Ciao 1 Is a Novel WD40 Protein That Interacts with the Tumor Suppressor Protein WT1*

(Received for publication, November 20, 1997, and in revised form, February 6, 1998)

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The Wilms tumor suppressor protein, WT1, is a transcription factor capable of activating or repressing transcription of various cellular genes. The mechanisms involved in regulating the transcriptional activities of WT1 are beginning to be unraveled. It appears that physical interactions of other cellular proteins (p53 and par-4) with WT1 can modulate the function of WT1. Here, we report the identification and cloning of a novel WT1-interacting protein termed Ciao 1, a member of the WD40 family of proteins. Ciao 1 specifically interacts with WT1 both in vitro and in vivo. This interaction alters the mobility of a WT1-DNA complex in gel shift assays, and results in a decrease in transcriptional activation mediated by WT1. Ciao 1 does not inhibit binding of WT1 to its consensus nucleotide sequence and does not affect the repression activity of WT1. Thus, Ciao 1 appears to specifically modulate the transactivation activity of WT1 and may function to regulate the physiological functions of WT1 in cell growth and differentiation.

WT1, the Wilms’ tumor suppressor protein, is a zinc finger-containing transcription factor that is capable of activating or repressing transcription depending on cell type and promoter context (1–4). Consistent with its predicted tumor suppressor function, mutations within the gene for WT1 have been detected in ~10% of sporadic Wilms’ tumors (5) and reintroduction of wild type WT1 into Wilms tumor cell lines expressing aberrant WT1 results in growth suppression (6). The gene for WT1 yields four alternatively spliced mRNAs (A, B, C, and D) that encode proteins of 52–54 kDa (7, 8). The A and B WT1 isoforms can bind to the EGR-1 (early growth response) consensus site (9); however, isoforms C and D, containing an extra three amino acids (Lys-Thr-Ser) between the third and fourth zinc fingers, recognize distinct DNA sequences (1, 10), suggesting that they may regulate a different set of genes.

The WT1 protein is expressed in the developing genitourinary system and plays a critical role in the development of organs such as the kidneys and gonads (reviewed in Refs. 3, 5, and 11). In addition to the role of WT1 in transcription regulation, other functions have also been proposed. Analyses of the subnuclear localization of WT1 led to a hypothesis that WT1 C and D protein isoforms may play a role in RNA processing (12). The overexpression of the WT1 B isoform has been shown to induce apoptosis (13), whereas the WT1 A isoform can bind to and stabilize p53, and subsequently inhibit p53-mediated apoptosis (14). In addition, WT1 B and D isoforms have been shown to decrease CDK2 and CDK4 kinase activity and block cell cycle progression through G1 phase (15).

In cell lines that constitutively express WT1, most of the WT1 protein is found in protein complexes with molecular masses ranging from 100 to 669 kDa (16). This suggests that the high order protein complexes may involve WT1 self-association (17, 18) and/or association with other cellular proteins such as p53 (16). Binding of p53 to WT1 results in a modulation of the transcriptional activity of WT1. Inasmuch as null mutations of WT1 cause embryonic lethality in mice due to failure in kidney development (19) but mice null for p53 are morphologically normal (20), it is likely that proteins other than p53 are also involved in transcription regulated by WT1. Consistent with this hypothesis, we have recently identified and cloned a cellular protein, par-4, and have demonstrated its in vivo association with WT1. The physical interaction of par-4 with WT1 is correlated with its ability to affect both the transcription and growth suppression functions of WT1 (21, 22).

