Subunit Communications Crucial for the Functional Integrity of the Yeast RNA Polymerase II Elongator (γ-Toxin Target (TOT)) Complex*

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In response to the Kluyveromyces lactis zymocin, the γ-toxin target (TOT) function of the Saccharomyces cerevisiae RNA polymerase II (pol II) Elongator complex prevents sensitive strains from cell cycle progression. Studying Elongator subunit communications, Tot1p (Elp1p), the yeast homologue of human IKK-associated protein, was found to be essentially involved in maintaining the structural integrity of Elongator. Thus, the ability of Tot4p (Kti12p) to interact with the HAT subunit Tot5p (Elp3p) of Elongator and with core Elongator Tot5p (Elp5p) is dependent on Tot1p (Elp1p). Also, the association of core-Elongator (Tot1–3p/Elp1–3p) with HAP (Elp4–6p/Tot5–7p), the second three-subunit subcomplex of Elongator, was found to be sensitive to loss of TOT1 (ELP1) gene function. Structural integrity of the HAP complex itself requires the ELP4/TOT7, ELP5/TOT5, and ELP6/TOT6 genes, and elp6Δ/Tot6Δ cells as well as elp4Δ/tot7Δ cells can no longer promote interaction between Tot5p (Elp5p) and Tot2p (Elp2p). The association between Elongator and Tot4p (Kti12p), a factor that may modulate the TOT activity of Elongator, requires Tot1–3p (Elp1–3p) and Tot5p (Elp5p), indicating that this contact requires a preassembled holo-Elongator complex. Tot4p also binds pol II hyperphosphorylated at its C-terminal domain Ser5 raising the possibility that Tot4p bridges the contact between Elongator and pol II.

Microbial rivalry between Kluyveromyces lactis killer strains and sensitive Saccharomyces cerevisiae cells relies on secretion of zymocin, a heterotrimeric (αβγ) protein toxin complex that acts as a cell cycle blocker in G1 (1–3). Zymocin docking involves interaction of its α-subunit, an exo-chitinase, with S. cerevisiae cell wall chitin (4, 5), and anti-zymotic activity resides within the γ-subunit, also termed γ-toxin (6, 7). In an effort to identify the intracellular γ-toxin target (TOT) process, seven TOT genes were found to abrogate toxicity when mutated (8, 9). TOT1–3 and TOT5–7 are identical with ELP1–6 coding for Elongator, an RNA polymerase II (pol II)-associated histone acetyltransferase (HAT) complex (8–15). In addition, loss of KTI11, KTI13, and SIT4 results in tol phenotypes characteristic for TOT mutants, implying that these genes may also be linked to Elongator function (16–18). Tot4p (Kti12p) can be found promotor-associated, and it contacts Elongator and pol II (8, 19, 20). Since both removal and overproduction of Tot4p induce zymocin resistance, Tot4p is likely to influence Elongator by modulating its TOT activity (16, 20). Holo-Elongator contains the core complex, Elp1–3p (Tot1–3p), and HAP, a second heterotrimer composed of Elp4–6p (Hapl3p/Tot5–7p) (8, 9, 13–15). Elp1p is the largest subunit within core-Elongator and homologous to human IKK-associated protein, an IkB kinase scaffold protein and a member of a five-subunit protein complex (21, 22). Elp2p is a WD40 protein homologous to murine STAT3-interacting protein StIP1 (12, 23, 24), and Elp3p is the HAT subunit (11, 25) whose activity requires the HAP complex (26). Consistent with a role in transcription, Elongator facilitates pol II activity through chromatin and stably associates with hyperphosphorylated pol II (H10) (10, 27). Its HAT activity is essential for Elongator to function as TOT and HAT-minus scenarios yield zymocin resistance (8, 14). Together with the finding that the TOT function can be dissociated from Elongator by mutagenesis of its HAT gene without affecting other Elongator properties (9), the HAT function of Elongator plays a key role in mediating zymocytosis. As judged from the observations that (i) pol II-driven transcription is down-regulated in zymocin-treated cells (8, 9), (ii) pol II underassembly and general pol II defects elicit zymocin-hypersensitivity (9, 28), (iii) interfering with pol II C-terminal domain (CTD) modification alters the response of a cell to zymocin (9), and (iv) the phospho-states of pol II are imbalanced in zymocin-treated cells (29), zymocin may work by hijacking the TOT function of Elongator to convert it into a global pol II inhibitor. Genetic analyses have shown that deletion of any one of the ELP/TOT genes phenocopies the full range of elp/tot phenotypes induced by elp3 point mutations that drastically reduce the HAT activity of Elongator (8, 12, 25). Thus, it has been speculated that the functional integrity of Elongator is compromised in these deletants, leading to non-productive Elongator HAT scenarios (12, 25).

