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Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation

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Phosphorylation is one of the major post-translational mechanisms by which the activity of transcription factors is regulated. We have investigated the role of phosphorylation in the regulation of nucleic acid binding activity and the nuclear translocation of WT1. Two recombinant WT1 proteins containing the DNA binding domain with or without a three amino acid (KTS) insertion (WT1ZF+KTS and WT1ZF–KTS) were strongly phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) *in vitro*. Both PKA and PKC phosphorylation inhibited the ability of WT1ZF+KTS or WT1ZF–KTS to bind to a sequence derived from the WT1 promoter region in gel mobility shift assays. The binding of WT1ZF–KTS to an EGR1 consensus binding site was also inhibited by prior PKA and PKC phosphorylation. We also demonstrate the RNA binding activity of WT1, but this was not altered by phosphorylation. PKA activation by dibutyryl cAMP in WT1-transfected cells resulted in the reversal of WT1 suppression of a reporter construct. Although WT1 protein is predominantly localized to the nucleus, this expression pattern is altered upon PKA activation, resulting in the cytoplasmic retention of WT1. Accordingly, phosphorylation may play a role in modulating the transcriptional regulatory activity of WT1 through interference with nuclear translocation, as well as by inhibition of WT1 DNA binding.

Keywords: cellular translocation/DNA binding/ phosphorylation/transcription factor/Wilms tumor

Introduction

The Wilms tumor suppressor gene WT1 encodes a transcription factor which is a member of the zinc finger protein family. Mutations in WT1 have been found in a subset of patients with Wilms tumor and in patients with a syndrome (Denys–Drash) characterized by nephropathy, urogenital anomalies and a predisposition towards developing Wilms tumor (reviewed in Coppes *et al.*, 1993). The WT1 gene spans >50 kb and contains 10 exons (Haber *et al.*, 1991). Two alternative splice sites are utilized in the WT1 gene to generate four RNA transcripts. The first splice introduces 17 amino acids just proximal to the first of four zinc fingers, and the second results in three amino acids (lysine, threonine and serine; KTS) inserted between the third and fourth zinc fingers (Haber *et al.*, 1991). The product of the WT1 gene exhibits structural features common to transcription factors, including a C-terminal DNA binding domain composed of the four zinc fingers and an N-terminal proline–glutamine-rich regulatory domain. The C-terminal region of WT1 (zinc fingers 2–4) shows a 60% amino acid sequence homology to the three zinc fingers of the early growth response (EGR1) protein family, which contains three zinc fingers (reviewed in Sukhatme, 1990). Like other EGR1 family members, WT1 DNA binding protein (–KTS form) can recognize a common GC-rich DNA sequence (5′-GCGGGGGCG-3′) and binds to this sequence, albeit with lower affinity than the EGR proteins (Rauscher *et al.*, 1990; Madden *et al.*, 1991). This specific DNA binding activity results in the transcriptional suppression of reporter constructs in transient transfection assays (Madden *et al.*, 1991). So far, several WT1 binding sites have been identified in the promoter regions of certain genes, including insulin-like growth factor II (IGF-II; Drummond *et al.*, 1992), IGF-I receptor (Werner *et al.*, 1993), platelet-derived growth factor A-chain (PDGF-A; Gashler *et al.*, 1992), transforming growth factor-β (TGF-β; Dey *et al.*, 1994), Pax-1 (Ryan *et al.*, 1995), retinoic acid receptor (Goodyer *et al.*, 1995), colony stimulating factor I (Harrington *et al.*, 1993), *bcl*-2 and *c*-myc (Hewitt *et al.*, 1995). Each of these promoter activities can be repressed by WT1 in cotransfection assays. However, with the possible exception of IGF-II, the natural downstream target genes regulated by WT1 are still unknown. Both the +KTS and –KTS forms of WT1 are capable of footprinting regions in the WT1 promoter (Campbell *et al.*, 1994; Rupprech *et al.*, 1994) and functioning as transcriptional repressors of WT1 promoter-regulated reporter constructs (Rupprech *et al.*, 1994). Thus, in addition to regulating downstream targets, WT1 may act as a negative regulator of its own expression. Alteration of transcriptional function may play a pivotal role in the development of Wilms tumors. Loss of function of WT1 protein can result from mutations within the zinc finger domains of the gene which destroy the DNA binding activity of the protein, or in the more N-terminal regions of the protein to block transcriptional repression or activation.

WT1 may also bind to RNA (Larsson *et al.*, 1995). In eukaryotic cells, numerous RNA binding proteins and small RNAs play key roles in the post-transcriptional regulation of gene expression, including pre-mRNA splicing and polyadenylation. RNA binding proteins can also form stable ribonucleoprotein (RNP) complexes that serve to transport and localize RNAs. Mutations in RNA binding proteins are known to account for some genetic disorders and, potentially, malignancy (reviewed in Burd and Dreyfuss, 1994). Although a function for WT1 RNA

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binding is not yet clear, WT1 may play a role in post-transcriptional processing of RNA. This is supported by co-localization of WT1 with splicing factors and the association of WT1 with one or a limited number of components in spliceosomes and coiled bodies (Larsson *et al.*, 1995). WT1 may also be autoregulatory. The WT1 locus is complex and is characterized by antisense transcription of an RNA termed WIT-1 (Huang *et al.*, 1990), which is expressed in the same temporal and spatial manner as WT1 (Yeger *et al.*, 1992). WT1 binding sites occur upstream of the major transcription start of WT1. They can bind WT1 protein *in vitro* (Campbell *et al.*, 1994) and regulate appropriate reporter constructs *in vivo*. It remains possible that WT1 could also interact with the antisense WIT-1 RNA.

Protein phosphorylation has been recognized as one of the major mechanisms regulating a diverse range of cellular processes. Phosphorylation can regulate transcription factor activity by affecting DNA binding activity either positively or negatively. For example, the transactivation of myogenin, which belongs to a family of helix–loop–helix proteins that activate muscle transcription through binding to a conserved DNA sequence or E box (Murre *et al.*, 1989; Chakraborty *et al.*, 1991), is negatively regulated by fibroblast growth factor (FGF) and protein kinase C (PKC). FGF and PKC induce phosphorylation of a specific site, Thr87, in the DNA binding domain of the protein, which results in a loss of DNA binding activity and inactivation of the transcriptional activity of the protein (Li *et al.*, 1992). Phosphorylation also plays an important role in the regulation of nuclear translocation. Nuclear protein import is a critical control point in the regulation of nuclear gene expression following signal transduction. Many transcription factors are synthesized and reside in the cytoplasm in an inactive form, but under certain conditions are subject to specific activation mechanisms which allow nuclear translocation. Protein phosphorylation may be an essential component for nuclear translocation or may result in sequestration in the cytoplasm, thereby preventing access to their cognate genes. For example, protein kinase A (PKA)-catalyzed phosphorylation results in the cytoplasmic retention of $p68^{c\text{-}rel}$ (Mosialos *et al.*, 1991). Phosphorylation, on the other hand, can also accelerate nuclear import, as seen with SV40 T-antigen phosphorylation by casein kinase II (CKII; Rihs *et al.*, 1991).

