Inquiro, the UAB undergraduate science research journal

Volume 2007 | Number 1 Article 9

2007

Identification of Proteins that Physically Interact with the Cell Cycle Regulated Ubiquitin Ligase G2E3

Christina Ho

David F. Crawford

Identification of Proteins that Physically Interact with the Cell Cycle Regulated Ubiquitin Ligase G2E3

Christina Ho, David F. Crawford

A putative ubiquitin ligase was identified in a screen for genes that are transcriptionally regulated in response to the G2 DNA damage checkpoint. This protein, which we have named G2E3, is regulated by multiple mechanisms including transcription, ubiquitin-mediated degradation, and subcellular localization. The subcellular localization of G2E3 is dynamic, relocalizing in response to DNA damage, and shuttling between the nucleus and cytoplasm. We have identified an ubiquitin ligase that contributes to the instability of G2E3. To better understand the function of G2E3, we have performed a yeast two-hybrid analysis which successfully identified three interacting proteins. We plan to identify other interacting proteins by a variety of methods. G2E3 is homologous to a *Drosophila* protein known as pineapple eye, or pie. Several important proteins have been shown to physically interact with pie, including proteins that regulate cell cycle progression and DNA damage responses. We hypothesized that G2E3 would interact with the human orthologs of pie-interacting proteins. Our plan was to test whether some of these proteins could be shown to interact by using co-immunoprecipitation and GST-pulldown assays. Nine human cDNAs were obtained from OpenBiosystems. We attempted to PCR-amplify these and clone them into a myc-tagged mammalian expression vector and a GST-tagged bacterial expression vector. We successfully cloned 4 cDNAs into pcDNA3myc and 4 cDNAs into pGEX-2T. Our mammalian cell co-IP experiments yielded no apparent interactions but the GST-pulldown analysis indicated that G2E3 interacts with two important DNA damage regulatory proteins, ERCC1 and Rad51. Additional studies are underway to test the significance of these potential interactions.

INTRODUCTION

Using a microarray screen, molecules that are transcriptionally regulated by the G2 DNA damage checkpoint were identified. Among the molecules identified in this screen was a putative ubiquitin ligase that we refer to as G2E3. We have demonstrated that this protein is regulated by many mechanisms. Included are cell cycle and DNA damage dependent transcriptional regulation, protein lability mediated by ubiquitination, and regulated subcellular localization by nuclear and nucleolar localization, DNA damage-dependent nucleolar delocalization, and CRM1-independent nuclear export. We have identified an ubiquitin ligase that ubiquitinates G2E3 and several other interacting proteins, allowing predictions about the function of G2E3.

G2E3 is very similar to two *Drosophila* proteins identified as pineapple eye (pie) and CG9756. A map of interacting proteins involved in cell cycle regulation and DNA damage responses (Stanyon, et al) demonstrated that pie interacts with numerous molecules that are involved in these processes. A schematic demonstrating these interactions follows.

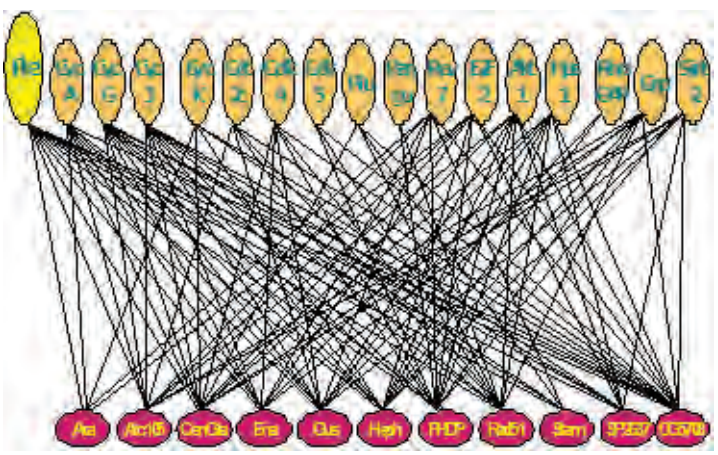

Figure 1. Identification of proteins that interact with *Drosophila* Pineapple Eye. We predicted that G2E3 might interact with the human homologs of these *Drosophila* proteins. To test this, we obtained cDNAs for several of these proteins and tested their ability to interact with G2E3 by co-immunoprecipitation and GST pull-down. Two proteins appeared to interact with G2E3 by GST pull-down.

