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Transcriptional Repression Mediated by the WT1 Wilms Tumor Gene Product

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The *wt1* gene, a putative tumor suppressor gene located at the Wilms tumor (WT) locus on chromosome 11p13, encodes a zinc finger–containing protein that binds to the same DNA sequence as EGR-1, a mitogen-inducible immediate-early gene product that activates transcription. The transcriptional regulatory potential of WT1 has not been demonstrated. In transient transfection assays, the WT1 protein functioned as a repressor of transcription when bound to the EGR-1 site. The repression function was mapped to the glutamine- and proline-rich NH_2-terminus of WT1; fusion of this domain to the zinc finger region of EGR-1 converted EGR-1 into a transcriptional repressor.

THE SEARCH FOR THE WILMS TUMOR (WT) gene on chromosome 11p13 has yielded a complementary DNA (cDNA) clone (WT1) that has characteristics of a tumor suppressor gene (*1, 2*). The *wt1* gene is mutated or deleted in a subset of sporadic and hereditary Wilms tumors (*2, 3*) and is expressed in the condensing mesenchyme, renal vesicle, and glomerular epithelium of developing kidney, suggesting that WT1 functions in normal kidney differentiation (*4*). The *wt1* gene encodes a protein that contains four zinc fingers and a glutamine- and proline-rich NH_2-terminus, structural motifs associated with sequence-specific binding to DNA, and transcriptional regulatory functions (*5*).

The *wt1*-encoded protein recognizes the same DNA sequence element (5′-CGCCCCCGC-3′) as the EGR-1 protein, a transcription factor that contains three zinc fingers and shares >65% amino acid sequence similarity with WT1 in the zinc finger region (*6*). After induction by a variety of cell surface stimuli, EGR-1 (also known as NGFI-A, T1S-8, Krox 24, and zif268) (*7, 8*) rapidly accumulates in the nucleus, binds to the EGR site, and activates the transcription of target genes whose products are required for mitogenesis and differentiation (*9, 10*). The proteins encoded by all members of the EGR family are positive activators of transcription when bound to their cognate sequence in target genes (*9, 10*).

To determine the function of WT1 in transcriptional regulation when it is bound to the EGR site, transient transfection assays were carried out with expression vectors (Fig. 1A) that contained the full-length coding region of *wt1* (CMV-WT1) and *egr-1* (CMV–EGR-1) under the control of the cytomegalovirus immediate-early promoter (*11*). As controls, stop codons were introduced into the coding regions of each gene to generate the plasmids CMV-WT1 (TGA) and CMV–EGR-1 (TGA). Of the two reporter plasmids used in these experiments (Fig. 1A), the first contained three synthetic EGR binding sites upstream of the minimal c-*fos* promoter (*10*) linked to the chloramphenicol acetyltransferase (CAT) gene (p3XEBS-CAT), whereas the second contained 1 kb of the murine EGR-1 promoter (*12*) upstream of the CAT gene (pEGR-1.1.2-CAT).

Analysis of CAT activity (*13–15*) in NIH 3T3 fibroblasts cotransfected with the expression and reporter plasmids revealed the expected stimulation of the p3XEBS-CAT vector by CMV–EGR-1 (*10*), but not by the control CMV–EGR-1 (TGA) (Fig. 1B). However, the basal level of transcription was reduced in cells cotransfected with CMV-WT1 and p3XEBS-CAT (Fig. 1B). Neither CMV–EGR-1 nor CMV-WT1 affected a CAT reporter plasmid that lacked EGR binding sites (*16*).

To determine whether WT1 inhibited the ability of EGR-1 to activate p3XEBS-CAT, varying ratios of the CMV-WT1 and CMV–EGR-1 plasmids were cotransfected with p3XEBS-CAT (Fig. 1B). At a 1:1 ratio of CMV-WT1 and CMV–EGR-1, activation by EGR-1 was reduced, and at a 2:1 ratio, activation by EGR-1 was abolished. Thus, WT1 repressed both basal and EGR-1–induced transcription from p3XEBS-CAT. The same experiments performed in human embryonic kidney–derived 293 cells revealed transcriptional activation of the p3XEBS-CAT reporter plasmid by CMV–EGR-1 and repression by CMV-WT1 (Fig. 1B).