There are several potential phosphorylation sites within the WT1 DNA binding domain. These consensus sequences for phosphorylation are conserved in the DNA binding domain of members of the EGR zinc finger proteins. Studies have shown that phosphorylated forms of EGR1 bind to its cognate element more efficiently than nonphosphorylated forms (Huang and Adamson, 1994). Accordingly, we have determined whether WT1 can be phosphorylated *in vitro*, and if this alters the DNA binding activity of the different isoforms of WT1. We show that WT1 can be phosphorylated *in vitro* by kinases present in tissue extracts. WT1 can also be phosphorylated by cAMP-dependent PKA, PKC and CKII *in vitro*. Phosphorylation by these kinases inhibits the ability of WT1 proteins to bind cognate DNA elements, but not RNA transcripts. Transient transfection assays show that the activation of PKA relieves the transcriptional repression

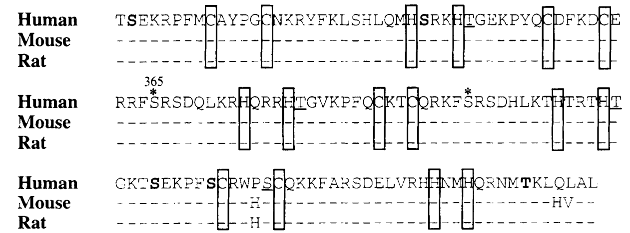

Fig. 1. Comparison of human, mouse and rat WT1ZF protein sequences. Cysteine (C) and histidine (H) residues involved in zinc coordination are boxed. The potential PKC phosphorylation sites are shown in bold. Underlined residues represent consensus sites for both PKC and PKA. (*) The amino acids that are possible phosphorylation sites for PKC, PKA and CKII. Serine 365 of the human WT1 protein is indicated by number.

activity of WT1. PKA activation also alters the predominant nuclear staining pattern of WT1 and results in the sequestration of WT1 protein in the cytoplasm. WT1 is partially co-localized with p53 proteins in the nucleus; however, p53 does not co-localize with WT1 into cytoplasm in response to forskolin treatment. Taken together, these results suggest that PKA phosphorylation may negatively regulate the transcriptional activity of WT1 by either sequestering WT1 protein in the cytoplasm and/or inhibiting WT1 DNA binding.

Results

WT1 proteins are phosphorylated in vitro

An examination of the human WT1 amino acid sequence for consensus sites of phosphorylation by known protein kinases (reviewed in Pearson and Kemp, 1991) reveals a number of potential phosphorylation sites for PKA and PKC in the C-terminal domain of WT1 (Figure 1). Two CKII consensus sites are also present. To test if WT1 can be phosphorylated by protein kinases *in vitro*, we expressed the C-terminal portion of two WT1 isoforms (amino acid residues 284–446 or 284–449) in bacteria as hexahistidine-tagged proteins (Figure 2A). Two forms of the truncated protein with or without a three amino acid insertion (KTS) between zinc fingers 3 and 4 (WT1ZF+KTS and WT1ZF–KTS respectively) were first tested for their ability to be phosphorylated by nuclear extracts. Nuclear extracts from adult rat kidney, spleen and liver can phosphorylate WT1 proteins in the presence of [^{32}P]ATP *in vitro* (Figure 2B). To investigate further the phosphorylation of WT1, a panel of serine/threonine kinases, including PKA, PKC, CKII and p34 cdc2, were used to phosphorylate WT1 proteins. Both forms of WT1 can be phosphorylated by all four kinases, albeit to varying degrees (Figure 2C). WT1 proteins are strongly phosphorylated by PKA and PKC (Figure 2C, lanes 1–4), but only weakly phosphorylated by CKII and p34 cdc2 (Figure 2C, lanes 5–8). A calculation of phosphate incorporation (Table I) showed that PKC phosphorylation resulted in more phosphate incorporated into the WT1 proteins than that incorporated as a result of PKA activity. As expected, phosphorylation by CKII and p34 cdc2 is much less efficient. Thus, the C-terminal domain of WT1 is preferentially phosphorylated by PKA and PKC *in vitro*.

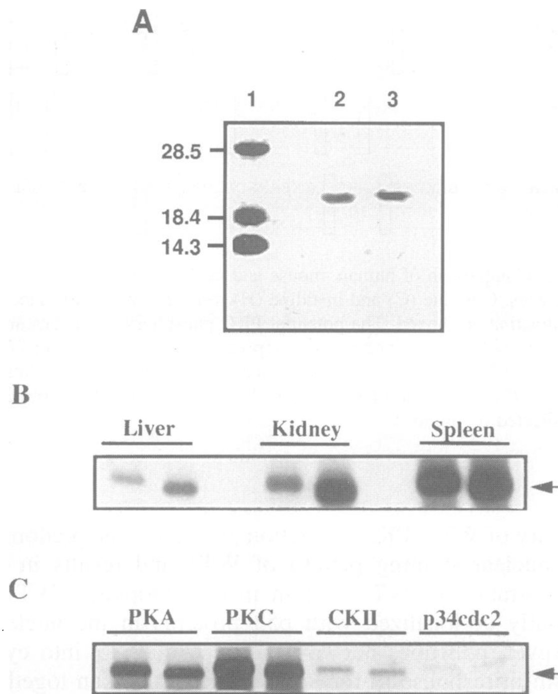

Fig. 2. *In vitro* phosphorylation of the C-terminus of WT1 proteins. (**A**) WT1ZF–KTS (lane 2) and WT1ZF+KTS (lane 3) were analyzed by SDS–PAGE followed by staining with Coomassie blue. Lane 1 contains molecular weight markers. Recombinant WT1ZF proteins were phosphorylated by rat nuclear extracts from liver, kidney and spleen as sources of kinases (**B**) and by serine/threonine kinases, including PKA, PKC, CKII and p34 cdc2 (**C**). The detailed experimental procedures are described in Materials and methods. The phosphorylated WT1ZF+KTS (lanes 1, 3 and 5 in B; lanes 1, 3, 5 and 7 in C) and WT1ZF–KTS (lanes 2, 4 and 6 in A; lanes 2, 4, 6 and 8 in C) are indicated by arrowheads.