To study TOT/Elongator function further, we analyzed subunit communications within the complex by co-immune precipitation (co-ip). We found Elongator subunit Tot1p (Elp1p) to be essential for Tot2-Tot3 (Elp2-Elp3) and Tot2-Tot5 (Elp2-Elp5) protein-protein interactions. Also, the association of core-Elongator (Tot1–3p/Elp1–3p) with the HAP complex was dependent on Tot1p (Elp1p). The interaction between Elongator and Tot4p (Kti12p) required Tot1–3p (Elp1–3p) and Tot5p (Elp5p), suggesting that Tot4p contacts Elongator as a preassembled holo-

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‡ The abbreviations used are: TOT, γ-toxin target; pol II, RNA polymerase II; HAT, histone acetyltransferase; HAP, histone acetyltransferase-associated protein(s); CTD, C-terminal domain; IIα, hyperphosphorylated pol II; II0, hyperphosphorylated pol II; co-ip, co-immune precipitations; HA, hemagglutinin; IKK, IκB-related kinases.

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complex. Tot4p also bound pol II hyperphosphorylated at its CTD Ser5 position, implying that it may bridge Elongator and pol II.

EXPERIMENTAL PROCEDURES

Strains, Media, General DNA Techniques, and K. lactis Zymocin Methods—All yeast strains used are listed in Table I. Routine yeast growth media,YPD (1% yeast extract, 2% peptone, 2% dextrose) and synthetic dextrose (SD) medium (0.67% yeast nitrogen base, 2% dextrose), were as described by Sherman (30). Yeast DNA transformation utilized the lithium acetate protocol of Gietz et al. (31). Construction of TOT2 -end deletions involved PCR amplification of TOT2 open reading frame fragments (promoter primer, 5′-CGC GAT TTA AGA-3′; S3-5′-CGT CCA GGG ATA AAA CTG TCA AAG TAT GGA GGC ACC AAA-3′) using plasmid template pFF10, a synthetic dextrose (SD) medium (0.67% yeast nitrogen base, 2% dextrose), and 4,5′-dCTP-GTA TTA AGA-3′; S3-5′-CGT CCA GGG ATA AAA CTG TCA AAG TAT GGA GGC ACC AAA-3′) using plasmid template pFF10, a synthetic dextrose (SD) medium (0.67% yeast nitrogen base, 2% dextrose), and 4,5′-dCTP. All yeast strains used are listed in Table I.

