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A biochemical approach to define the interactome for calpain2 in endothelial cells

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Current repositories for protein-protein interactions and high throughput screening methods focus on individual gene products and do not consider the significance of calcium induced conformational changes. These limitations suggest the need for alternative strategies to better define the calpain2 interactome. Affinity capture coupled with LC-MS/MS and proteomic analysis of the recovered proteins provides a powerful approach to identify protein-protein interactions for the heterodimeric calpain2. CAPN2 (rat) was modified to be catalytically incompetent (C105A) and fused with a C-terminal 15 residue peptide optimized for biotinylation by the biotin protein ligase, BirA. The resulting CAPN2*, heterodimerized with truncated CAPNS1, was purified from *E. coli*, and biotinylated *in vitro*. Biotinylated calpain2* served as 'bait' for streptavidin affinity capture of calpain2 and its interacting proteins from lysates of bovine aortic (BAEC) and human umbilical vein (HUVEC) endothelial cells (ECs). Protein-calpain2 complexes were formed in the presence of calcium to allow EGTA elution of interacting proteins and LC-MS/MS analysis in the absence of an abundance of bait peptides. Capture of the well characterized calpain inhibitor protein calpastatin (CAST), and a known substrate, vimentin provide proof of concept and validates the conformational integrity of the bait calpain2*. Significant overlap between datasets (two from BAEC and one HUVEC) is also encouraging. Of numerous other proteins including several annexins, ANXA1 was confirmed as a substrate for calpain2. Findings are expected to contribute to continuing efforts in the field to better characterize calpain2's selection of substrates and may reveal other important clues to calpain's localization and regulation.

Why pursue the calpain2 interactome?

- contributes to defining criteria for substrate selection/susceptibility;
- contributes to improved substrate prediction methods;
- aids in defining context specific biological roles;
- has potential to discover mechanisms for localization of the enzyme/protein;
- has potential to discover new contributors to regulation of its proteolytic activity and/or non-proteolytic functions.

Why choose endothelial cells (EC)?

♦ ***in vivo* phenotypic evidence (mouse)**

Defects in formation and repair of blood vessels occur with overexpression of calpastatin (CAST), the specific endogenous inhibitor of a subset of calpains, including calpain2. This implies that calpain's catalytic activity contributes to angiogenesis and vascular repair but its role(s) are undefined. e.g. Peltier, J *et al* (2006) J Am Soc Nephrol 17: 3415-3423; LeTavernier, B. *et al* (2012) Arterioscler Thromb Vasc Biol 32: 335-342; Miyazaki, T. et al. (2015) Circ. Res. 116: 1170-1181.

♦ **A role in EC sprouting (3D culture)**

Shear stress in the presence or absence of sphingosine1P stimulated a calpain2 dependent initiation of EC sprouting. Mechanism may involve proteolysis of vimentin upstream of MT1-MMP translocation to the membrane. Kang, H., et al., J Biol Chem (2011) 286: 42017-26; Kwak,HI et al (2012) Angiogenesis 15: 287-303.

♦ **Calpain as an integrator of signals regulating vascularization**

Calpains are implicated in the response of ECs to a variety of hormonal and/or cytokine signals including angiotensin, VEGF, CXCL-10 and NO, and potentially play an integrative role, although mechanistic details of calpain's role(s) are lacking.

e.g. Bodnar, R.J., C.C. Yates, and A. Wells (2006) Circ Res,. 98: 617-625; Bodnar, R.J. et al. (2009) J Cell Sci, 122: 2064-2077; Hoang, M.V. et al. (2010) PLoS One 5: e13612; Hoang, M.V., L.E. Smith, and D.R. Senger (2011) Biochim Biophys Acta 1812: 549-57, Scalia, R., et al. (2011) Circ. Res. 108: 1102-1111.

What do interaction databases currently provide?

