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Physical and Functional Interaction between the HCMV IE2 Protein and the Wilms' Tumor Suppressor WT1

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Human cytomegalovirus (HCMV) is a major renal pathogen in congenitally infected infants and renal allograft recipients. It has been shown that human kidney cells of glomerular, tubular, and vascular origin were all infected by HCMV *in vitro*. It has previously been demonstrated that the IE2 protein of HCMV directly associates with the zinc finger domain of Egr-1. The zinc finger region of WT1 is a sequence-specific DNA-binding domain which also recognizes the consensus DNA binding site (5′-CGCCCCCGC-3′) of Egr-1, thus suggesting a possible interaction between WT1 and IE2. Here we demonstrate that HCMV IE2 binds to the C-terminal region of WT1 containing zinc finger domain *in vivo* as well as *in vitro* and that WT1 can inhibit IE2-driven transactivation of the responsive promoter. Our results suggest that WT1 may be able to regulate the functional activity of HCMV IE2. Furthermore, these data may provide new insights into the possible involvement of HCMV in WT1-related pathogeneses.

Human cytomegalovirus (HCMV) can produce life-threatening complications in immunocompromised patients and can induce pathologic changes in a number of human organ systems. HCMV inclusions are found in the liver, lungs, and kidneys of HCMV viremic patients examined by biopsy or at autopsy (1, 2). In addition, high titers of HCMV are shed in urine during congenital or neonatal HCMV infection, and histologic examination of kidney sections from congenitally infected infants has revealed viral inclusions in the renal tubules (3). It has been shown that human kidney cells of glomerular, tubular and vascular origin can all be infected by HCMV *in vitro* (4–6).

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Similar to other herpesviruses, HCMV expresses its genes in three temporal classes: immediate early (IE), early, and late. The IE genes encode the first set of proteins expressed upon infection, some of which are involved in fine tuning the virus life cycle (7). One of the best examined IE genes of HCMV is IE2. This 86-kDa phosphonuclear protein appears to be a fine regulator of viral gene expression between the immediate-early and later stages of the HCMV life cycle. IE2 is a very potent transactivator of many cellular and viral promoters. It binds not only to cellular transcription factors such as TBP, TFIIB but also to tumor suppressor proteins such as p53 and Rb (8–12). It has also been reported that IE2 gene product can cooperate with another IE gene product, IE1 and adenoviral E1A to transform primary baby rat kidney (BRK) cells (13).

WT1, the Wilms' tumor suppressor protein, is a zinc finger-containing transcription factor that is capable of activating or repressing transcription depending on both cell type and promoter context (14, 15). Inactivation of WT1 has been correlated with the incidence of Wilms' tumor, a pediatric nephroblastoma, and Denys–Drash syndrome, which is characterized by severe genitourinary disorders. The WT1 gene is deleted or mutated in approximately 10% of sporadic Wilms' tumors and in nearly 100% of Denys–Drash patients. It has been reported that WT1 physically interacts with several cellular and viral proteins such as p53, par-4, and adenoviral oncoprotein E1B 55K (16–18). The interactions with these proteins can influence the transcriptional or biological activity of WT1.

Previously, we have demonstrated the binding of the IE2 protein to the zinc finger domain of Egr-1 (19). The zinc finger region of WT1 is a sequence-specific DNA-binding domain which recognizes the same consensus binding site (5′-CGCCCCCGC-3′) as Egr-1, therefore suggesting a possible interaction between WT1 and IE2. Here we show that IE2 binds to the C-terminal region of WT1 *in vivo* as well as *in vitro*, and that WT1 suppresses the transactivation capability of IE2.