| Strain | Description | Source |
|--------|-------------|--------|
| K. lactis AWJ137 | a leu2 trp1 [k1 k2'] | (8) |
| S. cerevisiae FY1679-08A | MATa ura3–52 leu2A1 trp1A63 his3A200 GAL | Euroscarf, Frankfurt, Germany |
| FYF2y-a | As FY1679–08A, but TOT2/ELP2-(c-myc)6; SpHIS5 | (20) |
| FYF3y | As FY1679–08A, but TOT3/ELP3-(c-myc)6; SpHIS5 | (8) |
| FYF5y | As FY1679–08A, but TOT5/ELP5-(c-myc)6; SpHIS5 | (8) |
| FYF4y-a | As FY1679–08A, but TOT4/ELP4-(c-myc)6; SpHIS5 | (20) |
| DJy4-b | As FY1679–08A, but TOT4/KTI12-(HA)6; KITRP1 | This study |
| FYF2y3-dt | As FFY2y-a, but TOT3/ELP3-(HA)6; KITRP1 | (20) |
| FYF2y3-dt-1d | As FFY2y3-dt, but tot1Δelp1Δ:KILEU2 | This study |
| FYF2y5-dt | As FFY2y-a, but TOT5/ELP5-(HA)6; KITRP1 | (20) |
| FYF2y5-dt-6d | As FFY2y5-dt, but tot1Δelp6Δ:KILEU2 | This study |
| FYF2y5-dt-7d | As FFY2y5-dt, but tot7Δelp7Δ:KILEU2 | This study |
| FYF4y-dt | As FFY4-a, but TOT5/ELP5-(HA)6; KITRP1 | (20) |
| FYF4y-dt-2d | As FFY4-dt, but tot2Δelp2Δ:KILEU2 | This study |
| FYF4y-dt-3d | As FFY4-dt, but tot3Δelp3Δ:KILEU2 | This study |
| FYF4y-dt-6d | As FFY4-dt, but tot6Δelp6Δ:KILEU2 | This study |
| FYF4y-dt-1d | As FFY4-dt, but tot1Δelp1Δ:KILEU2 | This study |
| FYF3y4-dt | As FYF3y, but TOT4/KTI12-(HA)6; KITRP1 | (20) |
| FYF3y4-dt-2d | As FYF3y4-dt, but tot2Δelp2Δ:KILEU2 | This study |
| FYF3y4-dt-3d | As FYF3y4-dt, but tot3Δelp3Δ:KILEU2 | This study |
| FYF3y4-dt-5d | As FYF3y4-dt, but tot5Δelp5Δ:KILEU2 | This study |
| FYF2y-a | As FY1679–08A, but TOT2A-(HA)6; KITRP1 | This study |
| FYF2y2-D1 | As FY1679–08A, but TOT2A-(HA)6; KITRP1 | This study |
| FYF2y2-D2 | As FY1679–08A, but TOT2A-(HA)6; KITRP1 | This study |

FIG. 1. Dependence of Elongator subunit interactions on TOT1, TOT2, TOT6, and TOT7 gene function. As shown in A, TOT2-Tot5 protein-protein interaction depends on TOT1, TOT6, and TOT7, wt, wild type. As shown in B, Tot4-Tot5 protein-protein interaction depends on TOT2 and TOT6. Protein extracts obtained from the indicated strains were subjected to co-ip using the anti-c-Myc antibody 9E10. The immune precipitates were probed with anti-HA antibody to detect Tot5p (A and B) and with anti-c-Myc antibody to detect Tot2p (A) and Tot4p (B). Protein positions are indicated by arrows. MSMD denotes molecular size markers (Benchmark24 protein ladder, Invitrogen) in kilodaltons.

RESULTS

To analyze subunit communication within TOT/Elongator, we constructed strains in which one subunit gene was deleted and different subunits were c-Myc- and HA epitope-tagged. Co-ip was then done in an effort to study protein-protein interaction. As illustrated in Fig. 1A, tot6Δ and tot7Δ cells were found to lose the ability of Tot5p (Elp5p) to associate with core-Elongator subunit Tot2p (Elp2p). Thus, TOT6 (ELP6) and TOT7 (ELP4) influence the structural integrity of the HAP complex, and deletion of either gene causes it to lose its capa-
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ability to interact with core-Elongator. The fact that protein-protein interaction between the two three-subunit entities also becomes distorted upon deletion of TOT1 (ELP1) (Fig. 1A) can be regarded as evidence that Tot1p (Elp1p) is crucial in mediating this inter-complex communication. Consistently, the ability to co-precipitate Tot5p (Elp5p) by the HAT subunit Tot3p (Elp3p), was lost when Tot1p (Elp1p) was removed (not shown). Together with the fact that Tot5-Tot3 (Elp5-Elp3) protein-protein interaction is not compromised by TOT2 (ELP2) deletion (19), this indicates that the HAP complex communicates with core-Elongator mainly by virtue of Tot1p (Elp1p)-mediated contact(s). Tot4-Tot5 protein-protein interaction, however, does require TOT2 (ELP2) and again Tot6 (ELP6) function (Fig. 1B). As for the contact between Tot4p and core-Elongator, this may indicate that Tot2p (Elp2p) directly communicates with Tot4p, and indeed, if Tot2p is missing or C-terminally truncated, Tot4p can no longer associate with the HAT subunit Tot3p (Elp3p) of Elongator (see below). Thus, both Tot4p and the HAP complex contact core-Elongator in a different manner that is likely to happen in parallel and not to be mutually exclusive.

