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EDD, the Human Hyperplastic Discs Protein, Has a Role in Progesterone Receptor Coactivation and Potential Involvement in DNA Damage Response*

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The ubiquitin-protein ligase EDD encodes an orthologue of the *hyperplastic discs* tumor suppressor gene, which has a critical role in *Drosophila* development. Frequent allelic imbalance at the EDD chromosomal locus in human cancers suggests a role in tumorigenesis. In addition to a HECT (homologous to E6-AP carboxyl terminus) domain, the EDD protein contains a UBR1 zinc finger motif and ubiquitin-associated domain, each of which indicates involvement in ubiquitinylation pathways. This study shows that EDD interacts with importin α5 through consensus basic nuclear localization signals and is localized in cell nuclei. EDD also binds progesterone receptor (PR) and potentiates progestin-mediated gene transactivation. This activity is comparable with that of the coactivator SRC-1, but, in contrast, the interaction between EDD and PR does not appear to involve an LXXLL receptor-binding motif. EDD also binds calcium- and integrin-binding protein/DNA-dependent protein kinase-interacting protein, a potential target of ubiquitin-mediated proteolysis, and an altered association is found between EDD and calcium- and integrin-binding protein/DNA-dependent protein kinase-interacting protein in response to DNA damage. The data presented here demonstrate a role for EDD in PR signaling but also suggest a link to cancer through DNA damage response pathways.

The EDD gene, the apparent human orthologue of the *Drosophila melanogaster* gene "*hyperplastic discs*" (*hyd*), was originally isolated as a progestin-induced gene (1). Some mutations in *hyd* result in hyperplasia of larval imaginal discs, suggesting *hyd* functions as a tumor suppressor gene. The proposed function of the HYD protein in *Drosophila* in initiation, maintenance, and/or termination of cell proliferation (2) points to a pivotal role in coordinating the balance between cell cycle progression and differentiation. Many pathways controlling these processes are highly conserved through evolution, and consequently mutations in orthologous genes can have hyperplastic or tumorigenic effects in both mammals and *Drosophila*. *Notch* gene mutations, for example, result in hyperplasia of the embryonic nervous system in *Drosophila* and have also been linked to human leukemia and breast cancer (3), whereas the Patched gene product is required for correct *Drosophila* development and if mutated in humans causes developmental abnormalities together with predisposition to basal cell carcinoma (4). Similarly, tumors are produced in both *Drosophila* and mice upon deletion of the *large tumor suppressor* gene (*lats*) (5). Based on these precedents, recent studies have sought to determine the normal biological functions of EDD and whether it has a role in human cancer (1, 6).[1]

Although the cellular functions of the EDD and HYD proteins are unknown, significant homology exists between their carboxyl termini and those of E6-AP and related proteins (7, 8). These HECT[2] (for homologous to E6-AP carboxyl terminus) family proteins form a subclass of ubiquitin-protein ligases (E3 enzymes) playing a role in the ubiquitinylation cascade that catalyzes the covalent attachment of ubiquitin to specific substrate proteins, targeting them for proteolysis. Unlike other ubiquitin ligases, which possess a RING domain, HECT ligases reversibly bind ubiquitin via a conserved cysteine residue within the HECT domain and directly transfer ubiquitin to the substrate (9). The biochemical properties of *in vitro* translated EDD protein provide evidence that EDD is a human ubiquitin-protein ligase (1). However, protein substrates for EDD in the cell have yet to be defined. It is now clear that ubiquitin-mediated proteolysis is essential for the regulation of many key cellular pathways including control of cell cycle progression (10–12), cellular signal transduction (13–15), DNA damage responses (16), and transcriptional control (17). Of particular note, the proteins targeted by ubiquitinylation include molecules important in oncogenesis such as the transcription factors NF-κB (18), N-Myc (19), and the tumor suppressor p53 (20,

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[1] C. K. W. Watts, unpublished data.

[2] The abbreviations used are: HECT, homologous to E6-AP carboxyl terminus; E3, ubiquitin-protein ligase; ER, estrogen receptor; aa, amino acid(s); GFP, green fluorescent protein; GST, glutathione *S*-transferase; VDR, vitamin D receptor; PR, progesterone receptor; CIB, calcium- and integrin-binding protein/DNA-dependent kinase-interacting protein; DBD, DNA binding domain; DNA PK, DNA-dependent protein kinase; EGFP, enhanced green fluorescent protein; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; HEK, human embryonic kidney.

21). In addition, there are examples where the E3 ligases themselves are either tumor suppressor genes (22) or oncogenes (23).

An additional role for several HECT ubiquitin ligases as steroid receptor transcriptional co-regulators has recently emerged. The progesterone receptor (PR), like other members of the nuclear steroid receptor family, acts as a ligand-dependent transcriptional regulator by binding to specific promoter sequences and recruiting a variety of co-regulator proteins to its ligand-dependent COOH-terminal activation domain (AF-2) (24, 25). Transcriptional coactivation is usually dependent upon direct interaction between nuclear receptor and coactivator, most often via L*XX*LL motifs within the coactivator. In this way coactivators are proposed to form a bridge connecting the transcription factor with the basal transcription machinery (26), and some also contribute enzymatic activities (27, 28). Such co-regulators may have roles in oncogenesis and in hormone resistance. AIB1 (SRC-3), for example, is commonly overexpressed and amplified in breast cancers, with higher expression in ER-positive breast cancer cell lines (29).

Recently, the HECT domain proteins yeast Rsp5 and its human orthologue hRPF1 (30) and E6-AP (31) were identified as having PR coactivation activity. The mechanism for this activity is unknown, but, like SRC-1, E6-AP interacts directly with PR. We now report an interaction between EDD and PR and show that EDD enhances PR transcriptional activity, providing for the first time a link between progestin-regulated EDD gene expression and PR function. In addition, yeast two-hybrid library screening was used to identify other EDD-interacting proteins including the nuclear import protein NPI-1 (importin α5), calcium- and integrin-binding protein/DNA-dependent kinase-interacting protein (CIB), and other nuclear proteins, with observations confirmed by a range of other approaches. Accordingly EDD was found to be predominantly nuclear. These studies therefore provide novel information on the functional role of EDD in the nucleus and point to possible multiple roles for this large HECT family ligase.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—EDD cDNAs used for *in vitro* translation, transfection, and yeast two-hybrid screening are shown in Fig. 1*A*. cDNAs encoded the full-length protein EDD (aa 1–2799), the NH_2-terminal domain EDDF1 (aa 1–889), the central domain EDDF2 (aa 889–1877), the carboxyl domain EDDF3 (aa 1877–2799), the NH_2-terminal plus central domains EDDF4 (aa 1–1877), and the central plus COOH-terminal domains EDDF5 (aa 889–2799). EDDM, EDDF3M, and EDDF5M contain a mutation (Cys-2768 → Ala) at the active site cysteine necessary for E3 ligase activity in HECT proteins. For mapping of the EDD NH_2 terminus, restriction fragment cloning was used to generate *in vitro* translation constructs expressing EDD aa 1–577 (EDDF1a), 578–889 (EDDF1b), 1–419 (EDDF1c), and 420–889 (EDDF1d) (Fig. 2*A*). For yeast two-hybrid screening, EDD cDNA fragments used as baits were cloned from pBluescript-EDD (1) in frame with the Gal4 DNA binding domain (DBD) of the pAS2.1 vector (CLONTECH, Palo Alto, CA). For *in vitro* translation, EDD-derived cDNAs were transcribed from pBluescript (Amersham Biosciences), pSG5 (Stratagene, La Jolla, CA), or pRcCMV (Invitrogen, Groningen, Netherlands) vectors. For EDD protein expression in mammalian cells, constructs in pRcCMV have been previously described (1) and additional constructs for expression of FLAG epitope-tagged EDD were generated in the pSG5 vector. A GFP reporter vector (pGFP20; Dr. S. Aota, Osaka, Japan) was co-transfected to monitor transfection efficiency. A bacterial plasmid expressing GST fused to amino acids 263–538 of human importin α5 (NPI-1) was obtained from Peter Palese (Mount Sinai School of Medicine, New York, NY). A GST fusion of mouse importin β1 (PTAC97) was expressed and purified as described previously (32). Full-length CIB was cloned from the pACT2 vector into the pGEX2T vector for GST-CIB fusion protein expression in bacteria and into the pCMVTag2C vector for mammalian expression of FLAG-tagged protein. For expression of GFP-tagged EDD in mammalian cells, full-length EDD was cloned into pEGFP-C1 (for NH_2-terminal EGFP tag) and pEGFP-N1 (for COOH-terminal EGFP tag, CLONTECH). For transient expression of progesterone receptor, phPR1 vector encoding human PR B was obtained from P. Chambon (INSERM, Strasbourg, France). A PRE-luciferase reporter vector (pMSGluc) was constructed by insertion of a MMTV-LTR promoter in the pGL3-Basic vector (Promega Corp.). The phPR1 vector was used to clone the PR(AB) (aa 1–546) and PR(CDE) (aa 456–933) regions into pGEX4T2 for GST fusion protein expression. For transient expression of vitamin D receptor (VDR), pCMV-VDR along with pOS_2-luc reporter vector were obtained from G. Leong (Garvan Institute, Darlinghurst, Australia). Estrogen receptor was expressed from pCMV-ER (V. C. Jordan, Northwestern University Medical School, Chicago, IL) and pERE-TK-GL3 reporter vector was obtained from M. Parker (Imperial Cancer Research Fund, London, UK). For *in vitro* translation, SRC-1 was cut from pCR3.1-SRC1A (B. O'Malley, Baylor College, Houston, TX) and cloned into pBluescript.

