JCB Report

TANGLED1: A Microtubule Binding Protein Required for the Spatial Control of Cytokinesis in Maize

Laurie G. Smith,* Suzanne M. Gerttula,* Shengcheng Han,* and Joshua Levy‡

*Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, California 92093; and ‡Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract. Spatial control of cytokinesis in plant cells depends on guidance of the cytokinetic apparatus, the phragmoplast, to a cortical "division site" established before mitosis. Previously, we showed that the *Tangled1* (*Tan1*) gene of maize is required for this process during maize leaf development (Cleary, A.L., and L.G. Smith. 1998. *Plant Cell.* 10:1875–1888.). Here, we show that the *Tan1* gene is expressed in dividing cells and encodes a highly basic protein that can directly bind to microtubules (MTs). Moreover, proteins recognized by anti-TAN1 antibodies are preferentially associated with the MT-containing cytoskeletal structures that are misoriented in dividing cells of *tan1* mutants. These results suggest that TAN1 protein participates in the orientation of cytoskeletal structures in dividing cells through an association with MTs.

Key words: cytokinesis • phragmoplast • preprophase band • microtubules • plant cytoskeleton

Introduction

Because plant cells are surrounded by walls that immobilize them within tissues, the orientations in which they divide during development constrain patterns of organ growth and are critical for establishing the cellular organization of plant tissues. Plant cells achieve cytokinesis through the action of a phragmoplast, a cytoskeletal structure composed of microtubules (MTs)[1] and F-actin, which directs the formation of a new cell wall between daughter nuclei after mitosis (Staehelin and Hepler, 1996). The phragmoplast arises initially in isolation from the parental wall and plasma membrane, and expands centrifugally as cytokinesis proceeds. The location at which the new cell wall will fuse with the parental wall is governed by an interaction between the expanding phragmoplast and a cortical "division site" established before mitosis (Gunning, 1982; Smith, 2001). This site is marked throughout prophase by a cortical band of MTs and actin filaments, the preprophase band (PPB), which probably plays an essential role in establishing the division site during prophase (Wick, 1991; Smith, 2001). Very little is known in molecular terms about the nature of the cortical division site or how the phragmoplast interacts with it during cytokinesis.

To better understand the spatial control of cytokinesis in plant cells, we have analyzed the function of a gene in maize, *Tangled1* (*Tan1*), which is required for this process. Throughout the development of *tan1* mutant leaves, the majority of cells in all tissue layers divide in abnormal orientations (Smith et al., 1996). Analysis of the cytoskeleton in dividing leaf cells of *tan1* mutants showed that all the cytoskeletal structures involved in cell division are formed and appear structurally normal, but are not oriented normally within dividing cells. Abnormally oriented cell divisions can be attributed mainly to the failure of most phragmoplasts to be guided to the former PPB site (Cleary and Smith, 1998). Here, we present an initial molecular characterization of the *Tan1* gene and its protein product. In combination with our previous analysis of the *tan1* mutant phenotype, the results suggest a direct role for TAN1 in orienting cytoskeletal structures during cell division.

Materials and Methods

Plant Material

tan-Mu1 was isolated from a *Mutator* mutagenized population (Smith et al., 1996). *tan1-py1* was obtained from the Maize Genetics Stock Center. The ethyl methanesulfonate–induced *tan-gt1* allele was a gift from Sharon Kessler and Neelima Sinha (University of California at Davis, Davis, CA).

The online version of this article contains supplemental material.

Address correspondence to Laurie G. Smith, Section of Cell and Developmental Biology, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0116. Tel.: (858) 822-2531. Fax: (858) 534-7108. E-mail: lsmith@biomail.ucsd.edu

[1]*Abbreviations used in this paper:* APC, adenomatous polyposis coli; MT, microtubule; PPB, preprophase band.

Nucleic Acid Isolation and Gel Blot Analysis

Genomic DNA isolation from leaf tissue and Southern blots was carried out according to standard protocols (Chen and Dellaporta, 1994; Ausubel et al., 2000). Blots were hybridized at 65°C in 0.25 M $NaPO_4$, pH 7.2, with 2% SDS and washed in 0.2× SSC with 0.2% SDS (high stringency) or at 54°C in 0.5 M $NaPO_4$, pH 7.2, with 7% SDS and washed at 54°C in 100 mM $NaPO_4$, pH 7.2, with 5% SDS (low stringency). Total RNA was extracted using Trizol reagent (GIBCO BRL) and enriched for poly A^+ RNA using the PolyATtract mRNA isolation system (Promega). Northern blots were carried out as described by Luehrsen (1994). To demonstrate equal loading, Northern blots were stripped and reprobed with a 700-bp PstI-SacI fragment of the ubiquitin clone pSKUBI (Christensen et al., 1992), a gift from P. Quail (US Department of Agriculture Plant Gene Expression Center, Albany, CA).