The yeast Elongator subunit Tot1p (Elp1p) has been shown to be related to IKAP, a scaffold protein that associates with IxB kinase and that is the largest subunit of the human Elongator complex (21, 24). To assess its role in mediating the structural integrity of Elongator, we required data concerning individual Elongator subunit interactions in the presence and absence of TOT1 (ELP1). Thus, the ability of Elongator subunit Tot2p (Elp2p) to co-precipitate the HAT subunitTot3p (Elp3p) of Elongator was fully dependent on the presence of a functional TOT1 (ELP1) gene (Fig. 2A). Similarly, Elongator-associated factor Tot5p (Elp5p) is required for the interaction with the HAP component Tot3p (Elp3p) (Fig. 2B). Together with our previous findings that the interactions between Tot1p (Elp1p) and Tot3p (Elp3p) as well as Tot5p (Elp5p) and Tot3p (Elp3p) are insen-
pol II forms. H5, anti-CTD-P (Bentley laboratory), and anti-CTD-S2-P (6% SDS-PAGE and immunoprobed with anti-CTD (H14), anti-CTD-P (Bentley laboratory), and anti-CTD-S2-P (H5) antibodies to detect both hyperphosphorylated and hypophosphorylated pol II forms. ip, immune precipitate.

Sensitivity to Tot2p (Elp2p) (19), our observations suggest that Tot1p (Elp1p) may play a role as a scaffold to which Tot3p (Elp3p) and Tot2p (Elp2p) assemble onto to form core-Elongator. Moreover, Tot1p (Elp1p) can be considered to mediate the contact between core-Elongator and the HAP complex since this inter-subcomplex communication is lost in the absence of Tot1p (Elp1p). As for the capability of Tot3p (Elp3p) to co-ip Tot4p, both Tot1p (Elp1p) and Tot2p (Elp2p) are required (Fig. 3). Together with the surprising observation that this also holds true for a functional TOT5 (ELP5) gene (Fig. 3), these data strongly indicate that the association of Tot4p with Elongator requires a completely assembled six-subunit holo-complex.

Tot2p (Elp2p) contains eight WD40 domains and was hypothesized to serve the structural integrity of Elongator (12). Intriguingly, however, TOT2 (ELP2) deletion does not compromise Tot1-Tot3 (Elp1-Elp3) and Tot3-Tot5 (Elp3-Elp5) protein-protein interaction, indicating that communication between both of these core-Elongator subunits and a representative HAP component is insensitive to Tot2p (15, 19). As for the established role TOT2 (ELP2) plays in the association of Tot4p with Elongator (19), we tested epistasis of TOT2 (ELP2) deletion alleles coding for C-terminally truncated variants (Fig. 4A, pTOT2Δ1–2) for their abilities to mediate Tot4-Tot3 protein-protein interaction by co-ip. As illustrated in Fig. 4C, none of these truncations lacking up to one WD40 domain was able to restore Tot4-Tot3 protein-protein interaction in a tot2Δ (elp2Δ) background, whereas full-length epistatic TOT2 (ELP2) mediated association of Tot4p with Tot3p (Elp3p) in a way indistinguishable from chromosomally encoded wild-type TOT2 (ELP2) (Fig. 4C). Anti-HA Western analysis of protein extracts obtained from yeast strains expressing the truncations as HA-tagged proteins revealed that the Tot2p (Elp2p) variants were synthesized (Fig. 4D), suggesting that their incapability in mediating co-ip between Tot4p and Tot3p (Elp3p) in tot2Δ (elp2Δ) cells was not due to protein instability or lack of protein synthesis. Thus, the function of Tot2p in mediating association of Tot4p with Tot3p (and core-Elongator) resides in its extreme C terminus. Consistently, the appropriate truncation alleles conferred zymocin resistance in killer eclipse assays, suggesting that this region plays a role in TOT function and K. lactis zymocin toxicity, too (Fig. 4B). Taken together, these data indicate that Tot2p (Elp2p) mediates at least in part the contact between Tot4p and Elongator. However, for inter-complex communication and interaction between Tot1p (Elp1p) and Tot3p (Elp3p), it appears to be dispensable (15, 19). As for the zymocin mode of action, which is abrogated in the absence of the C terminus of Tot2p, our findings indicate that zymocin requires Tot4p to be associated with Elongator, providing further evidence that Tot4p influences the TOT function of Elongator (19, 20).