The p3XEBS-CAT reporter plasmid contains EGR binding sites in an artificial promoter context and exhibits a low basal level of transcriptional activity (Fig. 1B). This low basal level made it difficult to assess the potency of WT1 as a transcriptional repressor in cotransfection assays (Fig. 1B). To circumvent this problem, we used the pEGR-1.1.2 promoter, which contains several EGR binding sites of varying affinities as well as other genetic regulatory elements (*12*) in their natural context. The WT1 vector efficiently repressed the normal, high basal levels of transcriptional activity of pEGR-1.1.2-CAT in NIH 3T3 cells (Fig. 1C), independent of whether the cells were incubated in high (10%) or low (0.5%) serum after transfection (*16*).

The WT1 mRNA transcript is subject to alternative splicing (*1, 2, 17*) (Fig. 2A), in one case resulting in the insertion of lysine, threonine, and serine (KTS) between the third and fourth zinc fingers. A WT1 protein that contains this insertion does not bind to the EGR-1 site (Fig. 2B) (*6*). A second alternative splice results in the insertion of 17 amino acids in the region immediately NH_2-terminal to the zinc finger domain (*1, 17*) and does not affect DNA binding activity (Fig. 2B). To determine the effect of these amino acid insertions on the ability of WT1 to repress transcription, the four possible combinations of the spliced variants were produced by site-directed mutagenesis (*18*), tested for DNA binding activity (Fig. 2B) (*19*) and expression in COS-1 cells (Fig. 2, legend), and assayed for repression by cotransfection with pEGR-1.1.2-CAT (Fig. 2C). The proteins that contained the KTS insertion (WT1-KTS, WT1-17AA-KTS) did not repress transcription of the reporter plasmid (Fig. 2C) or bind to the EGR-1 site (Fig. 2B). However, repression was observed in the absence (WT1) or the presence (WT1-17AA) of the 17–amino acid insertion. Thus, binding to the EGR-1 site is re-

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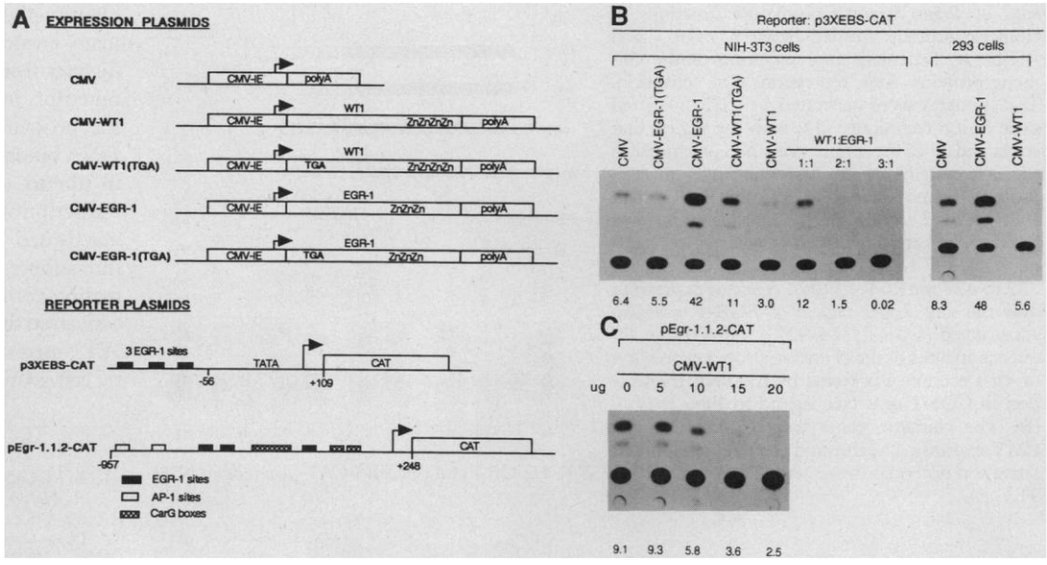

