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In a search for genes with a role in hormonal control of cell proliferation, we cloned a novel progestin-induced gene, EDD (previously DD5) that appears to be the human homologue of the *hyd* tumor suppressor gene of *Drosophila melanogaster*. By amino acid homology with other known proteins it is likely that the EDD protein is an E3 ubiquitin-protein ligase, enzymes which target one or more key proteins for destruction by ubiquitin-mediated proteolysis. The interaction between EDD and two potential ubiquitination substrates was further characterised. Evidence was obtained suggesting that one of these might be regulated by the proteasome while the other most likely serves to localize EDD in the cell. This was supported by cellular fractionation experiments using HEK293 cells over-expressing EDD. These cells are also a valuable resource in studying the role of EDD in the cell. Finally a panel of breast tumor and matched normal DNA was obtained and the microsatellite panel was expanded for future loss of heterozygosity studies. A system for mutation detection from cancer cell line mRNA was also established.

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ANNUAL SUMMARY- Year 2

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INTRODUCTION

In a search for genes with a role in hormonal control of cell proliferation, we have cloned a novel progestin-induced gene, EDD (previous designation DD5). It is hypothesized that EDD has a role in the control of cell growth and differentiation as it appears to be the human homologue of the *hyperplastic discs* (*hyd*) gene of *Drosophila melanogaster*. When mutated, *hyd* is either lethal or causes abnormal or hyperplastic growth in *Drosophila* larval imaginal discs and defective germ cell development. While the biochemical roles of *hyd* and EDD are unknown, by amino acid homology with other known proteins it is likely that the EDD protein is a ubiquitin-protein ligase (E3), enzymes which target one or more key proteins for destruction by ubiquitin-mediated proteolysis. The target proteins of EDD would be expected to have profound effects on cell cycle control or cell signalling. The project is designed to :

- define the normal function of EDD and its targets and relate this role to development and progression of breast cancer;
- determine the effects of EDD on breast cancer cell cycle progression;
- determine the role of EDD in progestin-induced growth stimulation of breast cancer cells; and
- determine the effect of mutations in EDD or dysregulation of EDD expression on tumor phenotype and tumor progression.

Over the past two years significant progress has been made towards these aims with demonstration of ubiquitin ligase properties for EDD and the identification of several potential target proteins. Further, we have developed mammalian cell lines with which to study the function of EDD and its effects on cell division. A new role for EDD emerged in year 1 and this formed the basis of a successful grant proposal and promises to shed light on the role of EDD in the progesterone response. Perhaps the most exciting development has been demonstration of disruption around the EDD genetic locus in several cancers and the establishment of methods to further characterize these disruptions. In the final year of this grant, we will come to know more about the role of EDD in ubiquitin-mediated proteolysis and other cellular processes and about the involvement of EDD in cancer.

BODY OF REPORT

TASK 1: To determine the ability of EDD protein to form a thioester bond with ubiquitin *in vitro* (months 1-6)
This work was completed and published in year 1.

