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TITLE: Elucidating the Mechanism of Gain of Toxic Function From Mutant C1 Inhibitor Proteins in Hereditary Angioedema

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## 1. INTRODUCTION:

HAE is autosomal dominant. Cells, heterozygous for the SERPING1 mutation, express both mutant and WT C1INH proteins. HAE is clearly a loss-of-function disease. Plasma functional C1INH levels in symptomatic HAE patients, however, are very low - far less than the predicted 50%. We have shown that this is due, at least in part, to an additional acquired GOTF defect caused by the mutant protein that interferes with the secretion of WT C1INH. Our overall hypothesis is that mutant C1INH proteins exert a variable GOTF phenotype that inhibit secretion of WT C1INH protein and worsen disease severity. This hypothesis suggests that abrogating the GOTF should result in clinical benefit. Two aims will address complementary sub-hypotheses. Aim #1 will assess the mechanisms of the GOTF with a hypothesis that misfolding of mutant C1INH protein in the ER causes impairment of WT C1INH secretion. Elucidating the mechanism of the mutant C1INH-induced GOTF opens the possibility for new therapeutic approaches designed to abrogate the GOTF. Aim #2 will use a synthetic biology approach to assess the structural determinants of mutant C1INH-induced GOTF with a hypothesis that unique structural characteristics of C1INH make it more susceptible to GOTF than other serpins.

## 2. KEYWORDS:

Hereditary angioedema, C1 inhibitor, serpin, mutation, secretion, aggregates, misfolding

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

- 1) To define the mechanism how mutant C1INH proteins inhibit secretion of normal C1INH.
  - a) To identify the role of C1INH polymerization in the inhibition of secretion of normal C1INH (months 1-20; 50% completed)
  - b) To identify the role of ER stress in the inhibition of secretion of normal C1INH (months 12-32; 5% completed)
  - c) To identify whether abrogating the GOTF phenotype decreases evidence of C1INH polymerization or ER stress (months 24-36; 0% completed)
- 2) To analyze the structural requirements for the mutant C1INH protein-induced GOTF.
  - a) To assess the ability of alpha<sub>1</sub>-AT mutations engineered into C1INH to induce a GOTF (months 1-24; 25% completed)
  - b) To assess the GOTF in chimeric C1INH/alpha<sub>1</sub>-AT proteins with C1INH or alpha<sub>1</sub>-AT mutations to induce a GOTF (months 6-36; 10% completed)

### What was accomplished under these goals?

#### Aim #1

We demonstrated in year 01 that we could detect C1INH polymers in transfected cells, and that mutant C1INH formed small polymers in transfected cells. In this past year, we concentrated on determining whether wild-type C1INH was trapped in these polymers. To accomplish this, we

sought to detect the wild-type C1INH by labeling it with a FLAG-tag and using anti-FLAG antibodies to specifically blot wild-type C1INH in the pathologic polymers.. A FLAG tag was placed into the wild-type C1INH cDNA located immediately 3' of the signal peptide. The FLAG-tagged C1INH protein was expressed and secreted and could be detected using anti-C1INH antibodies; however, we were unable to detect it using an anti-FLAG antibody. Suspecting that this problem was due to steric hindrance, we pursued multiple ways to overcome the problem, ultimately finding that insertion of a 3x-FLAG sequence between the 97th and 98th codon of the C1INH allowed detection of the labeled protein. The first 97 amino acids of C1INH define the N-terminal glycosylation domain and have been shown to be unimportant for C1INH function.

We then successfully demonstrated that transfection of COS-7 cells with either 3x-FLAG-WT-C1INH plus WT-C1INH or 3x-FLAG-WT-C1INH plus G162R-Mu-C1INH resulted in decreased secretion of the 3x-FLAG-WT-C1INH when cotransfected with the mutant cDNA. This was an important confirmation of our hypothesis and preliminary data, utilizing a more physiologic system. The results are illustrated in Figure 1 below.

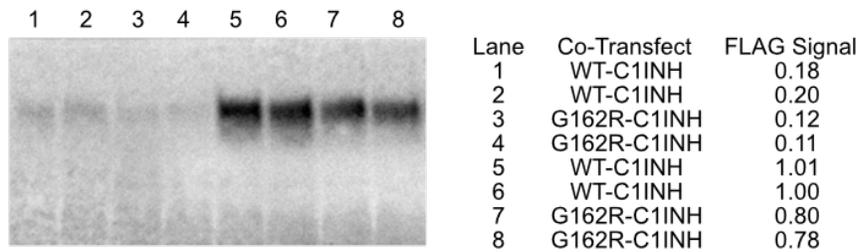


Figure 1. COS-7 cells were co-transfected with 3x-FLAG-WT-C1INH plus either WT-C1INH or G162R-C1INH. Secreted 3x-FLAG-WT-C1INH was visualized by immunoblotting with anti-FLAG. Lanes 1-4 = 24 hour supernatant; lanes 5-8 = 48 hour supernatant.