Public databases such as BioGRID and IntAct provide curated lists of reported or described protein-protein interactions. Much of the accumulated data is provided by high throughput proteomic studies screening 'orfeomes' or expressed cDNA libraries for interacting partners. For heterodimeric calpain2, with its two distinct conformations controlled by Ca^{2+} binding, much data reported for CAPN2 and CAPNS1 are likely misleading. Of the 50 or 59 binding partners listed for CAPN2 or CAPNS1 respectively at thebiogrid.org (03-Jun-2016) only 9 were shared and thus potentially also bind to the functional heterodimer.

from BioGRID overlap of CAPN2 and CAPNS1 interactors (June 2016)

- calpain1 large subunit (CAPN1)
- aldehyde dehydrogenase 7 family, member A1
- asparagine synthetase (glutamine-hydrolyzing)
- protein disulfide isomerase family A, member 4
- platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45kDa)
- polycomb protein eed
- proline synthetase co-transcribed homolog (bacterial)
- serine/threonine protein kinase 26
- tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon (14-3-3E)

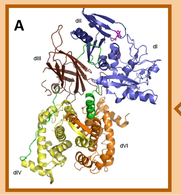
from IntAct (June 2016)

CAST for binding CAPN2 and CAPN1 —but not found in CAPNS1 list

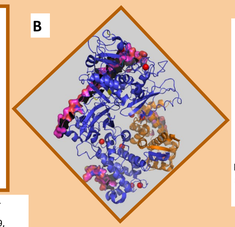
Databases organized by gene product interactions are not optimal for curating interactors for calpain2. Calpain.org lists reported (and predicted) substrates as curated from the literature but most studies lack data as to the affinity for interactions or kinetics for specific sites cleaved.

Rationale for an *in vitro* affinity capture strategy

- ♦ Control of calpain2 conformation with calcium/EGTA
- ♦ Calpain2 selects partners on the basis of binding affinity, kinetics and abundance in the 'prey' pool
- ♦ Applicable to a variety of cell lysates or tissue extracts
- ♦ Potential to discover interactors in the absence of calcium

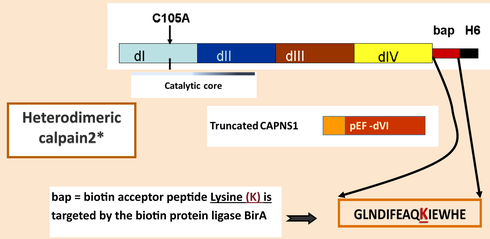


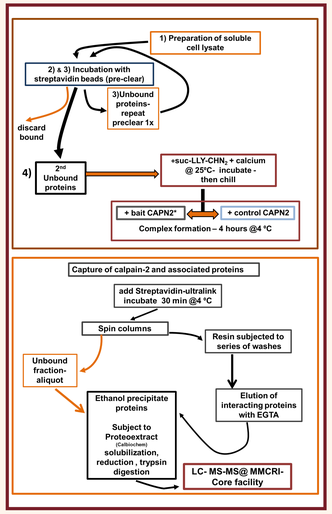
A. Calpain2— inactive in the absence of calcium (PyMol cartoon based on pdb 1DFO- Hosfield et al 1999, EMBO J 18,6880)



B. Calpain2 complex with CAST-domain-IV

illustrates the substantial conformational change of calpain2 induced by Ca^{2+} binding and can be reversed by chelation of Ca^{2+} with EGTA . (Pymol cartoon based on pdb 3BOW– Hanna et al 2008, Nature 456;409)





AFFINITY CAPTURE procedure

- Pre-clear removes endogenously biotinylated proteins
- Irreversibly inhibit endogenous calpains with suc-LLY-CHN_2
- Calpain2* added to ~250 nM
- Prey proteins at 70-100 fold excess by total µg
- $[Ca^{2+}]$ is gradually decreased during washes prior to elution with EGTA

Table 1: Number of proteins captured and identified by ProteinPilot™ analysis of MS/MS data files for each experiment.

NOTE: for BAEC2 and HUVEC-1 SCIEX-5600-Triple TOF increased depth of coverage.