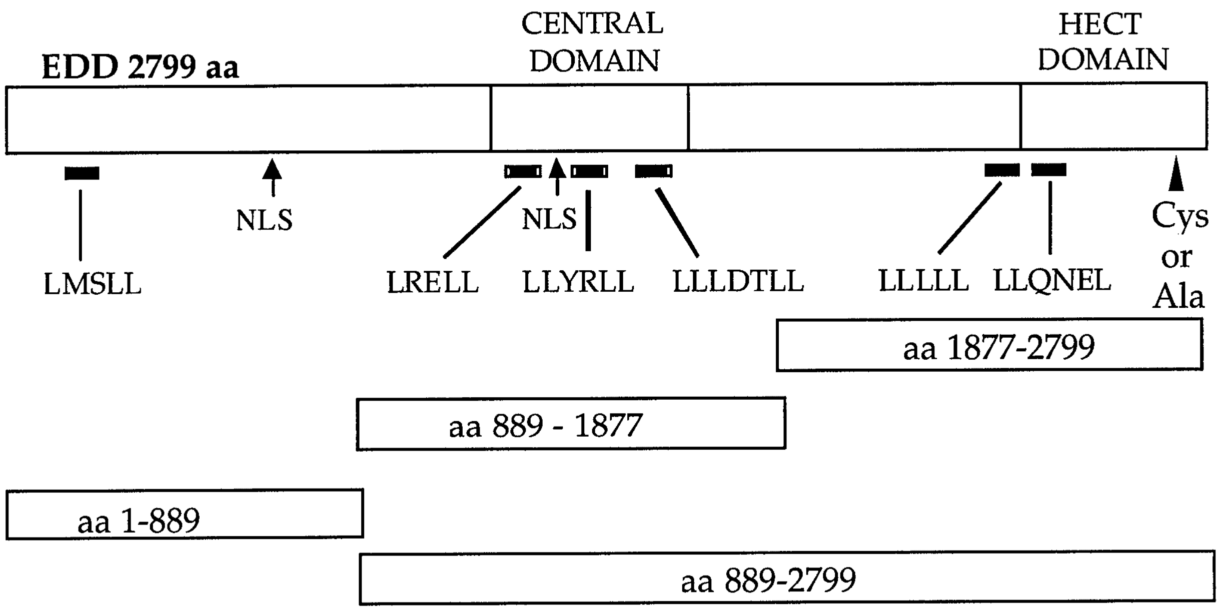

Figure 1. Schematic representation of the EDD protein sequence and overlapping deletion mutants.
Shown are the two domains highly conserved between EDD and HYD (CENTRAL and HECT) and the conserved cysteine residue (Cys) which has been mutated to alanine (Ala) in the ubiquitination-defective mutant. Three co-activator binding LLXXXL motifs, two of which coincide with two of the five receptor-binding LXXLL motifs, are also indicated, along with two putative nuclear localisation signals (NLS). (aa, amino acids). Yeast two-hybrid bait constructs are indicated in the lower half of the diagram.

TASK 2: To identify substrates for EDD-mediated ubiquitination (months 6-36).

During year 1, a range of proteins were tested for interaction with EDD via co-immunoprecipitation. However the main strategy for isolation of EDD-interacting proteins is to use yeast two hybrid library screening. Two human cDNA libraries, both cloned into the pACT2 vector, are available for screening in our laboratory, one derived from human placental mRNA and another derived from breast carcinoma mRNA. In year one, full length EDD cDNA was cloned downstream of the GAL4 DNA-binding domain in the pAS2.1 vector and used to screen three million clones from the placental cDNA library for interacting proteins. The C2768A mutant form of EDD was used to prevent degradation of potential interacting substrates. Two potentially interacting proteins, the human homologue of yeast SRP-1 (importin $\alpha 1$) and calcium-integrin binding protein (CIB), also known as kinase interacting protein (KIP), were identified as proteins which enable reconstitution of GAL4 promoter-binding activity, thus driving *HIS3* and *LacZ* reporter gene expression in the yeast strain Y190. Importin $\alpha 1$ is a protein essential for nuclear transport and may regulate passage of EDD between the nucleus and cytoplasm, while CIB/KIP appears to be involved in cell signalling and also interacts with DNA-dependent protein kinase, suggesting a second, nuclear role.

GST pull down confirmed interaction between EDD and importin $\alpha 1$ and CIB/KIP. Importin $\alpha 1$ interacts most strongly with the amino two-thirds of EDD, which contains two bipartite nuclear localization signals (NLSs) while CIB/KIP binds to the carboxyl third of EDD. EDD was also shown to co-immunoprecipitate with importin $\alpha 1$ from T-47D cells.