This assay provides a highly robust platform on which to test the impact of different mutant proteins on WT-C1INH secretion. We used it to look at the impact of various mutant C1INH constructs. The table below shows the results of this experiment.

Origin of mutation	Co-Transfection Construct	A1AT Equiv.	Replicate 1	Replicate 2
n/a	WT-C1INH	n/a	1.00	1.01
HAE	C183Y-Mu-C1INH	n/a	0.94	0.84
HAE	G162R-Mu-C1INH	n/a	0.57	0.57
HAE	V196D-Mu-C1INH	n/a	0.71	0.71

All 3 HAE disease causing mutations impaired the secretion of 3x-FLAG-WT-C1INH. We then examined whether there was evidence of the 3x-FLAG-WT-C1INH in the oligomers formed during co-transfection with G162R-Mu-C1INH by blotting with anti-FLAG. Cell lysates were separated on native blue gels. While the co-transfection with the mutant C1INH gave the

expected increase in C1INH polymers, we did not see evidence of the 3x-FLAG-WT-C1INH in the higher molecular weight bands. This experiment was repeated numerous times, as we had anticipated that the wild-type C1INH that we confirmed was not secreted would be found in the polymers.

Ultimately we accepted that this part of our hypothesis was incorrect. We then reasoned that the wild-type C1INH that was formed must be targeted for degradation in the proteasome in the presence of the mutant C1INH.

We therefore cotransfected cells with 3x-FLAG-WT-C1INH plus mutant C1INH cDNA in the presence or absence of a lactacystin, a proteasome inhibitor. As shown in figure 2, blocking degradation of C1INH by the proteasome resulted in a clear increase in the amount of FLAG-tagged C1INH detected in the cell lysate.

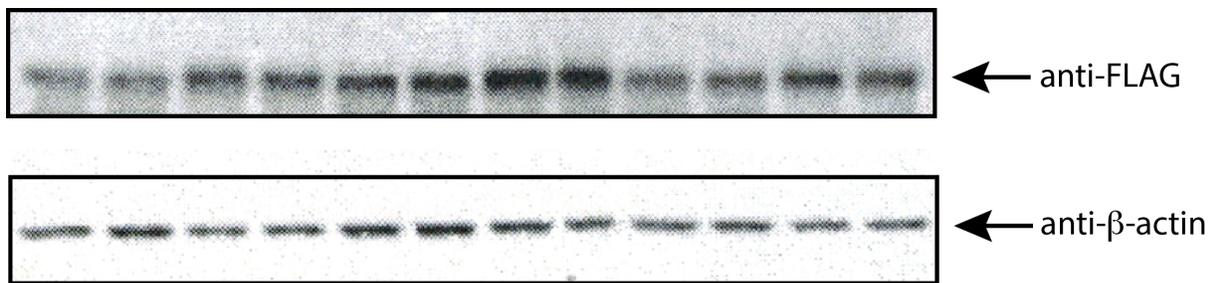


Figure 2. HEK293 cells were transfected with 3x-FLAG-C1INH plus either WT-C1INH (lanes 1-4), G162R mutant C1INH (lanes 5-8) or empty vector (lanes 9-12). Cells were treated with media alone (lanes 1-2, 5-6, 9-10) or lactacystin (lanes 3-4, 7-8, 11-12). Lysates were immunoblotted using anti-FLAG (top) or anti-β-actin (bottom). Lysates were immunoblotted using either anti-FLAG (top) or anti-β-actin (bottom).

Based on these results, we now conclude that mutant C1INH protein induces the GOTF with decreased secretion of wild-type C1INH by enhancing the proteosomal degradation of wild-type C1INH. We suspect that this is due to ER stress.

During this past year we have also begun to look for evidence of ER stress in cells transfected with mutant C1INH. We designed and optimized a real-time quantitative RT-PCR assay for BiP (Grp78). Treating COS-7 cells transfected with wild-type C1INH and then treated with brefeldin for 4 hours resulted in a 270% increase in BiP mRNA 48 hours after transfection and a 253% increase 72 hours after transfection. We observed, however, no change in the BiP levels in cells transfected with wild-type versus mutant C1INH cDNA. More surprisingly, we also did not observe any increase in BiP mRNA following transfection with α1-antitrypsin NHK, which has been previously reported to lead to increased expression of BiP.