In this study, we characterize the physical and functional interactions between WT1 and a novel protein, Ciao 1, which is a member of a family of proteins that contain so-called WD40 or β-transducin repeats (reviewed in Refs. 23 and 24). Proteins containing WD40 repeats have a wide range of diverse biological functions including signal transduction, cell cycle regulation, RNA splicing, and transcription (23). We demonstrate the physical interaction between Ciao 1 and WT1 both in vivo (mammalian cells and yeast) and in vitro. Significantly, interactions of Ciao 1 with WT1 results in a change in the mobility of a WT1-DNA complex in vitro and causes a dose-dependent decrease in WT1-mediated transcriptional activation. The functional importance of Ciao 1 is further supported by the observation that its putative yeast homolog is an essential gene. Taken together, these findings suggest that Ciao 1 is an important cellular regulator whose functions may include modulation of transcription mediated by WT1.
Materials and Methods

Plasmids

The pEGR-TKCAT reporter plasmid contains three Egr-1 binding sites inserted in tandem 5' of the herpes simplex virus TK promoter in plasmid pBLCAT2 (17). pCMV-FLAG-Ciao 1 was constructed by fusing full-length Ciao 1 to the FLAG moiety in the pB4T-4 vector. pB4T-4 contains the sequence encoding the FLAG epitope cloned into the Bov-His site of pBluescript SK (gift from J. Licht, Mount Sinai School of Medicine, New York, NY). A HincII/KpnI fragment containing the FLAG-Ciao 1 coding sequence was subsequently subcloned into the Bov-His site of the pCMV-neo-Bam expression vector. The WT1 (17) and EGR-1 (1) expression plasmids have been described previously. pGST-Ciao 1, pGST-p53, and pGST-RACK 1 were constructed by cloning full-length Ciao 1, p53, and RACK 1 cDNA, respectively, in frame in the pGEX-2TK vector. For in vitro transcription/translation of WT1, pSP64- WT1(A) and -WT1(D), -WT1-1–182, and -WT1-207–242 were used as described previously (17). pSP64-WT1(C) and -WT1(D) were similarly constructed (gift from J. Licht). In vitro transcription/translation of YY1 was performed using the plasmid pGem7zf(+)-YY1 described previously (25).

Isolation of WT1-interacting Proteins

Library Screening through Yeast Two-hybrid System—Escherichia coli and yeast were manipulated essentially as described (26). EGY48 (MATa trp1, ura3-52 leu2-3,112; lac1-15, 3a; his3::Tn10, hsdR, leuB600, trpC9830, lacD74, strA, gai1, his4B436) as described (27). The full-length WT1 cDNA was inserted into pEG202 at EcoRI site, and the generated plasmid pEG-WT1 was used as the bait. The oligonucleotide-primed HeLa cDNA yeast expression library, a generous gift from Dr. Russ Finley (Massachusetts General Hospital), was screened with pSP64-WT1 or pGem7zf(+)-YY1 and 5 μl was incubated with immobilized GST fusion proteins for 1 h at room temperature as indicated. The beads were washed five times with 1 ml of washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 200 μg/ml ethidium bromide). Bound proteins were eluted with Laemmli sample buffer, separated through a 10% SDS-polyacrylamide gel, and visualized by autoradiography.

Gel Shift Assays

Gel shift assays were performed using radiolabeled oligonucleotides containing either a consensus WT1 binding site (5'-TGGGACTCCTGGCC- CCAGCCCCGGCCG-3') or a consensus YY1 binding site (5'-AGGTCT- TCCATTTGGAGCCGG-3') and were essentially performed as described previously (29). The assay was programmed with pSP64-WT1 or pGem7zf(+)-YY1 and 5 μl was incubated with immobilized GST fusion proteins for 1 h at room temperature as indicated. The beads were washed five times with 1 ml of washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 200 μg/ml ethidium bromide). Bound proteins were eluted with Laemmli sample buffer, separated through a 10% SDS-polyacrylamide gel, and visualized by autoradiography.

Analysis of WT1 and Ciao 1 Interactions with Glutathione S-Transferase (GST) Fusion Proteins

GST fusion proteins were purified as described (30), and the yield of each protein was determined by SDS-PAGE analysis and Coomassie Blue staining. GST proteins bound to glutathione-agarose beads (Sigma) were separated by electrophoresis through a 10% polyacrylamide gel, transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with 0.1 μg/ml monoclonal anti-FLAG antibody (Eastman Kodak Corp.). Blots were incubated with alkaline phosphatase-coupled goat anti-mouse antibody (Sigma), and immunoreactive proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI).