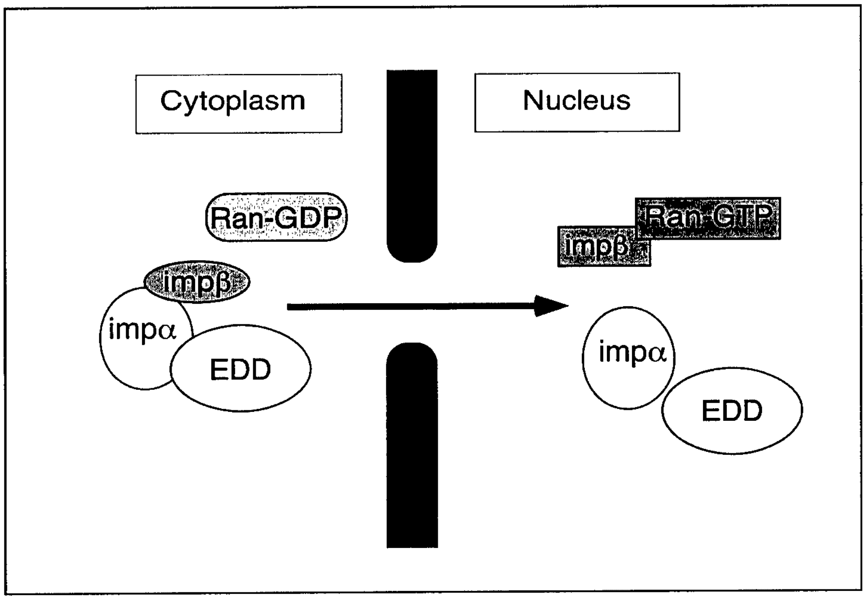

Figure 2. Schematic diagram depicting proposed method of nuclear import for EDD protein.
In the classical importin pathway, importin α binds an NLS in the import substrate (EDD) and forms a bridge with importin β, which in turn effects docking at the nuclear pore and transport into the nucleus. Once inside the nucleus, RanGTP causes dissociation of the complex.

EDD-importin α1 interactions have now been characterized further using GST pull downs and newly generated constructs for EDD *in vitro* translation. These studies indicated that the NLS in the N-terminal portion of EDD was likely to the site of importin α interaction with EDD. If EDD is transported into the nucleus via this mechanism, it is expected that importin β would also be complexed with EDD. GST pull downs confirmed that EDD and importin β can also interact. Through collaborative studies we established that EDD may interact with a second importin α fanily member, Rch1.

One possible reason for the association of EDD with importin α1 or CIB/KIP is to target these proteins for ubiquitin-mediated proteolysis. Experiments were carried out to test this hypothesis. T47D cells were incubated in the presence or absence of the proteasome inhibitor MG132. Cell lysates were analysed for levels of importin α1, CIB/KIP or as a control the known proteasomal substrate, p27. As expected for a ubiquitinated substrate, the levels of p27 and CIB/KIP were higher in lysates from cells treated with proteasome inhibitors. However the levels of importin α1 remained unaltered by proteasome inhibitors suggesting that the most likely explanation for this interaction is that it enables nuclear localisation of EDD (Fig. 2). This preliminary study is currently being extended to obtain direct evidence for CIB/KIP ubiquitination in cells and for the involvement of EDD in this process.

The use of the large full length EDD bait protein (~300 kDa) in this system may be problematic due to inefficient expression, explaining the low number of positives detected in the first screen so two other baits (which consist of amino acids 889-1877 and 1877-2799 respectively (Fig.1)) were used to screen the available cDNA libraries for interacting proteins. Screening two million placenta library clones with the central domain bait resulted in the isolation of a cDNA encoding a subunit of RNA pol II. This association has been confirmed so far only within the yeast two-hybrid system. The association may require other factors and might be important in light of the possible role of EDD as a transcriptional regulator (see 1999 report). In addition, five clones potentially encoding novel proteins were isolated. Three of these have been selected for further study. Interestingly one of these contains peptide motifs suggestive of a role in transcriptional control. As these are novel proteins, the lack of available reagents makes them less tractable to study. However the cDNAs obtained from two-hybrid screening were cloned into mammalian expression vectors which can also be used in conjunction with EDD constructs to study interactions by mammalian two-hybrid assay (Promega Checkmate System). As part of this project 15 fusion constructs were generated, including deletion mutants of EDD and positive control partner constructs. These vectors are a valuable resource for over-expression of the novel EDD-interacting proteins for co-immunoprecipitation and ubiquitination studies.

Due to a lack of proteins found to interact with the carboxyl third bait (EDD aa 1877-2799, C2768A mutant), new baits have been generated (Fig. 1). Specifically, these are EDD 1-889, EDD 1877-2799wt, EDD 889-2799wt and EDD 889-2799 C2768A mutant. These baits are currently being used to screen cDNAs from a breast tumor library.

TASK 3: To determine the effects of EDD under- and over-expression on cultured breast cancer cells (months 6-24).