Yeast Two-hybrid Assay for EDD-interacting Proteins—The cDNAs for full-length EDD mutant and carboxyl domain mutants (EDDM and EDD5M) were screened against a human placenta cDNA library in the pACT2 vector by the yeast two-hybrid method (Matchmaker 2, CLONTECH). Stable transformants of *Saccharomyces cerevisiae* strain Y190 expressing EDD fusion protein were transformed with the library using the lithium acetate method and 2–3 $\times$ 10^6 primary transformants selected on $His^-Leu^-Trp^-$ plates. Following a second round of selection on the same medium, colonies were assayed for β-galactosidase activity using a filter-based assay. Interacting plasmids that were positive for β-galactosidase only in the presence of the EDD bait plasmids were transformed into *Escherichia coli* DH5α cells for further analysis. Manual sequencing was carried out using ^{33}P-end-labeled primer in conjunction with the fmol Cycle sequencing kit (Promega Corp., Madison, WI). Sequences were analyzed by Blast searches of the GenBank™ and EMBL data bases and predicted proteins analyzed for motifs using the ISREC Profile Scan Server (www.isrec.isb-sib.ch).

For semiquantitation of protein interactions, CG1945 yeast cells containing pAS2.1-EDD constructs were mated with Y187 yeast cells harboring pACT2-derived plasmids. Diploids were selected on Leu^-Trp^- plates and used to inoculate cultures, which were grown to saturation, diluted 1:10, and grown for 16 h. Yeast cells were harvested for protein, and β-galactosidase activities were determined in a liquid chemiluminescence assay (Tropix Galacto-Light System, Applied Biosystems).

Recombinant Protein Binding Assays—GST-tagged fusion proteins were prepared from *E. coli* strain BL21 according to established protocols (Amersham Biosciences protocol handbook). Soluble fusion proteins were bound to glutathione-agarose and quantitated via Coomassie Blue staining against protein standards. ^{35}S-Labeled EDD protein and mutants or SRC-1 were synthesized in a coupled *in vitro* transcription/translation system (TnT Quick, Promega) and 10–20 μl of reaction mixture was diluted in 1% Triton X-100 lysis buffer (1) and incubated with 5 μg of GST, GST-importin α5, GST-PR(CDE), or GST-CIB coupled to glutathione-agarose beads at 4 °C for 2 h. Beads were collected by centrifugation, washed extensively in lysis buffer, and resuspended in SDS-PAGE sample buffer. After boiling, bound protein was visualized following SDS-PAGE and autoradiography.

Cell Culture and Transient Transfection—HEK 293 and T-47D were maintained as previously described (1). MCF-7 cells were maintained in RPMI medium (Life Biotechnologies) containing 10% serum in 5% CO_2. For overexpression by transient transfection, 3 $\times$ 10^6 HEK 293 cells were plated in minimal essential medium with Hanks' salts containing 10% serum in 15-cm Petri dishes. The following day pRcCMV-EDD (10 μg) was added to the cells along with 30 μl of FuGENE reagent (Roche Diagnostics, Castle Hill, New South Wales, Australia).

Localization of GFP-tagged and Endogenous EDD Protein—HEK 293, Chinese hamster ovary, MCF-7, or T-47D cells were seeded in six-well plates at 1–2 $\times$ 10^5 cells/well. Cells were transfected with 2 μg of pEGFP-EDD or empty vector DNA and the following day split to chamber slides for 24–48 h. Slides were washed in PBS, fixed in 3.7% paraformaldehyde, washed in PBS, and mounted in 90% glycerol. GFP was visualized by fluorescence microscopy. For immunostaining, HEK 293 cells or EDD-transfected HEK 293 cells (WT30) were embedded in paraffin. Sections were de-waxed and rehydrated before unmasking in EDTA/citrate buffer and then stained with goat anti-EDD antibody N19 (Santa Cruz Biotechnology, Santa Cruz, CA). EDD signal was detected using DAKO LSAB Plus Link and Label (DAKO Corp.) with liquid 3,3′-Diaminobenzidine Plus (DAKO Corp.) as substrate. Counterstaining was performed with hematoxylin.

Protein Interactions in Cell Lysates—For extracts of total cellular protein, cells were harvested in 1% Triton X-100 lysis buffer as previously described (1). Extraction of nuclear proteins and cytoplasmic s100

fractions from T-47D and MCF-7 cells was carried out according to published methods (33).

For GST fusion protein pull-down of endogenous or recombinant EDD from cell lysates, 0.5–1 mg of total protein was incubated with 5 μg of GST or GST fusion protein bound to glutathione beads for 1–2 h at 4 °C. Beads were washed extensively in 1% Triton X-100 lysis buffer and bound proteins resolved by SDS-PAGE and detected by immunoblotting with EDD antisera (1). For immunoprecipitation, 10 μl of importin α5 antiserum (Peter Palese, Mount Sinai School of Medicine, New York, NY) was incubated with 0.5–1 mg of cell lysate (4 °C for 1 h). Antibody conjugates were captured on protein A-Sepharose beads (4 °C for 1 h) and washed extensively in lysis buffer. Bound proteins were resolved by SDS-PAGE followed by immunoblotting with EDD antisera.

Stable HEK 293 cells overexpressing EDDM were transfected with pCMVTag2C-CIB or empty vector using FuGENE 6 reagent (Roche) for 24 h. Following 6 h of incubation in the presence of MG132, cells were harvested and lysates prepared and 1 mg of total protein incubated with anti-FLAG antibody M2 coupled to Sepharose (Sigma) for 2 h at 4 °C. Beads were recovered by centrifugation and washed extensively in lysis buffer. Western blotting for EDD has been described (1).

Nuclear Receptor Transactivation Assays—HEK 293 or COS7 cells were plated in six-well plates (2×10^5 cells/well) and the medium changed to 2% charcoal-stripped fetal calf serum the following day. Transfection was carried out using 3–4 μl of FuGENE 6 reagent (Roche) with 1–2 μg of DNA comprising 90 ng of receptor expression vector, 450 ng of luciferase reporter vector, and 1.2 μg of EDD, EDDM, or SRC-1 cDNAs in either pRcCMV or pSG5, or empty vector, and 200 ng of GFP expression vector pGFP20. The following day cells were split into 96-well plates (7×10^3 cells/well) or 6-well plates (1.4×10^5 cells/well), and drugged 24 h later. After another 24 h cells in 96-well plates were assayed for luciferase activity (Luclite reagent, Packard Bioscience, Meriden, CT) and cell number (Wst-1 reagent, Roche). In some experiments cell number was monitored using the CellTiter96® proliferation assay (Promega Corp., Madison, WI). Cells in six-well plates were analyzed for GFP expression by fluorescence microscopy to determine transfection efficiency and also used for preparation of protein lysates so that protein expression levels of various constructs could be compared. In experiments where there was significant variation in cell number or GFP expression, these parameters were used to normalize luciferase activity. In some experiments pRSV-β-gal or pRL-TK (Promega Corp.) vectors were transfected in place of pGFP20 and transfection efficiency monitored by assaying for β-galactosidase or *Renilla* luciferase activity, respectively.