Whole cell extracts were prepared from transfected cells. CAT activity was assayed as described (25) and quantitated with a Beckman LS6500 scintillation counter. Proper amounts of cell extracts were used to measure CAT activity to ensure that the assays were performed within linear range. For all the data presented, at least three independent transfections and CAT assays were performed.

Immunoprecipitation/Western Blotting Assays

293s were cotransfected with 15 μg of pcMVFLAG-Ciao 1 and 15 μg of pRSV or with 15 μg of pRSV, 48 h after transfection, cells were lysed in lysis buffer (25 μM HEPES, pH 7.0, 0.25 mM NaCl, 2.5 mM EDTA, 0.5 mM dithiothreitol, 10 mg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40) for 30 min on ice. Extracts were incubated with antibodies overnight, and immune complexes were collected with protein A-Sepharose beads at 4 °C for 1 h. The beads were washed eight times with lysis buffer and the proteins eluted with Laemmli sample buffer. Protein were separated by electrophoresis through a 10% polyacrylamide gel, transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with 0.1 μg/ml monoclonal anti-FLAG antibody (Eastman Kodak Corp.). Blots were incubated with alkaline phosphatase-coupled goat anti-mouse antibody (Sigma), and immunoreactive proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI).

Isolation of WT1-interacting Proteins—WT1 represses and activates transcription depending on promoter and physiological context (1–4). A simple model to account for its dual functionality is that interactions with different cellular proteins may determine its activity. We used the yeast “two-hybrid” assay (27, 32) to identify proteins that interact with WT1. Using full-length WT1(A) fused to the DNA-binding domain of Lex A (Lex A-WT1) as a “bait,” we screened approximately 2 × 106 independent colonies from a HeLa cell two-hybrid library, and identified several candidate WT-1-interacting proteins. These proteins interacted with WT1, but not with another zinc finger transcription factor YY1 (25) or the p65 subunit of P53 kinase (33) in yeast. Analysis of the predicted amino acid sequence of one of the cloned candidate proteins, F1, showed that F1 contained previously described WD40 repeat motifs (23, 24). We subsequently isolated a full-length cDNA (1.4 kb), which we will refer to here as Ciao 1 (“bridge” in Chinese; GenBank™ accession number U63810). We believe that this clone encompasses the complete coding region of Ciao 1, as inasmuch as a translation initiation sequence is found (GGGCGAGATCG) that conforms well with the Kozak consensus sequence (RNNATGG, where N represents nucleotides A, C, G, or T, and R represents an A or T).
G; Ref. 34). Furthermore, the consensus AUG can be efficiently used in a rabbit reticulocyte lysate transcription/translation system to produce a protein of a molecular weight similar to that predicted from the amino acid sequence (data not shown).

Ciao 1 contains 7 putative WD40 motifs, which were compared with the WD40 consensus (23, 24, 35) and were also aligned to obtain a Ciao 1 WD40 consensus sequence. This comparison shows that Ciao 1 is a member of the WD40 family of proteins (Fig. 1A). A search of the data base using the predicted amino acid sequence of Ciao 1 reveals the presence of a putative homolog in yeast Saccharomyces cerevisiae of 330 amino acids (GenBank™ accession no. U51030). Alignment of the human Ciao 1 amino acid sequence with the putative yeast homolog shows that they share 42% identity and 64% similarity over the entire polypeptide (Fig. 1B).

Physical Interaction between Ciao 1 and WT1—As described above, a partial clone of Ciao 1, H3–45, was shown in the yeast two-hybrid assay to interact specifically with WT1 but not with another zinc finger-containing transcriptional repressor YY1. To determine whether full-length Ciao 1 could also specifically interact with WT1 and to confirm the WT1/H3–45 interaction observed in yeast, the GST affinity matrix-based assay (30) was used as an independent approach to assess the specificity of the interactions. Full-length p53, Ciao 1, and RACK 1, another WD40-containing protein which binds activated protein kinase C (36), were fused to GST, and the fusion proteins were allowed to interact with in vitro translated, 35S-labeled WT1(A). After extensive washing, bound WT1 was separated by SDS-PAGE and visualized by autoradiography. As shown in Fig. 2A, WT1 was retained specifically by GST-Ciao 1 (lane 2) and GST-p53 (lane 3) as expected (16), but not by GST-RACK 1 (lane 4) or by GST alone (lane 5). The RACK 1 protein is composed almost entirely of seven WD40 motifs, a structure very similar to that of Ciao 1, thus serving as a stringent control for the specificity of the WT1/Ciao 1 interaction. Taken together, these data suggest that the interaction between WT1 and Ciao 1 is specific.