Fig. 1. Regulation of transcription from EGR-1 binding site–CAT reporter plasmids by EGR-1 and WT1. (**A**) Expression vectors for use in transient transfection assays contained the full-length protein coding regions of human WT1 (*11*) or murine EGR-1 (*10*), the cytomegalovirus immediate-early promoter, and SV-40 polyadenylation signals (*25*). The approximate locations of the EGR-1 binding sites and other regulatory elements are shown. (**B**) Calcium phosphate–mediated transfections were performed in murine NIH 3T3 fibroblasts or human embryonic kidney 293 cells (*13, 14*). Each dish of cells was transfected with p3XEBS-CAT (2 μg), the indicated expression plasmid (5 μg), and a β-galactosidase expression vector (1 μg) as an internal control for transfection efficiency (*15*). The total amount of CMV vector in each transfection mixture was kept constant at 20 μg by addition of CMV vector alone. When the EGR-1 and WT1 plasmids were mixed (WT1:EGR-1) the amount of EGR-1plasmid was kept constant at 5 μg and the WT1 plasmid concentration was 5 μg (1:1), 10 μg (2:1), and 15 μg (3:1). Forty-eight hours after transfection, cell extracts were prepared and aliquots [normalized for transfection efficiency via assay of β-galactosidase activity (*26*)] were used for determination of CAT activity (*13, 14*). (**C**) The EGR-1.1.2-CAT plasmid (0.5 μg) was transfected into NIH 3T3 cells with the indicated amounts of CMV-WT1 plasmid. The total amount of CMV vector remained constant at 20 μg in each transfected dish.

quired for repression by WT1 and repression appears to be unaltered by the 17–amino acid insertion.

To localize the domains of WT1 required for repression, a set of proteins truncated at their NH_2- and COOH-termini was prepared (*18*) and tested for DNA binding activity (Fig. 2B), expression in COS-1 cells (comparable amounts of protein were noted; see legend to Fig. 2), and effect on transcription in cotransfection assays (Fig. 2C). The two proteins that contained NH_2-terminal deletions (WT1,179–429 and WT1,294–429) did not repress transcription even though each contained an intact DNA binding domain and bound to the EGR-1 site in vitro (Fig. 2B). A protein that contained a COOH-terminal truncation (WT1,1–364) that deleted the last two zinc fingers did not bind to the EGR-1 site (Fig. 2B) and did not repress transcription. Deletion of amino acids 179 to 294 in the NH_2-terminal proline- and glutamine-rich region created a protein that bound to the EGR-1 site but did not repress transcription. These results (summarized in Fig. 2A) suggest that the DNA binding domain of WT1 is necessary but not sufficient for transcriptional repression and that the glutamine- and proline-rich NH_2-terminus of