Considerable difficulty was encountered when developing an inducible expression system for EDD. As an alternative a human embryonic kidney cell line, HEK-293, previously shown in our laboratory to be amenable to protein expression studies, was transfected with EDD constructs in the pRcCMV constitutive vector. As reported last year, the HEK-293 expression system enabled us to discover an additional and unexpected role for EDD: its apparent ability to function as a steroid receptor transcriptional co-regulator. Further, we have established several stable cell lines over-expressing either EDD or ubiquitination defective EDD, as well as empty vector transfected lines as controls. These lines have already proven a valuable resource for characterising the EDD protein and for co-immunoprecipitation studies. For example they were used to confirm interaction of EDD with importin $\alpha 1$. These cell lines were also used for cellular fractionation experiments and through these we were able to support our hypothesis that EDD is likely to function in the nucleus. An additional set of mammalian expression constructs for FLAG-tagged EDD driven by an SV40 promoter were generated and expression confirmed by transient transfection in several cell lines. In the final year of the grant these important resources will be used to study potential EDD-interacting proteins and assess these as potential ubiquitination targets.

If EDD is a tumor suppressor gene we predict that over-expression of EDD in breast cancer cells should affect their growth. We are currently taking several approaches to examine this, including analysis of the effects of EDD over-expression on cell cycle progression and on colony formation under selective pressure.

TASK 4: To determine the expression of EDD in breast tumor specimens (months 6-18).

Initial attempts to perform immunohistochemistry on paraffin embedded cell blocks were not successful due to a high level of background staining by the antisera. The antisera was affinity purified and tested through collaborations with a local laboratory. The affinity purified antibody was also found to be unsuitable for immunohistochemistry and consequently we have initiated development of additional antisera by collaborative agreement.

Previously EDD mRNA was quantitated in 16 breast cancer cell lines and 2 normal breast cell lines by Northern blot analysis. Interestingly, the lowest levels were displayed by normal breast cell lines and EDD protein could not be detected in these cell lines. Breast tumor cell lines displayed a range of mRNA expression levels and all had detectable levels of EDD protein. Further analysis of some cell lines by FISH showed amplification of the chromosomal region containing EDD in 2 of these lines.

Although it had been planned to quantitate EDD gene expression in breast tumor samples, given the possible tumor suppressor role of EDD, studies to determine the frequency of loss of heterozygosity or allelic imbalance at the EDD locus were given first priority. Given the availability to us of matched normal and tumor samples from a range of other cancers, these samples were used initially. We used microsatellite allelotyping of DNA extracted from tumors and matching normal tissues to determine whether chromosomal aberrations such as loss of heterozygosity (LOH) at the EDD locus on 8q22.3 (8) are common (see 1999 report). We demonstrated that allelic imbalance occurred at high frequency, particularly involving a microsatellite within the EDD gene, in ovarian cancers (notably in the serous subtype), hepatocellular carcinomas, squamous cell carcinomas of the tongue and metastatic melanoma. These results are exciting as they are consistent with the presence of a tumor suppressor gene at or very near the EDD locus and suggest that the EDD gene may have a common role in the progression of several human cancers. This approach will be extended to breast cancer where to date the only relevant data we have indicate that 2 of 16 breast cancer cell lines have apparent LOH at the EDD locus as judged by homozygosity for all six microsatellite markers. This panel of markers has been expanded to include a larger area of chromosome 8q and we have also obtained a series of matched normal and breast tumor DNA samples that are currently being tested for LOH at the EDD locus.

We have recently embarked on the next exciting stage of this project, that is, investigating whether in cases where LOH occurs, there is mutation of the remaining copy of the EDD gene. Initially, breast and ovarian cancer cell lines displaying apparent LOH at the EDD locus will be analyzed for mutations in EDD by direct sequencing of RT-PCR products from EDD cDNA. The protocol for these experiments has been developed and this work has already led to the identification of a mRNA splice variant. Mutation detection will be extended to tumor samples showing LOH and in this case PCR-amplified exons from tumor DNA will be analyzed by SSCP analysis with the assistance of a local collaborator.

APPENDICES

1. Key Research Accomplishments

- Characterization of the interaction between EDD and two other proteins, importin $\alpha 1$ and CIB/KIP.
- The region of EDD interacting with importin $\alpha 1$ was mapped to the N-terminal NLS of EDD.
- Obtained evidence that importin $\alpha 1$ is unlikely to be a ubiquitination target of EDD but rather is important to the nuclear function of EDD.
- Obtained preliminary data suggesting that CIB/KIP might be a ubiquitination target of EDD. Developed systems to test this further.
- Generation of 15 plasmid constructs for EDD and yeast two-hybrid positives suitable for use in the mammalian two-hybrid system and for over-expression in mammalian cell lines.
- Generation of 4 new EDD baits for use in the yeast two-hybrid system.
- Over-expression of EDD in HEK-293 cells and use of these cells to localize EDD in the cell.
- Assembly of a panel of breast tumor and matched normal DNA samples.
- Expansion of the microsatellite marker panel.
- Establishment of methodology for sequencing EDD cDNA from cancer cell lines for mutation detection.
- Establishment of key collaborative ties.