WT1 is expressed both in normal cells and in Wilms tumors as a mix of four alternatively spliced protein isoforms A–D. We asked whether Ciao 1 can interact with all four isoforms of WT1. As shown in Fig. 2B, all four WT1 isoforms interacted with GST-Ciao 1 (lanes 1–4) and GST-p53 (lanes 7–10) but not with GST alone (lane 5). The RACK 1 protein does not interact with the WT1 isoforms (lanes 13–16). To map the region of WT1 that mediates its interaction with Ciao 1, we produced 35S-labeled WT1-(1–182) and WT1-(307–429) containing the putative WT1 repression and zinc finger DNA binding domains, respectively (4). GST-Ciao 1 interacted specifically with the carboxyl-terminal zinc finger domain of WT1 (lane 6), but did not bind the...
Ciao 1 Modulates WT1-mediated Transcriptional Activation

In Vivo Interaction between Ciao 1 and WT1—
To determine if WT1 and Ciao 1 can interact in vivo, we expressed both FLAG tagged Ciao 1 (FLAG-Ciao 1) and WT1 in 293 cells, an embryonic kidney cell line. Cells were lyzed, and WT1 was immunoprecipitated with a polyclonal anti-WT1 antibody (Santa Cruz Biotechnology, CA). Subsequent Western analysis with an anti-FLAG monoclonal antibody revealed a band (Fig. 3, lane 1) corresponding to the FLAG-Ciao 1 fusion protein. In contrast, immunoprecipitation with normal rabbit serum alone did not result in the appearance of the FLAG-Ciao 1 band (lane 3). In addition, FLAG-Ciao 1 was not detected following immunoprecipitation/Western analysis in 293 cells expressing FLAG-Ciao 1 but lacking transfected WT1 (lane 2). Because the presence of the FLAG-Ciao 1 band is dependent on WT1 expression, this result suggests that co-precipitation of FLAG-Ciao 1 is not likely due to cross-reactivity of the WT1 antibody. These data show that when Ciao 1 and WT1 are expressed in the same cell, the two can form a complex that can be detected by co-immunoprecipitation. Together with the yeast two-hybrid and GST-binding data, these results strongly argue that WT1 and Ciao 1 can physically interact with one another.

WT1 and Ciao 1 Tissue and Cellular Expression—
For the WT1/Ciao 1 interaction to be physiologically relevant, we expected both proteins to be expressed in some of the same tissues. We screened adult human poly(A)+ mRNA from a variety of tissues for both WT1 and Ciao 1 expression by Northern blot. Consistent with previous observations (37–39), a 3.6-kb WT1 mRNA species is strongly expressed in spleen, testis, and ovary, weakly expressed in heart and kidney, and very weakly expressed in prostate and colon. We detected two major species of approximately 4.4 and 1.5 kb of mRNA that hybridized with the Ciao 1 probe in all tissues, indicating that the expression of Ciao 1 mRNA is ubiquitous (data not shown).