Proteasome Inhibition Experiments—For proteasome inhibition experiments, HEK 293 cells were plated at 3×10^5 cells/well of a six-well dish in minimal essential medium with Hanks' salts supplemented with 10% fetal bovine serum (Invitrogen). After 48 h, medium was replaced with medium containing 20 μM MG132 (Calbiochem) or Me_2SO vehicle for 2–6 h. A monoclonal antibody for Western blotting against CIB was kindly provided by U. P. Naik (University of North Carolina, Chapel Hill, NC).

Treatment of Cells with DNA Damaging Agents—MCF-7 cells were incubated in RPMI containing 0.5% fetal bovine serum for 18 h before addition of phleomycin (Cayla, Toulouse, France) at 100 μg/ml, hydroxyurea (Calbiochem) at 2 mM, or PBS vehicle, for 6 h. Cells were harvested for total protein or nuclear protein extracts as described above.

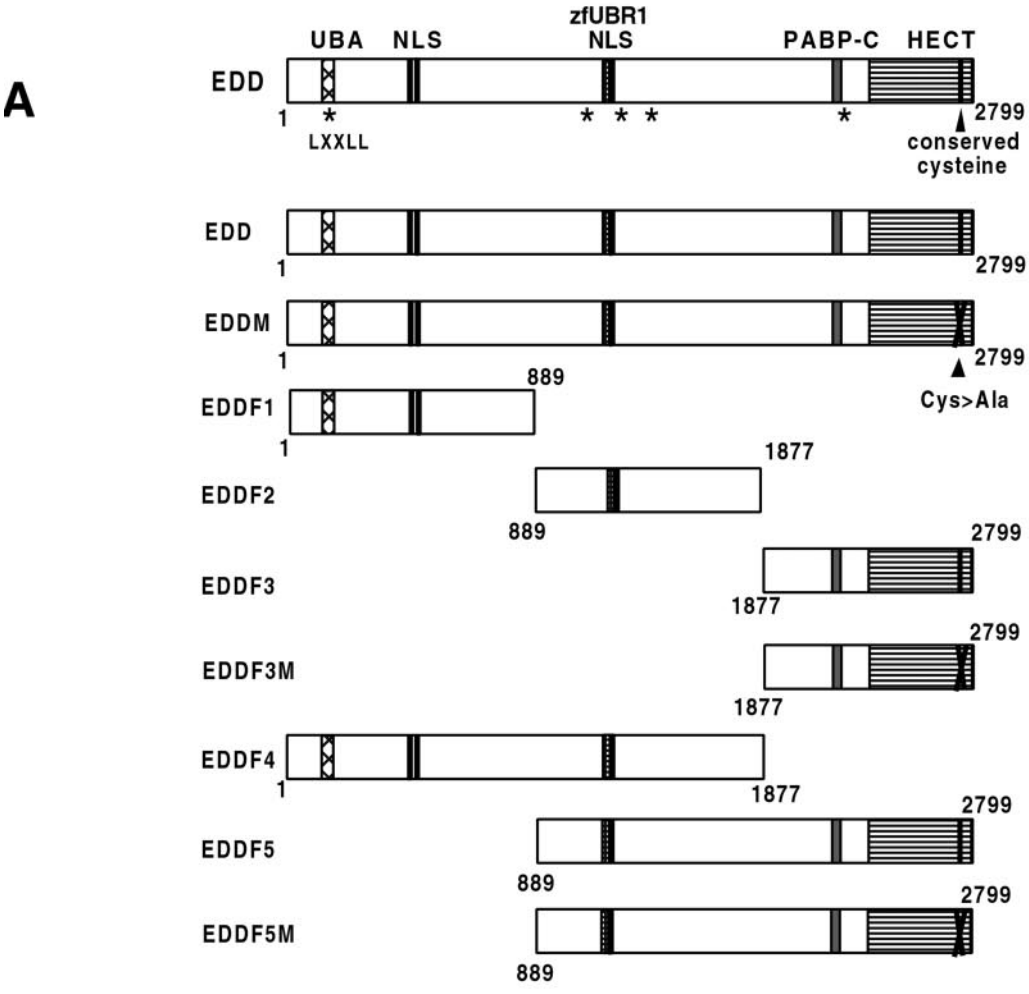
RESULTS

Domain Structure of EDD—Previous analysis of the EDD sequence showed the presence of a carboxyl-terminal HECT domain, identifying EDD as a member of the HECT family of ubiquitin protein ligases (1, 8) (Fig. 1*A*). Further examination of the central domain of EDD that is also highly conserved with HYD revealed a stretch of 68 amino acids (aa 1177–1245) that is 95 and 100% conserved with HYD and mouse EDD, respectively (Fig. 1*B*) and which shows a high degree of alignment with calossin (pushover), a calmodulin-binding protein important for neurotransmission and male fertility in *Drosophila* (34, 35). Contained within this region is a cysteine/histidine-rich putative zinc finger domain, zf-UBR1 (Pfam PF02207; Ref. 36), originally identified in the N-end rule E3 ubiquitin ligase UBR1p/N-recognin from a range of species (37–39). The consensus sequence $CX_{12-16}CX_2CX_{8-10}CX_2CX_{4-5}HX_2HX_{11-14}CXCX_{4-14}C$ is reminiscent of the more common RING domain, which is also found in a distinct region of UBR1p. Both types of zinc-binding domains are proposed to have roles in protein-protein interaction, with the RING domain having an established role in ubiquitinylation (40, 41). This central region of EDD also contains a potential bipartite nuclear localization sequence (NLS) (KL**KR**TSPTAYCDCWE**K**C**K**C**K**, aa 1402–1602), whereas another putative NLS resides in the NH_2-terminal region (**RK**KMLEKARAKN**KK**P**K**, aa 502–517) upstream of a potential SV40 large T antigen-like NLS (PYKRRR, aa 630–635) (42). Also within the NH_2-terminal region of EDD is another region conserved with HYD known as a UBA domain (aa 188–225), found in proteins with various roles in ubiquitinylation pathways and thought to form a protein-protein interaction interface (43). The UBA domain binds mono- and multi-ubiquitin chains and thus may be involved in regulation of protein ubiquitinylation (44). Amino-terminal to the HECT domain (aa 2391–2455) lies still another region likely to mediate protein interactions. This 60-amino acid stretch shows 50% homology to a region within the carboxyl terminus of poly(A)-binding proteins from a range of species (1, 45). The x-ray structure of this domain in both poly(A)-binding proteins and EDD has recently been determined and forms a protein interaction interface consisting of four α helices (45, 46).

Interaction between EDD and Importin α5—As the EDD protein sequence contains a number of features that are likely to provide protein-protein interaction interfaces, we used candidate gene and yeast two-hybrid approaches to identify interacting proteins that may be ubiquitinylation targets of EDD or other associating proteins with a role in EDD function. First, yeast two-hybrid screening of a human placental cDNA expression library was performed against baits encoding full-length EDD or fragments containing one or more potential interaction domains of EDD (see Fig. 1*A*). Screening with full-length EDDM (C2768A mutant) identified two independent cDNAs encoding the nuclear import protein importin α5 (NPI-1; Refs. 47 and 48), one full-length and the other encoding amino acid 229 to the carboxyl terminus (amino acid 538). Importin α has a specific role in nuclear import, by recognizing NLSs, implying both that one or more of the potential NLSs within EDD are indeed functional, and that EDD may have a role in the nucleus, with importin α involved in transporting EDD from the cytoplasm to the nucleus.

Importin α Interacts with a Region of EDD Containing Nuclear Localization Signals—A strong interaction was found between NPI-1/importin α5 and full-length EDD, with full-length importin α5 interacting more strongly than the amino-truncated protein isolated by two-hybrid screening (Fig. 2*A*). This difference might be explained if EDD, like other proteins that contain basic NLSs, is recognized by the armadillo repeats of importin α; only four of seven such repeats are present in the truncated importin α5 clone. Pull-down experiments showed that a GST-importin α5 fusion protein encoding amino acids 263–538 was able to bind to *in vitro* translated EDD and mutants encoding the NH_2-terminal one third of EDD but not to the central or carboxyl-terminal regions of EDD (Fig. 2, *B* and *C*). This suggested that the putative NLS in the central region of EDD was not functional in nuclear import. GST-importin α5 also interacted with endogenous EDD (T-47D cells) and recombinant EDD expressed in HEK 293 cells (Fig. 2*D*), precipitating a considerable proportion of the available EDD protein. Further, anti-importin α5 antisera also immunoprecipitated EDD protein from these lysates, showing that EDD and importin α5 interact *in vivo* (Fig. 2*D*).