Table I. Phosphate incorporation into WT1ZF proteins by serine/threonine kinases

Kinase	Phosphate incorporation (mol/mol protein)	
	WT1ZF+KTS	WT1ZF–KTS
PKA	1.2×10^{-1}	7.6×10^{-2}
PKC	3.8×10^{-1}	2.2×10^{-1}
CKII	5.9×10^{-3}	6.4×10^{-3}
p34 cdc2	8.2×10^{-3}	1.1×10^{-2}

Inhibition of WT1 DNA binding following PKA, PKC and CKII phosphorylation in vitro

The phosphorylation of transcription factors can dramatically alter their ability to interact with DNA. For example, the STAT family of proteins cannot bind their cognate DNA elements unless they are phosphorylated on tyrosine residues (reviewed in Darnell *et al.*, 1994). To determine if phosphorylation alters the capability of WT1 to interact with DNA, we used gel mobility shift assays to test the effect of kinase phosphorylation on DNA binding by WT1. The bacterially expressed and chromatographically purified WT1ZF+KTS and WT1ZF–KTS proteins were first subjected to kinase reactions and a portion of the total reaction used for DNA binding assays. In the presence of PKA but in the absence of ATP both WT1ZF+KTS and WT1ZF–KTS are able to interact with a WT1 oligo-

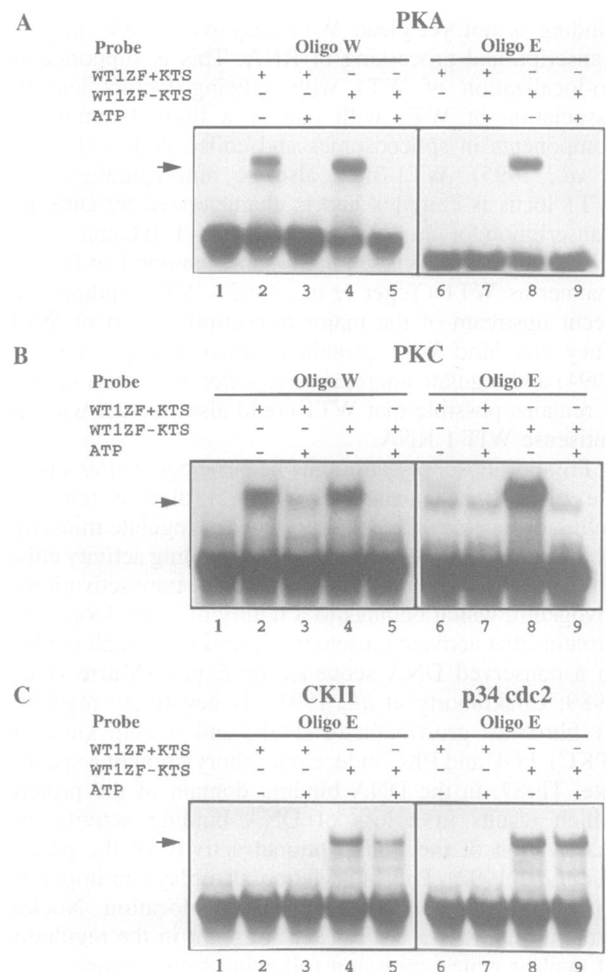

Fig. 3. Gel mobility shift assays with phosphorylated and unphosphorylated WT1ZF proteins. Bacterially produced +KTS and –KTS forms of WT1ZF proteins were treated with PKA (**A**), PKC (**B**), CKII or p34 cdc2 (**C**) in the presence (+) or absence (–) of ATP, and tested in gel mobility shift assays using either an oligo W probe or an oligo E probe. The labeled DNA–protein complexes are indicated by arrowheads. Lane 1 in (A), (B) and (C) contains probe only.

nucleotide binding site (oligo W; Figure 3A, lanes 2 and 4). WT1ZF–KTS can also bind to the EGR1 site (oligo E; Figure 3A, lane 8). However, upon addition of ATP, the binding of WT1ZF+KTS and WT1ZF–KTS to oligo W is severely impaired (Figure 3B, lanes 3 and 5), and the binding of WT1ZF–KTS to oligo E was nearly abolished (lane 9). In a similar assay, PKC phosphorylation also strongly inhibited the binding of both forms of WT1 to oligo W (Figure 3A, lanes 3 and 5) and WT1ZF–KTS binding to oligo E (Figure 3B, lane 9). The DNA binding activity of WT1ZF–KTS to oligo E is decreased by ~50% after the protein is phosphorylated by CKII (Figure 3C, lanes 4 and 5), but p34 cdc2 phosphorylation had no significant effect on WT1 interaction with oligo E (Figure 3C, lanes 8 and 9).

A limited number of PKC phosphorylation sites are involved in the regulation of WT1ZF–KTS DNA binding activity

As PKC had the most dramatic effect on WT1 DNA binding activity, we carried out kinetic experiments to determine if the effect of PKC phosphorylation on