WT1 is a nuclear protein but is retained in the cytoplasm upon activation of protein kinase A (40). To determine Ciao 1 subcellular localization, Ciao 1 was expressed in 293 human embryonic kidney cells as a fusion protein with a FLAG epitope attached to the amino terminus (FLAG-Ciao 1). Cells were fractionated into cytoplasmic and nuclear preparations (41), and the presence of Ciao 1 was determined by Western blot. As shown in Fig. 4, FLAG-Ciao 1 was detected in both the cytoplasmic and nuclear fractions (lanes 1 and 2). Co-transfection with WT1 and analysis by Western blot with a polyclonal anti-WT1 antibody (Santa Cruz Biotechnology) revealed greater than 90% of the transfected WT1 protein in the nuclear fraction (Fig. 4, lanes 3 and 4). As a quality control for the fractionation procedure, the cytoplasmic and nuclear lysates were probed for the expression of the cytoplasmic protein p70s6k (342) with an anti-p70s6k polyclonal antibody. p70s6k was found only in the cytoplasmic fraction (Fig. 6, lanes 5 and 6), indicating that there was little carryover of cytoplasmic proteins into the nuclear fraction. We have recently produced a polyclonal anti-Ciao 1 antibody. Western analyses of a variety of human cell lines with this antibody revealed a band corre-
and detected by Western blot using cytoplasmic lysates were prepared from 293 cells expressing trans-
pEGR3TKCAT and RSV-WT1 resulted in a dose-dependent de-
nated by WT1 (Fig. 6)

RSV-WT1 A, we observed a 13-fold transcription activation medi-

no effect on pEGR3TKCAT reporter activity (Fig. 6)

GST-Ciao 1 caused an increase in the mobility of the WT1 

complex, resulting in the formation of a new complex 2 (lanes

YY1 binding site oligonucleotide (lane 3)

unlabeled WT1 oligonucleotide (lane 9)

rupted by addition of unlabeled YY1 oligonucleotide (lane 10)

lanes 14–16

in vitro

transcribed/translated WT1 forms a complex with a 32P-
labeled oligonucleotide containing a consensus WT1 binding 

sequence (Fig. 5A, complex 1). The formation of this complex 
can be specifically inhibited by addition of a 200-fold excess of 
unlabeled WT1 oligonucleotide (lane 2) but not by addition of a 
YY1 binding site oligonucleotide (lane 3). Addition of purified 

GST-Ciao 1 caused an increase in the mobility of the WT1-DNA 

complex, resulting in the formation of a new complex 2 (lanes

4–9). Complex 2 formation could also be inhibited by addition of 
unlabeled WT1 oligonucleotide (lane 9), but was not dis-
rupted by addition of unlabeled YY1 oligonucleotide (lane 10), 
indicating that this new complex contains the WT1 protein. As 
expected, complex 2 was supershifted by the addition of a 

polycional anti-WT1 antibody that resulted in formation of complex 3 (lane 11), which could also be specifically inhibited 
with the unlabeled WT1 oligonucleotide (lane 12) but not with 
the unlabeled YY1 oligonucleotide (lane 13). No change in 

the mobility of complex 2 was observed by addition of normal rabbit 
senm (lanes 14–16). At present, it is not known if the increase in 

mobility of complex 2 is the result of a change in conformation 

of the WT1-DNA complex as the result of addition of Ciao 1, 

and/or that certain WT1-associated proteins present in com-

plex 1 are being removed upon binding of Ciao 1 to WT1.

The formation of complex 2 is caused by Ciao 1 but not the 

GST portion of the fusion protein, inasmuch as addition of GST 
alone did not result in complex 2 (Fig. 5A, lanes 17–19). As an 
additional specificity control, in vitro transcribed/translated YY1 was incubated with 
32P-labeled YY1 oligonucleotide to form a specific YY1-DNA complex (Fig. 5B, lane 1). Formation of this complex was inhibited by the addition of a 200-fold excess of unlabeled YY1 oligonucleotide (lane 2) but not by unlabeled WT1 oligonucleotide (lane 3). Addition of purified 

GST-Ciao 1 (lane 4) or GST alone (lane 5) had no effect on the 
mobility of the YY1-DNA complex, indicating that the effect of 

GST-Ciao 1 on the WT1-DNA complex is specific.

