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Gα12/13 Regulate the Expression of Calreticulin

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Abstract

There are large bodies of evidence linking the Gα12/13 family of G proteins to oncogenic signaling and tumor development. Gα12 and Gα13 contribute to the development of cancer through several avenues including regulating the expression of several proteins through a series of transcription factors. One gene previously unknown to be affected by Gα12/13 is *calr*. The protein product of this gene, calreticulin, is linked to cancer development in part because of its demonstrated ability to negatively regulate cellular adhesion through transcriptional regulation of e-cadherin. Data from protein separation and immunoblotting experiments demonstrate increased calreticulin expression predominantly in the nuclear and mitochondrial fractions of human embryonic kidney cells made to express wild type and constitutively activated forms of Gα12 and Gα13. This suggests that Gα12 and Gα13 may regulate the expression of calreticulin, a protein previously unknown to be affected by Gα12 or Gα13. Future work will examine the mechanism behind this newly discovered relationship and its effects on the expression of epithelial-cadherin and loss of cellular adhesion.

1. Introduction

G proteins are heterotrimeric, composed of alpha (α), beta (β), and gamma (γ) subunits and can be found in the intracellular fluid, of most eukaryotic cell types. These subunits play a critical role in several signaling pathways and cellular functions. G proteins transduce chemical information from G protein coupled receptors (GPCRs) to molecules downstream, initiating their respective signaling pathways. GPCRs function as guanine exchange factors (GEFs) for Gα subunits, stimulating these proteins to release a bound molecule of guanosine diphosphate (GDP). The process of Gα releasing GDP and binding GTP activates the protein. When GTP is hydrolyzed back to GDP, the α subunit reattaches to the β and γ subunits and the protein becomes inactive. The human genome contains genes that encode 16 different Gα subunits, all of which fall into 4 families: S, I, Q, and 12/13. Several recent investigations have linked aberrant G protein signaling to cancer development, particularly the Gα12/13 family[1].

Gα12 and Gα13 have been demonstrated to contribute to oncogenic development by increasing transcriptional activation of several cancer-promoting genes regulated by the various transcription factors including SRF, YAP, NF-kappaB, and AP-1[2,3]. The overactivation of these pathways can lead to uncontrolled cell proliferation, angiogenesis, and metastasis. One gene previously unknown to be affected by Gα12/13 is *calr* which encodes protein product calreticulin, a 46kDa, Ca^{2+}-binding protein native to the endoplasmic reticulum that aids in the folding and transportation of other proteins. The expression of calreticulin is associated with the development of several cancer types. Specifically, in breast cancer, increased calreticulin expression is correlated with an advanced stage, the development of metastasis, and decreased chance of metastasis-free survival[4]. In pancreatic cancer, calreticulin has been shown to promote epithelial to mesenchymal (EMT) transition, a key process in the development of metastasis[5]. Cells overexpressing calreticulin displayed an increase in cellular migration, and a decrease in expression of e-cadherin, a key protein in the EMT transition[6]. Calreticulin expression is dependent upon intracellular Ca^{2+} stores and signaling through transcription factors activator protein 1 (AP-1) and activator protein 2 (AP-2)[7]. In this manuscript, we present data from protein separation and immunoblotting experiments demonstrating increased calreticulin

expression in human embryonic kidney cells made to over express wild type and constitutively activated forms of Gα12/13. This suggests Gα12/13 may regulate the expression of calreticulin.

2. Methods

2.1 DNA Constructs

All constitutively activated (designated QL) and Myc epitope tagged forms of Gα12 and Gα13 were engineered by PCR mutagenesis. Primary PCR products were gel-extracted and purified using the Wizard SV system (Promega, Madison, WI). Secondary PCR reactions were conducted to add 5'-end restriction sites to allow for insertion into mammalian expression vector pcDNA3.1 and cloning. All constructs were sequenced for verification (Genewiz, South Plainfield, NJ).