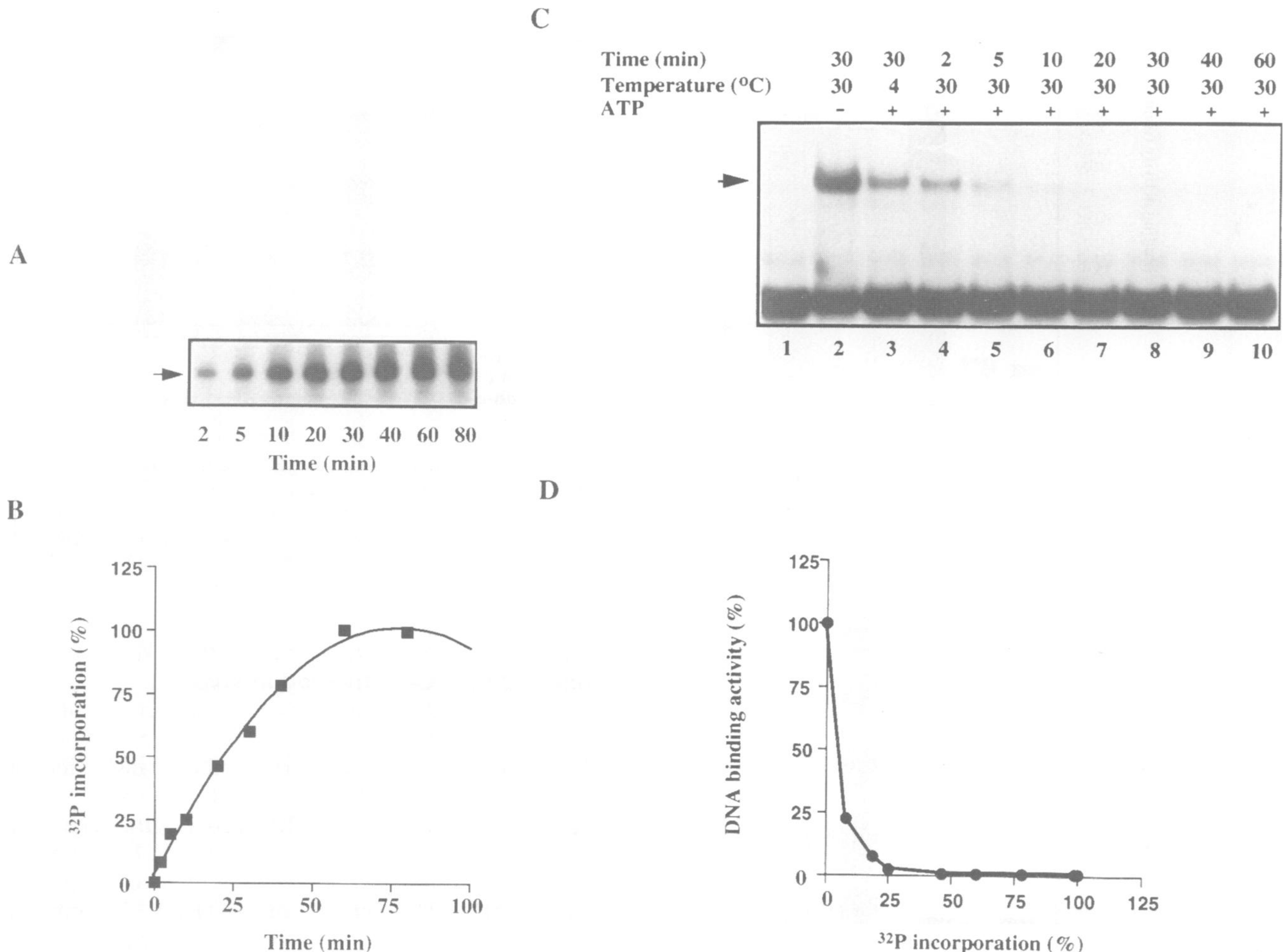

Fig. 4. Kinetics of WT1ZF phosphorylation by PKC and its effect on DNA binding activity. (**A**) Recombinant WT1ZF–KTS was subjected to *in vitro* PKC phosphorylation for varying times as indicated, followed by SDS–PAGE and autoradiography as described in Materials and methods. (**B**) Phosphate incorporation was quantified by a phosphorimager and is expressed as a percentage (%) relative to maximum ^{32}P incorporation. (**C**) WT1ZF–KTS was incubated with PKC, the necessary cofactors and ATP at 30°C for different periods of time as indicated. Reactions were stopped and combined with DNA binding buffer and ^{32}P-labeled oligo E probe, followed by a 30 min incubation on ice. Samples were subjected to a gel mobility shift assay (see Materials and methods). As a control (lane 2), no ATP was added to the reaction. (**D**) DNA binding activity is expressed as a percentage (%) relative to the DNA binding activity of unphosphorylated WT1ZF–KTS [control sample in (C), lane 2]. Quantification was determined by a phosphorimage analysis.

WT1ZF–KTS DNA binding was proportional to the amount of phosphate incorporated. After a 2 min kinase reaction, ~8% of maximal ^{32}P incorporation into WT1ZF–KTS was achieved (Figure 4A and B). The reaction was ~50% maximum at 20 min, and reached a plateau at 60 min. DNA binding and gel mobility shift assays were performed under the same conditions. WT1ZF–KTS DNA binding activity after phosphorylation by PKC at different times (Figure 4B and C) shows that after only 6% of the phosphate is incorporated WT1ZF–KTS has <30% of the DNA binding activity of the unphosphorylated protein. Incorporation of phosphate to 8% reduced WT1ZF–KTS DNA binding by 78%, while 24% of phosphate incorporation almost completely abolished the DNA binding activity of WT1ZF–KTS. A comparison of the kinetics of phosphate incorporation with DNA binding shows that the decrease in DNA binding activity of WT1ZF–KTS is not proportional to the increase in WT1ZF–KTS phosphorylation. Rather, because as little as 6% phosphate incorporation can severely reduce the DNA binding activity of WT1ZF–KTS, it seems likely that only one or very few specific phosphorylation sites are involved in the regulation of WT1ZF–KTS DNA binding.

Phosphorylation does not significantly change the ability of WT1ZF to bind to RNA

As WT1 has recently been suggested to bind RNA (Larsson *et al.*, 1995), we determined whether the RNA binding activity of WT1 could be demonstrated *in vitro* and, if so, whether this could be compromised by phosphorylation. A WIT-1 cDNA clone, pGB16 (Huang *et al.*, 1990), was used as a DNA template to generate RNA probes by *in vitro* transcription in the presence of [^{32}P]CTP. The RNA transcripts were then combined with hexahistidine-tagged WT1ZF+KTS and WT1ZF–KTS proteins coupled to Ni-NTA resin, and the RNA binding activities of the proteins were assessed. An extract made from bacteria transformed with the vector pQE10 alone and prepared in the same fashion as WT1ZF proteins was used as a control (Figure 5A, NC). An unrelated hexahistidine-tagged protein, the oxygenase domain of nitric oxide synthase (NOS), was also used as a control. The NC and NOS controls

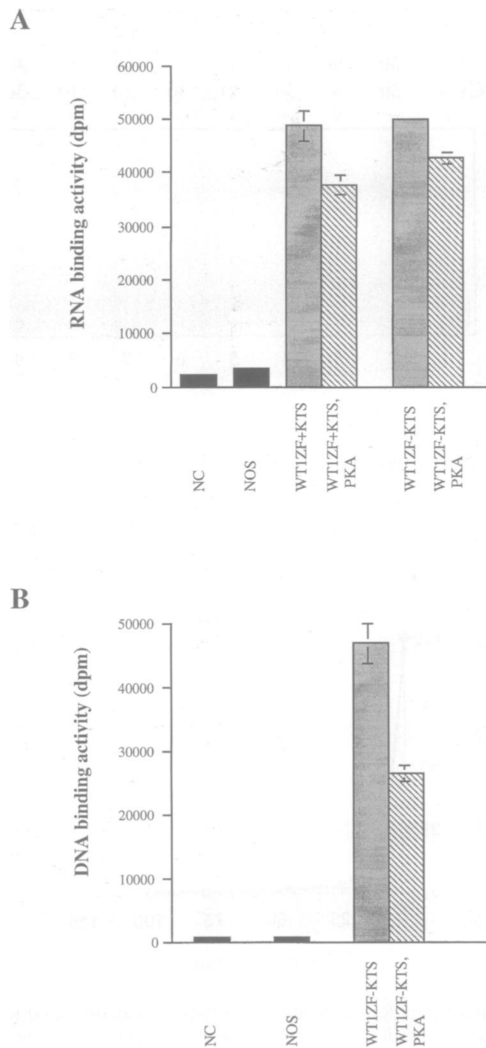

Fig. 5. Effects of PKA phosphorylation on WT1ZF RNA (**A**) and DNA (**B**) binding activities. Black bars represent control samples, as indicated. Unphosphorylated and phosphorylated WT1ZF proteins are indicated by gray and striped bars respectively. Binding activities are expressed by dpm from RNA or DNA–protein complexes. Each experiment was performed in triplicate.

showed only background RNA binding (Figure 5A). However, WT1ZF proteins showed >20-fold RNA binding activity over background, suggesting that both forms of zinc finger protein are capable of binding WIT-1 antisense transcripts to a similar extent. The RNA binding assays were also performed with recombinant WT1ZF–KTS and WT1ZF+KTS proteins after phosphorylation with PKA. The results (Figure 5A) showed that phosphorylation did not lead to a drastic change in WT1ZF RNA binding. The RNA binding activities of WT1 remained at ~78 and ~86% for WT1ZF+KTS and WT1ZF–KTS respectively after the proteins were phosphorylated by PKA. This experiment was repeated several times with consistent results. The effect of PKA phosphorylation on WT1ZF–KTS DNA binding was also assessed by this method using oligo E to make a comparison with RNA binding results (see Materials and methods). Following PKA phosphorylation, the DNA binding of WT1ZF–KTS was decreased (Figure 5B) in accordance with the results of the gel mobility shift assays.