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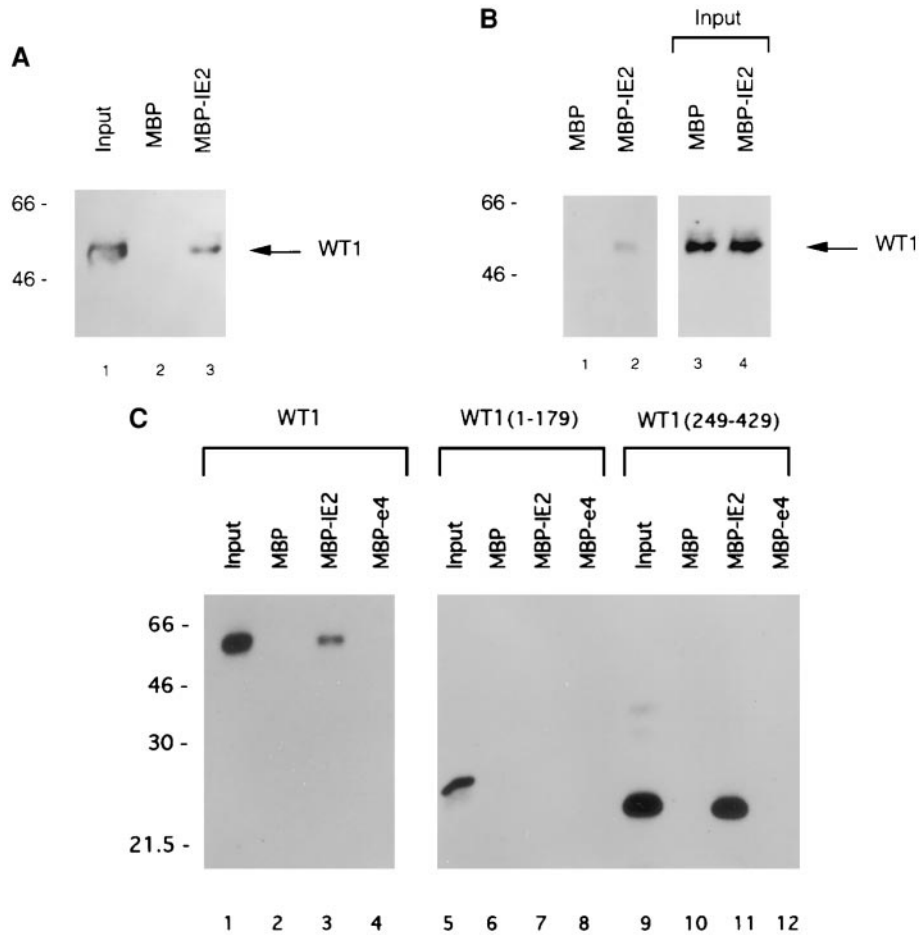

FIG. 1. (A) Association between WT1 and IE2. MBP fusion proteins were expressed in *E. coli* and bound to amylose resin were generated. Total cellular extracts were prepared from 293T cells transfected with a WT1 expressing vector (pCMV-WT1) or a control plasmid (pCMV) Two days after transfection, cells were lysed, incubated with a resin containing MBP alone (lane 2) or MBP-IE2 (lane 3), and pulled down with amylose resin. The bound proteins were detected by immunoblotting with a polyclonal antibody specific for WT1. One-fifth of the cell lysate was used as a marker for WT1 (lane 1). (B) *In vivo* interaction of WT1 with IE2. 293T cells were transfected with a mammalian expression vector producing the MBP–IE2 fusion protein (pCI-MBP-IE2) (lanes 1 and 3) together with pCMV-WT1. As a control, an expression vector express MBP alone (pCI-MBP) used. (lanes 2 and 4) Two days after transfection cell were lysed and pulled down with amylose resin. The bound proteins were detected by immunoblotting with a polyclonal antibody specific for WT1. One fifth of the cell lysate was used as a marker for WT1 (lanes 3 and 4). (C) Identification of WT1 domain interacting with IE2. Various domains of [^{35}S]methionine-labeled WT1 were generated by *in vitro* transcription and translation. Labeled proteins were incubated with an affinity matrix containing MBP (lanes 2, 6, and 10), MBP–IE2 (lanes 3, 7, and 11) or MBP–exon 4 (lane 4, 8, and 12) synthesized in *E. coli*. The bound proteins were subjected to SDS–PAGE and visualized by autoradiography. One fifth the amount of each domain of labeled WT1 used in the binding reaction was used as a marker (lanes 1, 5, and 9).