Cloning and Sequence Analysis of Tangled

The 2.5-kb *Mu1*-hybridizing SstI fragment cosegregating with the *tan1* phenotype was cloned from a size-selected library of SstI-digested genomic DNA from a homozygous mutant constructed in Lambda Zap (Stratagene). Full-length genomic and cDNA clones were isolated using the 600-bp *Tan1* fragment (see Fig. 1 A) to screen a B73 genomic DNA library (a gift from Pioneer Hi-Bred, Johnston, IA) and a B73 vegetative shoot tip cDNA library (a gift from B. Veit and S. Hake, US Department of Agriculture Plant Plant Gene Expression Center). The sequences of three different cDNAs and one full-length genomic clone were assembled using MacVector software (v6.5). Sequencing of genomic PCR products amplified from the *tan-gt1* allele revealed the presence of a point mutation near the end of exon 2. A combination of PCR and Southern blotting was used to identify a 6-kb insertion of unknown identity in the first intron of the *tan-py1* allele.

Protein and Antibody Production

Polyclonal rabbit antibodies were raised against a COOH-terminal TAN1 peptide (CGLKQRPGYSLTVRTVSSKISSR) coupled to keyhole limpet hemocyanin at Covance Research Products (Denver, PA) using their standard protocols. Antibodies were affinity-purified on peptide-coupled SulfoLink beads (Pierce Chemical Co.) as described by Harlow and Lane (1988). For the peptide competition experiments (see Fig. 5, O and P), 1.5 μg of affinity-purified COOH-terminal peptide antibody in 200 μl of PBS with 1 mg/ml BSA was absorbed with beads coupled to ~33 μg of peptide and used without further dilution for cell labeling experiments. mAbs were raised against the portion of TAN1 encoded by exons 1 and 2 (expressed as a glutathione *S*-transferase [GST] fusion protein from the pGEX-4T-2 vector [Amersham Pharmacia Biotech] and cleaved from glutathione *S*-transferase with thrombin protease) at Covance Research Products according to their standard protocols. The His-TAN1 fusion protein used for MT overlay assays was constructed by cloning a full-length *Tan1* cDNA in frame with the histidine tag of pQE-30 (QIAGEN).

Protein Extraction and Analysis

Plant extracts were prepared by homogenizing fresh tissue with an Omni TH homogenizer on ice in an extraction buffer of 100 mM Tris, pH 7.4, 10% sucrose, 5 mM EGTA, 5 mM EDTA, and a cocktail of protease inhibitors from Roche Molecular Biochemicals. Crude extracts were centrifuged at various speeds for 10 min in an Eppendorf microcentrifuge at 4°C. Proteins were separated by SDS-PAGE as described by Ausubel et al. (2000). Western blots were carried out as described by Harlow and Lane (1988), using the affinity-purified COOH-terminal peptide antibody at 5–10 μg/ml or hybridoma TAN75 culture supernatant at 1:20, and alkaline phosphatase–conjugated secondary antibodies (Promega). To confirm equal loading of proteins (see Fig. 3, C and D), blots were reprobed with antiactin mAb C4 (ICN Biomedicals) and detected with alkaline phosphatase–conjugated anti–mouse Ig as described above.

The MT overlay assay was based on the method of Saunders et al. (1997). Proteins were separated on SDS-PAGE gels and transferred to polyvinyldifluoride membranes (Millipore). Membranes were blocked in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% BSA for 1 h and washed three times in PEM buffer (80 mM Pipes, 1 mM EGTA, 0.5 mM $MgCl_2$, 10% glycerol, 5 mM DTT) containing 0.5% BSA and protease inhibitors. MTs were polymerized from bovine brain tubulin (Cytoskeleton) in PEM buffer, diluted to 10 μg/ml in PEM buffer containing protease inhibitors, 0.5% BSA and 1 μm taxol, and incubated with filters for 2 h at room temperature After washing three times in TBST with 0.5% BSA (TBST/BSA), filters were incubated with primary antibodies (TAN75 or anti–β-tubulin mAb DM1B [Amersham Pharmacia Biotech] diluted in TBST/BSA). Subsequent washes and detection were carried out as described above for Western blots.

Immunolocalization of TAN Proteins

Leaf primordia ≤1 cm in length were removed from 2–3-wk-old seedlings, fixed as described in Cleary and Smith (1998), and squashed onto coverslips essentially as described in Wick et al. (1981). Alternatively, tissue slices were plunge frozen in liquid nitrogen–cooled propane, freeze substituted, embedded in butylmethylmethacrylate resin, sectioned at 3 μm, and incubated in acetone to remove the resin as described by Baskin et al. (1996). These methods gave very similar results. Coverslips/slides were blocked in PBS with 5% normal goat serum (PBS/NGS), incubated with primary antibodies (anti-COOH peptide at 5–10 μg/ml, TAN75 supernatant at 1:50 in PBS/NGS) for 2 h at room temperature, washed in PBS with 0.2% Triton X-100, incubated with appropriate FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), and washed again as above. For double labeling experiments, coverslips/slides were then blocked in PBS with 5% normal mouse serum (PBS/NMS) and incubated for 2 h in Cy3-conjugated anti–β-tubulin mAb Tub 2.1 (Sigma-Aldrich) diluted into PBS/NMS (incubations were always done in this order to ensure that FITC labeling coinciding with MTs could not result from cross-reaction of the FITC secondary antibodies with the tubulin antibody). After washing as described above, coverslips/slides were mounted in Vectashield (Vector Laboratories) and viewed on a E-600 microscope using EF-4 FITC HYQ and G-2A filter sets and a CFI Plan Apo 60× 1,4 NA objective (Nikon). Images were captured on an MDS-120 digital camera (Eastman Kodak Co.) and processed using Adobe® Photoshop™ v4.0.