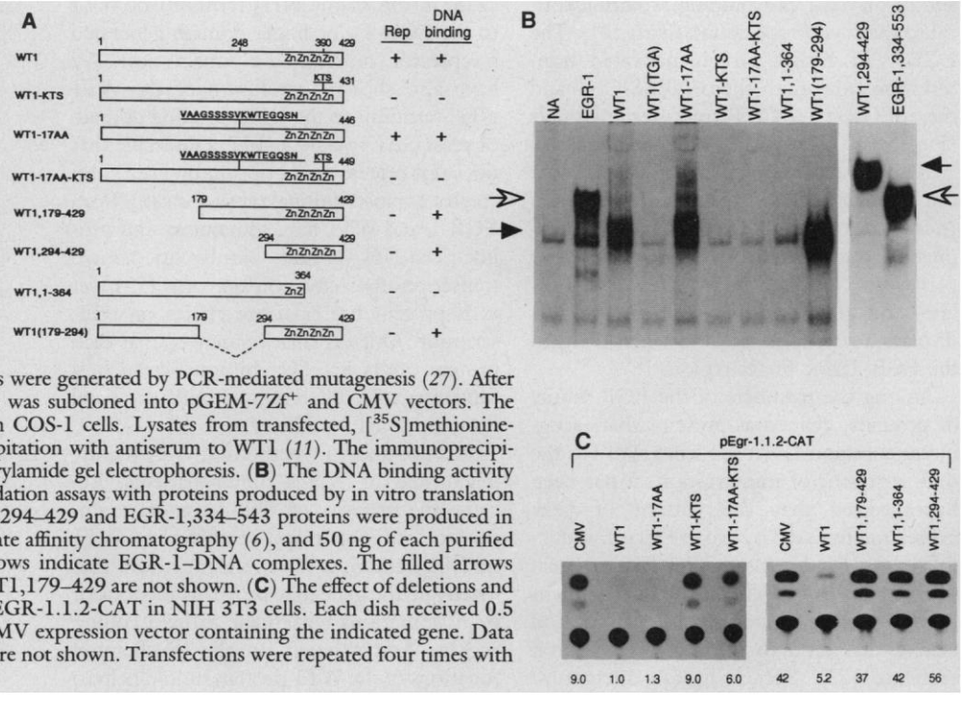

Fig. 2. Analysis of the domains of the WT1 protein required for transcriptional repression. (**A**) A schematic representation of the four possible WT1 proteins that result from alternative mRNA splicing. Rep, transcriptional repression. Each amino acid insertion, KTS at position 390, and VAAGSSSSVKWTEGQSN at position 248, was introduced separately or together into the coding region of WT1 by site-directed mutagenesis (*18, 27*). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The truncation and internal deletion mutants were generated by PCR-mediated mutagenesis (*27*). After DNA sequence analysis, each mutated gene was subcloned into pGEM-7Zf$^+$ and CMV vectors. The mutated *wt1* genes were stably expressed in COS-1 cells. Lysates from transfected, [^{35}S]methionine-labeled cells were analyzed by immunoprecipitation with antiserum to WT1 (*11*). The immunoprecipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis. (**B**) The DNA binding activity of each protein was determined by gel retardation assays with proteins produced by in vitro translation in rabbit reticulocyte lysates (*28*). The WT1,294–429 and EGR-1,334–543 proteins were produced in *Escherichia coli* and purified with nickel chelate affinity chromatography (*6*), and 50 ng of each purified protein were used per lane. The open arrows indicate EGR-1–DNA complexes. The filled arrows indicate WT1-DNA complexes. Data for WT1,179–429 are not shown. (**C**) The effect of deletions and insertions in WT1 on transcription from pEGR-1.1.2-CAT in NIH 3T3 cells. Each dish received 0.5 μg of the reporter plasmid and 15 μg of CMV expression vector containing the indicated gene. Data for the internal deletion (WT1[179–294]) are not shown. Transfections were repeated four times with comparable results (*15*).

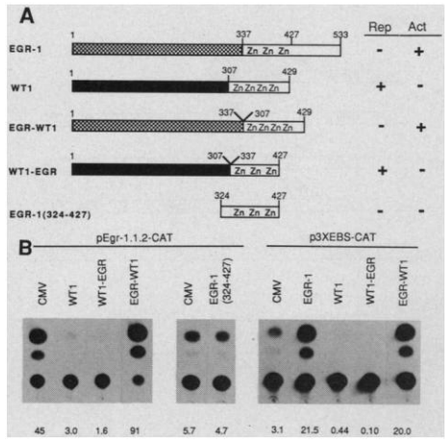

Fig. 3. Transcriptional regulatory functions of chimeric proteins formed between EGR-1 and WT1. (**A**) Schematic representation of the chimeric proteins. Rep, repression; Act, activation. The chimeras were generated by PCR-mediated gene fusion techniques (*27*) with the region that contained all of the amino acids NH_2-terminal to the first cysteine of the first zinc finger in each protein (amino acids 1 to 337 for EGR-1 and amino acids 1 to 307 for WT1). This region was fused to the heterologous zinc finger region (amino acids 307 to 429 for WT1 and amino acids 337 to 427 for EGR-1) (*20*). A vector expressing only the zinc finger region of EGR-1 was also constructed (EGR-1[324–427]). After DNA sequence analysis of the chimeras, proper expression of each protein was tested by transient transfection in COS-1 cells (see legend to Fig. 2) (*11*). (**B**) The chimeric genes were cloned into the CMV expression vector and their repression and activation potentials were determined with the pEGR-1.1.2-CAT and p3XEBS-CAT plasmids in NIH 3T3 cells.