We first tested whether WT1 interacts with IE2, using a maltose binding protein (MBP)-IE2 fusion protein and total cellular extract prepared from 293T cells transfected with a WT1 expression vector, pCMV-WT1. MBP-IE2 was expressed in *E. coli*, purified, and bound to an amylose resin as previously described (12). Total cellular extract was prepared 2 days post transfection using the ELB buffer (50 mM Hepes, pH 7.0/250 mM NaCl/0.5 mM EDTA/0.1% Nonidet P-40) as described by Maheswaran *et al.* (17) For protein binding assays, 500 ng of the MBP fusion proteins, on beads, were rocked for 3 h at 4°C with 150 μl of the total cellular extract in a final volume of 500 μl buffer A (140 mM NaCl/100 mM Tris–HCl, pH 7.4/0.5% Nonidet P-40/1 mM EDTA). The beads were then washed three times in 1 ml of buffer A, pelleted at 12,000*g* for 30 s, and boiled in 2× SDS–PAGE sample buffer. Bound proteins were released and analyzed by Western blot analysis with a polyclonal antibody specific for WT1 (C19, Santa Cruz Biotech., Santa Cruz, CA, US). The 52-kDa WT1 protein was preferentially retained by the MBP-IE2 resin, but not by the resin containing MBP alone (Fig. 1A, lanes 2 and 3). This result suggested that the IE2 protein might physically bind to the intact 52-kDa WT1 protein.

To confirm a physical interaction between WT1 and IE2 in mammalian cells, we constructed a mammalian expression vector, pCI-MBP-IE2, which produces a MBP-IE2 fusion protein from the HCMV IE promoter. As a control, the same expression vector containing only the MBP sequence, pCI-MBP, was constructed. To construct pCI-MBP and pCI-MBP-IE2, the *malE* gene was cloned from the pMal-c2 plasmid (New England Biolabs, Beverly, MA, US) by a polymerase chain reaction using a pair of oligonucleotide primers, 5′-GCTAGCCTCGAGACCATGAAAACTGAAGAAGGT (upstream primer) and 5′-AAGCTTGCCTGCAGGTCGAC (downstream primer). The amplified DNA fragment was cloned into the *Nhe*I/*Eco*RI site of pCI-neo (Promega, Madison, WI), resulting in pCI-MBP. pCI-MBP-IE2 was generated by cloning the *Eco*RI IE2 fragment (isolated from pMBP-IE2) into the *Eco*RI site of pCI-MBP. These expression vectors were transfected into 293T cells together with pCMV-WT1. Total cellular extracts were prepared using the ELB buffer and MBP-fusion proteins were purified by MBP-affinity chromatography. Bound proteins were detected according to ECL Western blotting protocols with a polyclonal antibody to WT1. As shown in Fig. 1B, WT1 was specifically copurified from cell lysates transfected with a MBP-IE2 expression vector (lane 2), while no cellular proteins reacted with the antibody when a control vector expressing MBP was transfected (lane 1). This result, together with the above *in vitro* binding data strongly, suggested that WT1 and IE2 could interact with one another both *in vivo* as well as *in vitro.*

To identify the domain of WT1 that interacts with the IE2 protein, another *in vitro* binding assay was performed using the MBP-IE2 protein and three *in vitro* translated [^{35}S]methionine-labeled WT1 proteins; the full-length WT1 protein (1–429) and two deletion proteins, WT1 (1–179) containing only the N-terminal domain and WT1 (249–429) containing the C-terminal zinc finger domain. An intact 52-kDa [^{35}S]methionine-labeled WT1 protein was retained by the MBP-IE2 resin (Fig. 1C, lane 3), but not by the resin containing MBP alone (Fig. 1C, lane 2) or the MBP-e4 (Fig. 1C, lane 4), which is identical to pMBP-IE1 described previously by Kim *et al.* (20), used as another control protein. *In vitro* translated and radiolabeled WT1 (249–429) was bound to the MBP-IE2 resin (Fig. 1C, lane 11), but not to the control resins (lanes 10 and 12). On the contrary, WT1 (1–179) was not retained by any columns (lanes 6–8). Taken together, these results show a direct interaction between IE2 and the C-terminal region of WT1 which contains a zinc finger domain.

To determine the functional consequences of the interaction between WT1 and IE2, transient transfection experiments were carried out to analyze the effects of WT1 on transcriptional activation activity of IE2. IE2 has been shown to be a promiscuous transactivator of various heterologous promoters. As shown in Fig. 2A, IE2 activated gene expression from the truncated PDGF-A promoter in a dose dependent manner in BHK21 cells. Activation was specific for IE2 because IE1, another immediate-early protein of HCMV, had little effect on the PDGF-A promoter under the same condition. Because the truncated PDGF-A promoter used in this study contains two overlapping EGR-1/WT1 consensus sequences, we first examined the effect of WT1 on the promoter in BHK21. As shown in Fig. 2B, WT1 alone had little effect on the expression from the promoter in BHK21 cells. However, the cotransfection of the WT1 and IE2 expression vector (pCMX-WT1 and pEQ326), decreased the IE2-mediated transactivation of the truncated PDGF-A promoter in a dose-dependent manner. Repression was specific for wild type WT1 because WT1 (249–429) lacking the N-terminal domain had no effect on the transactivation function of IE2 (Fig. 2C). To rule out the possibility that the observed inhibition of IE2-mediated transactivation by WT1 is due to the inhibitory effect of WT1 on IE2 expression from the cotransfected plasmid, we tested the effect of WT1 on the major immediate early promoter (MIEP) of HCMV driving IE2 in the expression vector. As shown in Fig. 2D, WT1 had no significant effect on the MIEP of HCMV. Thus the decrease in IE2-mediated transactivation caused by WT1 was not due to a reduction in IE2 expression level.