Table 1	Eluted from bait	control	unbound
BAEC-1	23	7	120
BAEC-2	63	41	760
HUVEC-1	146	84	1300

Dataset-BAEC-1 Table 2

Unused	total	%coverage		# peptides 95%
31.27	31.27	42.3	sp|P20811|ICAL_BOVIN	16
			not detected-unbound	
			not detected-control	
11	11	49.1	sp|P46193|ANXA1_BOVIN	5
6.2	6.2	26.3	unbound	3
2	2	21.4	unbound	1
			not detected-control	
9.63	9.63	31.9	sp|P04272|ANXA2_BOVIN	5
27.72	27.72	62.2	unbound	15
21.06	21.06	49.9	unbound	11
			not detected-control	
1.68	1.68	37.3	sp|P13214|ANXA4_BOVIN	1
			not detected-unbound	
			not detected-control	
4.38	4.38	38.5	sp|P79134|ANXA6_BOVIN	2
			not detected-unbound	
			not detected-control	
2.17	2.17	14.3	sp|P36225|MAP4_BOVIN	1
2	2	16	unbound	1
2	2	23	unbound	1
			not detected-control	
2.37	2.37	26.6	sp|P48616|VIME_BOVIN	2
10	10	31.8	unbound	5
7.66	7.66	30.9	unbound	4
			not detected-control	
14.52	14.52	52.8	sp|Q9XSJ4|ENOA_BOVIN	10
35.88	35.88	82.7	unbound	20
24.69	24.69	62.2	unbound	15
			not detected-control	

Dataset– BAEC-1: SCIEX QStar

Table 2

- ♦ Calpastatin (ICAL) (CAST) -highest score
- ♦ Annexins (ANXA) 1,2,4 and 6 recovered from calpain2—not detected in control. ANXA1 was confirmed as a substrate. (see Figure below)
- ♦ Microtubule associated protein 4 (MAP4), Vimentin (VIME) and EnolaseA (ENOA)

HUVEC-1 and BAEC-2: SCIEX-5600-Triple TOF Tables 3,4 & 5

Table 3 - CAST & CAPN

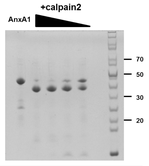
- ♦ CAST captured (3 for 3)
- ♦ Capture of CAPN2, CAPN1 and CAPNS1—may result from their interaction with CAST—or indicate multimeric association.

Table 4 - Annexins

- ♦ ANXA1,2,4,& 6 captured (3 for 3); however difference from control was inconsistent

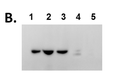
Table 5 - Selected other proteins

Confirmation of ANXA1 cleavage by calpain2



EXPERIMENT

ANXA1 (human recombinant with N-terminal His6, 125 µM) **was incubated with calpain2** at 0.25, 0.125, 0.05 or 0.025µM for 15 min, 22°C **prior to SDS treatment.**



Panel A: Stained gel of undigested ANXA1 (3 µg) **and calpain treated samples. Panel B: Western blot probed with HRP-conjugated anti-His antibody to detect the N-terminus of ANXA1. ANXA1 incubated** (15 min) **with Ca^{2+}** (lanes: 1,4 = 0.5 µg, 2 = 1 µg, 5= 0.3µg) and **calpain2** (lanes 4,5) **or with EGTA** (lane 3= 0.5 µg). Loss of the N terminus is consistent with cleavage site defined by Wang and Creutz (1994, Biochemistry 33:275) With thanks to Dr. K. Lauber for gift of the ANXA1 plasmid described in Blume, K.E., et al.2012, J. Immunol. 188: 135.

Table 3

	Unused	total	%coverage		# peptides 95%		Unused	total	%coverage		# peptides 95%
**	149.1	149.1	81.5	sp|P20810|ICAL_HUMAN	107		60.45	60.45	44.7	sp|P20811|ICAL_BOVIN	44
				not detected-unbound						not detected –unbound	
				not detected-control						not detected-control	
*	43.94	43.94	40	sp|P17655|CAN2_HUMAN	25		49.02	49.1	54.6	sp|Q27971|CAN2_BOVIN	40
	46.33	46.34	37.4	unbound	28		37.48	37.49	34.9	unbound	24
	10.17	10.18	9.4	control	6		27.24	27.24	15.3	control	14
*	31.65	31.75	23.7	sp|P07384|CAN1_HUMAN	16		7.16	7.19	7.5	sp|Q27970|CAN1_BOVIN	4
	9.93	10.12	14.7	unbound	6					not detected-unbound	
				not detected-control						not detected-control	
*	12.02	12.02	46.6	sp|P04632|CPNS1_HUMAN	7		5.64	5.74	24.7	sp|P13135|CPNS1_BOVIN	6
	3.15	3.28	28	unbound	2		4.1	4.13	26.6	unbound	2
				not detected-control			4.2	4.21	16.4	control	4