We also assessed whether mutant C1INH would cause an increase in BiP protein. Figure 3 shows the BiP protein levels in cells transfected with either WT-C1INH or G162R-Mu-C1INH. The lysates were blotted for BiP, then stripped and re-probed for the housekeeping gene beta-actin.

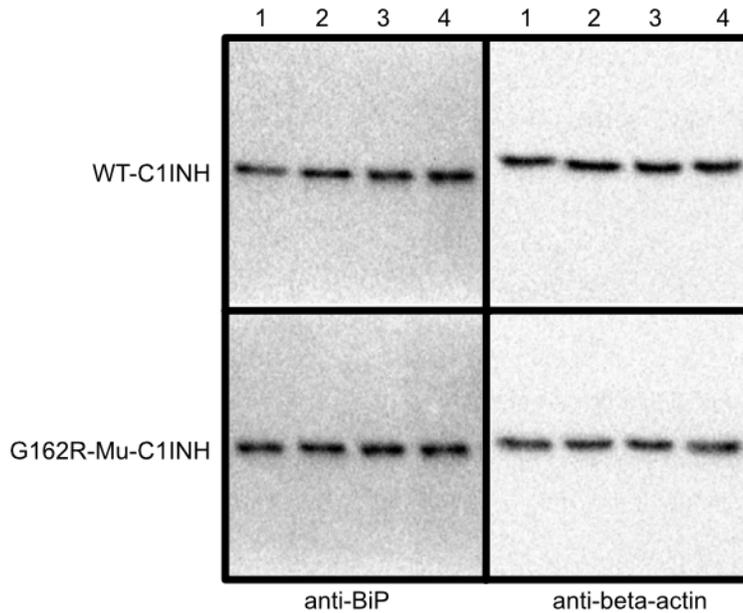


Figure 3. Cells were transfected with WT-C1INH or G162R-Mu-C1INH and the lysate recovered and blotted for BiP (left). Membranes were then stripped and reprobed for beta-actin (right). Lanes 1-2 used 1.5 ug of plasmid DNA while lanes 3-4 used 2 ug of plasmid DNA.

The cells transfected with the G162R-Mu-C1INH appear to have increased BiP (particularly when normalized to the beta-actin levels) compared to the cell transfected with WT-C1INH. The BiP level was 27% higher at 1.5 ug DNA and 14% higher at 2 ug. We also measured BiP mRNA by TaqMan real-time quantitative RT-PCR, and saw small increases in the mRNA level in cells transfected with G162R-Mu-C1INH.

In order to enhance the sensitivity of our measurements, we developed a promoter-reporter assay that uses the BiP promoter elements and a firefly luciferase reporter. Cells were also transfected with a Renilla luciferase reporter under the control of an endogenous promoter (PGL4.74) to normalize the results. Cells were cotransfected with wild-type C1INH plus the BiP-Firefly luciferase plasmid, the TK-Renilla luciferase plasmid, plus either wild-type C1INH, G162R C1INH. As a control we also cotransfected cells with wild-type A1AT plus either wild-type A1AT or mutant NHK A1AT (which is known to cause ER stress).

Co-transfection	Firefly luciferase	Renilla luciferase	Ratio	Fold Increase
Wild-type C1INH	182,447,728	70,714	2,580	
	201,994,160	70,589	2,862	
G162R C1INH	392,510,400	30,097	13,042	4.72
	470,603,200	37,152	12,666	
Wild-type $\alpha$ 1AT	113,137,152	35,348	3,201	
	98,634,424	35,799	2,755	
NHK $\alpha$ 1AT	196,457,680	30,761	6,387	2.24
	260,361,520	37,479	6,947	

As shown in the table, co-transfection with G162R mutant C1INH resulted in a 4.72 fold increase in BiP promoter activity compared to wild-type C1INH. In contrast, NHK A1AT, which is known to increase ER stress, resulted in a 2.24-fold increase in BiP promoter activity.

Thus, we have evidence now that mutant C1INH proteins induce a GOTF by causing ER stress and degradation of wild-type C1INH in the proteasome.

### Aim #2

We successfully made novel C1INH mutants that mirrored the equivalent A1AT mutations that cause A1AT deficiency based on the homology between these two serpins to determine equivalent residues. Using these novel C1INH mutants, we asked whether we would still see a GOTF. We utilized the assay to measure secretion of 3x-FLAG-WT-C1INH. The results are shown in the table below:

Origin of mutation	Co-Transfection Construct	A1AT Equiv.	Replicate 1	Replicate 2
n/a	WT-C1INH	n/a	1.00	1.01
A1AT	E429K-Mu-C1INH	Z	0.74	0.68
A1AT	H421D-Mu-C1INH	King's	0.66	0.67
A1AT	S148F-Mu-C1INH	Silyama	0.71	0.54

Surprisingly, we saw that introducing A1AT mutation equivalents into C1INH also hindered the secretion of 3x-FLAG-WT-C1INH. This is particularly notable because the A1AT mutations do not have an autosomal dominant effect on WT A1AT secretion. These results appear to support our hypothesis that the structure of C1INH is uniquely susceptible to GOTF, leading to the autosomal dominant pathophysiology.