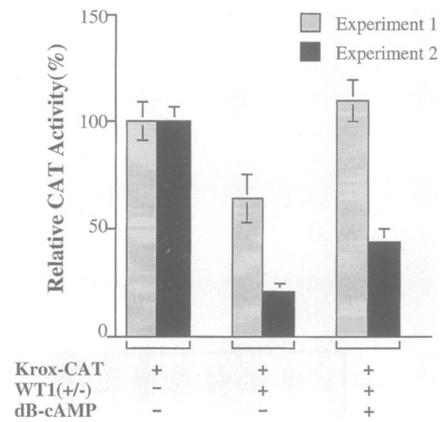

Fig. 6. Release of repressor activity of WT1 by PKA. Transfections and CAT assays were performed as described in Materials and methods. Data are expressed as the percentage of CAT activity relative to the reporter plasmid after normalizing for β-galactosidase. The error bars represent the standard deviation of three independent transfections of each sample as indicated. Experiments 1 and 2 are two independent transfections.

Transcriptional suppressor activity of WT1 is inhibited by PKA activation in vivo

Our data implied that the transcriptional activity of WT1 may be negatively regulated by PKA phosphorylation. To address this possibility, we performed co-transfection and chloramphenicol acetyl transferase (CAT) assays in cells treated with dibutyryl cAMP (dB-cAMP). The CAT reporter construct (Krox–CAT) contains a WT1-responsive promoter derived from the 5′ region of Krox 24 (Lanoix *et al.*, 1991). The activity of the Krox 24 promoter was repressed by the WT1 (+/–) isoform, following cotransfection into 3T3 cells (Figure 6). However, the transcriptional repression by WT1 was largely relieved following dB-cAMP treatment (5 μM for 16 h) prior to harvesting cells (Figure 6). This system does not allow us to distinguish whether the release of suppression by PKA activation is due to the inhibition of WT1 DNA binding or the alteration of WT1 nuclear translocation.

PKA activation alters the cellular localization of WT1

To determine whether the localization of the WT1 protein could be altered by phosphorylation, we examined the localization of WT1 in a stable transfectant of 56A1 cells (C2) expressing a full-length WT1 (+/+) isoform. The 56A1 cell line is a human glomerular visceral epithelial cell line immortalized by SV40 T-antigen (Delarue *et al.*, 1991). 56A1 parental cells express low levels of endogenous WT1, while C2 cells overexpress both WT1 mRNA and WT1 protein. When C2 cells were stained with C19, a polyclonal antibody raised against the C-terminal 19 amino acids of WT1 and analyzed by fluorescence microscopy, a typically diffuse, predominantly nuclear staining pattern (Englert *et al.*, 1995; Larsson *et al.*, 1995) was observed (Figure 7a). The majority of the C2 cells showed very bright nuclear staining, but a small number (20%) of the cells were weakly stained to a comparable expression level of parental cells (56A1; data not shown). This small population of C2 cells probably expresses only endogenous WT1. Of 200 cells from at least five fields that were counted (Table II), 172 cells showed exclusive nuclear

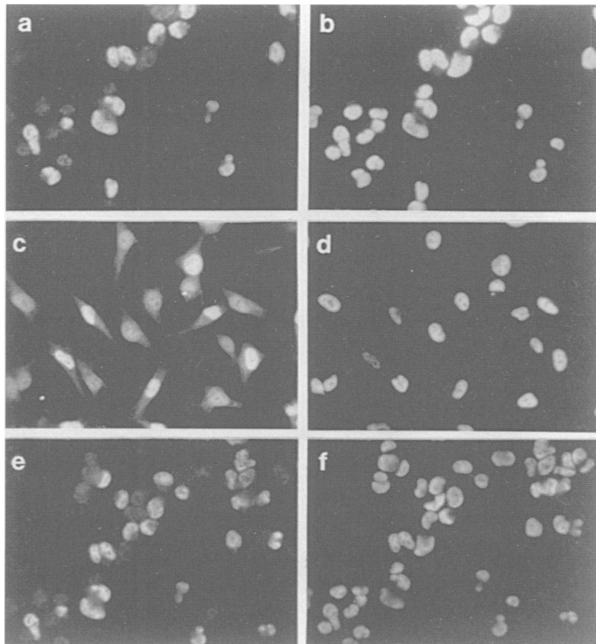

Fig. 7. Localization of WT1 and p53 proteins. C2 cells were double stained for WT1 (C19) (**a**, **c** and **e**) and p53 (DO1) (**b**, **d** and **f**), following no treatment (a and b), forskolin treatment (c and d) and PMA treatment (e and f).

Table II. Indirect immunostaining of C2 cells with anti-WT1 antibody

Treatment	Expression pattern	
	Nuclear	Nuclear and cytosolic
No treatment	172/200	28/200
Forskolin	3/200	197/200
PMA	176/200	24/200

staining and 28 cells showed both nuclear and weak cytoplasmic staining.

To investigate whether the nuclear localization of WT1 is regulated by PKA phosphorylation, we treated C2 cells with forskolin, a strong PKA activator. After 24 h, almost all the cells showed cytoplasmic and nuclear staining (Figure 7c). Less than 2% of the cells expressed WT1 exclusively in the nucleus (Table II). The intensity of cytoplasmic staining of each cell is about the same regardless of the expression level of WT1 in the nucleus. This result suggests that PKA phosphorylation may inhibit the translocation of WT1 into the nucleus, leading to increased cytoplasmic retention of the protein. To determine whether the cytoplasmic sequestration of WT1 is restricted to the activation of PKA, C2 cells were treated with 40 nM phorbol-12 myristate-13-acetate (PMA) for 16 h to activate PKC, and then stained with antibody C19. No alterations in WT1 localization were observed compared with untreated cells (Figure 7e). Of 200 cells, 176 showed exclusive nuclear staining and 24 cells showed both nuclear and weak cytoplasmic staining (Table II), suggesting that PKC activation does not result in the alteration of WT1 localization.

The physical association of WT1 and p53 proteins has been demonstrated previously in baby rat kidney cells and primary Wilms' tumors (Maheswaran *et al.*, 1993). This protein interaction was detected by sequential immuno-precipitation and immunoprecipitation–Western analyses of cellular lysates. As confocal microscopy has made possible the high-resolution analysis of protein co-localization in whole cells, we performed double staining on C2 cells with C19 antibody and DO1, a monoclonal antibody against p53 protein. We analyzed by fluorescence microscopy the expression patterns of WT1 and p53. C2 cells uniformly express a high level of p53 protein in the nucleus (Figure 7b), while the expression level of WT1 varied between cells as mentioned above (Figure 7a).