We have demonstrated that (i) HCMV IE2 protein binds to WT1 *in vivo* as well as *in vitro;* (ii) C-terminal region of WT1 is required for this protein-protein interaction; and (iii) full-length WT1 can prevent the IE2-mediated transactivation of the truncated PDGF-A promoter.

The interaction between these proteins appears to be mediated by the C-terminus of WT1 which contains the four C2H2-type zinc fingers. It has previously been suggested that zinc fingers, in addition to binding DNA and RNA, may mediate protein–protein interaction. For instance, the zinc fingers of YY1 have been shown to be involved in physical interactions with Sp1, and p300, and CREB (21–23). In addition, the zinc fingers of Egr-1 have been shown to interact with the HCMV IE2 (19). The finding that the C-terminus of WT1 is required for the interaction with IE2 is reminiscent of the WT1-p53 and WT1-par4 interaction reported previously (16, 17). In these studies, the zinc fingers of WT1 were shown to interact with p53 or par4, and these protein–protein interactions were shown to play a role in WT1-mediated transcriptional repression or activation. It thus appears that the zinc finger domain of WT1 may be targeted by several proteins which may regulate the functional activities of WT1.

We have shown that WT1 can prevent transactivation of the truncated PDGF-A promoter by IE2. This functional consequence of WT1–IE2 interaction is similar to that of Rb–IE2 interaction previously reported

