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# Mechanisms and Integration of Signal Pathway: A Role for Calpains?

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**Final Report for Period:** 09/1997 - 08/2000**Submitted on:** 06/07/2004**Principal Investigator:** Croall, Dorothy E.**Award ID:** 9723636**Organization:** University of Maine**Title:**

Mechanisms and Integration of Signal Pathway: A Role for Calpains?

**Project Participants****Senior Personnel****Name:** Croall, Dorothy**Worked for more than 160 Hours:** Yes**Contribution to Project:****Post-doc****Graduate Student****Name:** Xiong, Wei**Worked for more than 160 Hours:** Yes**Contribution to Project:**

supported as a graduate research assistant 50% time Fall 1997; examined calpain-2 substrate preference using spectrin mutants

**Name:** Hatch, Harold**Worked for more than 160 Hours:** Yes**Contribution to Project:**

began as undergraduate student employee ('97-'98) and became a MS student fall '98 completed degree August 2000. Supported as a graduate research assistant Fall '99-August '00. Project involved the role of an IQ motif in calpain function

**Name:** Rauch, Steven**Worked for more than 160 Hours:** Yes**Contribution to Project:**

began studies of calpain-substrate recognition using an expressed model peptide summer '00

**Undergraduate Student****Name:** Bailey, Andrew**Worked for more than 160 Hours:** Yes**Contribution to Project:**

Visiting student from East Anglia University for 'year in America', subsequently pursued PhD in UK. He examined spectrin clavage by calpain-2

**Name:** Schott, Will**Worked for more than 160 Hours:** Yes**Contribution to Project:**

his required undergraduate research project examined the interaction between calpain and calpastatin

**Name:** Moffett, Katherin**Worked for more than 160 Hours:** Yes**Contribution to Project:**

her required undergraduate research project began exploring zebrafish as a model for studying calpain expression and function

**Name:** O'Hazo, Megan**Worked for more than 160 Hours:** Yes**Contribution to Project:**

assisted graduate student in studies of the role of calpain's IQ motif

**Name:** Wong, Kelli

**Worked for more than 160 Hours:** Yes

**Contribution to Project:**

visiting student employed summer '00 to assist others

**Technician, Programmer**

**Name:** Morrill, Wendy

**Worked for more than 160 Hours:** Yes

**Contribution to Project:**

research technician 50% time Sept '97-August '99; assisted in general lab management and cloning of a library of calpain fragments

**Name:** Prince, Alison

**Worked for more than 160 Hours:** Yes

**Contribution to Project:**

research technician 20% time, Sept. '97-May '98; assisted in general lab management and cloning of a library of calpain fragments

**Other Participant**

**Research Experience for Undergraduates**

**Organizational Partners**

**Other Collaborators or Contacts**

John S. Elce  
Sabbatical Host Queen's University  
Kingston, Ontario

**Activities and Findings**

**Research and Education Activities: (See PDF version submitted by PI at the end of the report)**

**Findings: (See PDF version submitted by PI at the end of the report)**

**Training and Development:**

Enhancement of undergraduate education at the University of Maine:

This award provided research opportunities and experience for six (three male and three female) undergraduates at the University of Maine. All majors in Biochemistry are required to complete 6 credits of research for their 'capstone'; three of the involved undergraduates fulfilled their requirement as described here. Sept 97 û Aug '98 Harold Hatch was an undergraduate student employee while PI was on sabbatical; he aided the part-time technician in her efforts to clone calpain subdomains. Sept. 98 û May '99 Andrew Bailey (visiting student from East Anglia U., U.K.) attempted to complete the work unfinished by a graduate student to examine the substrate specificity of calpain-2 using selected mutants of spectrin. Sept. 98 û May '99 Will Schott (capstone) expressed and isolated the calpain fragment containing the crosslinking site for the essential calpastatin peptide. Sept '99 û Aug '00 Katherin Moffett (capstone) completed the preliminary work on calpain activity in zebrafish embryos. Sept '99 û Dec. '99 Megan O'Hazo (capstone) assisted then graduate student Harold Hatch with his studies of IQ-motif mutants. Ms. Kelly Wong, was an undergraduate student employee (a visiting student from Colgate University) in summer '00; she provided general assistance to everyone in the laboratory. Lab group meetings are a required part of research experience in my laboratory thus each student also gained experience in reading the literature, critically, and in making presentations on journal articles and on their experimental progress.