Consistent with copurification of Elongator and pol II (10, 24), c-Myc-tagged Tot2p (Elp2p) and, to a lesser extent, c-Myc-tagged Tot5p (Elp5p), were able to associate with pol II form I10 using co-ip (Fig. 5A). Similarly, c-Myc-tagged Tot4p interacted with pol II form I10 (Fig. 5A). Remarkably, as judged from utilizing different anti-pol II antibodies, the interaction between Tot4p and pol II was solely restricted to form I10 hyperphosphorylated at Ser5 within the CTD repeat (Fig. 5B). In contrast, neither pol II form I1A (hypophosphorylated on its CTD) nor pol II form I10 (hyperphosphorylated at the Ser5 of CTD) were co-precipitable with HA-tagged Tot4p (Fig. 5B). Also, lack of interaction could not be overridden by subjecting yeast cells to cross-linking prior to protein extraction and co-ip to amplify weaker interactions (Fig. 5B). Thus, Tot4p interacts with pol II form I10 hyperphosphorylated at the Ser5 of CTD, a
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modification that is transcriptionally characteristic for a post-initiation event and for pol II0 engaged in promoter clearance and/or early transcript elongation (37–39).

DISCUSSION

Taken together, our findings can be hypothetically incorpo-
rated in a working model (Fig. 6) in which the assembly of holo-Elongator requires the association of preformed core-Elo-
gator with the HAP complex. This inter-complex communica-
tion largely relies on Tot4p (Elp1p), which may serve as a scaffold protein. Fully assembled holo-Elongator is capable to contact Tot4p (Fig. 6), an Elongator-associated factor that is not a structural subunit but rather transiently contacts Elongator, presumably to promote its interaction with elongation-competent pol II0. Consistent with this, Tot4p associates both with core-Elongator subunits and HAP components (8) as well as with pol II form II0 hyperphosphorylated at the Ser5 of CTD. Since this modification occurs before preinitiation complex for-
mation (37–39) and since Tot4p is able to occupy the promoter rather than the coding sequence of the ADH1 gene in chroma-
tin immune precipitations (19), Tot4p may be recruited to and communicate with pol II form II0 engaged in promoter escape and/or early transcript elongation (39). One interpretation of such a scenario includes the conclusion that Tot4p mediates the association of Elongator with pol II to yield HAT-productive holo-enzymes ready for efficient transcript elongation. Consist-
et with such a role for Tot4p as a loading factor, its removal yields tot/elp phenotypes indistinguishable from TOT/Elonga-
tor mutants (8, 9). Moreover, multicopy TOT4 induces zymocin resistance and intermediate tot phenotypes, indicating that excess Tot4p levels affect Elongator function, too (8, 16, 20). According to the identification of a putative ATP/GTP binding P-loop motif in the N terminus of Tot4p that is necessary for protein function, Tot4p may be a G-protein (20). Together with the fact that a deletion of KTI13 (ATS1) encoding a putative GTP exchange factor (40) results in zymocin resistance and tot phenotype expression (18) that can be suppressed by multicopy 

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