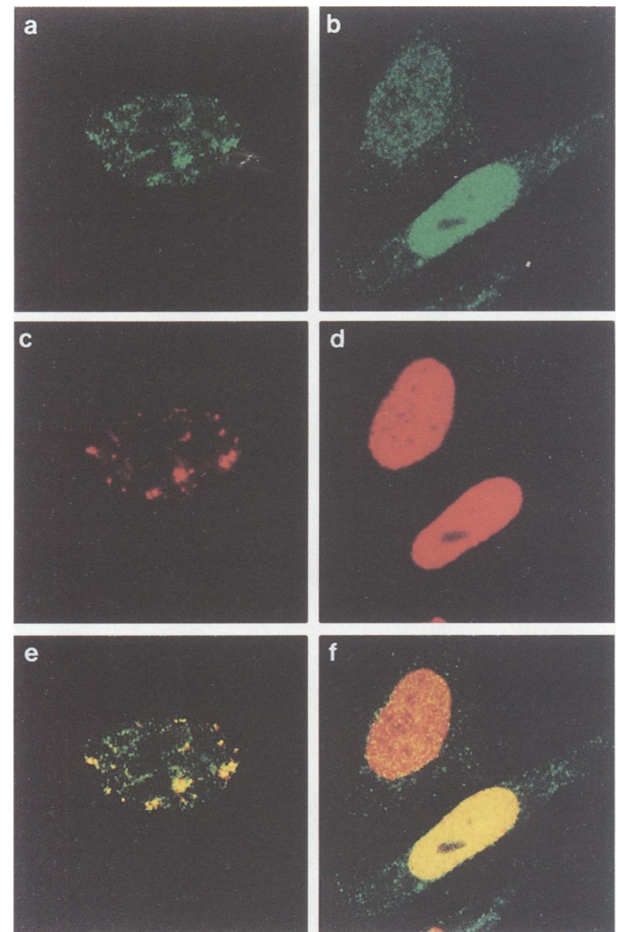

Fig. 8. Co-localization of WT1 and p53 proteins. Untreated (**a**, **c** and **e**) and forskolin-treated (**b**, **d** and **f**) C2 cells were double stained for WT1 (a and b) and p53 (c and d). Images (e) and (f) are created by overlaying (a) and (b) with (c) and (d) respectively.

When individual cells were analyzed by confocal microscopy for WT1 and p53 co-localization, similar expression patterns of these two proteins were observed in the nucleus (Figure 8a and c). In fact, co-localization of the WT1 and p53 proteins occurs and was observed by superimposing the WT1 and p53 images (seen as yellow areas in Figure 8e). This co-localization of WT1 and p53 probably reflects the proposed functional relationship of these two proteins (Maheswaran *et al.*, 1993, 1995). Interestingly, in contrast to WT1 (Figures 7c and 8b), p53 localization in C2 cells was not altered after treatment with forskolin or PMA. P53 remained exclusively in the nucleus after the forskolin (Figures 7d and 8d) or PMA (Figure 7f) treatments, indicating that p53 does not relocate with WT1 in response to PKA phosphorylation.

Discussion

The presence of two alternative splice sites in the WT1 gene generates four isoforms of WT1 protein, allowing for an increased level of functional complexity. The DNA binding activity of the WT1 protein is quite distinct for two of the four isoforms of the protein. The –KTS isoform of WT1 (WT1–KTS; but not the +KTS isoform, WT1+KTS) can bind to a consensus sequence containing the EGR1 recognition site and can act as a transcriptional repressor (Rauscher *et al.*, 1990; Madden *et al.*, 1991). However, both forms of WT1 are able to interact with different DNA elements, including sequences derived from the putative promoter of the WT1 gene itself (Campbell *et al.*, 1994; Rupprech *et al.*, 1994). Recent studies have shown that the WT1+KTS protein recognizes an extended sequence of the EGR1/WT1–KTS binding core sequence within the PDGF A-chain gene but does not bind to the nonextended EGR1 binding motif (Wang *et al.*, 1995). These results clearly indicate that the different spliced products of WT1 function through altered DNA binding specificity and regulatory properties. Here we show that phosphorylation may provide an additional level of modulation of the specific DNA binding activity of WT1. Both +KTS and –KTS forms of WT1ZF proteins were phosphorylated *in vitro* by nuclear extracts from rat kidney, spleen and liver, and by different purified serine/threonine kinases including PKA, PKC and CKII. The finding that WT1 is a good substrate for PKA and PKC but not for CKII and p34 cdc2 is not surprising, because there are about seven to 11 potential phosphorylation sites for PKA and PKC and only two for CKII in the zinc finger region (Figure 1). Although no consensus site for p34 cdc2 was found in this DNA binding domain, incubation of WT1 with this kinase resulted in a weak phosphorylation of WT1. This could be because of the existence of unconventional phosphorylation site(s), or result from nonspecific *in vitro* phosphorylation.

Phosphorylation can positively or negatively affect the binding of sequence-specific transcription factors to DNA (reviewed in Hunter and Karin, 1992). The inhibition of DNA binding activity by phosphorylation appears to be a common mechanism regulating many transcription factors. Here, we are able to show that the phosphorylation of bacterially expressed WT1 DNA binding proteins by PKA and PKC blocks the ability of WT1 to interact with its binding sites. Upon phosphorylation by PKA or PKC, the ability of WT1ZF–KTS to bind to an EGR1 binding site (oligo E) was nearly abolished. This phosphorylation event also greatly reduced the DNA binding activity of both +KTS and –KTS forms of WT1ZF protein to a WT1 binding site (oligo W). Although CKII can phosphorylate WT1 only weakly, this phosphorylation also influences the DNA binding of WT1ZF–KTS, reducing by ~50% the ability of the protein to bind to oligo E. In contrast, p34 cdc2 phosphorylation does not significantly affect the ability of WT1ZF–KTS to bind oligo E, suggesting that specific phosphorylated residues may be responsible for the inhibition of DNA binding. To assess whether the inhibition of DNA binding by phosphorylation could be attributed to specific site(s) or to the nonspecific phosphorylation, the kinetics of phosphate incorporation into WT1ZF–KTS by PKC were compared with the DNA binding activity. The results (Figure 4) suggest that the phosphorylation of a specific site(s) is likely to be responsible for the inhibition of DNA binding.

Most of the potential PKA and PKC phosphorylation sites within the WT1 DNA binding domain are highly conserved amongst WT1 proteins from different organisms (Call *et al.*, 1990; Gessler *et al.*, 1990; Buckler *et al.*, 1991; Madden and Rauscher, 1993). Some of them are also conserved within the related proteins of the EGR family (Joseph *et al.*, 1988; Sukhatme *et al.*, 1988; Rangnekar *et al.*, 1990; Muller *et al.*, 1991; Patwardhan *et al.*, 1991). Based on the crystal structure of the EGR1 protein (Pavletich and Pabo, 1991), two of the serine residues, Ser365 and Ser393 (in zinc fingers 2 and 3 respectively), are adjacent to two of the arginine residues involved in making contacts with the guanine residues of the DNA binding site. In addition, at least one of the serine residues (Ser365) that is a potential target for phosphorylation by PKA, PKC and CKII may also be involved in contacting the DNA backbone. One might envisage that either of these contacts could be perturbed by phosphorylation. Indeed, there is a report that serine residue 365 is a PKA phosphorylation site *in vivo* (Sharma *et al.*, 1994). Moreover, in *N*-nitroso-*N'*-methyl urea-induced rat embryonal kidney tumors, which resemble Wilms tumors, a C^{1091}–T mutation converting Ser365 to Phe was found (Sharma *et al.*, 1994). In addition, a mutation affecting this same Ser residue has been reported in a human leukemia cell line, CEM. Functional studies have revealed that the DNA binding activities of this mutant protein were severely impaired (Bickmore *et al.*, 1992).