Ciao 1 Can Inhibit WT1-mediated Transcriptional Activation— WT1 A and B isoforms have been shown to activate a herpes simplex virus TKCAT reporter construct containing three EGR1/ WT1 binding sites (pEGR,TKCAT) (17). As expected, when we transactivated the pEGR,TKCAT reporter into 293 cells along with RSV-WT1 A, we observed a 13-fold activation mediated by WT1 (Fig. 6A, lane 2), similar to that demonstrated previously (17, 21). Addition of pCMV-FLAG-Ciao 1 or pCMV alone had no effect on pEGR,TKCAT reporter activity (Fig. 6A, lanes 4 and 5). Co-transfection of increasing amounts of pCMV-FLAG-Ciao 1 with pEGR,TKCAT and RSV-WT1 resulted in a dose-dependent de-
crease in WT1-mediated transcriptional activation (Fig. 6A, lanes 6–8). Addition of 5 μg of pCMV-FLAG-Ciao 1 resulted in a 3-fold decrease in activation (Fig. 6A, lane 8), whereas co-transfection of the same amount of pCMV expression vector with pEGR,TKCAT and RSV-WT1 had no effect on WT1-mediated activation (Fig. 6A, lanes 9–11).

WT1 and the closely related transcription factor, EGR1, share a common DNA binding site (9). To ensure that modulation of WT1-mediated transcriptional activation by FLAG-Ciao 1 is not due to a nonspecific function of the protein, we assayed for the effect of FLAG-Ciao 1 on EGR1-mediated transcriptional activation. We found that addition of 1–5 μg of pCMV-

FLAG-Ciao 1 or pCMV alone had no effect on EGR1-mediated transcriptional activation (data not shown). These data suggest that the effect of Ciao 1 on WT1 transcriptional activation function is specific and requires a protein-protein interaction between Ciao 1 and WT1.

One possible explanation for the decrease in WT1-mediated transcriptional activation upon co-expression of Ciao 1 could be that Ciao 1 somehow affected the level of expression of the transfected WT1. We examined cell lysates of 293 cells transfected with pRSV-WT1 A and pCMV-FLAG-Ciao 1 for WT1 and FLAG-Ciao 1 expression by Western blot (Fig. 6B). Transfection of 1–5 μg of pCMV-FLAG-Ciao 1 resulted in a steady increase in expression of a ~48-kDa protein corresponding to FLAG-Ciao 1. However, this increase in expression of FLAG-

Ciao 1 did not decrease the WT1 protein level (Fig. 6B). Thus, the ability of Ciao 1 to inhibit WT1-mediated transcriptional activation is not due to a reduction in WT1 protein level.

**DISCUSSION**

We have identified and cloned a novel member of the WD40 family of proteins, Ciao 1, which interacts with the tumor suppressor protein WT1. We demonstrated the Ciao 1/WT1 interaction in vitro using GST-based assays and in vitro (mam-

malian and yeast cells) by co-immunoprecipitation and the yeast two-hybrid assay. Ciao 1 mRNA was ubiquitously expressed in adult human tissues, and the protein product was detected in both nuclear and cytoplasmic preparations. Func-

tionally, Ciao 1 can modify a WT1-DNA complex and can spe-
cifically inhibit WT1-mediated transcriptional activation. Taken together, our findings suggest that Ciao 1 is a candidate protein that may regulate WT1 transcription and physiological functions in vivo.

The Ciao 1 protein of 339 amino acids is a member of an ancient family of proteins, consisting of repeating domains termed β-transducin-like or WD40 repeats (23, 24, 35). A search of the data base revealed the existence of a putative yeast homolog of human Ciao 1 also containing seven WD40 repeats with a similar consensus sequence. The presence of this homolog in yeast and mouse (as determined by Northern blot) shows that the gene is conserved during evolution and suggests that the protein is functionally important. Indeed, we find that the putative yeast Ciao 1 is essential for viability.²

² Y. Shi, unpublished results.