Online Supplemental Materials

Supplementary Materials including details of the relationship between TAN1 and vertebrate adenomatous polyposis coli (APC) proteins, and additional biochemical characterization of TAN proteins, can be found at http://www.jcb.org/cgi/content/full/152/1/231/DC1.

Results

Cloning of Tangled1 Using a Transposon Tag

The *tan-Mu1* mutation arose in a stock with active *Mutator* transposons. A *Mu1*-hybridizing 2.5-kb SstI fragment that cosegregated with the mutant phenotype was cloned and used as a probe to isolate three cDNAs and a full-length genomic clone. Sequencing of these clones revealed the gene structure illustrated in Fig. 1 A. The *Mu1* insertion in *tan-Mu1* coincides approximately with the presumed start site of transcription, as estimated from the size of *Tan1* mRNA (~1.5 kb, see below). Southern blot analysis and sequencing of genomic PCR products showed that the *tan1-py1* mutant allele contains an ~6-kb insertion of unknown identity in the first intron of the gene, and the ethyl methanesulfonate–induced *tan-gt1* allele contains a premature stop codon near the end of the second exon, which would truncate the TAN1 protein to <10 kD.

The 600-bp fragment shown in Fig. 1 A hybridizes to the 2.5-kb SstI fragment in genomic DNA of homozygous mutant individuals, a 1.2-kb fragment in their homozygous wild-type siblings, and to both fragments in heterozygous siblings (Fig. 1 B). This is the expected pattern of hybridization for a fragment of the *Tan1* gene, since the wild-type allele should be smaller than the *tan1-Mu1* allele by the size of *Mu1* (1.3 kb). Proof that the 600-bp fragment is a part of the *Tan1* gene came from the analysis of somatic revertant sectors. Because *tan1* mutant leaves have a rough crepe papery texture, large wild-type sectors in mutant leaves, such as that illustrated in Fig. 1 D, are readily visible. Four such sectors showed the result illustrated in Fig. 1 C: the 600-bp fragment hybridizes to a 2.5-kb SstI

