FUNCTIONAL CHARACTERIZATION OF HUMAN CALCIUM-DEPENDENT CYSTEINE PROTEASE, CALPAIN 1 (μ-CALPAIN), USING RNA INTERFERENCE TECHNOLOGY

By

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ABSTRACT

Calpains are calcium-dependent cysteine proteases consisting of two major isoforms, m-calpain and μ-calpain, which have been implicated in aspects of carcinogenesis. However, the individual physiological function and biochemical mechanism for these two isoforms involved in cell motility are not clear. In the present study, *in vitro* synthesized human μ-calpain specific siRNAs were able to significantly knockdown μ-calpain expression. Accordingly, stable MCF-7 cell lines expressing the functional μ-calpain siRNAs were established. Casein zymography revealed that the enzymatic activity of μ-calpain was reduced in comparison with that of the control cell line. The cells with an 80 % knockdown of μ-calpain expression displayed reduced cell mobility and significant morphology changes. In addition, knockdown of μ-calpain decreased the proteolytic products of filamin and talin, suggesting that their proteolysis could be one of the key mechanisms by which μ-calpain regulates cell migration. Thus siRNAs can function as calpain isoform-specific inhibitors for the study of isoform functions and related intracellular signaling in carcinogenesis, and may have potential for therapeutic use.

RÉSUMÉ

Les calpaines font partie de la famille des protéases à cystéine. Elles sont décrites comme des protéases neutres à activités calcium dépendantes. Au sein des calpaines, on distingue différents isoformes dont la μ -calpaine et la m-calpaine. Ces dernières sont connus dans divers aspects de la cancérogenèse. Cependant, la fonction physiologique et le mécanisme biochimique de ces deux isoformes, aussi impliqués dans la motilité des cellules, demeurent ambiguës. Dans la présente étude, des siRNAs, synthétisés in vitro, spécifiques de μ-calpaine humaine ont supprimé de manière significative l'expression de μ-calpaine. Ensuite, une lignée stable de cellules MCF-7 exprimant des siRNAs fonctionnels de μ-calpaine a été établie. La zymographie de la caséine de cette lignée stable démontra que l'activité enzymatique de leur μ-calpaine a été réduite en comparaison de celle du témoin négatif. De plus, les cellules démontrant une suppression de 80 % de l'expression de leur μ-calpaine démontraient une mobilité cellulaire réduite et des changements morphologiques importants. Enfin, la suppression de l'expression de μ-calpaine a diminué les produits protéolytiques de filamine et de taline, suggérant que leur protéolyse pourrait être l'un des principaux mécanismes par lesquels la μ-calpaine régulerait la migration de cellules. Ainsi, il a été démontré que les siRNAs exerce un rôle d'inhibiteurs spécifiques des isoformes de calpaine. Ils sont utiles pour étudier la physiologie cellulaire et certains processus de signalisation intracellulaire impliqués dans la cancérogenèse. Leur potentiel dans le développement d'une thérapie est prometteur.

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LIST OF ABBREVIATIONS

aa	amino acid (s)
BLAST	basic local Alignment search tool
BSA	bovine serum albumin
dsRNA	double-stranded RNA
EC	enzyme commission
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid (editic acid)
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
ERs	estrogen receptors
et al.	[*et alia*] and others
FAK	focal adhesion kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase gene
IS	insertion sequence
IUBMB	nomenclature committee of international union of biochemistry and molecular biology
LhRNA	long hairpin RNA
MAP2	microtubule-associated protein 2
MARCKS	myristoylated alanine-rich C kinase substrate
MLCK	myosin light chain kinase
MMPs	matrix metalloproteases
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
NS	N-terminal Extension
Oligo	oligodeoxyribonucleotide
^{32}P	radiolabelled phosphorus
PBH	PalB Homologous
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PMSF	phenyl methane sulfonyl fluoride
RISC	RNA induced silencing complex
RNAi	RNA interference
rpm	revolutions per minute
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
Tween-20	polyoxyethlene - sorbitan monolaurate

I. Rational and Objectives

Calpains are a family of cytosolic cysteine proteases, which are activated by a number of processes, including increased intracellular Ca^{2+}, phosphorylation and proteolysis. Two well-characterized human calpain family members, μ-calpain and m-calpain, play important roles in various biological processes, including integrin-mediated cell migration, cytoskeletal remodeling, cell differentiation and apoptosis. So far, due to the lack of isoform-specific inhibitors, there is no clear evidence to prove the distinct functions of each calpain experimentally. However, RNAi provides a useful tool to study gene functions through knockdown of target RNA expression. It is necessary to test the concept that RNAi can be used to study the functions of proteases in an isoform-specific manner, and also might be useful for the development of therapeutic approaches through inhibiting the expression of a disease-related target gene.