Graduate education : Mr. Wei Xiong was supported by this award Sept. 97- Jan '98 when he left to work in industry. The failure of Mr. Xiong to complete his thesis research was very costly to my program. He was awarded a non-thesis master's degree in May '98. Harold Hatch began

an MS program in summer of '98 and completed his degree in August 2000 studying the IQ-motif mutants. This award provided support for Mr. Hatch as a research assistant Sept '99- August 2000; this was crucial for his timely completion of his degree. Steven Rauch, M.D. had elected to pursue his MS in my laboratory in May '00 but was encouraged to take an alternate path in August 2000.

Other Personnel Involved at the University of Maine Two part time employees (female) were supported by this award. Sept 97-May '98 Wendy Morrill 50% time technician and Alison Prince 20% time. The failure of my department to provide adequate supervision to part time staff during my sabbatical leave resulted in less than adequate progress by Ms. Morrill. She did retain her position at 50% time until August 1999.

At Queen's University: Although I had no formal responsibilities towards personnel at Queen's U. as the only female of faculty status on the 6th floor of Botterrell Hall I found myself being consulted regularly by undergraduate and graduate students for both discussions of science and for career advice.

### **Outreach Activities:**

#### **Journal Publications**

Potter, D.A., Tirnauer, J.S., Jansenn, R., Croall, D.E., Hughes, C.N., Mier, J., Maki, M. and Herman, I.M., "Calpain regulates cell spreading and function of the cortical actin cytoskeleton.", *J. Cell Biol.*, p. 647, vol. 141, (1998). Published

Dutt, P., Arthur, J.S.C., Croall, D.E. and Elce, J.S., "m-calpain subunits remain associated in the presence of calcium.", *FEBS Lett.*, p. 367, vol. 436, (1998). Published

Hosfield, C.M., Ye, Q., Arthur, J.S.C., Hegadorn, C., Croall, D.E., Elce, J.S., Jia, Z., "Crystallization and X-ray crystallographic analysis of m-calpain, a calcium-dependent protease.", *Acta Cryst.*, p. 1484, vol. D55, (1999). Published

Croall, D.E., Moffett, K.A. and Hatch, H., "Casein zymography of calpain using a 4(2-hydroxyethyl)-1 piperazineethansulfonic acid-Imidazole buffer.", *Analytical Biochemistry*, p. 129, vol. 304, (2002). Published

Dutt, P., Croall, D.E., Arthur, J.S.C., DeVeyra, T., Williams, K., Elce, J.S.E. and Greer, P.A., "m-calpain is required for preimplantation embryonic development in mice.", *Molecular and Cellular Biology*, p. , vol. , ( ). Submitted

Croall, D.E. and Hatch, H., "The regulation of calpain activity by calcium: a role for an IQ-motif.", *Biochim. Biophys. Acta*, p. , vol. , ( ). in preparation

#### **Books or Other One-time Publications**

Croall, D.E., ""Affinity chromatography methods for purifying calpains."" (1999). Book, Published

Editor(s): J.S. Elce

Collection: Calpain Methods and Protocols

Bibliography: *Methods in Molecular Biology* 144, 33-40. Humana Press

#### **Web/Internet Site**

#### **Other Specific Products**

#### **Contributions**

#### **Contributions within Discipline:**

Calcium ions are used as messenger molecules that regulate many important intracellular processes. We need to learn how calcium's 'messages' are interpreted by cells in order to understand how cells move, divide, grow, become cancerous, or die. A variety of calcium binding proteins function to respond to calcium's 'message' and my work is focussed on one family of these proteins; the proteolytic enzymes called calpains. The family of calpains may be as large as ~15 enzymes although only 3 or 4 of these enzyme isoforms have been studied in any

detail. The most well studied types of calpain (calpains 1 and 2) are ubiquitously present in cells from fruit flies to humans. Genetic studies have linked several novel calpains to specific disease processes. For example, inactivation of calpain-3 is the cause of an inherited disease, Limb-Girdle muscular dystrophy (type IIA). Loss of calpain-9 is linked to oncogenesis and variations in calpain-10 were identified as a risk factor for type 2 diabetes. Each of these results suggests that calpains have very important biological functions.