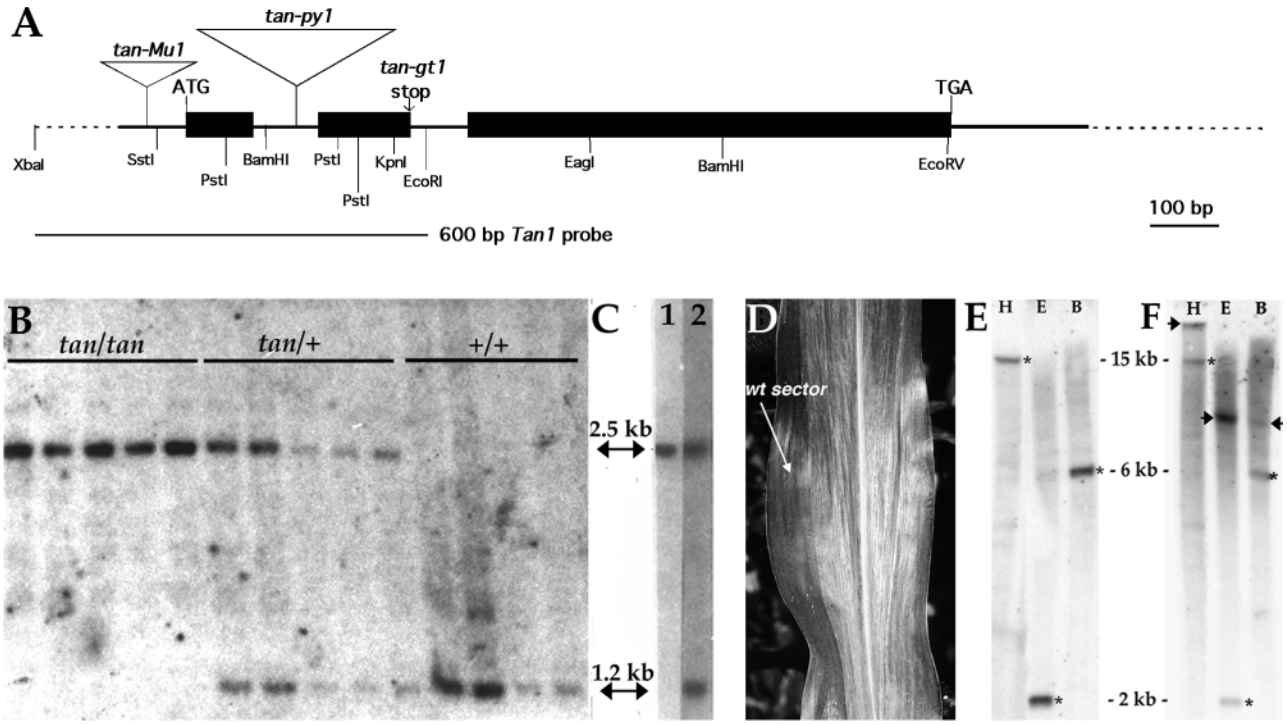

Figure 1. Cloning of *Tan1*. (A) Structure of the *Tan1* gene, showing the transcribed region as a solid line and exons as filled bars. Insertions in the *tan1-Mu1* and *tan1-py1* alleles (not drawn to scale) and the premature stop codon in the *tan1-gt1* allele are shown. (B) SstI-digested DNA from individuals of the indicated genotypes in a *tan1-Mu*; segregating family was probed with the 600-bp *Tan1* fragment illustrated in A. (C) SstI-digested DNA from a wild-type sector in a mutant leaf (lane 2) and adjacent mutant tissue (lane 1) was probed with the *Tan1* fragment illustrated in A. (D) Leaf from a *tan1-Mu1* mutant plant showing a large wild-type (wt) sector. (E and F) A619 DNA digested with HindIII (H), EcoRV (E), and BglII (B), hybridized with the same *Tan1* fragment, and washed at high stringency (E) or low stringency (F). *Fragments that hybridize at low and high stringency; arrows point to fragments that hybridize only at low stringency.

fragment in DNA from mutant tissue (Fig. 1 C, lane 1) and to both 2.5 and 1.2-kb fragments in DNA from the adjacent wild-type sector (Fig. 1 C, lane 2). Thus, appearance of the wild-type phenotype in this sector correlates with excision of *Mu1* from one of the two mutant alleles. At high stringency, the 600-bp *Tan1* probe hybridizes to a single fragment of maize genomic DNA digested with various restriction enzymes (Fig. 1 E, asterisks). At low stringency, this probe hybridizes to the same fragment (Fig. 1 F, asterisks) and to one additional fragment (Fig. 1 F, arrows).

Tan1 Gene Expression Is Correlated with Cell Division

Northern blot analysis using the 600-bp *Tan1* probe illustrated in Fig. 1 A was carried out to examine the pattern of *Tan1* gene expression. As illustrated in Fig. 2 A, this probe hybridizes to a single mRNA of ~1.5 kb in wild-type vegetative shoot tips consisting of leaf primordia and the bases of immature leaves, the shoot apical meristem, and unexpanded stem tissue (highly enriched in actively dividing cells). This mRNA is greatly reduced in *tan1-Mu1* mutants and to a lesser extent in *tan1-py1* mutants. In addition, a smaller transcript is observed in *tan-py1* mutants (and their wild-type siblings, mostly *tan-py1* heterozygotes), presumably a product of aberrant RNA splicing (Fig. 2 A, asterisks). *Tan1* mRNA accumulation is only slightly reduced in shoot tips of homozygous *tan-gt1* mutants, consistent with the presence of a premature stop codon in this allele. As shown in Fig. 2 B, compared with the 0–2-cm shoot segment enriched in dividing cells (Fig. 2 B, lane 1), *Tan1* mRNA is vastly reduced in the 3–5-cm shoot segment composed mainly of postmitotic expanding leaf cells (Fig. 2 B, lane 2), and in the 6–8-cm segment composed mainly of fully expanded leaf cells that are differentiating (Fig. 2 B, lane 3), as well as in mature (fully expanded and differentiated) leaf tissue (Fig. 2 B, lane 4). In addition, *Tan1* is strongly expressed in other tissues enriched in dividing cells: ear primordia (Fig. 2 B, lane 5), and embryos (Fig. 2, lane 6). Thus, *Tan1* gene expression correlates with cell division in the shoot.

Tan1 Encodes a Highly Basic Protein with Microtubule Binding Activity

The *Tan1* cDNA sequence (sequence data are available from GenBank/EMBL/DDBJ under accession no. AF305892) contains an open reading frame encoding a predicted protein of ~41 kD that is highly basic with a pI of 12.6. Analysis of this protein sequence reveals no strong homology with other proteins or motifs of known function, but a distant similarity was observed between 85% of the TAN1 protein and the basic regions of vertebrate APC proteins (see supplemental material available at http://www.jcb.org/cgi/content/full/152/1/231/DC1). The basic region of APC has been shown to bind to tubulin in vitro and associate with MTs in vivo (Polakis, 1997), suggesting that TAN1 might perhaps be an MT binding protein.