Two major calpain isoforms, μ-calpain and m-calpain, have been demonstrated to regulate cell motility by using conventional protease inhibitors that have no isoform specifity. Fibroblasts from knockout mice with both μ-calpain and m-calpain deficiencies also display impaired ability in cell migration without distinguishing the individual roles of each calpain. Taking advantage of the RNAi technology developed in recent years, I will exploit the particular function of the human μ-calpain in cell migration. My main efforts are to design isoform specific siRNAs based on rational siRNA design, to prove the feasibility of application of RNAi in studying human μ-calpain experimentally, instead of using protease inhibitors, and to demonstrate that μ-calpain alone controls cell motility, possibly by proteolysis of focal adhesion molecules. Since improper expression of μ-calpain is associated with many human diseases and/or disorders, the research has a great potential not only to develop methodology for basic research, but also to provide therapeutic approaches for medical practice. This basic working procedure may be applied to study the functions of other calpain members and essentially any protein.

II. Literature Review

Proteases are all those enzymes that catalyze the cleavage of peptide bonds in proteins. Consequently, they play very important biological roles by regulating the function of proteins. They are essential to various physiological processes such as inflammation, infection, fertilization, allergic reactions, cell growth and death, blood clotting, tumor growth and bone remodeling (Barrett, 1994). Molecules that inhibit the action of proteases are important in the treatment of human diseases.

Proteases make up almost 2% of human gene products. They are classified into four groups: serine proteases, cysteine (thiol) proteases, aspartic proteases and metalloproteases. Recently, the calcium-dependent cysteine protease, calpain, has been implicated in a wide range of cellular functions including apoptosis, proliferation and cell migration. In particular, calpain-regulated cell motility is a central process involved in many pathophysiological events including tumor invasion, embryonic development, and wound healing (Goll et al., 2003). Two ubiquitious calpain isoforms, μ-calpain and m-calpain have been implicated in adhesion and migration phenomena. However, defining a clear physiological function for calpain with respect to individual isoforms *in vivo* has remained elusive. In order to analyze isoform-related physiological functions of calpain, inhibitors specific for calpain, especially those specific for each calpain homologue, are crucial. So far, various calpain inhibitors have been developed, but none of them is selective for only one of the calpain isoforms. In contrast, RNA interference (RNAi) has the potential to allow a sequence-specific destruction of target RNA. It has been widely used for gene function manipulation and may have a potential for drug development.

In this section of literature review, I will give an introduction of each family of proteases followed by a focus on the calcium-dependent cysteine protease, calpain, regarding its involvement in cell migration. RNA interference methodology is also discussed to help us better understand this newly emerged biotechnology used in this study.

2.1 General Aspects of Proteases

2.1.1 Nomenclature and Classification

At the present time, around 600 distinct proteases have been recognized, including over 200 that are expressed in mammals. New ones are being discovered continuously. The high and accelerating rate of discovery and research on this area demands a clear system of naming and classification. This need can be achieved by the combined application of two partially overlapping systems, the MEROPS system of protease clans and families (http://merops.sanger.ac.uk/) and the Enzyme Commission (EC) recommendations on enzyme nomenclature created by the Nomenclature Committee of International Union of Biochemistry and Molecular Biology (IUBMB) (http://www.chem.qmul.ac.uk/iubmb/).

2.1.1.1 Nomenclature

Proteolytic enzymes or proteases are enzymes of class 3, the hydrolases, and subclass 3.4, the peptide hydrolases or peptidases. IUBMB recommends to use the term *peptidase* for the subset of peptide bond hydrolases (E.C 3.4.), referring to all enzymes that cleave peptide bonds. The widely used term *protease* is synonymous with *peptidase*. *Peptidases* comprise two groups of enzymes: the endopeptidases (EC 3.4.21-99) and the exopeptidases (EC 3.4.11-19). The term *proteinase* is also used as a synonym for *endopeptidase* (Beynobm and Bond, 1989) (http://www.chem.qmul.ac.uk/ iubmb/enzyme /EC3/4/).