2.2 Cell Culture and Transfections

Human embryonic kidney cells (HEK293) were grown in 10cm plates in Dulbecco's modified Eagle medium (DMEM, Corning Life Sciences, Tewksbury MA) containing 10% fetal bovine serum (Gibco, Gaithersburg, MD). Cells were transfected with 500µg of cDNA plasmid encoding Myc epitope tagged forms of Gα12 or Gα13 at 80-90% confluence. Cells transfected with 500µg of pcDNA3.1(-) served as the control. Cells were washed in phosphate buffered saline and harvested at approximately 48 hours post transfection.

2.3 Cell Fractionation and Western Blot Analysis

Following the wash and harvest, cells were suspended in a lysis buffer (5 mM HEPES pH 7.55, 250 mM mannitol, 0.5 mM EGTA & 0.1% BSA) first described by Lyssand and Bajjalieh 2007[8]. Cells were lysed via dounce (10 loose passes followed by 10 tight passes). Cellular components were separated via differential centrifugation. Unruptured cells were pelleted by centrifugation at 500 x g (Cellular Pellet). The resulting supernatant was removed and centrifuged at 2,000 x g to pellet nuclei (Nuclear Pellet). Again, the resulting supernatant was transferred and centrifuged at 11,000 x g to pellet mitochondria (Mitochondrial Pellet). All pellets were washed once in the lysis buffer. Samples were subjected to SDS-PAGE gels and immunoblotted with antibodies against the Myc epitope tag, calreticulin, and calnexin followed by alkaline phosphate-conjugated secondary antibodies. Developed blots were imaged using a Gel Logic 100 system (Kodak) equipped with Carestream Molecular Imaging gel analysis software.

3. Results

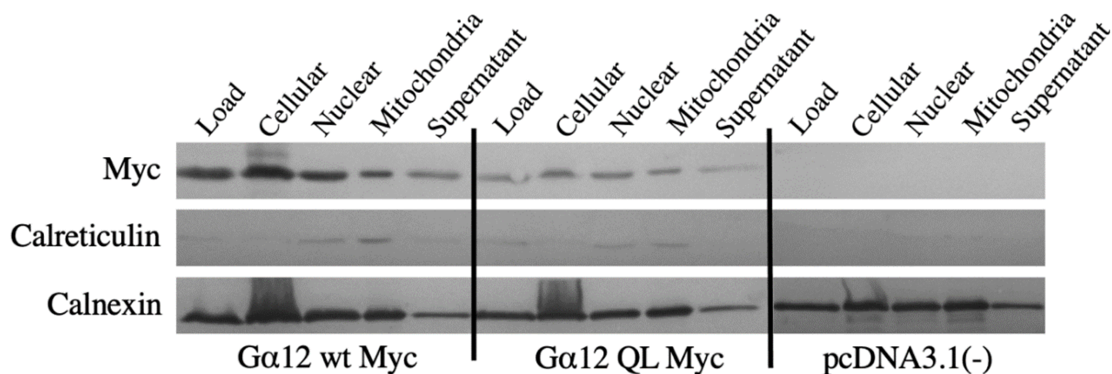

Image 1. Calreticulin expression is increased in HEK293 cells expressing Gα12 wildtype (wt) and Gα12 QL compared to the control.