Antibodies were raised against various fragments of TAN1 to provide tools for analysis of this protein. Fig. 3 A shows a Western blot of proteins extracted from wild-type

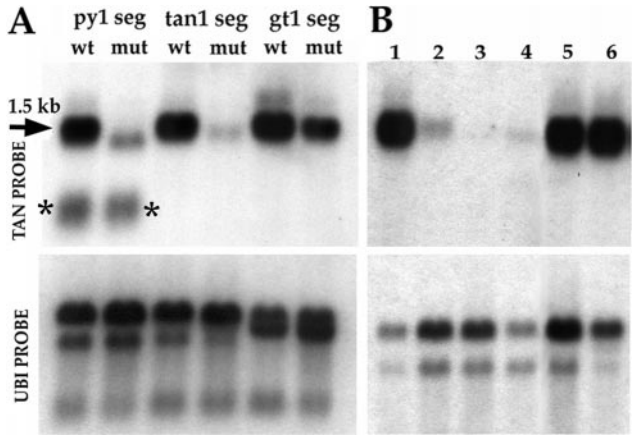

Figure 2. Northern blot analysis of *Tan1*. (A) The 600-bp *Tan1* fragment illustrated in Fig. 1 A was used to probe ~5 μg of poly A–selected RNA from vegetative shoot tips of mutants (mut) and their wild-type (wt) siblings in families segregating *tan1-py1*, *tan1-Mu1*, and *tan1-gt1*. *Aberrant transcripts seen only in *tan-py1* homozygotes and heterozygotes. (B) The same *Tan1* fragment was used to probe poly A–selected mRNA isolated from various wild-type maize tissues: (lanes 1–3) shoot segments of 2–3-wk-old seedlings from which all fully expanded leaves had been removed: the basal-most 2 cm (lane 1), 3–5 cm from the shoot base (lane 2), and 6–8 cm from the shoot base (lane 3); (lane 4) mature leaf tissue; (lane 5) embryos at 21 d after pollination; (lane 6) ear primordia 0.5–2-cm long. Blots were stripped and reprobed with ubiquitin to confirm RNA integrity and equal loading.

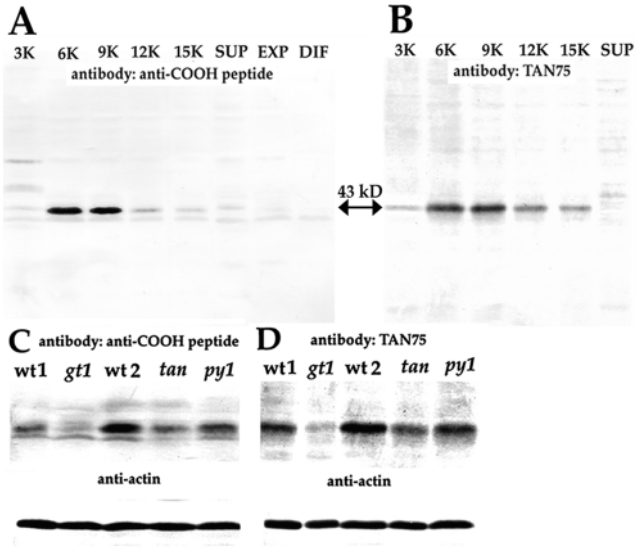

Figure 3. Western blot analysis of TAN1 protein. (A) Extracts from wild-type vegetative shoot tips of 2–3-wk-old seedlings were centrifuged sequentially for 10 min at the indicated speeds to produce a series of pellets and a final supernatant (SUP). In addition, 3,000–10,000 *g* pellets were isolated from extracts of 3–5-cm shoot segments (EXP) and 6–8-cm shoot segments (DIF) as defined in the legend to Fig. 2. A Western blot of these samples was probed with the affinity-purified anti–COOH-terminal peptide antibody. (B) A duplicate blot probed with mAb TAN75. (C) The anti-COOH peptide antibody was used to probe Western blots of 3,000–10,000 *g* pellets from vegetative shoot tip extracts of *tan-gt1* mutants and their wild-type siblings (wt1), *tan1-Mu1* and *tan1-py1* mutants in the A619 genetic background, and A619 wild-type individuals (wt2). (D) A duplicate blot probed with TAN75. Blots shown in C and D were reprobed with antiactin mAb C4 to confirm equal loading.

vegetative shoot tips probed with an affinity-purified antibody raised against a TAN1 COOH-terminal peptide. In Fig. 3 B, an equivalent blot is probed with an mAb raised against the NH_2-terminal portion of TAN1 encoded by exons 1 and 2 (TAN75). Both antibodies predominantly label a single protein band at ~43 kD, close to the predicted molecular mass of TAN1 (41 kD). Sequential centrifugation of crude cell extracts at gradually increasing speeds of 3,000–15,000 *g* shows that the majority of this protein sediments at 6,000–9,000 *g*, indicating that it is associated with a relatively large or dense structure in cell extracts. However, since this protein cannot be solubilized with detergents, it does not appear to be associated with a membrane-bound organelle (see supplemental material available at http://www.jcb.org/cgi/content/full/152/1/231/DC1). Parallel to results from the Northern blot analysis of *Tan1* mRNA, this protein is not detected in 3,000–10,000 *g* pellets from extracts of shoot segments composed of expanding (EXP) or differentiating (DIF) leaf tissue (Fig. 3 A).