Table 4 Annexins from HUVEC-1 and BAEC-2

	Unused	total	%coverage		# peptides 95%
**	31.99	31.99	54.6	sp|P04083|ANXA1_HUMAN	16
	53.79	53.8	66.8	unbound	27
	22.23	22.23	43.4	control	11
**	46.04	46.04	65.2	sp|P07355|ANXA2_HUMAN	24
	66.9	66.97	66.1	unbound	33
	21.02	21.03	37.8	control	11
	6.8	6.85	13.9	sp|P12429|ANXA3_HUMAN	3
	10.86	11.16	30.7	unbound	7
	2.01	2.01	5	control	1
**	6.44	6.5	14.4	sp|P09525|ANXA4_HUMAN	3
				not detected-unbound	
	1.15	1.2	0	control	0
	5.78	5.87	17.8	sp|P08758|ANXA5_HUMAN	3
	29.6	29.66	59.1	unbound	13
	2.09	2.09	3.8	control	1
**	52.23	52.23	43.2	sp|P08133|ANXA6_HUMAN	25
	40.31	40.44	38	unbound	18
	20.98	21.01	20.4	control	9

Unused	total	%coverage		# peptides 95%
50.33	50.33	67.9	sp|P46193|ANXA1_BOVIN	29
16.16	16.23	35.6	unbound	8
64.18	64.23	75.7	control	37
109.65	109.65	77.9	sp|P04272|ANXA2_BOVIN	62
89.29	89.38	82.6	unbound	45
116.06	116.64	76.4	control	67
22.17	22.24	46.4	sp|Q3SWX7|ANXA3_BOVIN	14
			not detected-unbound	
31.53	31.53	51.4	control	17
20.02	20.06	37.6	sp|P13214|ANXA4_BOVIN	10
			not detected-unbound	
35.16	35.16	58.6	control	19
48.58	48.76	64.8	sp|P81287|ANXA5_BOVIN	26
			not detected-unbound	
59.23	59.23	78.2	control	33
94.16	94.55	62.9	sp|P79134|ANXA6_BOVIN	51
7.19	7.43	8.5	unbound	4
117.02	117.02	69.4	control	61

Table 5 HUVEC

	88.8	88.8	36.5	sp|Q9NYU2|UGGG1_HUMAN	52
	12.6	12.8	7	unbound	6
	2.2	2.21	1.5	control	1
**	15.9	16	10	sp|P27816|MAP4_HUMAN	8
	22.5	22.6	16.5	unbound	11
				not detected-control	
**	62.5	62.5	67.4	sp|P08670|VIME_HUMAN	34
	66.8	66.9	69.5	unbound	35
	4.02	4.03	4.7	control	2
	3.66	3.81	1.9	sp|P21333|FLNA_HUMAN	2
	163	171	48.3	unbound	1
	1.85	1.92	1.6	control	1
	4.05	4.05	6.1	sp|P26038|MOES_HUMAN	2
	75.8	75.8	61.2	unbound	37
				not detected-control	
	5.57	5.66	3.8	sp|P18206|VINC_HUMAN	3
	65.9	66	44.2	unbound	35
				not detected-control	
	6.87	6.9	8.1	sp|P05556|ITB1_HUMAN	4
	31.8	32	28.3	unbound	19
	1.77	1.8	2.1	control	1
	4.54	4.63	2.1	sp|Q13813|SPTN1_HUMAN	3
	20.2	20.4	5.8	unbound	9
				not detected-control	
*	18.4	18.4	35.3	sp|P06733|ENOA_HUMAN	20
	80.7	80.8	82.5	unbound	55
	6.56	6.56	19.6	control	15
	6.83	6.88	13.3	sp|P62258|1433E_HUMAN	3
	32.5	32.5	74.1	unbound	17
	1.19	1.26	7.8	control	1