WT1 contains a discrete domain that mediates transcriptional repression. To determine whether this domain could repress transcription when fused to a heterologous DNA binding domain, we constructed chimeric proteins and tested their ability to repress transcription (*18*). We first established that the isolated zinc finger regions (*20*) of WT1 and EGR-1 bound to the EGR site with high affinity (Fig. 2B) but did not repress transcription (Figs. 2C and 3B). The chimeras were generated by fusing the NH_2-terminal 307 amino acids of WT1 to the EGR-1 zinc finger region (WT1-EGR) or the NH_2-terminal 337 amino acids of EGR-1 to the WT1 zinc finger region (EGR-WT1) (Fig. 3A). Each chimera was tested for expression in COS-1 cells (comparable amounts of protein were observed) and displayed DNA binding activity identical to their wild-type counterparts (*21*). The EGR-WT1 fusion protein activated transcription from both the p3XEBS-CAT and the pEGR-1.1.2-CAT reporter plasmids (Fig. 3B). Thus, the NH_2-terminus of EGR-1 contains an activation domain that can be transferred to a heterologous protein. In contrast, the WT1-EGR chimera repressed transcription from both reporter plasmids. Thus, the 307–amino acid, proline- and glutamine-rich segment of WT1 displays a repressor function when fused to the EGR-1 zinc finger region.

Among the members of the EGR family of proteins, defined as proteins that recognize a common DNA sequence, WT1 is the only repressor of transcription. It has been hypothesized that the pattern of gene expression initiated by growth factor–inducible, short-lived transcription factors (such as EGR-1) may be altered by developmentally regulated, tissue-specific transcription factors that bind to the same recognition sequence (*22*). Because it can antagonize transcriptional activation mediated by each of the EGR proteins (*16*), WT1 may function by antagonizing the actions of an inducible, immediate-early gene in vivo. WT1 may inhibit cell proliferation and thus initiate a tissue-specific program of gene expression. Clarification of the physiological function of WT1-mediated repression awaits identification of relevant target genes active during kidney development.

The mechanism of repression by WT1 is still unclear. Simple competition between the endogenous activator (EGR-1) and a transfected nonactivator (WT1) at a single DNA binding site is unlikely to be the primary mechanism, as the zinc finger domain alone is unable to repress transcription even though it binds DNA. The hybrid protein experiments support this view, because fusion of the NH_2-terminus of WT1 to the EGR-1 zinc finger domain generated a repressor, not simply a nonactivator. We have also shown that fusion of the WT1 NH_2-terminus to the DNA binding domain of yeast GAL4 (Gal4 1–147, a molecule that does not repress transcription by itself) also creates a transcriptional repressor (*21*). Both EGR-1 and WT1 have glutamine- and proline-rich NH_2-termini similar to known transcriptional activation domains (*23*), yet each protein has opposite effects on transcription. One explanation may be that each protein exerts its effect by interacting in a fundamentally different way with the basal transcriptional machinery.

It is not known whether *wt1* is frequently inactivated in Wilms tumor patients. Because the majority of Wilms tumors examined to date contain apparently normal WT1 mRNA transcripts (*1–4*), the mutations that inactivate the gene may be small deletions or point mutations within the protein coding region. Assays based on the biochemical functions of the WT1 protein should help to identify these mutations. Because the domain encoding the repression function is distinct from the DNA binding domain, a mutation in either may serve to inactivate the protein. Evidence suggesting that the DNA binding domain of WT1 is inactivated in tumors (*2*) raises the possibility that its transcriptional repression function is also inactivated. Mutations that render the tumor suppressor gene encoding p53 transformation-competent disrupt its transcriptional activation function (*24*). Whether the loss of WT1-mediated repression leads to neoplastic cell growth is not known.