As shown in Fig. 3, C and D, the 43-kD protein identified by both antibodies is reduced, but not eliminated, in mutants homozygous for each of the three *tan1* mutant alleles. Notably, the truncated form of TAN1 protein encoded by the *tan-gt1* allele (see Fig. 1 A) would not be recognized by the COOH-terminal peptide antibody and would not comigrate with full-length TAN1. Thus, it appears that the 43-kD protein recognized by anti-TAN1 antibodies in *tan-gt1* shoot tip extracts is the product of another gene, which is similar enough to TAN1 to be recognized by antibodies raised against nonoverlapping NH_2- and COOH-terminal fragments of TAN1 as well as three other polyclonal antibodies raised against other fragments of TAN1 covering its entire length (data not shown).

An overlay assay was used to show that TAN1 protein can bind directly to MTs. Fig. 4 A shows Coomassie blue–stained *Escherichia coli* extracts separated by SDS-PAGE before or after induction of a His-tagged TAN1 protein (arrow). After transfer to membranes, *E. coli* extracts were incubated with or without MTs polymerized from bovine brain tubulin. Subsequent detection with an anti–β-tubulin antibody showed that MTs bound to the His-tagged TAN1 protein (Fig. 4 A). A similar experiment with maize vegetative shoot tip extracts is shown in Fig. 4 B. Anti–β-tubulin detects endogenous maize tubulin with or without prior incubation of the blots with polymerized MTs. In addition, this antibody detects MTs bound to a 43-kD protein that comigrates with TAN1 and is reduced in abundance in the *tan-gt1* mutant extract. The residual MT-binding protein of the same molecular mass as TAN1 seen in the *tan-gt1* mutant extract presumably corresponds to the 43-kD protein in this extract recognized by anti-TAN1 antibodies (see Fig. 3, C and D), suggested earlier to be the product of a *Tan1*-related gene.

Proteins Recognized by Anti-TAN1 Antibodies Are Associated with the Cytoskeleton in Dividing Cells

Leaf primordium cells labeled with mAb TAN75 are shown in Fig. 5, A–G. This antibody labels cells at all stages of the cell cycle in a punctate manner. In interphase cells, antibody labeling is distributed uniformly through-

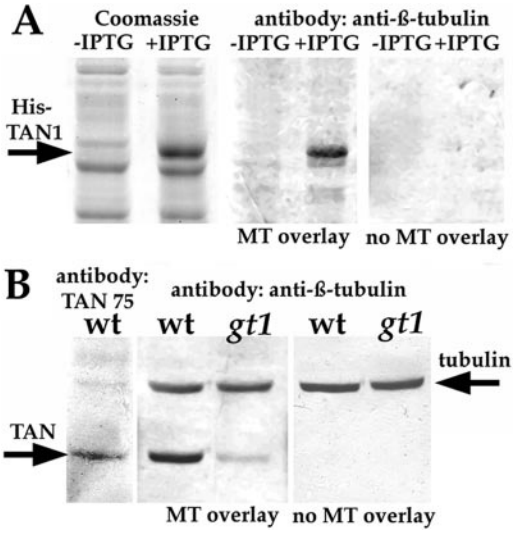

Figure 4. MT binding activity of TAN1 in a blot overlay assay. (A) Coomassie blue staining of total proteins extracted from *E. coli* before and after induction of His-TAN1 (arrow points to induced His-TAN1 protein). After transfer to membranes, *E. coli* proteins were incubated with or without MTs and then probed with monoclonal anti–β-tubulin. (B) Proteins recovered in the 3,000–10,000 *g* pellet from vegetative shoot tip extracts were transferred to membranes and probed with TAN75 or with anti–β-tubulin after incubation of membranes with or without MTs (arrows indicate the location of TAN proteins [43 kD] and of maize tubulin [55 kD]).

out the cytoplasm but is excluded from the nucleus (Fig. 5 A). Examination of thin sections confirmed that TAN75 labeling is not restricted to the cortex as interphase MTs are but is evenly distributed throughout the cytoplasm. In mitotic cells, TAN75 labeling is also observed throughout the cytoplasm but is preferentially associated with the cytoskeleton. A prophase cell labeled with TAN75 (Fig. 5 B) and anti–β-tubulin (Fig. 5 C) illustrates the coincidence of TAN75 labeling with the PPB. Similarly, mitotic cells labeled with TAN75 (Fig. 5, D and F) and anti–β-tubulin (Fig. 5, E and G) show that proteins recognized by TAN75 are predominantly associated with the spindle and phragmoplast. Fig. 5, H–N, shows that labeling of leaf primordium cells with the affinity-purified COOH-terminal peptide antibody produces very similar results.