Additionally, the term *family* refers to a set of homologous peptidases. The homology is shown by a significant similarity in amino acid sequence either to the type example of the family, or to another protein that has already been shown to be homologous to the type example, and thus a member of the family. The term *clan* indicates all the modern-day peptidases that have arisen from a single evolutionary origin of peptidases. It represents one or more families that show evidence of their evolutionary relationship by their similar tertiary structures, or by the order of catalytic-site residues in the polypeptide chain and often by common sequence motifs around the catalytic residues (Rawlings et al., 2004).

2.1.1.2 Classification

Currently, proteases are classified on the basis of three major criteria: (i) type of catalytic reaction; (ii) catalytic mechanism; (iii) evolutionary relationship with reference to structure (Barrett, 1994).

(i) Classification by Type of Catalytic Reaction

Based on the site at which they break the peptide chains, proteases can generally be grouped into two categories: 1) exopeptidases act only near the ends of polypeptide chains at the N or C terminus, or 2) endopeptidases cleave preferentially in the inner regions of peptide chains. Depending on their sites of action, either near the ends of polypeptide chains at the N or C terminus, and specifity of action, the exopeptidases can be subdivided into Aminopeptidases (EC 3.4.11), Dipeptidases (EC 3.4.13), Dipeptidyl- and Tripeptidyl- peptidases (EC 3.4.14), Peptidyl-dipeptidases (EC 3.3.15), and, Serine-type carboxypeptidases (EC 3.4.16), Metallocarboxypeptidases (EC 3.4.17), Cysteine-type carboxypeptidases (EC 3.4.18), and Omega peptidases (EC 3.4.19).

(ii) Classification by Catalytic Mechanism

Endopeptidases can be ordered further, according to the reactive groups responsible for their activity, into serine-, cysteine-, and aspartic- proteases and metallo proteases. Serine proteases have a serine residue involved in the active center, the aspartic proteases have two aspartic acids in the catalytic center, while cysteine-type proteases have a cysteine residue and metallo-proteases use a metal ion in the catalytic mechanism.

In addition to these four mechanistic classes, there is a section of the enzyme nomenclature that is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified and thus the possibility remains that novel types of proteases exist.

(iii) Classification by Evolutionary Relationship with Reference to Structure

This third classification is an extended system based on the similarities in amino acid sequences of proteases proposed by Rawlings and Barrett, who developed MEROPS

database of protease clans and families in 1996. There has been a long and strong argument for using the wealth of date on the amino acid sequences and three-dimensional structures of proteases in their classification. The fact is that simple and automated searches of the sequence databases readily returns lists of similar proteases, even in the absence of an ideal nomenclature. The structural similarities within a family of proteases commonly reflect a close evolutionary relationship; important similarities in catalytic mechanism and a wealth of biological meaning can be extracted from this. Detailed data of this classification can be acquired through the MEROPS website (http://merops.sanger.ac.uk).

2.1.2 General Introduction of Protease Inhibitors

In order to understand proteolytic activity in biological processes, synthetic and natural protease inhibitors are generated for characterization and regulation of protease activity *in vitro* and *in vivo*. Protease inhibitors are as diversified as the proteases themselves. They come in many different forms and can be grouped into two classes. The first group is active-site-specific, low-molecular-weight inhibitors that irreversibly modify an amino acid residue of the protease active site. For example, the serine proteases are inactivated by Phenyl Methane Sulfonyl Fluoride (PMSF), which reacts with the active serine, and by the chloromethylketone derivatives that react with the histidine of the catalytic triad. The second group is natural protein protease inhibitors that behave as tight-binding reversible or pseudo-irreversible inhibitors of proteases by preventing substrate access to the active site through steric hindrance (Neurath, 1989).

2.1.3 Introduction of Each Class of Proteases

2.1.3.1 Serine Proteases

Serine proteases are the enzymes that require a serine residue for their catalytic activity. On the basis of three-dimension structure, more than 20 families of serine proteases have been classified and further subdivided into six clans with common ancestors. The primary structures of proteases in the four clans, chymotrypsin, subtilisin, carboxypeptidase C, and Escherichia D-Ala-D-Ala peptidase A are totally distinct, suggesting that at least four separate evolutionary origins for serine proteases exit. The

first three clans have a similar catalytic mechanism as a result of containing a common Ser-His-Asp catalytic triad, whereas clans Escherichia D-Ala-D-Ala peptidase A, repressor LexA and CI_P endopeptidase appear to have distinctive mechanisms without the classical Ser-His-Asp triad. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11 (Rawlings and Barrett, 1994).