The amino-terminal one third of the EDD protein contains two potential basic NLSs: one bipartite and one simple. To determine the relative contributions of these motifs to importin α binding, a



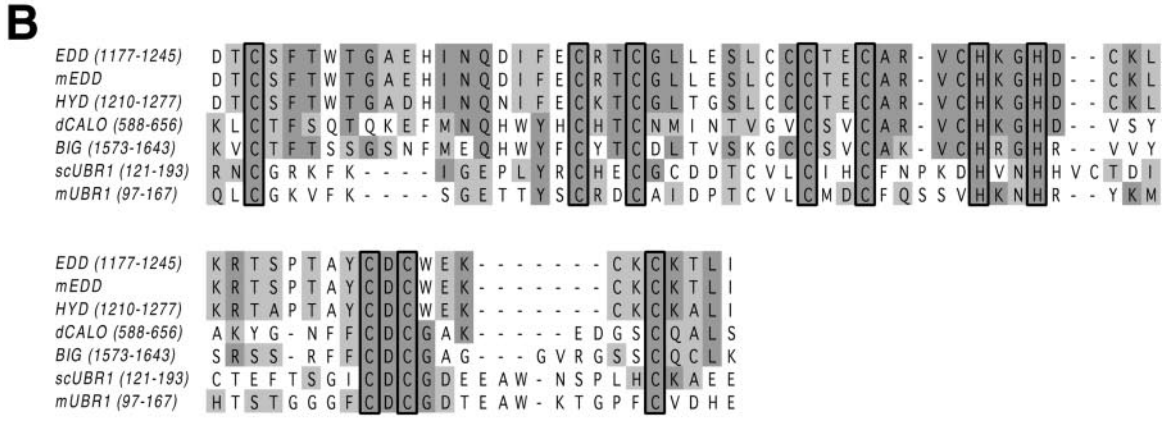


FIG. 1. **Structural features of the EDD sequence.** *A*, schematic diagram of EDD and its derivatives used for mammalian expression, yeast two-hybrid analysis, or *in vitro* translation. The UBA domain, three putative NLSs, a HECT domain, and domains with homology to N-recognin zinc finger (*zfUBR1*) or the carboxyl region of poly(A)-binding protein (*PABP-C*) are indicated. The positions of potential steroid receptor binding motifs (L*X*XLL) are indicated by *asterisks*. *Numbers* indicate amino acid positions of fragment breakpoints. The conserved cysteine within the HECT domain (Cys-2768) is mutated to alanine (*X*) in fragments EDDM, EDD3M, and EDD5M. *B*, potential zinc finger in EDD protein. A cysteine-rich domain shows similarity to *D. melanogaster* calossin (*dCALO*) and *Arabidopsis thaliana* BIG proteins and has a similar arrangement of conserved cysteine and histidine residues (*boxed*) as the zinc finger region first identified in N-recognin. N-recognin sequences shown are from yeast (*scUBR1*) and mouse (*mUBR1*). Identical residues are designated by *dark shading* and conservative substitutions by *lighter shading*.

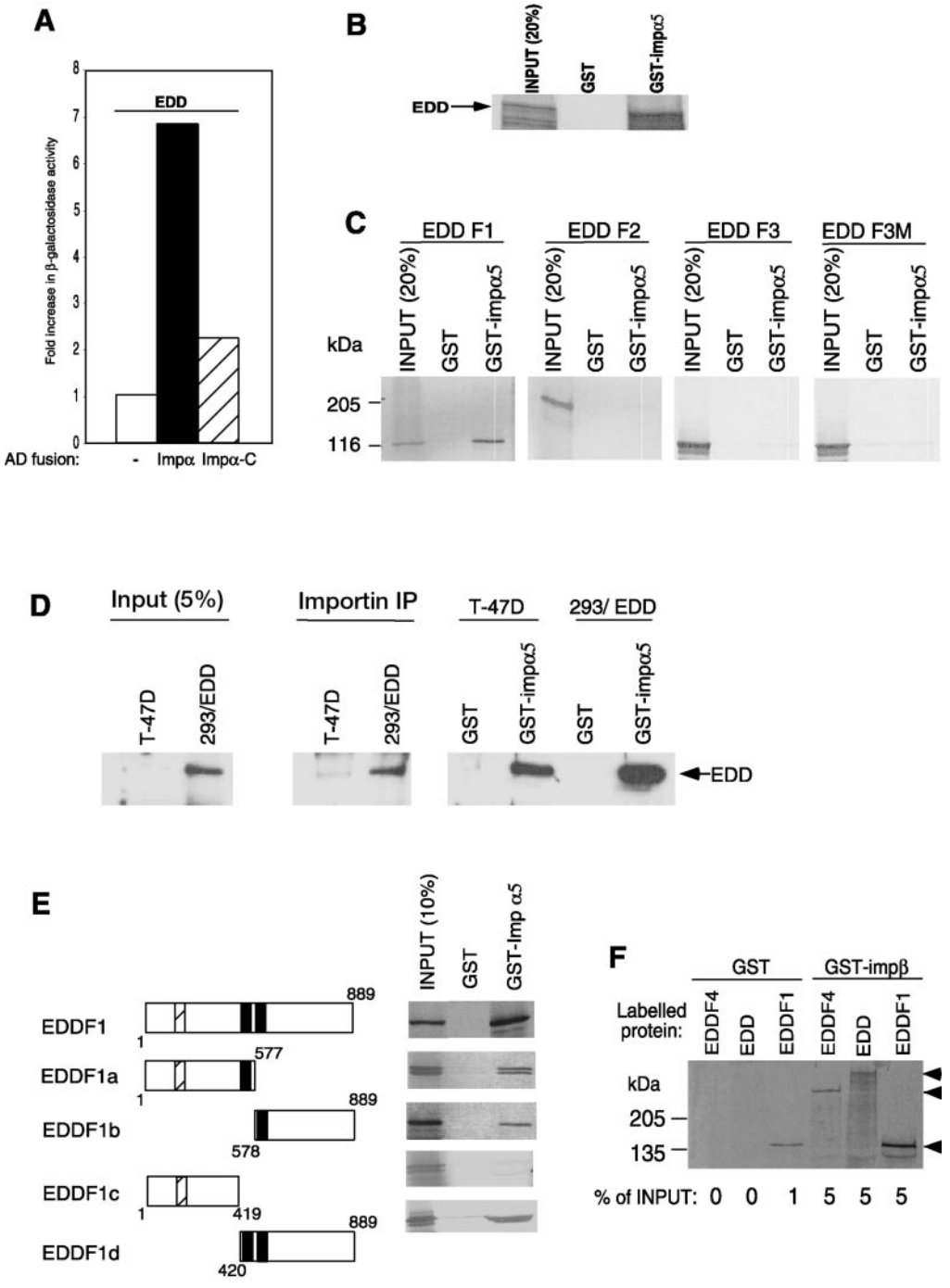


FIG. 2. **EDD interacts with importin α5 via two NLSs.** *A*, interaction of EDD with importin α5 in a yeast two-hybrid assay. The entire coding sequence of EDD was fused in-frame with the yeast GAL4 DBD. This construct or control vector pAS2.1 was co-expressed with either control vector (pACT2) or the GAL4 AD-importin α constructs encoding aa 1–538 (*Impα*) or 229–538 (*Impα-C*) in diploid yeast strain CG1945/Y187. Protein extracts were prepared from cultures of six independent colonies and assayed in duplicate for β-galactosidase activity (expressed as -fold increase over pAS2.1 vector control). *B* and *C*, *in vitro* interaction of importin α with EDD and mapping of interaction. *In vitro* translated ^{35}S-labeled EDD (*B*) or EDD fragments (*C*) were incubated with a purified GST-importin α5 fusion protein or with GST alone bound to glutathione-Sepharose beads, washed, and analyzed by SDS-PAGE and autoradiography. *D*, interaction of EDD with importin α in HEK 293 and T-47D cells. HEK 293 cells were stably transfected with a plasmid encoding full-length EDD protein (*293/EDD*). Extracts from these cells or T-47D cells were subjected to immunoprecipitation with anti-importin α5 antibody (*middle panel*) or incubated with either GST or GST-importin α5 fusion protein bound to glutathione-Sepharose beads (*right panel*). Bound proteins from both procedures were separated by SDS-PAGE and Western blotted for EDD. *E*, mapping interaction between importin α and individual NLSs of EDD. *In vitro* translated ^{35}S-labeled EDD derivatives from the NH_2-terminal region were incubated with GST-importin α5 or with GST alone bound to glutathione-Sepharose beads. Bound EDD was detected by SDS-PAGE and autoradiography. *F*, *in vitro* interaction of importin β with EDD. *In vitro* translated ^{35}S-labeled EDD and derivatives were incubated with GST-importin β fusion protein or with GST alone bound to glutathione-Sepharose beads. Bound EDD was detected by SDS-PAGE and autoradiography. Amounts of bound EDD relative to input are indicated as percentages *below* gel.

set of constructs for *in vitro* translation were made that contained one, both, or neither NLS. GST-importin α interacted with each NLS to some degree, and no interaction was seen in the absence of both signals (Fig. 2*E*). We therefore conclude that both NH_2-terminal signals are required for full binding potential.