The WD40 motif is thought to act as an interface for protein-protein interactions. The probable secondary structure is one of β-sheets separated by turns (23, 44). WD40 repeat-containing proteins possess a variety of cellular functions, including cell signaling, RNA splicing, transcription, meiosis and vesicular traffic (23). Of particular interest in this regard are WD40 proteins known to be involved in RNA polymerase II transcription. For instance, Drosophila TAF₆₈₀ contains six WD40 re-

peats and is an essential component of TFIID, a complex con-
taining the TATA-binding protein and associated TAFs, that is required for activated transcription (45). The yeast Tup1 pro-

Fig. 4. Subcellular expression of Ciao 1 and WT1. Nuclear and cytoplasmic lysates were prepared from 293 cells expressing trans-
fected FLAG-Ciao 1 and WT1. Proteins were separated by SDS-PAGE and detected by Western blot using α-FLAG, α-WT1, and α-p70s6k antibodies. Molecular size markers (kDa) are indicated on the left, and cytoplasmic and nuclear fractions are indicated by the letters C and N on the top.
tein contains six WD40 repeats and functions as a transcriptional repressor (46).

Tup1 is complexed with another protein, SSn6, and the Tup1-SSn6 complex has been shown to be a general repressor involved in the regulation of mating type $\alpha$-specific, haploid-specific, and glucose-repressible genes in yeast (47). Tup1-SSn6 is recruited to glucose-repressible promoters via interactions with the transcription factor Mig1 (47), which is related to mammalian WT1 in the zinc finger region (48). It had been shown previously that Tup1 fused to the LexA DNA binding domain can repress transcription from a reporter gene containing LexA DNA binding sites (49). Given the fact that Ciao 1 is a WD40 protein like Tup1, that it interacts with WT1 (which is related to Mig1), and that it suppresses WT1-mediated transcription, we thought it possible that Ciao 1 may function as a transcriptional repressor like Tup1. We therefore tested the intrinsic transcriptional regulating properties of Ciao 1 by fusing it to the yeast GAL4 DNA binding domain. However, expression of this fusion protein with a CAT reporter plasmid containing five GAL4 DNA binding sites did not affect CAT activity (data not shown). This is perhaps not surprising, as the COOH-terminal domain of Tup1 containing the WD40 repeats is not sufficient to mediate repression but is necessary for full repression activity of wild type Tup1 (49). Repression by the full-length Tup1 fused to the LexA DNA-binding domain occurs in the absence of functional SSn6 and the putative repression domain of Tup1 is mapped to the NH$_2$-terminal region. The WD40 repeats of Tup1 have been shown recently to be involved in a direct association with the $\alpha_2$ transcription factor (50). Thus, the WD40 repeats of Tup1 may play a role in positioning the repressor complex at specific promoters, whereas transcriptional repression is mediated by a different region of Tup1. Ciao 1 consists almost entirely of WD40 repeats, and, like Tup1, these repeats can interact with a DNA binding protein such as WT1, inhibit WT1 transcriptional activation activity, but themselves do not possess intrinsic transcriptional repression activity.

How then does Ciao 1 function to inhibit WT1-mediated transcriptional activation? We ruled out the possibility that Ciao 1 affects expression of the transfected WT1. We believe that the effect of Ciao 1 on transcriptional activation mediated by WT1 is specific because Ciao 1 fails to affect transcription

![Figure 5. Binding of Ciao 1 to a WT1-DNA complex. A, Ciao 1 can affect the mobility of a WT1-DNA complex. In vitro translated WT1 was incubated with $^{32}$P-labeled oligonucleotide containing a consensus WT1 binding site to form a specific complex (complex 1). Unlabeled competitor oligonucleotides (200-fold excess) containing either the WT1 consensus site or the YY1 consensus site were added as indicated by + or − signs on the top. GST-Ciao 1 was added either in a dose range of 1–0.032 µg (lanes 4–8) or as a standard amount of 1 µg (lanes 9–16) as indicated. Approximately 1 µg of GST alone was also added (lanes 17–19). Supershift experiments were performed with either an α-WT1 polyclonal antibody or preimmune rabbit serum as indicated. WT1-DNA complexes 1, 2, and 3 are indicated by arrows on the right. B, Ciao 1 does not affect a YY1-DNA complex. In vitro translated YY1 was incubated with $^{32}$P-labeled oligonucleotide containing a consensus YY1 binding site. Excess unlabeled YY1 or WT1 oligonucleotides and approximately 1 µg of GST-Ciao 1 or GST alone were added, as indicated by + and − signs on the top.