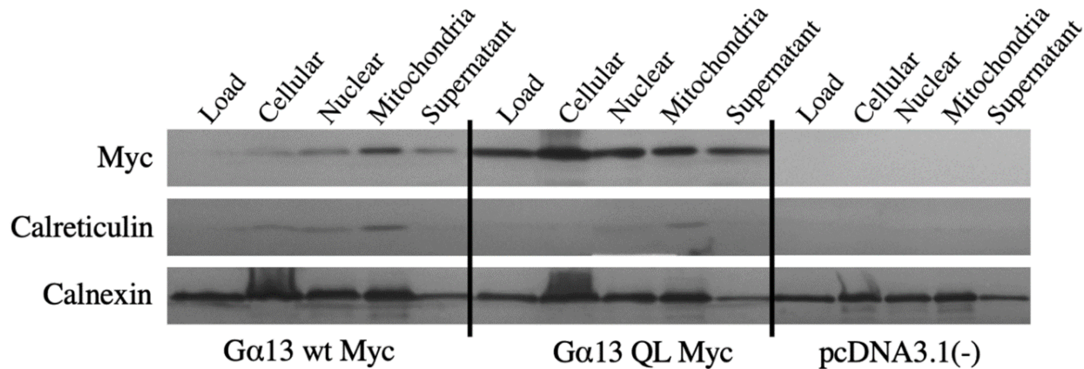

Image 2. Calreticulin expression is increased in HEK293 cells expressing Gα13 wildtype (wt) and Gα13 QL compared to the control

Protein separation and immunoblotting data from Image 1 demonstrates increased calreticulin expression in HEK293 cells expressing Gα12 wt Myc and Ga12 QL myc. In these samples, calreticulin was greatest in the mitochondrial fraction followed by the nuclear fraction. Data depicted in Image 2 shows that calreticulin expression is increased in HEK293 cells expressing Gα13 wt Myc and Gα13 QL myc. Calreticulin expression was greatest in the mitochondrial fraction followed by the nuclear fraction, consistent with data from Image 1.

4. Discussion

4.1 Ga12/13 Regulate the Expression of Calreticulin

Data from Image 1 and Image 2 demonstrates increased calreticulin expression in HEK293 cells transfected with Gα12/13 wt Myc and Gα12/3 QL Myc, most notably in the mitochondrial fraction. These results support the hypothesis that Gα12/13 regulate the expression of calreticulin. The mechanism behind this newly discovered regulation remains to be elucidated. One possibility is that Gα12/13 affect the expression of calreticulin through the transcription factor AP-1. Gα12/13 have previously been demonstrated to regulate the expression of other genes via AP-1 by interacting with upstream effector proteins[9]. It is possible that Gα12/13 may also regulate the expression of calreticulin via AP-1 because the *calr* promoter region harbors several AP-1 motifs[7]. Future experiments will investigate the validity of this hypothesis.

The exact mechanism by which Gα12/13 signal through AP-1 is unclear. AP-1 is a dimeric transcription complex and is often found to be dysregulated in several cancer types leading to tumor development and progression. The AP-1 complex is most commonly comprised of proteins from the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2)[10]. Gα12 signals through AP-1 by regulating the phosphorylation of c-Jun by c-Jun N-terminal Kinase (JNK) via a Rho meditated pathway[11]. In the case of Gα13, JNK activation is dependent on activation of p115RhoGEF[12]. Other lines of data suggest Gα13 activation of JNK is dependent on the binding Gα13 to JNK-associated leucine zipper protein (JLP)[13]. Further investigations will be needed to determine the specific nature of AP-1 activation in the Gα12/13 – calreticulin pathway.

4.2 Calreticulin Negatively Regulates the Expression of E-Cadherin

The regulation of calreticulin by Gα12/13 may have broader implications than previously understood. Gα12/13 and calreticulin have been demonstrated to affect e-cadherin at the functional and transcriptional levels respectively. Gα12/13 have been found to negatively regulate the adhesive function of e-cadherin by binding to the cytoplasmic region of the e-cadherin causing the release of β-catenin[14,15]. Alternatively, calreticulin has been shown to regulate the expression of e-cadherin by controlling intracellular Ca^{2+} levels leading to increased expression of slug, a known suppressor e-cadherin transcription[6]. The data we present combined with the associations between Gα12/13,

calreticulin, and e-cadherin demonstrated in previous research leads to the hypothesis that Gα12/13 may regulate the expression of e-cadherin via calreticulin.

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