We expected that EDD might be in a nuclear import complex

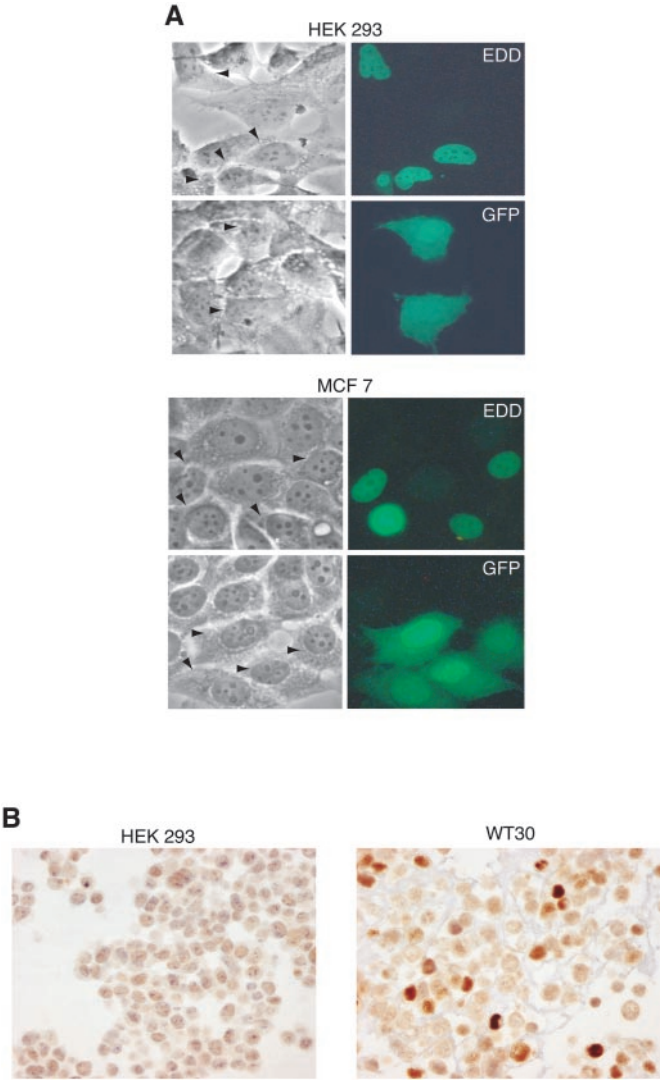


FIG. 3. **EDD is a nuclear protein.** *A*, subcellular localization of EDD-GFP in mammalian cell lines (original magnification, ×40). EDD cDNA was fused to the amino terminus of GFP and transiently transfected into HEK 293 (*upper panel*) or MCF-7 cells (*lower panel*). Transfected cells are indicated by *arrowheads* on bright field images. Although diffuse cellular staining was observed with GFP alone, strong nuclear GFP fluorescence was observed for EDD-GFP in both cell lines. *B*, immunostaining of cells with EDD antibody (original magnification, ×40). Nuclear staining seen for endogenous EDD in HEK 293 cells (*left panel*) is more intense in HEK 293 cells that overexpress EDD protein (*WT30*, *right panel*).

with importins α and β, so binding to the nuclear import partner importin β/p97 was also tested (Fig. 2*F*). GST-importin β bound to *in vitro* translated EDD and the amino two thirds or one third of the EDD protein, resulting in pull-down of ~5% of the available EDD protein. As the extracts used for *in vitro* translation contain endogenous importin α, binding of EDD and importin β is most likely mediated by the importin α/β heterodimer. Yeast two-hybrid analysis also indicated EDD interaction with both importin α1 (Rch1) and importin α3 (Qip1), although the interaction between EDD and importin α5 was markedly stronger (data not shown). Overall, the interaction between EDD and several importin α isoforms and importin β points to a role for EDD within the nucleus.

EDD Is a Nuclear Protein—To determine the cellular localization of EDD, mammalian expression vectors were made for EDD fused to the NH_2 or COOH terminus of GFP. Transfection of HEK 293 cells or MCF-7 breast cancer cells with the NH_2-terminal EDD-GFP fusion showed that fluorescence was restricted to the nucleus (Fig. 3*A*). Identical results were obtained with the COOH-terminal fusion (data not shown). In contrast, when cells from either line were transfected with pEGFP vector only, a diffuse pattern of staining throughout the whole cell was observed. Nuclear localization was confirmed when EDD-specific antibodies were used to stain sections of HEK 293 cells, which endogenously express EDD, or WT30 derivative of HEK 293 cells, which overexpress EDD (Fig. 3*B*). The same pattern of staining was seen for a second EDD-specific antibody (data not shown).

EDD Interacts with Progesterone Receptor B—Previous studies have demonstrated that the HECT-domain protein E6-AP interacts directly with PR-B through a region containing L*XX*LL motifs (31). These motifs, which are present in other transcriptional coactivators, are potentially involved in nuclear receptor interaction and coactivation (49). Because the EDD protein is nuclear and contains five L*XX*LL domains (at amino acids 248, 1102, 1255, 1398, and 2428), we tested the ability of EDD to interact with PR-B and regulate its function. First we performed GST-PR fusion protein pull-downs of EDD or *in vitro* synthesized EDD fragments. The NH_2-terminal AB region of PR contains a ligand-independent activation function 1, whereas the COOH-terminal CDE region of PR contains the hinge and DNA binding domains and a ligand-dependent activation function 2. The CDE region of PR, PR(CDE), interacted with endogenously expressed EDD from T-47D cells (Fig. 4*A*). This interaction was mapped using *in vitro* translated EDD protein fragments. A strong interaction was detected between the amino-terminal region of EDD (EDDF1, aa 1–889) and the CDE region of PR, being greater than that seen for SRC-1 (Fig. 4*B*). In these *in vitro* assays, interactions between PR(CDE) and either SRC-1 or EDDF1 were not affected by the PR ligand ORG2058 (data not shown). No significant binding was observed between PR and other fragments of EDD (Fig. 4*B* and data not shown).

The NH_2-terminal region of EDD contains one of the five L*XX*LL motifs so the interaction was mapped further to assess the involvement of this motif. EDD fragments EDDF1a–EDDF1d were tested for their ability to bind GST-PR(CDE). Although EDDF1a (aa 1–577) and EDDF1c (aa 1–419) contained the L*XX*LL motif, the strongest binding occurred between EDDF1b (aa 578–889) or EDDF1d (aa 420–889) and PR(CDE) (Fig. 4*C*), suggesting that binding is mediated by the region of EDD consisting of amino acids 420–889, which includes both NLSs but not the L*XX*LL motif. This also ruled out the involvement of the UBA domain in this interaction. Taken together, these data demonstrate an interaction between EDD and PR.

