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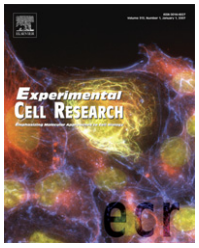

Research Article

G2E3 is a nucleo-cytoplasmic shuttling protein with DNA damage responsive localization

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ABSTRACT

G2E3 was originally described as a G2/M-specific gene with DNA damage responsive expression. The presence of a conserved HECT domain within the carboxy-terminus of the protein indicated that it likely functions as a ubiquitin ligase or E3. Although HECT domains are known to function in this capacity for many proteins, we demonstrate that a portion of the HECT domain from G2E3 plays an important role in the dynamic subcellular localization of the protein. We have shown that G2E3 is a nucleo-cytoplasmic shuttling protein with nuclear export mediated by a novel nuclear export domain that functions independently of CRM1. In full-length G2E3, a separate region of the HECT domain suppresses the function of the NES. Additionally, G2E3 contains a nucleolar localization signal (NoLS) in its amino terminus. Localization of G2E3 to the nucleolus is a dynamic process, and the protein delocalizes from the nucleolus rapidly after DNA damage. Cell cycle phase-specific expression and highly regulated subcellular localization of G2E3 suggest a possible role in cell cycle regulation and the cellular response to DNA damage.

Introduction

Cell cycle phase-specific expression of genes is a principal mechanism controlling cell division. In previous work, we identified numerous genes that are specifically expressed in G2-phase and mitosis, suggesting a role for the encoded proteins in mitotic regulation [1]. Many of these proteins are known to function in cell cycle regulation including cyclin B1 [2], Plk1 [3], Aurora-A [4], Cks2 [5], topoisomerase IIα [6], and Rab6-kinesin [7], while others are still uncharacterized. In addition, we demonstrated that many of these genes are down-regulated in response to genotoxic agents, which may contribute to G2 DNA damage checkpoint function. Included among the genes identified in this screen were several proteins that are known or predicted to function in the ubiquitination of other proteins, including two ubiquitin conjugating enzymes and a component of the anaphase promoting complex (APC/C). Also among the G2/M-specific proteins identified in this screen was a protein with a c-terminal HECT domain that we have named G2E3. HECT domains function as ubiquitin ligase catalytic domains and are named for their similarity to E6-associated protein, a ubiquitin ligase involved in degradation of p53 in cells infected with tumorigenic papillomaviruses.

We were particularly interested in characterizing G2E3 since ubiquitin mediated protein degradation serves as a major regulatory mechanism in the cell division cycle and checkpoints. For example, degradation of cyclin B1 and securin [8] following ubiquitination by the ubiquitin ligase APC/C is essential for completion of mitosis. Similarly, degradation of

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cyclin-dependent kinase inhibitors p21 and p27 following ubiquitination by the SCF complex is required for entry into S-phase [9,10]. Oscillations of APC/C and SCF complex activity, therefore, serve as another major mechanism that contributes to control of the cell cycle [11]. Although these two ubiquitin ligase complexes are central to cell cycle control, other ubiquitin ligases also participate in cell cycle and checkpoint regulation. For example, the ubiquitin ligase CHFR is necessary for function of the mitotic stress checkpoint which leads to arrest in early mitosis in response to microtubule inhibitors [12], a checkpoint also referred to as the antephase checkpoint [13]. Similarly, Mdm2-mediated ubiquitination of p53 [14] plays a critical role in the G1 DNA damage checkpoint. Ubiquitin-mediated degradation will undoubtedly regulate many other aspects of cell cycle and checkpoint function that are not yet understood, and we predict a role for G2E3 in these processes.

Many cell cycle regulatory genes are compartmentalized within the cell as a means of additional gene regulation. Nucleo-cytoplasmic exchange has been described for several molecules that regulate the cell cycle and stress responses including Cdc25C [15], cyclin B1 [16], and p53 [17]. A variety of mechanisms are employed to control migration of proteins between the nucleus and cytoplasm. For example, Cdc25C is retained in the cytoplasm by binding to 14-3-3 proteins [18] and exported from the nucleus in a CRM1-dependent manner [15] to block its accumulation in the nucleus during interphase. Cyclin B1 is exported from the nucleus during interphase, but this export is inactivated by phosphorylation prior to mitosis to allow its nuclear accumulation [19]. The shuttling of p53 between nucleus and cytoplasm is controlled by cytoplasmic retention and nuclear import [20], as well as regulated nuclear export that is activated by ubiquitination [21]. Nuclear export plays a major role in controlling the subcellular localization of several proteins, but the mechanisms employed are incompletely characterized. Nuclear export by CRM1/Exportin 1 is the mechanism that is most completely characterized, due in large part to the availability of a potent inhibitor known as leptomycin B (LMB). This export factor binds to a leucine rich element ($LX_{2-3}LX_{2-3}LXL$) that was first identified in the HIV-1 protein Rev and the protein kinase A inhibitor PKI [22,23]. To date, several other nuclear export mechanisms have been described [24–28] which function through other members of the karyopherin family, but little is known about these CRM1-independent nuclear export mechanisms.