I have devoted ~twenty years towards understanding the protein structure-function relationship for calpains-1 and 2 and towards elucidation of their physiological function(s). Support from this award for my sabbatical work at Queen's University contributed to successful crystallization and thus subsequent determination of the structure of inactive calpain-2 by Hosfield et al. (EMBO J. 18, 6880-6889, 1999). The availability of the enzyme structure, albeit the inactive form, provides many new opportunities for expediting our understanding of this family of enzymes. The vector I constructed for targeted deletion of the catalytic subunit of the ubiquitous calpain-2 (capn-2 gene) in mouse was subsequently utilized by the group at Queen's U. and has produced the unexpected result that deletion of capn-2 is embryonic lethal in very early embryogenesis in contrast to the capn-1 deletion that has no substantial phenotype. This information provides important insight into the non-redundancy of calpain isoform function, at least at early stages of mammalian development. The novel calpain phenotype resulting from site directed mutagenesis of an IQ-motif has spawned a new hypothesis as to the mechanism by which calpain recognizes and selects target proteins. While the role of 'exosites' are well known and characterized for a number of extracellular serine proteases, their existence in calpain had not previously been suspected. Further characterization of a substrate binding exosite could be significant for potential development of isoform selective substrates and/ or inhibitors; reagents that will be key for determining the physiological roles of each of these enzymes. In the course of our studies we also modified a zymographic assay for calpain to enhance detection of calpain-2 (and calpain-3). This methodology furthers our ability to investigate the physiological roles and regulation of calpain.

#### **Contributions to Other Disciplines:**

#### **Contributions to Human Resource Development:**

Personnel Involved at the University of Maine: 6 (3 male/2 female) undergraduates, 3 graduate students (male), 2 part time employees (female)

Sept. 97- Jan '98 Wei Xiong graduate student, rec'd non thesis master's degree May '98 initially employed Myriad Genetics, Utah

Sept 97-May '98 Wendy Morrill part time technician (50%), Alison Prince (20%)

Sept 97 - Aug '98 Harold Hatch Undergraduate student employee, pursued MS in my laboratory

August '98- Aug '99 Wendy Morrill (50% time technician)

Sept. '98 - Aug '00 Harold Hatch MS student (T.A./ R.A.); post completion of MS initially employed Millennium Pharmaceuticals, Cambridge MA

Sept. 98 - May '99 Andrew Bailey (visiting student from East Anglia U., U.K.) pursued PhD in UK post graduation

Will Schott undergraduate research, employed at the Jackson Laboratory, BarHarbor ME

Sept '99 - Aug '00 Katherin Moffett, undergraduate research; matriculating at Dartmouth Medical School

Sept '99 - Dec. '99 Megan O'Hazo, undergraduate research, matriculating at Tufts School of Dentistry

May '00 - Aug '00 Steven Rauch, M.D. MS student, (MS completed initial employment The Jackson Laboratory) Ms. Kelli Wong, undergraduate student employee (a visiting student from Colgate University)