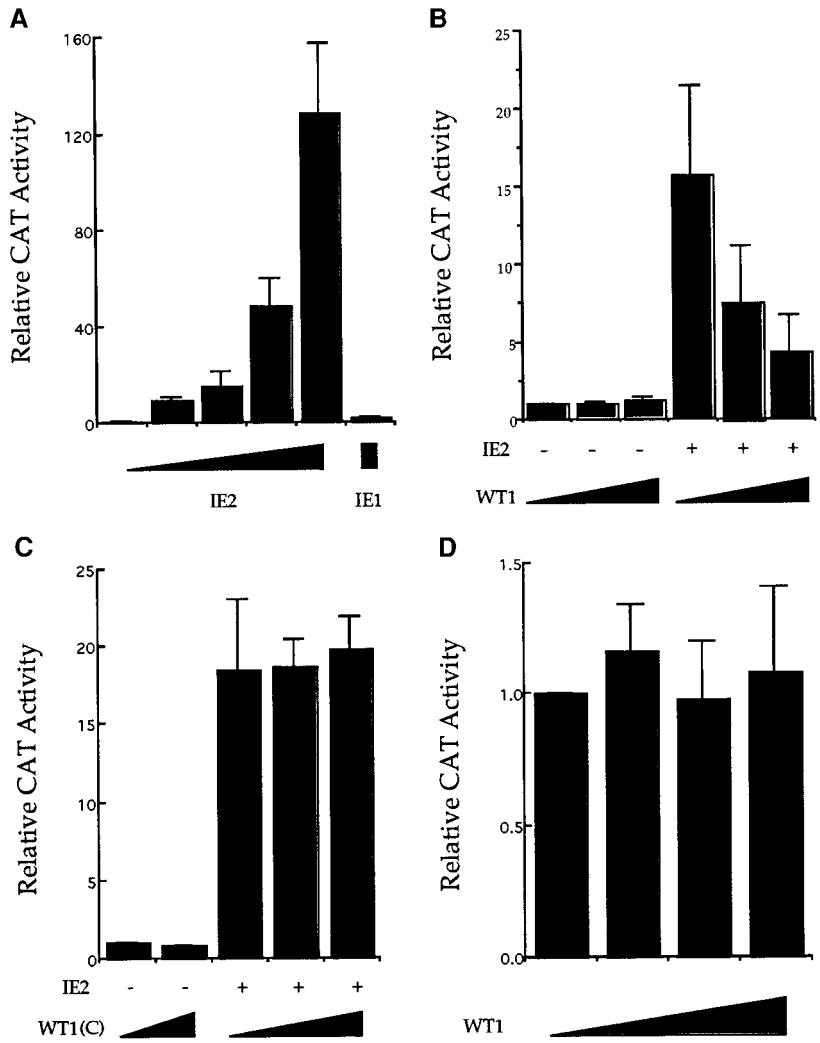

FIG. 2. (A) Effects of IE2 on gene expression from the PDGF A promoter (−262/+8). BHK21 cells were transfected by DEAE-dextran method with 2μg of a PDGF A promoter–CAT fusion construct and various concentrations (0.5, 1, 2, or 4 μg) of an IE2 expression vector pEQ326 (Biegalke and Geballe, 1991). The total amounts of DNA per transfection was always adjusted to 4μg by using pEQ 336 containing no IE coding sequence. As a control, 4 μg of an IE1 expression vector pEQ273 was also used. Forty-eight hours after transfection, cells were harvested and CAT activity determined. Fold activation was calculated by dividing the CAT activity from IE2- or IE1-transfected cells by that from pEQ336 transfected cells. (B) Effects of WT1 on gene expression from the PDGF A promoter (−262/+8). BHK21 cells were transfected with 2 μg of a PDGF A promoter-CAT fusion construct and 2 μg of an IE2 expression vector (pEQ326) together with various concentrations (2, 4, or 8 μg) of a WT1 expression vector (pCMX-WT1). As a negative control, pEQ336 was used. (C) Effects of the mutant WT1 expressing only the C-terminus on gene expression from the PDGF A promoter (−262/+8). BHK21 cells were transfected with 2 μg of PDGF A promoter-CAT fusion construct and 2 μg of an IE2 expression vector (pEQ326) together with various concentrations (2, 4, or 8 μg)of pCMX-WT1 (C) express only the C-terminus of the WT1. As a negative control, pEQ336 was used. (D) Effects of WT1 on gene expression from the HCMV major immediate early promoter (MIEP). BHK21 cells were transfected with 1 μg of a MIEP-CAT fusion construct (pC3.1 CAT) and various concentrations (1, 2, or 4 μg) of a WT1 expression vector. The total amounts of DNA per transfection was adjusted to 4 μg by using pCMX.

by Choi *et al.* (12). Similar to WT1, Rb directly binds to IE2 and negatively regulates transcriptional activation mediated by IE2. The IE2 gene product is required for expression of HCMV genes involved in the later stages of the life cycle. It thus appears that by binding to and inhibiting transactivational activity of IE2, tumor suppressor protein like WT1 and Rb may provide a means by which the host cell delays the replication of this virus.

Our studies have investigated the effects of WT1 on transcriptional activation by IE2. It will also be interesting to see whether IE2 can modulate the biological activity of WT1. Recently, it has been reported that adenoviral E1B sequesters WT1 along with p53 within a cytoplasmic body in adenovirus-transformed kidney cells (18). In the case of E1B, such interaction may result in the functional inactivation of both tumor suppressor gene products. Interestingly, there are func-

tional similarities between HCMV IE2 and adenovirus E1B oncoprotein. First, similar to E1B, IE2 can block apoptosis induced adenovirus E1A protein (24). Second, IE2, with IE1, can cooperate with adenovirus E1A protein to generate transformed foci of primary baby rat kidney cells with a level comparable to the activity of E1A plus E1B protein (13). Third, as in E1B, IE2 may interact with both p53 and WT1 (11). although the formation of a trimeric complex remains to be proven. Therefore, it will also be interesting to examine the consequences of IE2-WT1 interaction for cell growth.

Our results suggest that HCMV IE2 can interact with WT1. Although the biological significance of the interaction between IE2 and WT1 is not yet clear, the observation that HCMV can infect a variety of kidney cells implicates this protein–protein interaction in pathogenesis (4–6). Furthermore, WT1 mutation were identified in only 10–15% of Wilms' tumors, which suggests that other factors such as viral proteins may also contribute to the development of Wilms' tumor (14). Future studies of the interactions between HCMV and WT1 may increase our understanding the pathogenesis of their associated diseases.

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