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11. A detailed description of the construction of full-length WT1 expression vectors, production and characterization of the antisera to WT1, transfection and immunoprecipitation from COS-1 cells, and biochemical characterization of the WT1 protein will be published elsewhere (J. F. Morris, S. L. Madden, O. E. Tournay, D. M. Cook, V. P. Sukhatme, F. J. Rauscher, *Oncogene*, in press). Briefly, we used the WT33 cDNA clone of WT1 [K. M. Call *et al.*, *Cell* **60**, 509 (1990)], which is missing 84 amino acids of the NH_2-terminus including the initiator methionine, as a starting point for construction of a full-length coding region. The missing segment of the gene including the initiator methionine (*1, 2, 17*) and a Kozak consensus sequence was synthesized from synthetic oligonucleotides with overlap-extension polymerase chain reaction (PCR) (*6*), and the synthetic segment was fused to the WT33 gene at a unique Bst XI restriction site in WT33. After cloning, both strands of the synthetic region were sequenced. Full-length WT1 was produced by in vitro transcription–translation in rabbit reticulocyte lysates and was shown to bind to the EGR-1 sequence with high affinity. The completed *wt1* gene was cloned into the pCB6 vector (*25*), which contains the cytomegalovirus immediate-early promoter. A PCR error in one of the clones resulted in the introduction of a TGA stop codon at amino acid 12 in the coding sequence. This clone was used to generate the control plasmid CMV-WT1 (TGA). Polyclonal rabbit antisera were raised to two separate regions of the protein that were expressed and purified from *Escherichia coli*. One region encompassed amino acids 85 to 173 (anti-WT91) and the other encompassed amino acids 294 to 429 (anti-WTZF) (*6*).
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13. The cells used for transfection (murine NIH 3T3 fibroblasts and human embryonic kidney–derived 293 cells) were grown in Delbecco's modified Eagle's medium (DMEM) plus calf serum (10%). The cells were seeded in 100-mm dishes and transfected by the calcium phosphate–mediated coprecipitation technique (*14*), with a total of 25 μg of plasmid DNA. An internal control plasmid [pON260 (*26*)] that contained the β-galactosidase gene driven by

the CMV-IE promoter was added (1 μg) to each transfection cocktail. Forty-eight hours after transfection, cell extracts were prepared and assayed for β-galactosidase activity (*26*). Appropriately normalized amounts of cell extract were used for CAT assays (*14*). After autoradiographic exposure, the thin-layer chromotography (TLC) plates were scanned, and percent conversion values were calculated.
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15. Each transfection experiment was performed in duplicate at least four times. The percent conversion values shown represent the average of duplicates in a representative experiment. The variation among experiments was <10%. The transfection efficiency (as measured by β-galactosidase activity in cell extracts) varied <10% among all plates in a particular experiment. We have detected no toxic or growth inhibitory effects (as measured by reductions in β-galactosidase activity or cell number) as a result of expressing WT1 or EGR-1 in transient assays.
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18. The insertion of 3- and 17–amino acid segments into the WT1 coding sequence and the construction of chimeric *wt1–egr-1* genes was accomplished with sequential PCR-mediated mutagenesis (*27*). The amino acid sequence of the 17–amino acid insertion was taken from the LK15 clone of WT1 [M. Gessler *et al.*, *Nature* **343**, 744 (1990)]. After each round of PCR, the complete coding region of the gene was sequenced to guard against Taq polymerase–induced errors. Two of the deletion mutants of WT1 were created with unique Bam HI (WT1,179–429) and Xmn I (WT1,1-364) restriction sites in the WT33 coding sequence (*1*). The WT1,294–429 deletion corresponded to the previously described WTZF protein (*6*) and included the six histidine residues at the NH_2-terminus. The internally deleted protein WT1(179–294) was created by first cleaving the WT1 and WTZF (*6*) genes at unique Bam HI sites. The restriction sites were blunted by filling in with the large fragment of DNA polymerase, and the gene fragments were fused in a blunt-end ligation reaction. The fusion of WT1 and WTZF in this manner regenerated the proper frame of translation and resulted in the introduction of one additional arginine residue at the site of fusion. Each gene was cloned into pGEM7Zf+ vector and used to generate synthetic RNA by in vitro transcription. The RNAs were used to program rabbit reticulocyte lysates. The DNA binding activity of each protein was assessed by gel retardation assays with a ^{32}P-labeled oligonucleotide probe that contained an EGR binding site (*6, 28*).
19. The full-length WT1 and EGR-1 proteins displayed identical apparent affinity for the EGR binding site when measured in vitro by gel retardation assays. No detectable association was observed between EGR-1 and WT1 in coimmunoprecipitation assays (*21*).
20. For generating chimeric proteins we defined the zinc finger region of WT1 as extending from amino acid 307 to the natural stop codon at position 429. This stop codon occurs 11 amino acids after the last zinc finger in WT1. To ensure that this same number of amino acids was present after the last zinc finger in EGR-1, a stop codon was introduced at amino acid position 427 in the EGR-1 coding sequence (the natural stop codon in *egr-1* occurs 117 amino acids COOH-terminal to the last zinc finger). Thus, the EGR-1 zinc finger region we used for preparing chimeric proteins spanned amino acids 337 to 427. The zinc finger regions of these two proteins displayed identical affinities and DNA binding specificities for the EGR sequence (Fig. 2B).
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The Roles of the Subunits in the Function of the Calcium Channel