EDD Acts as a Transcriptional Coactivator for Nuclear Receptors—The nuclear localization of EDD and the observed interaction between EDD and PR-B, together with evidence from separate studies that other HECT-domain proteins such as yeast Rsp5, its human homolog hRPF1 (30), and E6-AP (31) have coactivator activity for nuclear receptors, prompted an investigation of whether EDD could enhance transcriptional activation by PR-B. To this end, HEK 293 and COS7 cells, which lack endogenous PR, were transfected with a PR expression vector (pSG5/hPRB-1) and the progestin-responsive MMTV-luciferase reporter construct together with expression vectors for EDD, or SRC-1 as a positive control. EDD consistently increased progestin (ORG2058)-induced luciferase activity 3–5-fold above control levels in both lines, an effect comparable with that of SRC-1 (Fig. 5*A*). In the absence of added ORG2058, EDD and SRC-1 also slightly increased the basal activity of the luciferase MMTV-LTR promoter, an effect more apparent in the COS7 cell line. We next tested whether the

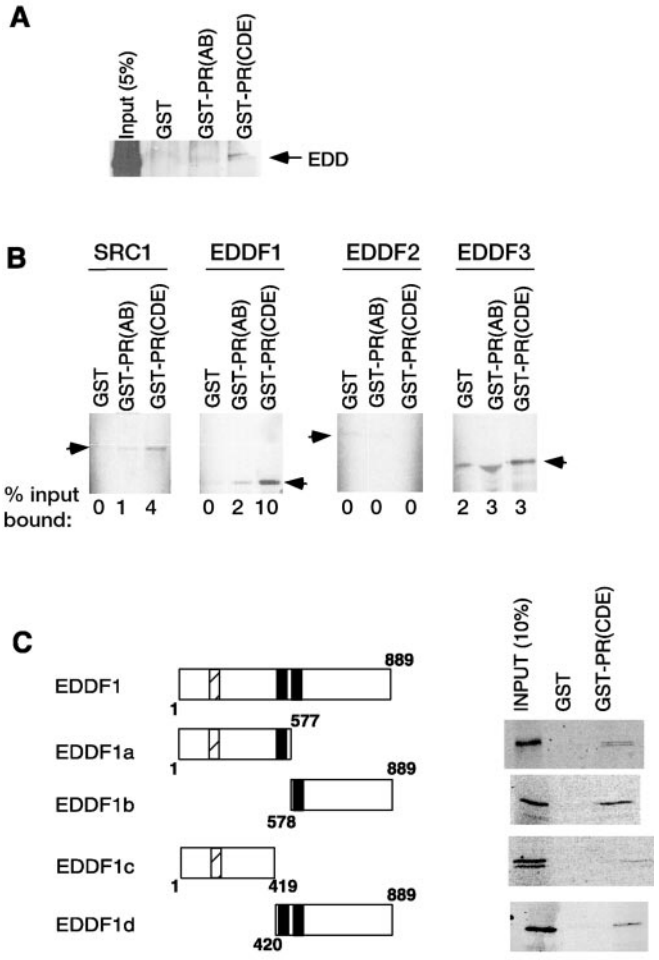


FIG. 4. **EDD interacts with PR-B.** *A*, interaction of EDD with PR in T-47D cells. Extracts from T-47D cells were incubated with either GST, GST-PR(AB) or GST-PR(CDE) fusion proteins bound to glutathione-Sepharose beads. Bound proteins were separated by SDS-PAGE and Western blotted for EDD. *B*, mapping interaction between PR and EDD. *In vitro* translated ^{35}S-labeled EDD derivatives or SRC-1 were incubated with GST-PR(AB) and GST(CDE) fusion protein or with GST alone bound to glutathione-Sepharose beads. PR-bound EDD fragments were analyzed by SDS-PAGE and autoradiography. Amounts of bound EDD or SRC-1 relative to inputs are indicated as percentages *below* gel. *C*, fine mapping of the interaction between PR(CDE) and the NH_2-terminal region of EDD. *In vitro* translated ^{35}S-labeled EDD derivatives from the NH_2-terminal region were incubated with GST-PR(CDE) fusion protein or with GST alone bound to glutathione-Sepharose beads. PR(CDE)-bound EDD fragments were analyzed by SDS-PAGE and autoradiography.

observed transcriptional effect of EDD was because of the ubiquitin ligase activity of EDD. When the ligase-defective EDD mutant (EDDM) was transfected, a comparable coactivator activity was observed, suggesting that the coactivator activity of EDD is independent of its ubiquitin ligase activity (Fig. 5*B*).

Importantly no effect of EDD on PR transactivation is seen in the presence of the progestin antagonist RU486 (data not shown), indicating specificity for ligand-bound receptor. In addition, there was no effect of EDD on reporter gene activity in the absence of PR, indicating a specific effect on transactivation by PR (data not shown). Transfection of increasing amounts of pRcCMV-EDD showed a clear dose response for effects on progestin-induced luciferase activity (Fig. 5*C*), and ORG2058 at all concentrations between 10 pM and 100 nM stimulated luciferase activity to a much greater extent when EDD was co-expressed (Fig. 5*D*). EDD co-expression resulted in a greatly enhanced response to low concentrations of progestins such that, without EDD transfection, 10 nM ORG2058 gave a maximal response, whereas this was exceeded at a 100-fold lower concentration, 100 pM, with EDD overexpression. These data reveal for the first time a cellular function for EDD as a nuclear receptor coactivator. Interestingly, EDD also enhanced transactivation by the VDR 3-fold (Fig. 5*E*). However, ER activity was not enhanced by EDD, whereas in the same experiment SRC-1 acted as a coactivator (Fig. 5*F*), demonstrating that EDD discriminates between steroid receptors. Together these data demonstrate that EDD serves as a coactivator in PR- and VDR-mediated transcription.

EDD Interacts with CIB, a Protein Potentially Involved in DNA Damage Responses—Further yeast two-hybrid screening was aimed at identifying other proteins involved in the ubiquitinylation or coactivation functions of EDD. When full-length EDDM or EDDF5M (aa 889–2799) were used as baits, three clones encoding calcium- and integrin-binding protein/DNA-dependent protein kinase-interacting protein (CIB/KIP) were isolated: two full-length and another encoding CIB/KIP aa 5–191. CIB is a protein with possible dual roles in the cytoplasm and nucleus (50–53). The interaction between CIB and full-length EDD initially detected in the yeast two-hybrid system (Fig. 6*A*) was confirmed by pull-down of *in vitro* translated EDD proteins with GST-CIB (Fig. 6*B*). Mapping of this interaction using *in vitro* translated EDD fragments showed that CIB interacts with the carboxyl-terminal portion of the EDD protein (EDDF3, EDDF3M; Fig. 6*C*). To obtain evidence for interaction in cells, FLAG-tagged CIB was expressed in HEK 293 cells overexpressing EDD and protein extracts were prepared. EDD protein was detected in FLAG immunoprecipitates from these lysates but not from those of vector-transfected cells (Fig. 6*D*, *left panel*). GST-CIB fusion protein also interacted with EDD in cell lysates prepared from nuclei of MCF-7 cells expressing endogenous levels of EDD (Fig. 6*D*, *right panel*, *Control*).

Because we observed EDD in nuclei, a possible nuclear role for CIB was investigated. As CIB was previously found to interact with the DNA damage-sensing enzyme DNA PK (50), lysates from MCF-7 cells treated with the radiomimetic phleomycin, which induces double strand breaks in DNA, were incubated with GST-CIB fusion protein. Capture of the bound protein revealed significantly less association between EDD and CIB when cells had been treated with phleomycin, when compared with untreated cells or cells treated with hydroxyurea, which causes DNA cross-linking (Fig. 6*D*, *right panel*). The change in binding was not because of decreases in EDD protein levels, which were unchanged (data not shown).

Being a binding partner of EDD, CIB is a possible target for ubiquitin-mediated degradation. Because proteins regulated in this manner are usually stabilized in cells in the presence of inhibitors of the 26 S proteasome, HEK 293 cells were treated with MG132, and the levels of CIB were ascertained by Western blot. As a positive control, the protein levels of the cyclin-dependent kinase inhibitor p27, a known substrate of the proteasome, were also monitored. Whereas p27 levels were enhanced in the presence of the proteasome inhibitor, the protein levels of importin α1 remained constant regardless of treatment (Fig. 6*E*). However, the levels of CIB followed the same pattern as those of p27, which suggests that CIB is a target of ubiquitin-mediated degradation, in turn raising the possibility of involvement of the E3 ligase activity of EDD in this process. These studies show that EDD interacts with a potential ubiquitinylation substrate, CIB, and that this interaction is sensitive to DNA damage. This is the first indication of protein interactions involving either EDD or CIB being responsive to DNA damage.

