, transcription dropped eventually to zero compared to non-injected cells (Fig. 7A).

Figure 6. Co-purification of the hSUG1 protein with TFIIH by affinity chromatography. XPCS2BA cells were transfected with a tagged XPB cDNA construct pSHE3HA. Immunoblot analysis (25 µl fractions) of WCE, immunopurified TFIIH eluted with a HA peptide and nickel-chelate affinity chromatography purified TFIIH. The monoclonal antibodies used to detect XPB, p62 and hSUG1 proteins are 1B3, 3C9 and 2SU, respectively. Note that in the WCE two XPB protein bands are visible. The upper band corresponds to the tagged XPB and is eluted with 100 mM imidazole or with the HA peptide. The lower band represents the endogenous non-tagged XPB protein. The specific retention of only the larger tagged XPB species on the affinity columns indicates that only one XPB molecule resides in a TFIIH complex.

with proper functioning of the XPB protein, since both transfection and microneedle injection of the tagged cDNA encoding the double-tagged molecule was able to correct the repair-deficient XP-B cells (Winkler et al., manuscript submitted). The tagged XPB protein was not significantly overexpressed in the stable transformants used. WCE extracts were prepared from XPCS2BA (XP-B) cells expressing the functional tagged XPB and fractionated by nickel-chelate affinity chromatography and immunopurification using a 12CA5 MAb anti HA-tag. Material that bound specifically to both columns was eluted by competition with 100 mM imidazole or with a HA peptide, respectively. hSUG1 was present in both column eluates which were strongly enriched for TFIIH (Fig. 6). Similar results were obtained when a nuclear extract was used to purify TFIIH, whereas a WCE from untagged HeLa cells did not contain detectable quantities of TFIIH subunits or SUG1 (data not shown). We determined that the HA column chromatography yields at least 1000-fold purification of TFIIH. In the reverse experiment, WCE extracts were prepared from HeLa cells expressing a HA-tagged mSUG1. Immunoblot analysis indicated that ~50% of the intracellular SUG1 is HA-tagged (data not shown). Both XPB and p62 were present in immunopurified HA-tagged mSUG1 complexes eluted with the HA peptide as analyzed by immunoblots (data not shown). In conclusion, these results clearly demonstrate that a fraction of SUG1 interacts with TFIIH in vitro in non-overexpressing human cells, however, the protein is not part of the final purified TFIIH complex that consists of at least nine subunits (XPB, XPD, p62, p52, p44, p34, cdk7, cyclin H and MAT1) (6).
identified to suppress a GAL4 mutation in yeast cells, SUG1 has since been known to interact in a ligand-enhanced manner with nuclear receptors (39,45). Moreover, SUG1 has a direct interaction with TBP which was thought to be a target for its transcriptional mediation in yeast (43). Clearly, in the light of our findings, TFIIH may also be involved. Since the most pure TFIIH preparations lacking SUG1 are active in the in vitro transcription and repair reactions it can be concluded that SUG1 is apparently neither indispensable for the core of the basal transcription initiation mechanism nor for NER.

**Functional implications of the SUG1–XPB interaction**

The sequence identity between ySUG1 and mSUG1 is 74% indicative of a very strong sequence conservation throughout evolution. The most highly conserved region (88% identity) between the two proteins is a central domain of 244 amino acids (residues 132–375), abbreviated as the AAA module (for ATPases associated with a variety of cellular activities; 48). Proteins of the AAA family have been linked with numerous cell functions, including cell cycle regulation, secretion, vesicle-mediated transport, peroxisome biogenesis, gene expression and proteasome activity and are found not only in yeast and higher eukaryotes but also in archaea and bacteria (48 and references therein). The members of this AAA protein family include: (i) the human proteasome component S7/MSS1 (52,53), also shown to modulate HIV Tat-mediated transcription, and the yeast homologue CIM5 (54); (ii) human TBP1 a protein that is closely related to TBP7 which binds the HIV Tat protein in vitro (55) and the yeast homolog YTA1 (56) (Fig. 1). Interestingly, the mSUG1 amino acid sequence is completely identical to a recently identified human subunit of the PA700 proteasome complex (44) and very closely related to the reported sequence of the human Trip1 protein. Trip1 was isolated in a two-hybrid screen in yeast using human Thyroid hormone receptor (TR-β1) as a bait (45).