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Dihydropyridine-sensitive voltage-dependent L-type calcium channels are critical to excitation-secretion and excitation-contraction coupling. The channel molecule is a complex of the main, pore-forming subunit α_1 and four additional subunits: α_2, δ, β, and γ (α_2 and δ are encoded by a single messenger RNA). The α_1 subunit messenger RNA alone directs expression of functional calcium channels in *Xenopus* oocytes, and coexpression of the α_2/δ and β subunits enhances the amplitude of the current. The α_2, δ, and γ subunits also have pronounced effects on its macroscopic characteristics, such as kinetics, voltage dependence of activation and inactivation, and enhancement by a dihydropyridine agonist. In some cases, specific modulatory functions can be assigned to individual subunits, whereas in other cases the different subunits appear to act in concert to modulate the properties of the channel.

THE DIHYDROPYRIDINE (DHP)-SENsitive Ca^{2+} channel protein of the skeletal muscle (SM) consists of five subunits, α_1, α_2, δ, β, and γ (*1*). The α_1 subunit contains the binding site for DHPs and other classical organic Ca^{2+} channel blockers (*2*), is the pore-forming subunit (*1–4*), and is indispensable for the function of the channel (*5–8*). A single α_2/δ mRNA encodes the precursor protein α_2/δ, which is proteolysed into the δ and α_2 subunits, linked by disulfide bonds (*1, 9*). The cDNAs of the α_2/δ, β, and γ subunits have been cloned from SM (*10–13*). Identical or homologous proteins are found in brain (α_2/δ, β), smooth muscle (α_2/δ, γ), and heart (α_2/δ, β) (*6, 10–15*). Functional Ca^{2+} channels can be expressed in *Xenopus* oocytes injected with RNA of the α_1 subunits from heart, smooth muscle, and brain (*6–8*); coexpression of SM α_2/δ and β with cardiac and brain α_1 subunit enhances the expressed Ca^{2+} currents, whereas the γ subunit is without effect (*6, 8*). The electrophysiological properties of the channel have been reported to be unaffected by the auxiliary subunits (*6, 8*). We have investigated this problem by coexpressing the Ca^{2+} channel subunits in various combinations in *Xenopus* oocytes and examining the macroscopic characteristics of the current through the emerging Ca^{2+} channels.

cRNAs of cardiac α_1 subunit and of SM α_2/δ, β, and γ subunits were synthesized in vitro and injected into *Xenopus* oocytes (*16*). After cRNA injection (4 to 5 days), we measured currents using the two-electrode voltage-clamp method, usually in a solution containing 40 mM $BaCl_2$ (*16*), because Ba^{2+} results in a larger current through the Ca^{2+} channel.

Uninjected oocytes displayed a small endogenous Ba^{2+} current, I_{Ba} (Table 1), that was insensitive to the DHP agonist (−) Bay K 8644 (Bay K) (*17*). A small, Bay K–sensitive I_{Ba} was observed in oocytes injected with the cRNA of the α_1 subunit alone (0 to −50 nA) (Fig. 1). Coexpression in the α_2/δ or β subunit with α_1 consistently enhanced the expressed currents, and coexpression of both α_2/δ and β with α_1 caused more than additive increase in the amplitude of I_{Ba} (Table 1). The γ subunit did not have a consistent effect on the amplitude of I_{Ba} (Table 1). Since the auxiliary subunits similarly enhance the expression of a brain Ca^{2+} channel (*6*), this phenomenon may be common to a variety of Ca^{2+} channels.

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