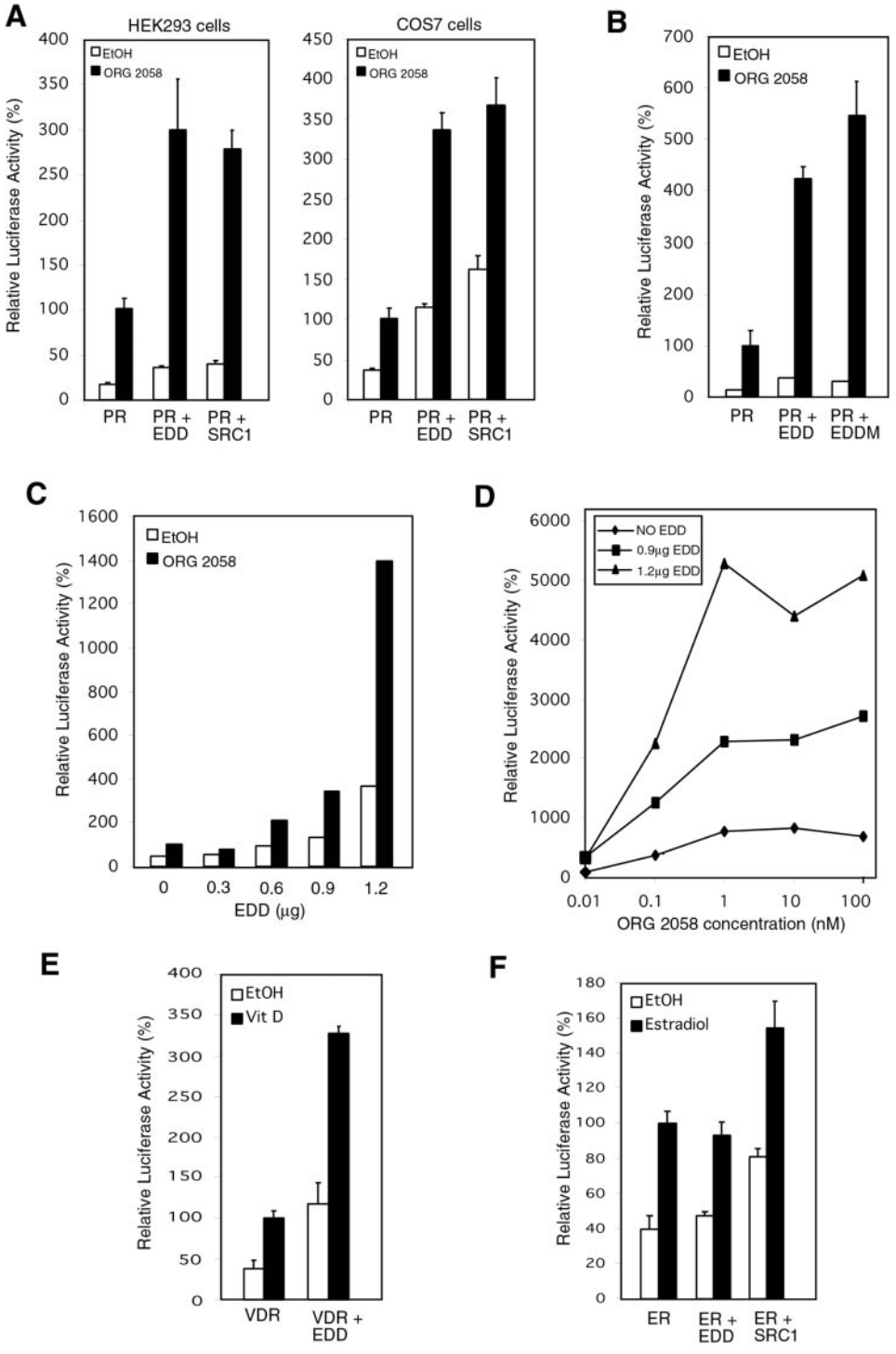


FIG. 5. **Enhancement of nuclear receptor transactivation activity by EDD.** Luciferase activity was corrected for cell number and transfection efficiency where appropriate (see "Experimental Procedures") and graphed relative to the value for liganded receptor alone, which was set at 100%. *A*, EDD enhances PR B transactivation activity. Reporter assays were carried out using either HEK 293 (*left*) or COS7 (*right*) cells in the presence of EDD, SRC1, or empty vector, transfection control plasmid (pGFP20), and either 1 nM synthetic progestin ORG2058 or equivalent ethanol vehicle (*EtOH*). *B*, mutation of the catalytic cysteine of EDD does not alter the effect of EDD on PR transactivation. Reporter assays were carried out using HEK 293 cells in the presence of EDD, EDDM, or empty vector and 10 nM ORG2058. *C*, EDD enhances PR reporter gene expression in a dose-dependent manner. HEK 293 cells were transfected for a standard reporter assay along with increasing amounts of a constitutive expression vector for either EDD or empty vector (*0*) in the presence of 1 nM ORG2058. The amount of DNA transfected was normalized to 1.2 μg with empty vector. Cell number was monitored using proliferation assay and transfection efficiency by co-transfection with pRL-TK followed by *Renilla* luciferase assay. *D*, effect of EDD on response to the synthetic progestin ORG2058. HEK 293 cells were transfected for reporter assay along with a transfection control plasmid (pRL-TK). Cells were harvested for luciferase assay following 24 h of treatment with increasing concentrations of ORG2058. *E*, enhancement of VDR reporter gene expression by EDD. HEK 293 cells were transfected with a constitutive expression vector for VDR and a vitamin D response element-containing luciferase reporter vector along with a constitutive expression vector for EDD or empty vector and a transfection control plasmid (pRL-TK). Cells were harvested for luciferase assay following 24 h of treatment with 10 nM 1,25-dihydroxyvitamin D_3 (*Vit D*). *F*, EDD does not enhance ER reporter gene expression. HEK 293 cells were transfected with a constitutive expression vector for ER and an ERE-containing luciferase reporter vector along with either a constitutive expression vector for EDD, SRC1, or empty vector and a transfection control plasmid (pGFP20). Cells were harvested for luciferase assay following 24 h of treatment with 100 nM 17β-estradiol.

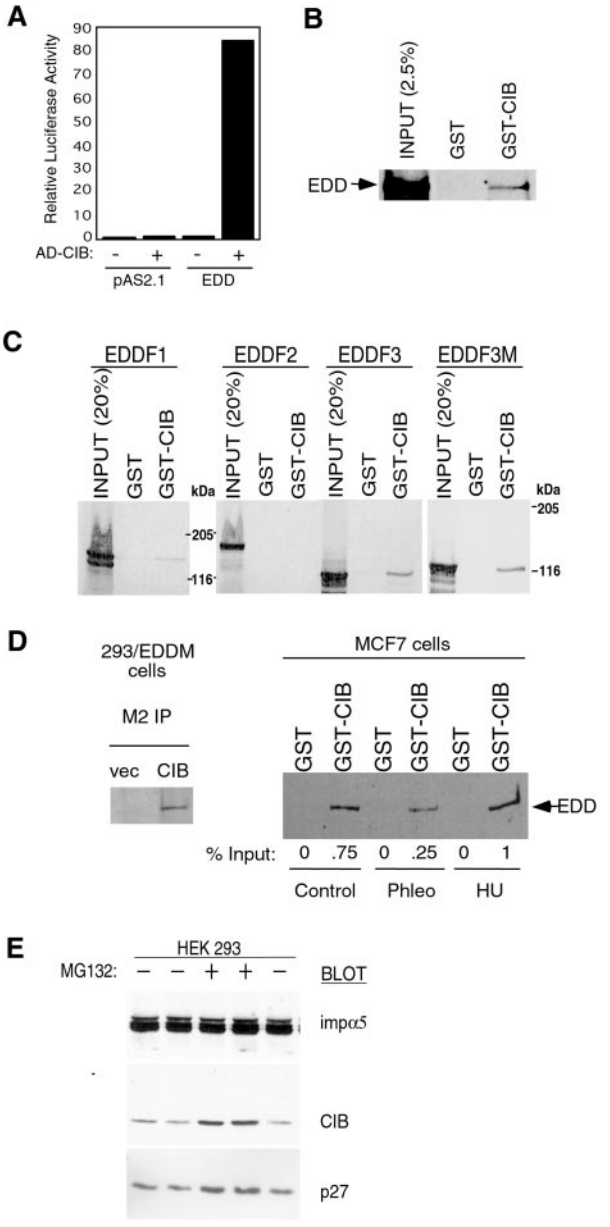


FIG. 6. **Interaction of EDD and CIB and the effect of DNA damage.** *A*, interaction of EDD with CIB in a yeast two-hybrid assay. The entire coding sequence of EDD was fused in-frame with the yeast GAL4 DBD. This construct or control vector pAS2.1 was co-expressed with either control vector (pACT2) or GAL4 AD-CIB in diploid yeast strain CG1945/Y187. *B*, *in vitro* interaction of CIB with EDD. *In vitro* translated ^{35}S-labeled EDD was incubated with GST-CIB fusion protein or with GST alone bound to glutathione-Sepharose beads. Bound EDD was analyzed by SDS-PAGE and autoradiography. *C*, mapping interaction between CIB and EDD. *In vitro* translated ^{35}S-labeled EDD derivatives were incubated with GST-CIB fusion or with GST alone bound to glutathione-Sepharose beads. Bound EDD fragments were analyzed by SDS-PAGE and autoradiography. *D*, interaction of EDD and CIB in HEK 293 and MCF-7 cells. *Left*, HEK 293 cells overexpressing mutant EDD were transfected with a plasmid encoding FLAG-tagged CIB or empty vector (*vec*). Extracts from these cells were subjected to immunoprecipitation using anti-FLAG antibody M2. *Right*, nuclear extracts prepared from MCF-7 cells following treatment with DNA damage agents phleomycin (*Phleo*) or hydroxyurea (*HU*) were incubated with either GST or GST-CIB fusion protein bound to glutathione-Sepharose beads. Bound proteins from both procedures were analyzed by SDS-PAGE and Western blotted for EDD, and amounts bound are indicated as percentages relative to input. *E*, potential regulation of CIB by the proteasome. HEK 293 cells were treated with the proteasome inhibitor MG132 (20 μM) or vehicle (Me_2SO) for 6 h and whole cell extracts analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and Western blotted for importin α5, CIB, and the known proteasomal target, p27.