Shared with BAEC-2

26.1	26.28	25	sp|P36225|MAP4_BOVIN	15
19.42	19.5	16.1	unbound	11
			not detected-control	
95.73	95.73	80.9	sp|P48616|VIME_BOVIN	56
70.6	70.63	75.1	unbound	36
81.9	81.9	77.7	control	51

- ♦ UGGG1 = UDP-glucose:glycoprotein glucosyltransferase – UGGT1
- ♦ MAP4 and VIME as above for BAEC-1
- ♦ FLNA =filamin A
- ♦ MOES = moesin
- ♦ VINC= vinculin
- ♦ ITB1 = integrin β1
- ♦ SPTN1 =non erythroid spectrin
- ♦ 14-3-3E - experimental evidence for one BioGRID listed interactor

Selected candidates

♦ **MAP4:** The less studied, more widely expressed, member of the tau/MAP2 family.

♦ Tau and MAP2 are substrates for calpain but structurally very distinct from MAP4.

♦ Significant portions of the protein are intrinsically disordered and thus are predicted to allow varied binding interactions with multiple binding partners beyond a role in binding with, and stabilizing, microtubules.

♦ siRNA evidence suggests a role in retroviral infection; MAP4 is implicated in positioning the mitotic spindle.

♦ **ANXA1:** Roles in cell migration, plasma membrane repair, several types of cancer, trafficking of TRPV6

♦ Differentiated functions are documented for the intact protein, the N-terminal peptide (2-26) and 33kDa truncated protein.

♦ N-peptides: 4-26 is found in the pancreatic cancer secretome; 2-26 functions as a ligand for the formyl peptide receptor.

♦ When and where is it a target for calpain2?

♦ Challenges: calcium-lipid binding alters the ANXA1 conformation to increase susceptibility to proteolysis and similar cleavage is catalyzed by other proteases.

♦ **UGGG1:** Second strongest candidate from HUVEC lysate is an ER lumenal protein with a key role for protein quality control (PQC). It functions by re-glucosylating non-native proteins.

♦ Precedence exists for a cytosolic ATPase-p97 to cooperate with UGGG1; this prevented transport of proteins with intermembrane defects to the Golgi.

♦ Proteolytic enzymes participate in PQC. Does a fraction of calpain2 participate in an ER quality control mechanism?

Proof of concept

- ♦ Uniquely biotinylated calpain2 functions as 'bait' to affinity capture Ca^{2+} dependent binding partners from lysates of endothelial cells.
- ♦ Chelation of Ca^{2+} with EGTA induced release of the captured proteins to improve analysis.
- ♦ Capture of the calpain inhibitor calpastatin, and known substrates– such as vimentin, filaminA, spectrin, and confirmation of annexinA1 as a substrate, validates success of this biochemical strategy.
- ♦ These data better represent calpain2 specific interactors when compared with those found using high-throughput, gene product screens found in existing database resources.

What's next?

Analyze a group of validated Ca^{2+} dependent interactors to

⇒ discover key insights into calpain2's selection of substrates;
⇒ generate a testable hypothesis for substrate selection;
⇒provide better data for furthering development of cleavage site prediction tools.

Perform affinity capture in the absence of Ca^{2+}

⇒ Does calpain2 have functional importance when it is not catalytically competent? Binding partners are expected to reveal insights into calpain2 localization and/or possible non-proteolytic roles.

Concerns and challenges

- ♦ **'Piggybacking' proteins:** Are calpains captured because they bind CAST? or does this suggest oligomers form? Further testing is required to distinguish direct from indirect capture.
- ♦ **Variations within control:** Does never frozen or frozen-thawed bait matter? Were samples cross contaminated (i.e. possible experimental error?) Does incubation time for sample precipitation matter? Others???

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