Recently, structural modeling identified an RNA recognition motif in WT1 (Kennedy *et al.*, 1996), but RNA binding was not demonstrated. Therefore, we tested the ability of WT1 to bind RNA and the effect of PKA phosphorylation on this activity. The WIT-1 gene transcript was chosen as a target RNA because it is expressed as an antisense to WT1 (Campbell *et al.*, 1994; Eccles *et al.*, 1994). We have speculated previously that WIT-1 might function as an RNA and play a role in WT1 transcriptional regulation (Campbell *et al.*, 1994). The *in vitro* RNA binding assay indicated that both WT1ZF+KTS and WT1ZF–KTS proteins can bind to WIT-1 antisense transcripts to a similar extent. Although the specific binding sequence(s) has not been elucidated, unlike WT1 DNA binding, the RNA binding of both forms of WT1ZF is not affected by PKA phosphorylation *in vitro*.

Our experiments suggest that phosphorylation may control the activity of transcription factors by regulating the accessibility to cytoplasmic signal-transducing systems or regulating nuclear uptake, or both. In general, proteins >45 kDa require a nuclear localization signal (NLS) in order to be targeted to the nucleus. NLSs have been identified as the sequences necessary and sufficient for nuclear localization. Although WT1 proteins lack the 'highly basic' NLS (defined as a stretch with five or more consecutive lysine and arginine residues; Boulikas, 1994), a potential NLS (LKRHQRRH) is found in the second zinc finger of the C-terminal domain. Interestingly, this short sequence is located four amino acids from the residue Ser365, the target for *in vivo* phosphorylation (Sharma *et al.*, 1994). Thus, it is feasible that PKA phosphorylation

modulates the transport of WT1 into the nucleus. Our experiments support this possibility. Following forskolin treatment, WT1 protein is now detected in the cytoplasm compared with the predominant nuclear staining pattern of untreated cells. Forskolin treatment did not change the p53 localization pattern. The sequestration of WT1 in the cytoplasm because of phosphorylation may contribute to the reversal of transcriptional repression by WT1. This is supported by the transient transfection assays (Figure 6) where the transcriptional repression by WT1 was partially released by PKA activation. Although this is the first indication that phosphorylation plays a role in the regulation of WT1 function, several key questions still remain to be addressed. It is unclear whether the inactivation of WT1 suppressor activity by PKA phosphorylation resulted from an inhibition of WT1 DNA binding or from the alteration of WT1 nuclear translocation. The effect of PKA activation on WT1 transcriptional repression may be a result of direct phosphorylation of the WT1 protein or could be caused by phosphorylation of other cellular components that regulate WT1 function.

WT1 has been found to associate with p53 *in vivo* by sequential immunoprecipitation and Western analyses (Maheswaran *et al.*, 1993). The biological significance of this association is not completely understood. However, a recent study suggested that WT1 protein stabilizes p53 (Maheswaran *et al.*, 1995). The ability of WT1 to stabilize p53 was associated with the inhibition of papillomavirus E6-mediated p53 degradation. Here we have demonstrated directly the co-localization of WT1 and p53 in cultured cells using confocal microscopy. The C2 cells, which are derived from SV40 T-antigen-transformed human newborn kidney cells stably transfected with a WT1 expression construct, are composed of two cell populations based on the expression level of WT1. The majority of cells express high levels of WT1, with a small subset expressing much less WT1 which probably represents endogenous WT1. p53 protein is expressed uniformly in C2 cells at a high level and therefore does not correlate with WT1 expression. However, because the C2 parental cell line, 56A1, is immortalized by SV40 T-antigen, it is likely that this results in stabilization of the p53 protein regardless of the expression status of WT1. C2 cells double labeled with WT1 and p53 antibodies and analyzed by confocal microscopy showed an overall similar expression pattern for these two proteins. By overlaying the images collected from the double-stained cells, partial co-localization of WT1 and p53 proteins in the nucleus could be ascertained. However, when WT1 protein was retained in the cytoplasm in response to PKA activation, p53 remained in the nucleus. Therefore the nuclear translocation of WT1 is regulated by a distinct pathway from that regulating p53. In conclusion, we have demonstrated that WT1 proteins can be phosphorylated by PKA, PKC, CKII and p34 cdc2 *in vitro*. Phosphorylation by PKA, PKC and CKII can negatively regulate the specific DNA binding activity of WT1ZF and probably involves a specific phosphorylation site(s). *In vivo* experiments suggest that PKA phosphorylation may play a role in modulating the transcriptional regulatory activity of WT1 through either the inhibition of DNA binding and/or cytoplasmic sequestration of WT1.

Materials and methods

In vitro kinase assays

Construction and expression of WT1ZF+KTS and WT1ZF–KTS proteins. DNA fragments encoding the DNA binding (zinc finger) domain of the WT1 protein (amino acid residues 284–446 or 284–449 corresponding to the –KTS or +KTS forms respectively) were isolated as *Nla*IV fragments from their respective cDNAs. The cDNA encoding the –KTS protein was constructed by site-directed mutagenesis of cDNA 31E1 (Huang *et al.*, 1990). The *Nla*IV fragments were cloned into the expression vector pQE10, which contains a hexahistidine tag (Qiagen). Recombinant protein was purified using nickel affinity chromatography according to the manufacturer's protocol. Briefly, *Escherichia coli* cells expressing the fusion protein were harvested and lyzed in a buffer containing 6 M guanidine hydrochloride. After low-speed (10 000 *g*) centrifugation, the supernatant was applied to a charged NTA resin column equilibrated with lysis buffer. The column was washed and the fusion protein eluted with buffers of stepwise lower pH which contain 8 M urea. The denatured protein was then dialyzed slowly against binding buffer containing Zn^{2+} [25 mM HEPES, pH 7.5; 50 mM KCl; 10 mM $ZnSO_4$; 0.1% NP-40; 1 mM dithiothreitol (DTT) and 5% glycerol] to assure correct refolding. Under optimal binding conditions at least 1% of the renatured protein bound DNA.

In vitro protein kinase assays. Nuclear extracts from adult rat kidney, spleen and liver were prepared essentially as described by Gorski *et al.* (1986). *In vitro* phosphorylation of WT1 was carried out in 20 mM HEPES (pH 7.6), 5 mM DTT, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 10% glycerol, 10 μg bovine serum albumin (BSA) and 37 μCi [γ-^{32}P]ATP (NEN; 6000 Ci/mmol). 2 μg WT1 protein and 5 μg nuclear extract were added to a total volume of 20 μl. Reactions were incubated at 37°C for 15 min, followed by purification of the WT1 protein according to the manufacturer's protocol (Qiagen). Phosphorylated WT1 proteins were resolved by SDS–PAGE. WT1 proteins were also phosphorylated with a panel of partially purified kinases, including PKA, PKC, CKII and p34 cdc2 (UBI) *in vitro*. 500 ng purified bacterial proteins were incubated in a 30 μl volume with 15–25 ng protein kinases. The kinase reactions containing 10 μCi [γ-^{32}P]ATP (NEN; 6000 Ci/mmol) and 0.25 mM cold ATP were carried out at 30°C for 30 min and stopped by the addition of 30 μl 2× SDS sample buffer. 10% of the total reaction (50 ng) was analyzed by 12% SDS–PAGE, followed by autoradiography. To quantify the phosphate incorporation, phosphorylated products were separated by SDS–PAGE. Gels were dried on Whatman paper, followed by autoradiography. The areas corresponding to the bands on the autoradiograph were excised and read by liquid scintillation counting.