DISCUSSION

This study demonstrates a new functional role for the nuclear protein EDD. A progestin-regulated gene, EDD itself has the ability to potentiate PR transcriptional activity. In addition, EDD may play a role in DNA damage signaling, as suggested by complex formation with CIB, a DNA PK-binding protein, an interaction that is sensitive to DNA damage.

Many *Drosophila* genes have been identified in which mutations result in hyperplasia or tumorigenesis. For several of these, mammalian orthologues have been identified that have similar properties. The close homology between the *D. melanogaster* tumor suppressor gene *hyd* and the human EDD gene (1) suggests the potential for involvement of EDD in human cancer. Further evidence comes from our studies showing overexpression of EDD and frequent allelic imbalance at the EDD locus in a variety of cancers.[1]

Although little is yet known about the precise biochemical roles of the HYD and EDD proteins, inferences can be made from the existence of highly conserved protein domains. The present study showed that EDD is a nuclear protein, most likely arising from a direct interaction with importin α via two NLSs within the NH_2 terminus of EDD. The HECT domain, which has reversible ubiquitin binding activity in EDD and other E3 ligases (1), is also associated with a separate role in transcriptional coactivation in related proteins; Rsp5/hRPF1 and E6-AP coactivate ligand-dependent nuclear receptor activity (30, 31), whereas Tom1p is required for transcriptional regulation of certain yeast genes (17) and UREB1 enhances transcription from the rat preprodynorphin gene (54) but suppresses p53 transactivation of target genes (55).

EDD was able to potentiate PR transactivation to a level comparable with that seen for the p160 coactivator SRC-1. EDD has a distinct selectivity profile, being able to coactivate PR and VDR but not ER, in a ligand-dependent manner. This is in contrast to the HECT ligase E6-AP, which coactivates a range of hormone receptors including ER, PR, androgen receptor, and glucocorticoid receptor (31). Rsp5 also shows some selectivity, coactivating transcription by PR and glucocorticoid receptor but not ER (30). EDD is unique among HECT ligases, however, in that enhancement of PR transactivation by EDD raises the intriguing possibility of a positive feedback loop, as EDD itself is a progesterone-regulated gene (1). Another consequence might be that the overexpression of EDD seen in some breast cancers could increase the sensitivity of PR-positive tumors to lower levels of progestins.

Coactivation by EDD, like E6-AP, Rsp5/hRPF1, and Tom1p, is independent of ubiquitin binding ability of the HECT domain (17, 30, 31). These findings are somewhat surprising in the light of evidence that ubiquitinylation is intimately involved in the process of transcriptional activation. Like many other transcription factors, several nuclear receptors including ER, PR, and VDR are down-regulated by the 26 S proteasome (56–60) and coactivator binding appears necessary for this degradation. Inhibition of the proteasome diminishes transcriptional activity by steroid receptors ER and PR (61), and more general implications come from studies showing that the 19 S proteasome subunit is required for transcription elongation (62). Furthermore, the carboxyl-terminal tail of RNA polymerase II itself is a target of ubiquitin-mediated proteolysis (16, 63). It may be that EDD and these other HECT ubiquitin ligases can still perform some function in the ubiquitinylation cascade without themselves having a catalytically active HECT domain. EDD appears to be the only E3 ligase to possess both a RING-like zinc finger domain and a HECT domain, and we cannot rule out the possibility that coactivation by EDD is mediated through the RING-like or UBA domains.

The mechanism of coactivation by E6-AP, as for the p160 family, has been attributed to direct coactivator-receptor interaction, providing either bridging or enzymatic activities to the transcriptional complex. Many steroid receptor coactivators possess histone acetyltransferase activity, but when compared with p300 little or no histone acetyltransferase activity was associated with EDD.[1] Two regions of E6-AP contain L*XX*LL receptor-binding motifs, and both of these regions interact with PR (31). A search of the EDD sequence revealed one NH_2-terminal, one COOH-terminal, and three centrally located L*XX*LL domains (Fig. 1*A*). Furthermore, the NH_2-terminal and centrally located motifs lie in regions of high homology to HYD. However, the NH_2-terminal motif, in the region with the strongest binding to PR(CDE), was not required for the interaction. Nevertheless, direct interaction between other NH_2-terminal sequences of EDD and PR may partially explain the observed effects of EDD on PR transactivation. In their studies on the role of ubiquitinylation in transcriptional enhancement, Salghetti *et al.* (64) found that mono-ubiquitinylation of the VP16 transcriptional activation domain was sufficient for transcriptional activity. Interestingly, another study found that the UBA domain might bind such mono-ubiquitinylated proteins and thus prevent the formation of multi-ubiquitin chains (65), raising a possible mechanism for coactivation via PR stabilization by EDD. However, we found that the UBA domain was not required for interaction between EDD and PR *in vitro*, although we cannot rule out a separate role for the UBA domain in PR coactivation by EDD.

In addition to the UBA domain, the zf-UBR1 domain of EDD is also likely to be involved in protein-protein interactions. The zf-UBR1 domain coincides with the type 1 site in UBR1 proteins, a binding site specific for N-end rule substrates with basic NH_2-terminal residues (39). This zf-UBR1 domain is critical for function of the calossin-like RING-H2 finger protein, BIG, and it therefore may also have a role in substrate recognition and binding in EDD family members. Other HECTs have substrate interaction domains distinct from the HECT domain (*e.g.* the WW domain (Ref. 63)). Unfortunately attempts to use the UBA and zf-UBR1 as baits for yeast two-hybrid analysis were unsuccessful because of autoactivation and indiscriminate binding, respectively, so the precise functions of these regions in the EDD protein and their role, if any, in coactivation remain elusive.

Using cell lysates EDD and CIB were shown to interact in MCF-7 and HEK 293 cells. CIB is 58 and 56% homologous with other EF-hand proteins calmodulin and calcineurin B, respectively, and may function as a calcium-dependent regulatory subunit of a kinase or phosphatase (51). Interactions of CIB with five other proteins have been described: DNA PK (50), integrin α_{IIb} (51), the cell cycle regulatory polo-like kinases, Snk and Fnk (52, 66), and presenilin 2 (67). Snk and Fnk are activated by progesterone in maturing frog oocytes (68) and have roles in both G_1 and mitotic phases of the cell cycle, and CIB could affect the activity of these kinases. CIB is found in both the nucleus and cytoplasm, and its subcellular localization can be influenced by association with its interacting partners and by calcium levels (52, 67), but its role in the nucleus is unexplored. Interaction with DNA PK would implicate CIB in the response to DNA double strand break sensing and repair. We found that GST-CIB pull-down of EDD after treatment with the radiomimetic phleomycin caused a decrease in the amount of associating EDD, whereas the levels of EDD remained unchanged. Interestingly a recent report links EDD and ubiquitinylation of another protein involved in DNA repair, the topoisomerase II-associated protein, TopBP1 (6). In the light of these data and our findings that CIB is a potential target of the proteasome, experiments are currently under way to determine the possible involvement of EDD and CIB in the cellular response to DNA damage.

These data, identifying a role for EDD in transcriptional control and DNA damage (6), together with our unpublished data[1] demonstrating embryonic lethality in $EDD^{-/-}$ mice and frequent allelic imbalance at the EDD locus in diverse human cancers, provide strong evidence that EDD plays a pivotal role in normal cellular physiology and when dysregulated has important consequences for development and potentially tumorigenesis. As such it shares properties with other *Drosophila* tumor suppressor genes with critical functions in mammalian biology and potential disease states.

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PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
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