Serine proteases are numerous and widespread among viruses, bacteria, and eukaryotes. The most commonly studied serine proteases are chymotrypsin, trypsin, elastase, and subtilisin. While cascades of serine protease activations control blood clotting and complement, other such proteases are involved in signalling pathways, in enzyme activations and in degradative functions in different cellular or extracellular compartments (Putnam et al., 2003). Thrombin is one of the best-studied trypsin-like serine proteases from a medical point of view because of its vital role in hemostasis and the induction of platelet aggregation and secretion. While thrombin inhibitors are used as anticoagulants, their action on platelet function disposes individuals to excessive bleeding suggesting that other inhibitors with specificity but without side effects are desirable. Another trypsin-like serine protease, Tryptase, is the main secretory product that is released from human mast cell upon activation. This protease has been demonstrated to act as an inflammatory mediator in different inflammatory and allergic disorders such as conjuctivitis, rhinitis, and especially asthma. Several synthetic tryptase inhibors, for instance APC366 and 1,2-Benzisothiazol-3-one 1,1-dioxide, are currently in use for clinical trials and are showing some cross-reaction with other serine proteases such as trypsin and elastase (Leung et al., 2000).

2.1.3.2 Aspartic Proteases

Aspartic proteases are the endopeptidases that depend on aspartic acid residues for their catalytic activity. They have been classified into three families, namely, pepsin, retropepsin, and enzymes from pararetroviruses. Most of the aspartic proteases belong to the pepsin family. The first pepsin family comprises digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as renin, and certain fungal proteases (*e.g.* penicillopepsin, rhizopuspepsin, endothiapepsin). The second family includes viral proteases such as the protease from the AIDS virus (HIV).

Aspartic proteases are characterized by an Asp-Thr-Gly motif in their active site and by an acid-base catalysis as their mechanisms of action, showing maximal activity at low pH (pH 3 to 4) (Rawlings and Barrett, 1995b).

Among various aspartic proteases, the human immunodeficiency virus (HIV-1 protease) has been excessively studied as an attractive drug target owing to its important role in the replicative cycle of HIV (Rodriguez-Barrios and Gago, 2004). Cathepsin D, another aspartic acid protease, functions as "house-keeping" protease for degradation of cellular or phagocytosed proteins for reprocessing. Consequently, it may be involved in a variety of diseases, including cancer and Alzheimer's disease. In order to further define the role of cathepsin D in human disorders and possibly lead to therapeutic agents, a series of effective nonpeptidic inhibitors have been generated. However, these inhibitors were not optimized for specificity with respect to other aspartic proteases (Leung et al., 2000).

2.1.3.3 Metalloproteases

The metalloproteases may be one of the older classes of proteases and are found in bacteria, fungi as well as in higher organisms. 30 families of metalloproteases differ widely in their sequences and their structures but the majority of enzymes contain a metal atom, usually a zinc atom which is catalytically active. On the basis of sequence comparison, many metalloproteases are found to contain the sequence His-Glu-Xaa-Xaa-His (HEXXH), which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid or a histidine (Rawlings and Barrett, 1995a).

One important metalloprotease is the zinc metalloprotease tumor necrosis factor-α convertase (TACE) that cleaves a membrane-bound protein (pro-TNF- α) and releases a 17-kDa proinflammatory and immunomodulatory cytokine, tumor necrosis factor (TNF-α) to the circulation. TNF-α plays an important signaling role in inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis, and Crohn' disease which make TACE an attractive target for therapeutical treatment (Ghezzi and Cerami, 2004). Matrix Metalloproteases (MMPs) are a family of structurally related zinc metalloproteases that degrade and remodel structural proteins. Studies implicate their crucial roles in the growth and spread of malignant tumors and development of chronic diseases such as

fibrosis, arthritis, multiple sclerosis and other inflammatory conditions. Currently, seventeen MMP isoforms with significant sequence homology have been discovered. By exploiting differences in the active sites based on the x-ray crystal structures, inhibitors for MMPs have been developed that are more potent and specific for particular isoforms of the MMP family (Bode and Maskos, 2003). However, lack of selectivity is still an unresolved issue for most MMP inhibitors.