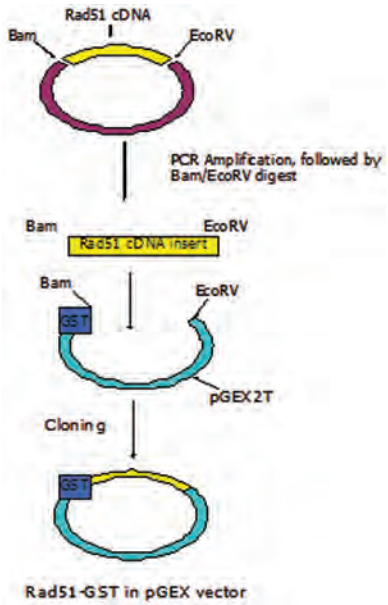

Figure 2. Schematic of construct preparation. We PCR amplified and digested cDNAs with restriction site sequences as primers. The insert was ligated into GST-tagged pGEX2T bacterial expression vector and cloned.

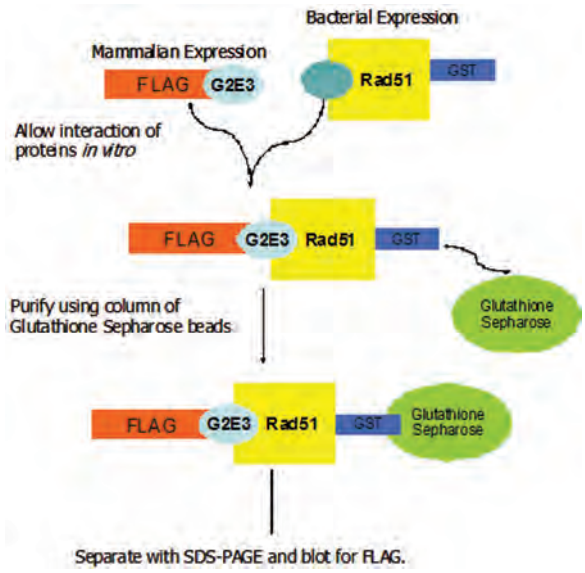

Figure 3. Schematic of recombinant GST-tagged protein purification. GST-tagged Rad51 (bacterial expression vector) and FLAG-tagged G2E3 (mammalian expression vector) were allowed to interact *in vitro*. GST-tagged protein was then purified with glutathione sepharose beads and washed. Following separation by SDS-PAGE, we probed for FLAG with α-FLAG M2.

MATERIALS AND METHODS

Construct Development

Nine human cDNAs were obtained from Open Biosystems. PCR amplification with primers containing restriction enzyme sites was performed. cDNAs and their corresponding primer sequences were as follows: Arix1 (5'-GAGGATCCTACGACTCGTGCGTGG and 5'-GGGAATTCGAGGCCGGCAGCTAG), ERCC-1 (5'-GGGGATCCGACCCTGGGAAGGACAA and 5'-GGGAATTCGGGATTACAGGCGGAAG), IRFBP-1 (5'-GAGGATCCGCGTCTGTGCAGGCG and 5'-GGGAATTCGCCTAGGGGTCCCGTT), Iroquois (5'-GGGGATCCTACCCGCAGTTTGG and 5'-GGGAATTCAGGCGCAGAAGGG), LMO4 (5'-GAGGATCCGTGAATCCGGGCAGC and 5'-GCGGGATATCTTAGCAGACCTTCTGGTCTG), PTB-1 (5'-GAGGATCCGACGGCATCGTCCCA and 5'-GGGAATTCCTAGATGGTGGACTT GGAGA), Rad51 (5'-GAGGATCCGCAATGCAGATGCAGC and 5'-GCGGGATATCTCAGTCTTTGGCATCTCC), SSB1 (5'-GAGGATCC GGTCAGAAGGTCACTGGAG and 5'-GGGAATTCGCGAACGTCACTGGTAGAG), and Stam (5'-GGAGATCTCCTCTTTTTGCCACCAA and 5'-GAGAATTCGGGTCCTATAGCAGAGCC). Inserts were digested with the appropriate restriction enzymes and cloned into pcDNA3myc (for mammalian cell expression) and pGEX2T or a related vector (for bacterial expression of GST-tagged protein). Recombinant plasmids were prepared for mammalian cell or bacterial expression as shown in Figure 2.