Ciao 1 Modulates WT1-mediated Transcriptional Activation
mediated by the WT1-related transcriptional activator, EGR1. It is possible that interaction of Ciao 1 with WT1 inhibits binding of WT1 to its cognate DNA binding sites. For instance, the interferon-inducible protein p202 inhibits transcriptional activation by c-Jun and c-Fos by binding to Jun-Jun homodimers or Jun-Fos heterodimers and blocking binding to DNA (51). We have identified the binding domain of WT1 for Ciao 1 to be within the COOH-terminal zinc finger domain shown previously to be necessary and sufficient to bind DNA (4, 52). In vitro translated WT1 can efficiently bind to a GC-rich oligonucleotide, described previously as an EGR1 consensus site (17). However, addition of recombinant Ciao 1 did not result in a decrease in WT1 binding to DNA but did result in a change in mobility of the WT1-DNA complex. These observations, together with the finding that Ciao 1 itself does not have intrinsic transcriptional repression activity, suggest that Ciao 1 may affect the transcriptional activity of WT1 by either causing a conformation change of the WT1 protein that masks its activation function, or by negatively interfering with the communication between the activation domain of WT1 and the basal transcriptional machinery.

Previous work on the Drosophila Esc protein, which contains six WD40 repeats, suggests that it blocks transcription by competing through its WD40 domains with the Drosophila TAFII80 for interaction with other components of the TFIID complex (53). It is possible that when recruited to the promoter through interactions with WT1, Ciao 1 may work in a manner similar to that for the Esc protein, perhaps by competing with human TAFII100, the human homolog of dTAFII80 (54, 55). At present, it is unknown whether Ciao 1 can interact with members of the RNA polymerase II basal transcription machinery. Studies are under way to identify and clone Ciao 1-interacting proteins. In sum, we have isolated a novel WD40 motif-containing protein that can physically interact with WT1, which results in a decrease in the transcriptional activation activity of WT1.

Finally, we have mapped the gene for Ciao 1 to chromosome 2q11.1–q11.2 (data not shown). This chromosomal region has not been described previously to be associated with Wilms' tumorigenesis, and it will be interesting to determine whether Ciao 1 mutations may be associated with a subset of Wilms' tumor patients in future studies. Given the known role of WT1 in the development of the genitourinary system (19), it is possible that mutations of Ciao 1 may be associated with urogenital malformation. Thus far, two potential candidate genes, familial nephronophthisis (NPHP1) and a putative human homolog to the mouse mutation Danforth's short tail (Sd), have been identified within the proximal 2q region that give rise to malformations of the urinary system. NPHP1 has been localized to chromosome region 2q13 (56), which is cytogenetically close to Ciao 1. A large 250-kb deletion is the most frequent mutation found in familial nephronophthisis patients, and point mutations within NPHP1 have been detected in some individuals (57). However, the presence of point mutations of NPHP1 has so far always been associated with a deletion on the other chromosome, suggesting the possibility that haploinsufficiency for additional gene(s) not yet identified may be involved in the phenotype. Thus, Ciao 1 would be an attractive candidate due to its association with the WT1 product. The mouse mutation Danforth's short tail (Sd) is a semidominant mutation, which affects the skeleton and urogenital system (58, 59). Sd is tightly linked to Pax-8 on mouse chromosome 2, and human PAX-8 is at 2q12 (60). A recombination between Sd and Pax-8 has been demonstrated (43), thereby excluding Pax-8 as the gene for Sd, but due to the tight linkage between Pax-8 and Sd, it is not unlikely that a human counterpart of Sd would be on proximal 2q. Further studies are required to determine if Ciao 1 is the human gene for Sd.

Acknowledgments—We thank members of the laboratory for helpful comments, and Jennie Staple for assistance in manuscript preparation. We acknowledge the expert technical help of Kate Nielsen and Winni Pedersen. We thank R. Brent (Massachusetts General Hospital) for the two-hybrid reagents, J. Licht (Mount Sinai School of Medicine) for plasmids, and J. Blenis and R. Tung (Harvard Medical School) for the anti-\(\text{p70}^{\text{S6K}}\) antibody.