Gel mobility shift assays

The effect of kinase phosphorylation on DNA binding by WT1ZF protein was tested by first treating WT1ZF proteins with kinases in the presence or absence of ATP *in vitro*. A portion of the reaction containing 500 ng protein was combined with DNA binding buffer [20 mM HEPES, pH 7.6; 5 mM DTT; 50 mM KCl; 10% glycerol; 2.6 μg BSA and 300 ng poly(dI-dC)]. ^{32}P-labeled oligonucleotide probe (40 000 c.p.m.) was added and incubated on ice for 30 min. The DNA–protein complexes were separated from free probes on 6% polyacrylamide native gels. Two oligonucleotide probes were used: one containing a consensus EGR1 binding sequence (oligo E; 5′-CTAGACCGGC**GCGGGGGCG**A-GGGCG-3′) and the second, a WT1 binding site (oligo W; 5′-CTAGA-GCCTACCTGCCCCTCCCTCCAAA-3′) derived from the promoter region of WT1 respectively. The latter binding element (oligo W) was identified by DNase footprinting and positioned at 2005 relative to the 5′-most *Pst*I site within the WT1 genomic sequence reported previously (GenBank X77549).

Kinetics of WT1 DNA binding activity after phosphorylation by PKC

To study the kinetics of WT1ZF DNA binding activity after phosphorylation by PKC, kinase reactions were performed as described previously, except that no radiolabeled ATP was added to the reaction. Reactions were stopped at 2, 5, 10, 20, 30, 40, 60 and 80 min by transferring 5 μl of the reaction to dry ice. Phosphorylated WT1ZF–KTS proteins were subject to gel mobility shift assays with ^{32}P-labeled oligo E probe. As the DNA binding assay was performed on ice for 30 min following the kinase assay, there may be residual PKC activity during the incubation. Therefore, the kinetics for WT1ZF–KTS phosphorylation by PKC were determined as follows. The PKC kinase reaction was carried out in the

presence of [γ-^{32}P]ATP and stopped at different time points by transferring 5 μl of total reaction to dry ice. Reactions were then incubated on ice for 30 min, followed by the addition of 2× SDS sample buffer. Phosphate incorporation and DNA binding activity were quantified by a phosphorimage analyses.

RNA binding assays

cDNA clone pGB16, containing most of the WIT-1 sequence (Huang *et al.*, 1990), was used as a DNA template and linearized by *Xba*I digestion. *In vitro* reactions were carried out with T7 RNA polymerase in the presence of [^{32}P]GTP under conditions recommended by Promega. Labeled probes were purified by 6% acrylamide–8 M urea gel and eluted in buffer containing 0.5 M NH_4OAc, 1 mM EDTA and 0.1% SDS overnight at 37°C. Probes were washed twice with binding buffer (20 mM HEPES, 5 mM DTT, 37 mM KCl) using a Centricon-50 (Amicon). 500 ng hexahistidine-tagged WT1ZF+KTS and WT1ZF–KTS proteins were added to 50 μl of a 50% slurry of Ni-NTA resin (Qiagen) in binding buffer. About 50 ng labeled RNA probe were combined with 40 μg BSA in 50 μl binding buffer and incubated with the protein–Ni-NTA resin on ice for 20 min. Protein–RNA complexes coupled with resin were pelleted by centrifugation for 10 s at 15 000 *g* and washed twice with 1 ml binding buffer. Radioactivity in the pellet was quantified by liquid scintillation counting. To investigate whether WT1ZF RNA binding is affected by protein kinase phosphorylation, WT1ZF proteins were first treated with PKA for 30 min in the presence of ATP and then subjected to RNA binding assays as described above. The effect of PKA phosphorylation on WT1ZF–KTS DNA binding was also assessed by this method. A DNA probe, oligo E, was labeled as described for the gel mobility shift assays. DNA binding assays were carried out using the same procedure as the RNA binding assays except using the DNA probe (oligo E).

Generation of the stable transfectant, C2

pCMVWT1F was constructed starting with a partial cDNA clone of WT1, 31E1 (Huang *et al.*, 1990), that was extended at the 5′ end by removing a *Xba*I–*Xho*I fragment from 31E1 and replacing it with a *Hin*dIII–*Xho*I fragment derived from a genomic clone. The entire WT1 insert was excised from pGEM3Z (Promega) using *Xba*I and *Hin*dIII, blunted with Klenow and ligated to the vector pCMVβ-gal (MacGregor and Caskey, 1989), from which the β-galactosidase had been removed by *Not*I digestion. Expression of the WT1 (+/+) isoform is driven by the cytomegalovirus (CMV) immediate early promoter. 56A1 cells (Delarue *et al.*, 1991) were plated at 3×10^5 cells per 100 mm dish and stably transfected with 500 ng pSV2* neo plus 20 μg pCMV or 20 μg pCMVWT1F per dish using calcium phosphate/DNA precipitation. At 3 days after transfection, the cells were fed with medium containing 250 μg/ml G418 and allowed to grow. Individual colonies were picked, maintained in G418-containing medium and tested for their expression of WT1 using immunofluorescence and the polyclonal anti-WT1 antibody described previously (Rackley *et al.*, 1993). C2 cells were shown to overexpress both WT1 mRNA and WT1 protein relative to the parental 56A1 cells (data not shown).

Indirect immunofluorescence and confocal microscopy

C2 cells grown on coverslips were washed with PBS, fixed with cold acetone for 10 min and rehydrated in PBS. Indirect immunofluorescent staining was performed by preincubating cells with blocking solution (3% BSA, 0.1% Tween 20 in PBS), followed by incubation with primary antibodies for 1 h at room temperature and rinsing with PBS. Secondary antibodies (FITC-conjugated anti-rabbit IgGs and biotin-conjugated anti-mouse IgGs) were applied for 30 min, cells were rinsed and Texas Red–avidin was added for 1 h. Cells were rinsed, mounted on glass slides with Vectashied (Vector) and analyzed using fluorescent microscopy. Protein localization in the double-labeled cells was analyzed using a Leica Aristoplan (Leica Inc., Malvern, PA) confocal laser scanning microscope. Images were collected with the same brightness and contrast.

Transient transfections

The CAT reporter construct, Krox–CAT, was kindly provided by D.Skup (Institut du Cancer de Montréal, Canada). Krox–CAT was created by replacing the *Eco*RI–*Sac*II fragment of pSV2CAT with the Krox 24 promoter fragment (1.5 kb). The WT1 (+/–) expression vector (RSVWT1) expresses WT1 under the control of the Rous sarcoma virus long terminal repeat. 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and plated at a density of 1×10^6 cells per 100 mm dish on the day before the experiment. Transfections were carried out in triplicate using DEAE–Dextran (Promega) with the Krox–CAT reporter plasmid (5 μg) and WT1 expression plasmid (6 μg). The β-galactosidase expression plasmid (RSV/β-gal) was also used to establish transfection efficiency and normalize the CAT activity. Cell extracts were harvested 72 h later by freeze–thaw lysis, and CAT activity was assayed by a nonchromatographic method (Sleigh, 1986).

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