2.1.3.4 Cysteine Proteases

Cysteine proteases are present in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized, including the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calcium-dependent calpains as well as several parasitic proteases. The activity of cysteine proteases depends on a His-Cys-Asp catalytic triad that is considered analogous to the Ser-His-Asp arrangement found in serine proteases. Based on their side chain specificity, cysteine proteases are broadly divided into four clusters: (i) trypsin-like, (ii) papain-like with preference to cleave at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Generally, cysteine proteases need neutral pH optima for activation and are active only in the presence of reducing agents such as HCN or cysteine (Turk et al., 1997).

Recently, calpain, the calcium-dependent cysteine protease, has received enormous research interest. Today, 14 calpain family members in humans have been recognized and implicated in various pathological processes such as neurodegenerative and neurological disorders, muscular dystrophy, cataract formation, type 2 diabetes mellitus, inflammatory and tumor metastasis. Two isoforms, μ-calpain and m-calpain, contribute to most of these disorders through their involvement in cell spreading and migration, cell cycle control, and apoptosis (Perrin and Huttenlocher, 2002). However, their precise physiological functions are still not clear.

For a long time, the study of the participation of calpain in physiopathological processes at the cellular level has been hampered by the lack of cell-permeable calpain-specific inhibitors able to discriminate between the Ca^{2+}-activated enzyme and other cysteine proteases. Inhibitors that can specifically target each calpain homologue are even more difficult to achieve. Hence, the results of calpain functional assays that rely on the

effects of a "calpain inhibitor" must be interpreted cautiously. While a combination of inhibitors with different known selectivities are used to distinguish calpain from other proteases, RNA interference (RNAi) for sequence-specific degradation of targeted mRNA appears to be a new approach for functional study of calpain isoforms.

2.2 Calpain–Ca^{2+}-Dependent Cysteine Protease

Calpain is a cytoplasmic nonlysosomal cysteine protease that requires calcium ions for activity (Perrin and Huttenlocher, 2002). Molecular biological studies have shown that calpain constitutes a superfamily, which exists ubiquitously in organisms ranging from humans to microorganisms. Calpain has attracted much attention because of the recent discovery of correlations between calpain and human diseases, together with elucidation of its three-dimensional structure and calcium-induced activation mechanisms. In the following section, the current status of mammalian calpain studies is summarized with emphasis on involvement of calpain in cell migration.

2.2.1 Classification of Calpain Family

Calpains [EC 3.4.22.17, clan CA family C2 (Sorimachi and Suzuki, 1998)], the Ca^{2+}-activated neural proteases (CANP), were originally identified as a unique class of calcium-dependent proteolytic enzymes in rat brain and skeletal muscle 30 years ago by Guroff and Huston (Guroff, 1964; Huston and Krebs, 1968). These proteases were later named calpains to reflect their calcium dependency and homology with the protease domain of the papain family of cysteine proteases. Further studies demonstrate that they form a large gene family that are widely expressed with both non-specific and tissue specific isoforms in higher organisms. Homologues of the calpain catalytic subunits are also present in lower organisms including nematodes, plants, fungi, yeast, flies, and bacteria (Yasuko et al., 1999). This conservation of the calpain protease domain suggests that the calpain proteolytic system confers important functions for many living organisms.

At present, the number of mammalian calpain members has grown to 14 at last count. They can be classified based on the mode of expression or tissue distribution. Calpains are usually expressed ubiquitously in the cytosol, but some are expressed in a

tissue-specific or preferred manner. Calpains 1, 2, 4, 5, 7, 10, and 13 are regarded as ubiquitous calpains, whereas calpains 3, 6, 8, 9, 11, and 12 are tissue-specific calpains. For example, calpain 3 is skeletal muscle specific and calpain 9 is digestive track specific (Huang and Wang, 2001). The gene products of all 14 members are summarized in Table 1.

Table 1. Mammalian calpain genes (adopted from http://ag.arizona.edu/calpains/nomenclature.html).