The HECT domain protein G2E3 is identified in GenBank as KIAA1333. In this work, we demonstrate that G2E3 accumulates in the nucleus of most cell types and specifically within the nucleolus of some cells. Nucleolar localization is highly regulated and this protein re-localizes to the nucleoplasm in response to DNA damage. We have defined the sequence responsible for directing the protein to the nucleolus and directing nuclear export of the protein in a CRM1-independent manner. Furthermore, we demonstrate that nucleolar localization is a dynamic process that occurs very rapidly. Although the HECT domain of G2E3 may function as a catalytically active ubiquitin ligase domain, this region of G2E3 also plays an important role in controlling the protein's subcellular trafficking. This unexpected function of a HECT domain has not previously been demonstrated.

Materials and methods

Cell culture

All cell lines were obtained from the ATCC. HeLa (human cervical cancer derived), Cos-7 (African Green Monkey kidney derived), SiHa (human cervical cancer derived), HEK293T (human embryonic kidney derived), and BSC-40 (African Green Monkey kidney derived) cells were cultured in DMEM supplemented with 10% FBS, 100 I.U. penicillin, and 100 μg/mL streptomycin. WI-38 primary foreskin fibroblasts were cultured in EMEM with 10% FBS, 100 I.U. penicillin, and 100 μg/mL streptomycin. All cell lines were maintained under standard environmental conditions. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Plasmids

All primers used for preparations of clones and mutants are listed in Supplementary Data. The full sequence of all constructs prepared for these experiments was confirmed by nucleotide sequencing to ensure that no unexpected mutations were introduced during construct preparation. Full-length G2E3 was generated by RT-PCR using HeLa mRNA, RACE cDNA library synthesis kit (Stratagene), Advantage HF2 high fidelity PCR kit (Clontech) and G2E3 primers (G2E3GFP5 and G2E3GFP3). The cDNA was cloned into pEGFP-C3 (Clontech) after digestion with *Xho*I and *Kpn*I. All other deletion mutants were cloned into pEGFP-C3 using *Xho*I and *Kpn*I. G2E3 c-terminal deletion mutants were cloned using G2E3GFP5 along with either G2E3-237R (2–237 mutant), G2E3-363R (2–363 mutant), G2E3-496R (2–496 mutant), G2E3-654R (2–654 mutant), and G2E3-674R (2–674 mutant) primers. N-terminal deletion mutants were generated using G2E3GFP3 along with either G2E3-287F (287–706 mutant) or G2E3-80F (80–706 mutant). A mutant containing amino acids 287–496 was generated using G2E3-287F and G2E3-496R primers. To facilitate preparation of a mutant lacking amino acids 18–35 (2–17/36–706 mutant), two separate PCR products were prepared and cloned adjacent to one another in-frame in the pEGFP-C3 vector. A fragment encoding amino acids 2–17 was generated by PCR using the G2E3GFP5 and G2E3-17R primers and cloned using *Kpn*I and *Xho*I. A second PCR product was generated using the G2E3-36F and G2E3-706XbaR primers. This fragment was cloned into the pEGFP-C3 plasmid already encoding amino acids 2–17. The resulting plasmid encodes amino acid 2–17 fused in-frame with amino acids 36–706 with an intervening *Kpn*I site (that encodes a GT dipeptide). Although we predicted that addition of two amino acid residues was unlikely to alter protein localization, we separately prepared a control construct encoding amino acids 2–35 fused to amino acids 36–706 with an intervening *Kpn*I site. This control is essentially wild-type G2E3 with a GT dipeptide between amino acids 35 and 36. This control was prepared in the same way using the primer pairs G2E3GFP5 with G2E3-35R and G2E3-36F with G2E3-706XbaR. A mutant with K30A and K31A mutations was prepared in the same way with the exception that the cDNA fragment encoding amino acids 2–35 was replaced by a fragment generated using the G2E3-GFP5 and G2E3-K30/31A-35R primers. cDNAs encoding NYD-SP6 and