What might be the functional implications of the SUG1–XPB interaction? SUG1 is a component of the 26S proteasome which has the capacity to interact with and degrade a diverse set of ubiquitinated proteins (27) but is also required for activation of proteins by processing inactive precursors (for reviews see 57–59 and references therein). The precise role of ATP hydrolysis in the mechanism of protein breakdown has not been identified, but may be linked to substrate unfolding and translocation into the proteolytic lumen of the 26S proteasome. To investigate the possibility that SUG1 bound to TFIIH is present in the proteasome form, we used proteasomal antibodies against S4 and MSS1 and showed that there is no immunoactivity in TFIIH fractions (Heparin 5PW, data not shown). Furthermore, using antibodies against ubiquitin we were unable to detect ubiquitinated forms of TFIIH. These findings render it unlikely that the fraction of TFIIH bound to SUG1 is undergoing proteolysis.

Alternatively, or in addition, SUG1 may act on its own or in a separate complex as an ATP-dependent molecular chaperone catalyzing protein conformational alterations (58). It is not excluded that SUG1 and the related ATPases MSS1 and TBP1 in this complex directly participate in the control of transcription (7,42, 43,45,55).

Our findings demonstrate that only a small fraction of all cellular SUG1 is in a complex with TFIIH. This points to a more universal function for SUG1. Size-fractionation experiments of total cell extracts showed that the majority of SUG1 migrates over

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**DISCUSSION**

**mSUG1, the homolog of ySUG1, interacts with XPB**

Our demonstration that SUG1 associates with TFIIH may explain its reported presence in the RNA polymerase II holoenzyme (7). However, in its active form, RNA polymerase II holocomplex does not appear to include SUG1 (27,50). The lowered affinity of SUG1 for the XPB bearing the XP11BE mutation, a mutation which lowers overall transcription levels, is consistent with the idea that SUG1 acts as a mediator of transcription (45). Originally

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**Figure 7.** Effect of wild-type mSUG1 on transcription. Micrographs showing the effect of microinjection of wild-type mSUG1 encoding cDNA (pCMSUG1) on RNA synthesis in normal human fibroblasts. The injected polykaryons (indicated with an arrow) of which one nucleus is injected demonstrates a strong inhibition of RNA synthesis (A, B) and a clear chromatin collapse (B). Nuclei are indicated with arrowheads.

and B). These unusual effects are very similar to the injection of a dominant-negative XPB mutant (37) suggesting that SUG1 overexpression may act via the same mechanism as mutant XPB. No such phenomena were seen when numerous other wild-type genes on the same vector were injected such as XPB, XPD and p62. This experiment suggests that overexpression of mSUG1 in mammalian cells confers a dominant-negative effect on transcription in vivo. This is consistent with a direct or indirect role of SUG1 in transcription (51).
a broad but higher range than expected for free SUG1, indicating that this protein resides in (large) complexes (60). In glycerol gradients S4 and MSS1 peak with the PA700 while SUG1 is found throughout the gradient (40). These findings suggest that there might be a link between the transcription/repair and proteasome machineries.

The microinjection experiments support a role of SUG1 in transcription in vivo, probably by saturation of essential transcription components. In fact, besides other targets, XBP may be one of the factors that are saturated when SUG1 is overexpressed after microinjection of many SUG1 cDNA copies under a strong promoter.

**Differential interaction between mSUG1, wild-type and mutant XBP**

It is likely that a mutation in a subunit of an intricate multifunctional complex such as TFIIH has multiple effects. The XP11BE frameshift mutation in the XBP C-terminus, leads to a virtually complete inactivation of the NER pathway (14). The equivalent mutation in yeast also resulted in a clear UV sensitivity (61,62) indicating that the C-terminus is essential for repair. We showed that TFIIH purified from XP11BE cells displays an impairment not only of the NER function but also at least partially of the basal transcription function. In addition, the mutation affected the XBP helicase activity (25). These biochemical data suggest that a conformational modification may be responsible for the decrease of XBP helicase activity resulting in a severe NER defect and a less strong impairment of transcription. Here we report that the XP11BE mutant protein has a diminished interaction with SUG1 which may contribute to the clinical, cellular and molecular defects in the patient, because SUG1-mediated effects on TFIIH will probably be diminished as well. Obviously, this may indirectly impinge on the repair and transcription function of the complex. Recent reports also show that interactions between the basal factors TFIIH and TFIIE and between TFIIH and TFIIA function of the complex. Recent reports also show that interactions between the basal factors TFIIH and TFIIE and between TFIIH and TFIIA are involved in the transcription process (57,58).

**REFERENCES**


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