Consistent with the results of Western blot experiments suggesting that these antibodies recognize a TAN1-related protein in *tan1* mutants, we found that cell labeling with TAN75 and the COOH-terminal peptide antibody is not eliminated or qualitatively altered in any of the *tan1* mutants, including *tan-gt1* mutants. However, peptide competition experiments confirm that the COOH-terminal peptide antibody specifically labels proteins sharing epitopes with TAN1: preincubation of the antibody with COOH-terminal TAN1 peptide–coupled beads reduced labeling to background levels (Fig. 5 P), but preincubation with beads coupled to an unrelated peptide had no effect (Fig. 5 O). From these results, in combination with the Western blot data and the observation that antibodies directed against nonoverlapping parts of TAN1 show equivalent cell labeling patterns, we conclude that these patterns reflect the intracellular distribution of a family of two or more related proteins that includes TAN1.

Discussion

Previous work showed that *Tan1* is required for spatial control of cytokinesis during maize leaf development. In

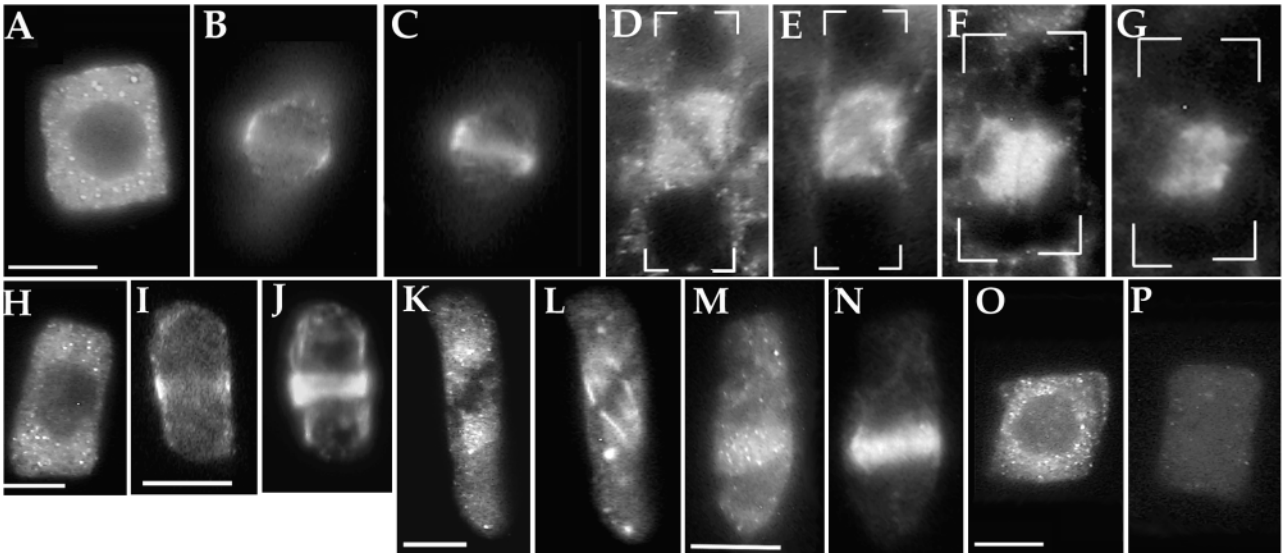

Figure 5. Labeling of wild-type leaf primordium cells with anti-TAN1 antibodies (D–G are 3-μm sections; all other images are of whole cells in leaf primordium squashes). (A) An interphase cell labeled with TAN75. A prophase cell is labeled with (B) TAN75 and with (C) anti-β-tubulin. A metaphase cell is labeled with (D) TAN75 and with (E) anti–β-tubulin. A cytokinetic cell is labeled with (F) TAN75 and with (G) anti-β-tubulin. (H) An interphase cell labeled with anti-COOH peptide. A prophase cell is labeled with (I) anti-COOH peptide and with (J) anti-β-tubulin. A metaphase cell is labeled with (K) anti-COOH peptide and with (L) anti-β-tubulin. A cytokinetic cell is labeled with (M) anti-COOH peptide and with (N) anti-β-tubulin. (O) An interphase cell labeled with anti-COOH peptide after incubation with beads coupled to a peptide unrelated to the TAN1 COOH terminus. (P) Another interphase cell labeled with anti-COOH TAN1 peptide after incubation with beads coupled to the COOH-terminal peptide. Bars, 10 μm.

tan1 mutant leaf cells, cytoskeletal structures involved in establishing planes of cell division (PPBs) and forming new cell walls (phragmoplasts) appear structurally normal but are frequently misoriented (Cleary and Smith, 1998). Here, we report the cloning of *Tan1* and initial molecular analysis of its protein product.