### **What opportunities for training and professional development has the project provided?**

Nothing to Report.

### **How were the results disseminated to communities of interest?**

Nothing to Report.

### **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we have several new goals. The first is to extend the results obtained using transfected COS-7 cells to a more clinically relevant cell. We will therefore use both primary human monocytes as well as human hepatoma cells and demonstrate that the GOTF is accompanied by ER stress.

The second goals will be to extend the studies looking at the differences between C1INH and A1AT by making chimeric proteins in which we swap different domains in order to try to define the critical structural elements that make C1INH so susceptible to GOTF.

#### **4. IMPACT**

##### **What was the impact on the development of the principal discipline(s) of the project?**

During this past year, our studies have had a significant impact leading to a number of new observations that increase our understanding of HAE. First, we developed techniques that will be useful in the study of HAE. Second, we unequivocally demonstrated that mutant C1INH induces a GOTF. Third, we showed that mutant C1INH induce ER stress. And fourth, we show that the GOTF is not restricted to natural HAE causing mutations but appears to be intrinsic to almost any disruption of the normal C1INH structure.

##### **What was the impact on other disciplines?**

Nothing to Report.

##### **What was the impact on technology transfer?**

Nothing to Report.

##### **What was the impact on society beyond science and technology?**

Nothing to Report.

#### **5. CHANGES/PROBLEMS:**

Nothing to Report.

##### **Changes in approach and reasons for change**

There are no anticipated modifications of the plans relative to the SOW.

##### **Actual or anticipated problems or delays and actions or plans to resolve them**

Although we had significant delays during the prior year, we made good progress this year and do not anticipate additional problems. We lost our technician this summer to graduate school, and we are still training his replacement.

##### **Changes that had a significant impact on expenditures**

Nothing to report.

##### **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

No changes. The most recent IRB approval was 09/13/2016.

## 6. PRODUCTS:

- **Publications, conference papers, and presentations**

Zuraw, B.L., Herschbach J., Christiansen, S.C. Heterogeneity in the Secretion of Mutant C1 inhibitor Proteins. Manuscript being readied for submission.

Zuraw, B.L., Herschbach J., Christiansen, S.C. Type I Mutant C1 inhibitor Proteins are Retained in the Endoplasmic Reticulum. Manuscript being readied for submission.

Zuraw, B.L., Kansakar, S. Herschbach J., Christiansen, S.C. Mutant C1 Inhibitor Proteins Cause a Gain of Toxic Function with Decreased Secretion of Wild-Type C1 Inhibitor. Manuscript being readied for submission.

- **Inventions, patent applications, and/or licenses**

None.

- **Other Products**

None

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### What individuals have worked on the project?

**Name:** Bruce Zuraw

**Project Role:** Principal Investigator

**Research Identifier:** bzuraw

**Nearest person month worked:** 3

**Contribution to project:** supervised project and other staff

**Funding Support:** None

**Name:** Marc Riedl

**Project Role:** Co-Investigator

**Research Identifier:** mriedl

**Nearest person month worked:** 1

**Contribution to project:** Building database of HAE patients who can be contacted for the study

**Funding Support:** None

**Name:** Jack Herschbach

**Project Role:** Research Technician

**Research Identifier:**

**Nearest person month worked:** 12

**Contribution to project:** constructed all plasmids and performed mutagenesis

**Funding Support:** None

**Name:** Shamir Kansakar

**Project Role:** Research Technician

**Research Identifier:**

**Nearest person month worked:** 10

**Contribution to project:** Performed native gels and working on stress response

**Funding Support:** None

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

U19 AI070535                      09/01/06-8/31/16              3.0 calendar months

NIH/NIAID

Epithelial GILZ in Inflammation and Remodeling

The aims of this project were to: 1) Assess the mechanism of GILZ down-regulation in the airway of asthmatics; 2) Elucidate the impact of decreased expression of GILZ on asthma exacerbations and airway remodeling following allergen challenge or HRV infection; and 3) Determine whether novel therapeutic strategies to increase GILZ will decrease airway inflammation and protect patients from asthma exacerbations and airway remodeling.

**What other organizations were involved as partners?**

Nothing to Report.

**8. SPECIAL REPORTING REQUIREMENTS:** None

**9. APPENDICES:**

None