2. Reportable Outcomes

Poster presentations

1. Characterisation of EDD: a novel progestin-regulated gene.
   Michelle J. Henderson, Amanda J. Russell, Gillian M. Lehrbach, Robert L. Sutherland and Colin K. W. Watts.
   3rd Peter Mac Symposium: The Initiation and Progression of Cancer. November 7th-10th, 1999. University of Melbourne, Melbourne, Australia.
2. Characterisation of EDD: a novel progestin-regulated gene.
   Michelle J. Henderson, Amanda J. Russell, Gillian M. Lehrbach, Samantha Hird, Robert L. Sutherland and Colin K. W. Watts.
   Australian Society for Medical Research 38th National Scientific Conference, Leura, NSW, Australia. November 27th-29th, 1999.
3. Involvement of the putative tumor suppressor gene EDD in human cancers.
   Jennifer Clancy, Michelle J. Henderson , Amanda J. Russell, Robert L. Sutherland and Colin K. W. Watts.
   12th Lorne Cancer Conference, February 10th-13th, 2000, Lorne, Victoria, Australia.
4. Characterisation of EDD: a progestin-regulated tumor suppressor gene.
   Colin K. W. Watts, Michelle J. Henderson, Jennifer L. Clancy, Amanda J. Russell, Gillian M. Lehrbach and Robert L. Sutherland.
   Advances in Human Breast and Prostate Cancer Keystone symposia. March 19-24, 2000. Incline Village, Nevada, USA.
5. Characterisation of EDD: a progestin-regulated gene.
   Michelle J. Henderson, Amanda J. Russell, Gillian M. Lehrbach, Robert L. Sutherland and Colin K. W. Watts.
   Nuclear Receptors 2000 Keystone Symposia. March 25-31, 2000. Steamboat, Colorado, USA.
6. Characterisation of EDD: a putative tumor suppressor gene.
   Michelle J. Henderson, Amanda J. Russell, Gillian M. Lehrbach, Robert L. Sutherland and Colin K. W. Watts.
   Era of Hope DOD Breast Cancer Research Program Meeting. June 8-12, 2000. Atlanta, Georgia, USA.

Oral presentations

1. Characterisation of a progestin-regulated putative tumor suppressor gene.
   Michelle J. Henderson, Jennifer L. Clancy, Amanda J. Russell, Gillian M. Lehrbach, Robert L. Sutherland and Colin K. W. Watts.
   Invited Speaker, 6th International Congress on Hormones and Cancer. Jerusalem, Israel, September 5-9, 1999.
2. Involvement of the putative tumor suppressor gene EDD in human cancers.
   Jennifer Clancy, Amanda J. Russell, Michelle J. Henderson, Robert L. Sutherland and Colin K. W. Watts.
   3rd Australian Cancer Gene Analysis and Mutation Detection Workshop. 15-18 June, 2000. Noosa, QLD, Australia.

Funding applications

The progress made so far in this project has enabled the preparation of several new grant applications in areas branching out from this research. Each of these were successful.

1. Title: Investigation of a putative tumour suppressor gene with ubiquitin-protein ligase and co-activator functions
   Agency: The Leo & Jenny Leukemia and Cancer Foundation of Australia
   PI: Dr. Michelle Henderson

Funded 2000

2. Title: Functional characterisation of a putative tumour suppressor gene and its role in human cancer
   Agency: New South Wales Cancer Council
   PIs: Dr. Michelle Henderson, Dr. Colin Watts

Awarded then funded 2000-2002 as part of a larger Program grant.

3. Title: Functional characterisation of a novel progesterone receptor co-activator and its role in breast cancer.
   Agency: US Army Breast Cancer Research Program IDEAS Awards
   PI: Dr. Colin Watts, AI: Dr. Michelle Henderson

Funded 2000-2002.

Research Opportunities

The progress made in this project resulted in the participation of the PI in national and international scientific conferences. In addition, important collaborative ties in several areas have been made possible.