The *Tan1* gene is expressed in regions of active cell division and encodes a highly basic protein distantly related to the basic MT-binding domain of vertebrate APC proteins. Antibodies raised against various fragments of the TAN1 protein specifically recognize a 43-kD protein band detectable only in extracts from regions of active cell division, which apparently includes at least one TAN-related protein in addition to TAN1 itself. A genomic DNA fragment shown to hybridize with the *Tan1* probe at low but not high stringency could correspond to a gene 80–90% identical to *Tan1* at the nucleotide and protein levels, sharing most if not all epitopes recognized by the anti-TAN1 antibodies employed in this study. Similarly, antibodies raised against individual maize profilins cross-react with other family members that are 85% identical at the amino acid and nucleotide levels (Kovar et al., 2000). Efforts are currently underway to identify TAN1-like proteins for direct comparison with TAN1.

TAN1 protein produced in *E. coli* and extracted from maize shoot tips can bind directly to MTs in an MT overlay assay. Furthermore, proteins recognized by anti-TAN1 antibodies are preferentially associated with the MT-containing structures in dividing cells that are misoriented in *tan1* mutants (PPBs, spindles, and phragmoplasts). The apparent lack of association of these proteins with MTs in interphase cells suggests that their interaction with the cytoskeleton is regulated in a cell cycle–dependent manner. Together, our results suggest that TAN1 protein participates in the orientation of cytoskeletal structures in dividing cells through an association with MTs. An interesting possibility is that TAN1 mediates interactions between these structures and the cell cortex that are necessary for their proper orientation, such as those that guide phragmoplasts to cortical division sites previously occupied by PPBs.

We thank Scott Hammer and Andy Teng for their help with analysis of *tan1* mutant alleles; Sharon Kessler, Neelima Sinha, and the Maize Genetics Stock Center for seeds; Tama Hasson for advice on preparation of protein extracts; and Bob Schmidt, Raffi Aroian, and members of the Smith lab for valuable input. Finally, special thanks to Sarah Hake for financial support and mentorship during the early stages of this work.

This work was supported by National Institutes of Health grant R01 GM53137 to L.G. Smith.

Submitted: 4 August 2000
Revised: 31 October 2000
Accepted: 7 November 2000

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. 2000. Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Baskin, T.I., D.D. Miller, J.W. Vos, J.E. Wilson, and P.K. Hepler. 1996. Cryofixing single cells and multicellular specimens enhances structure and immunocytochemistry for light microscopy. *J. Microsc.* 182:149–161.

Chen, J., and S.L. Dellaporta. 1994. Urea-based plant DNA miniprep. *In* The Maize Handbook. V. Walbot and M. Freeling, editors. Springer-Verlag, New York. 526–527.

Christensen, A.H., R.A. Sharrock, and P.H. Quail. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression, and transcript splicing and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* 18:6675–6689.

Cleary, A.L., and L.G. Smith. 1998. The *Tangled1* gene is required for spatial control of cytoskeletal arrays associated with cell division during maize leaf development. *Plant Cell.* 10:1875–1888.

Gunning, B.E.S. 1982. The cytokinetic apparatus: its development and spatial regulation. *In* The Cytoskeleton in Plant Growth and Development. C.W. Lloyd, editor. Academic Press, London. 229–292.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kovar, D.R., B.K. Drøbak, and C.J. Staiger. 2000. Maize profilin isoforms are functionally distinct. *Plant Cell.* 12:583–598.

Luehrsen, K.B. 1994. Northern blotting. *In* The Maize Handbook. V. Walbot and M. Freeling, editors. Springer-Verlag, New York. 572–574.

Polakis, P. 1997. The adenomatous polyposis coli (APC) tumor suppressor. *Biochim. Biophys. Acta.* 1332:F127–F147.

Saunders, R.D.C., M.C. Avides, T. Howard, C. Gonzalez, and D.M. Glover. 1997. The *Drosophila* gene abnormal spindle encodes a novel microtubule-associated protein that associates with the polar regions of the mitotic spindle. *J. Cell Biol.* 137:881–890.

Smith, L.G. 2001. Plant cell division: building walls in the right places. *Nat. Rev. Mol. Cell Biol.* In press.

Smith, L.G., S. Hake, and A.W. Sylvester. 1996. The *tangled1* mutation alters cell division orientations throughout maize leaf development without altering leaf shape. *Development.* 122:481–489.

Staehelin, L.A., and P.K. Hepler. 1996. Cytokinesis in higher plants. *Cell.* 84: 821–824.

Wick, S.M. 1991. The preprophase band. *In* The Cytoskeletal Basis of Plant Growth and Form. C.W. Lloyd, editor. Academic Press, London. 231–244.

Wick, S.M., R.W. Seagull, M. Osborn, K. Weber, and B.E.S. Gunning. 1981. Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells. *J. Cell Biol.* 89:685–690.