#### **Contributions to Resources for Research and Education:**

#### **Contributions Beyond Science and Engineering:**

#### **Categories for which nothing is reported:**

Organizational Partners

Activities and Findings: Any Outreach Activities

Any Web/Internet Site

Any Product

Contributions: To Any Other Disciplines

Contributions: To Any Resources for Research and Education

Contributions: To Any Beyond Science and Engineering

The  $\text{Ca}^{2+}$ -dependent proteolytic enzymes, calpain 1 and 2 are thought to contribute to controlling the levels or activities of various component proteins in signal transduction cascades. The two primary objectives of this award were 1) to engineer a calpastatin insensitive calpain and 2) to test a hypothesis describing the mechanism by which  $\text{Ca}^{2+}$  regulates calpain's activity. A corollary objective aimed to investigate calcium dependent protein-protein interactions between calpain sub-domains. Experiments towards objective 1) included site directed mutagenesis of the catalytic subunit of calpain-2 to reduce hydrophobicity of conserved residues surrounding C498; the site of crosslinking of a peptide inhibitor designed as a calpastatin mimic (Croall and McGrody 1994, *Biochemistry* 33, 13223-13230). Experiments towards objective 2) included mutation of two "IQ-motifs" within the catalytic subunit of calpain-2 to test the hypothesis that regulation of calpain by  $\text{Ca}^{2+}$ -binding would be analogous to how calmodulin regulates its target proteins and several subdomains of calpain-2 were cloned (as defined by selected exon boundaries in the absence of protein structural information) and expressed in *E. coli* to attempt to examine calcium-dependent interactions within calpain and between calpain and calpastatin. As the first year of the award supported a sabbatical leave at Queen's University (Kingston, Ontario) I also undertook a project to construct a knockout vector for the mouse calpain-2 catalytic subunit gene (*capn2*). The project was based on the prediction that targeted deletion of the calpain small subunit (*capn4*) would be embryonic lethal, which it was. As the graduate student supported by the preceding award had opted to pursue his MS rather than a PhD his project involved further characterization of the substrate specificity of calpain-2 utilizing a library of P2 site mutants of spectrin provided to us by Dr. Jon Morrow and Paul Stabach (Yale University). These activities overlapped with the beginning of the new award.

I participated and presented results of our efforts at the first FASEB Summer conference on 'The calpain system in health and disease' (June '99 –Copper Mountain CO); presented a poster at the ASCB annual meeting, Washington, D.C. in December '99; and presented two posters (one relating to the *capn-2* knockout with J. Elce group) at the Gordon Research Conference on "Proteolytic enzymes and their inhibitors: (July 2000, New London, NH). Construction of a zebrafish facility at U Maine in 1999 provides an opportunity to assess this organism as a potential model for deciphering calpain function. This model is particularly attractive for assessing the function of calpain-2 in early development and has promise for investigating isoform specific functions of calpains. Towards this aim I attended a two week summer course, "Neural development and genetics of zebrafish" at the Marine Biological Laboratory at Woods Hole in summer of 2000.

- Towards engineering of a calpastatin insensitive calpain: Of the mutants generated (L484A, V501A or F502Y) only F502Y was expressed and recoverable from E coli in an active form. Characterization of this enzyme showed no measurable difference in its susceptibility to inhibition by the calpastatin peptide. Our results, in conjunction with work of others localizing interactions of other conserved regions of calpastatin to domains IV and VI of calpain, led us to conclude that this approach would not be sufficient to rescue cells overexpressing calpastatin (Potter et al 1998, J Cell Biol) and thus was not pursued further.
- Site directed mutagenesis of putative “IQ-motifs”: Q554M-R558N had no impact on calpain function. In contrast, Q413S and/or R417Q produced enzymes with a novel phenotype; i.e. their ability to hydrolyze casein and their ability to autoproteolyze were uncoupled. Based on this novel finding we propose that the loop containing these IQ-motif residues (412-418 of calpain-2) plays a role in the Ca<sup>2+</sup> dependent regulation of calpain. In conjunction with a number of other facts about calpain we propose a new hypothesis that an exosite is likely to be significant for calpain’s recognition and binding to exogenous substrates. This hypothesis provides the basis for a future proposal.
- Library of cloned calpain fragments: We cloned nine fragments of calpain-2. Several fragments have also been expressed as gst-fusion proteins (pGEX vectors) to allow the possibility of co-purification of interacting peptides. Two of these fragments are “calcium sensor peptides” (csp) i.e. the first 3 EF hands of domains IV (csp4) and VI (csp6). Several products were expressed as insoluble forms. Elucidation of the structure of inactive calpain (subsequent to these efforts) provides better indicators of independent folding domains than the exon boundaries we had used, and several attempts to identify peptides capable of binding calpastatin or the calcium sensor peptides were unsuccessful.
- The construct for targeted deletion of capn-2 was utilized by Previn Dutt as his doctoral research project, directed by Dr. John Elce and Dr. Peter Greer at Queen’s University. The important, and surprising, eventual result being that capn2<sup>-/-</sup> appears to be lethal in early embryogenesis.
- Sharing my knowledge and experience with calpain purification (e.g. affinity chromatography via reactive red agarose) with the Queen’s group contributed to their success at solving the crystal structure of inactive calpain-2 . (Hosfield et al 1999).
- Calpains in zebrafish and their expression in development: An improved casein zymographic technique was developed to allow resolution of more than 2 calpain-like activities in early zebrafish embryos. Using zymography a preliminary study documents developmental changes in isoform expression from immediately post fertilization through 5 days.