Co-Immunoprecipitation

Rad51, ERCC-1, LM04, and SSB1 cDNAs were subcloned into myc-tagged mammalian expression vector and transfected into Cos-7 cells. After overnight incubation, cells were harvested with PBS + 3mM EDTA and lysed in complete mammalian cell lysis buffer. Equal amounts of whole cell lysate (200μg) were incubated for 1hr with FLAG M2 beads. Pellets were washed six times and then eluted by boiling in SDS sample buffer and separated by SDS-PAGE.

Immunoblotting

Following SDS-PAGE, proteins were transferred by electrophoresis to nitrocellulose membranes. The membranes were stained with Ponceau to confirm equal loading and then blocked in 5% milk. Blots were probed with primary antibody FLAG-M2 (Sigma) or Myc9E10 and then washed. HRP-conjugated secondary antibodies were added and incubated prior to washing. Blots were developed with ECL (Invitrogen).

Recombinant GST-tagged Protein Purification

BL21 (DE3) cells were transfected with pGEX2T constructs. After growth to log phase, protein expression was induced with 500μM IPTG. After 2 hr, cells were harvested. FLAG-tagged G2E3 or G2E3 mutants were transiently expressed in Cos-7 cells and 24 hr following transfection, cells were lysed. 1mg of whole cell lysates were incubated 1hr with 50μg purified recombinant protein along with glutathione-sepharose. Following washing, sepharose beads were boiled in SDS and separated by SDS-PAGE. See Figure 3.

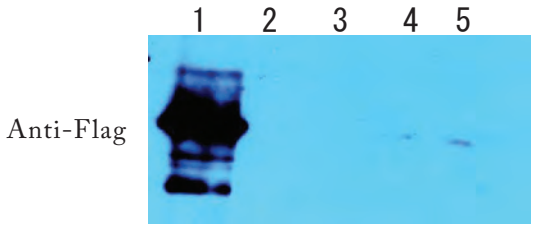

Figure 4. Mammalian cell expressed FLAG-tagged G2E3 was pulled down with GST-Rad51 and GST-ERCC1 but not GST alone. Lane 1) Input, 2) Input, 3) GST, 4) GST-Rad 51, 5) GST-ERRC1

GST Pulldown

Rad51 and ERCC1 cDNAs were subcloned into pGEX2T and transfected into BL21 cells. After growth to an O.D. of 0.6, bacteria were induced with 500μM IPTG for 1 hour. Bacterial cells were lysed and GST-tagged protein was purified over a glutathione-sepharose column. Following elution, protein was dialyzed to remove glutathione and quantified for total protein yield.

RESULTS AND CONCLUSIONS

Of nine cDNAs, we successfully cloned 4 into the pcDNA 3-Myc vector and 4 into the pGEX2T vector. No co-immunoprecipitation was observed with either G2E3 or 5CA (a G2E3 mutant). We did, however, observe interaction of G2E3 and GST-tagged Rad51 and ERCC1. There was no interaction with GST alone or any other negative controls.

We have identified a protein that physically interacts with G2E3. Rad51 is a human homolog of RecA, a recombinase in *E. coli*. This apparent interaction is under further investigation.

REFERENCES

- Brooks WS, Banerjeet S, Crawford DF. G2E3 is a nucleo-cytoplasmic shuttling protein with DNA damage responsive localization. Experimental Cell Research. 2007 Feb 15;313(4):665-76. Epub 2006 Dec 14.
- Crawford DF, Piwnica-Worms H. The G(2) DNA damage checkpoint delays expression of genes encoding mitotic regulators. Journal of Biological Chemistry. 2001 Oct 5;276(40):37166-77. Epub 2001 Aug 1.
- Einarson, M.B. Detection of Protein-Protein Interactions Using the GST Fusion Protein Pulldown Technique. In Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press. 2001; 18.55-18.59.
- Fields S, Sternglanz R. The two-hybrid system: an assay for protein-protein interactions. Trends Genet. 1994 Aug;10(8):286-92.Links
- Phizicky, E.M. and Fields, S. Protein-Protein Interactions: Methods for Detection and Analysis. Microbiological Reviews. 1995 March; 94-123.
- Stanyon CA, Liu G, Mangiola BA, Patel N, Giot L, Kuang B, Zhang H, Zhong J, Finley RL. A ***Drosophila*** protein-interaction map centered on cell-cycle regulators. Genome Biology. (2004); 12(5): R96