Calpain protein	Calpain gene	Other names	Tissue distribution	Amino acid residues	Species	Chromosome number	Genebank accession number
Calpain 1	*capn1*	μ-Calpain, CAPN1	Ubiquitous	714	Human	11q13	X04366
					Mouse	19	NM_007600
					Rat	NA	NM_019152
Calpain 2	*capn2*	m-Calpain, CAPN2	Ubiquitous	700	Human	1	NM_001748
					Mouse	NA	NM_009794
					Rat	NA	NM_017116
Calpain 3	*capn3*	nCL-1, p94, Lp82,	Skeletal muscle,	821	Human	15q15	X85030
		Lp85, Rt88	lens, retina		Mouse	2	NM_007601 AF091998
					Rat	NA	NM_017117 U96367
Small subunit 1	*capn4*		Ubiquitous	268	Human	19q13	NM_001749
					Mouse	NA	NM_009795
					Rat	NA	RNU10861
Calpain 5	*capn5*	htra3, nCL-3	Ubiquitous (high in colon, small intestine and testis)	640	Human	11q14	NM_004055
					Mouse	7	NM_007602
					Rat	NA	NA
Calpain 6	*capn6*	CAPNX, Calpamodulin	Placenta	641	Human	Xq23	NM_014289
					Mouse	X	NM_007603
					Rat	NA	AF067793
Calpain 7	*capn7*	palBH	Ubiquitous	813	Human	3p24	AB028639
					Mouse	NA	NM_009796
					Rat	NA	NA
Calpain 8[a]	*ncl-2*, *capn8*	nCL-2	Stomach mucosa	703	Human	NA	AA_043093[c]
					Mouse	NA	NA
					Rat	NA	D14479-D14480
Calpain 9	*capn9*	nCL-4	Digestive track	690	Human	1	NM_006615
					Mouse	NA	U89513
					Rat	NA	U89514
Calpain 10	*capn10*, (*capn8*)[b]	CAPN10, CAPN8[b]	Ubiquitous	672	Human	2q37	AF089088, AF089090 -96
					Mouse	NA	NM_011796 AF089089
					Rat	NA	AF227909
Calpain 11	*capn11*		Testis	702	Human	6p12	AJ242832
					Mouse	NA	NA
					Rat	NA	NA
Calpain 12	*capn12*		Ubiquitous (high in hair follicle)	720	Human	NA	NA
					Mouse	7	AJ289241
					Rat	NA	NA
Calpain 13[a]	*capn13*[a]	Sol H	Ubiquitous	1086	Human	16p13	U85647
					Mouse	17	NM015830
					Rat	NA	NA
Small subunit 2	*capn14*[a]		NA	248	Human	16	AC026802
					Mouse	NA	AK009171
					Rat	NA	NA

[a]Proposed name. [b]Old name. [c]Partial sequences only.
μ-calpain = protein heterodimer of calpain 1 and calpain 4
m-calpain = protein heterodimer of calpain 2 and calpain 4

2.2.2 Properties and Structure of Calpain

Two well-characterized members of this family are ubiquitous mammalian calpains, μ-calpain and m-calpain. They were named according to their calcium sensitivity with μ-calpain requiring micromolar concentrations and m-calpain requiring millimolar levels of Ca^{2+} for half-maximal activation *in vitro*. Each calpain is a heterodimer composed of a large unique 80-kDa catalytic subunit (encoded by calpain 1 and calpain 2 for μ-calpain and m-calpain, respectively) and a small common 30-kDa regulatory subunit (encoded by calpain 4). Calpain activity is tightly regulated by its ubiquitously expressed endogenous inhibitor calpastatin.

Based on amino acid sequence comparisons, calpains were originally subdivided into six domains (I–VI). The crystal structure of m-calpain in the absence of Ca^{2+} further redefined the presence and organization of four distinct domains in the large subunit and two domains in the small subunit. As shown in Fig. 2.1, domain I is a 19-residuce N-terminal pro-domain region that is cleaved by intermolecular autolysis before or following activation. The protease domain II is further divided into subdomains IIa and IIb, with its substrate binding cleft in between. The catalytic triad residue Cys-105 is on IIa, wherease His-262 and Asn-286 are on IIb. In the absence of calcium, the distance between the catalytic Cys and His residues is too far to constitute a functional catalytic triad Cys105-His262-Asn286. This inactive conformation of domain II is also stabilized by interaction of domain I with domain VI on one side and interaction of IIb with domain III, especially in an acidic loop, on the other side. It appears that the calcium-induced conformational changes draw subdomain IIa and IIb together. Domain III is not homologous to any other known proteins and is structurally similar to a Ca^{2+} and phospholipid-binding C2 domain (Tompa et al., 2001). Domain V might be required for interaction with membrane phospholipids. Both domain IV and VI contain five sets of EF-hand Ca^{2+} -binding motifs. The first four EF-hands are similar to those found in calmodulim and can bind calcium. The extreme COOH-terminal fifth EF-hand motif in IV and VI cannot bind Ca^{2+} but interacts with each other to form a heterodimer

comprising a big catalytic subunit and a small regulatory subunit for μ-calpain and m-calpain (Strobl et al., 2000).

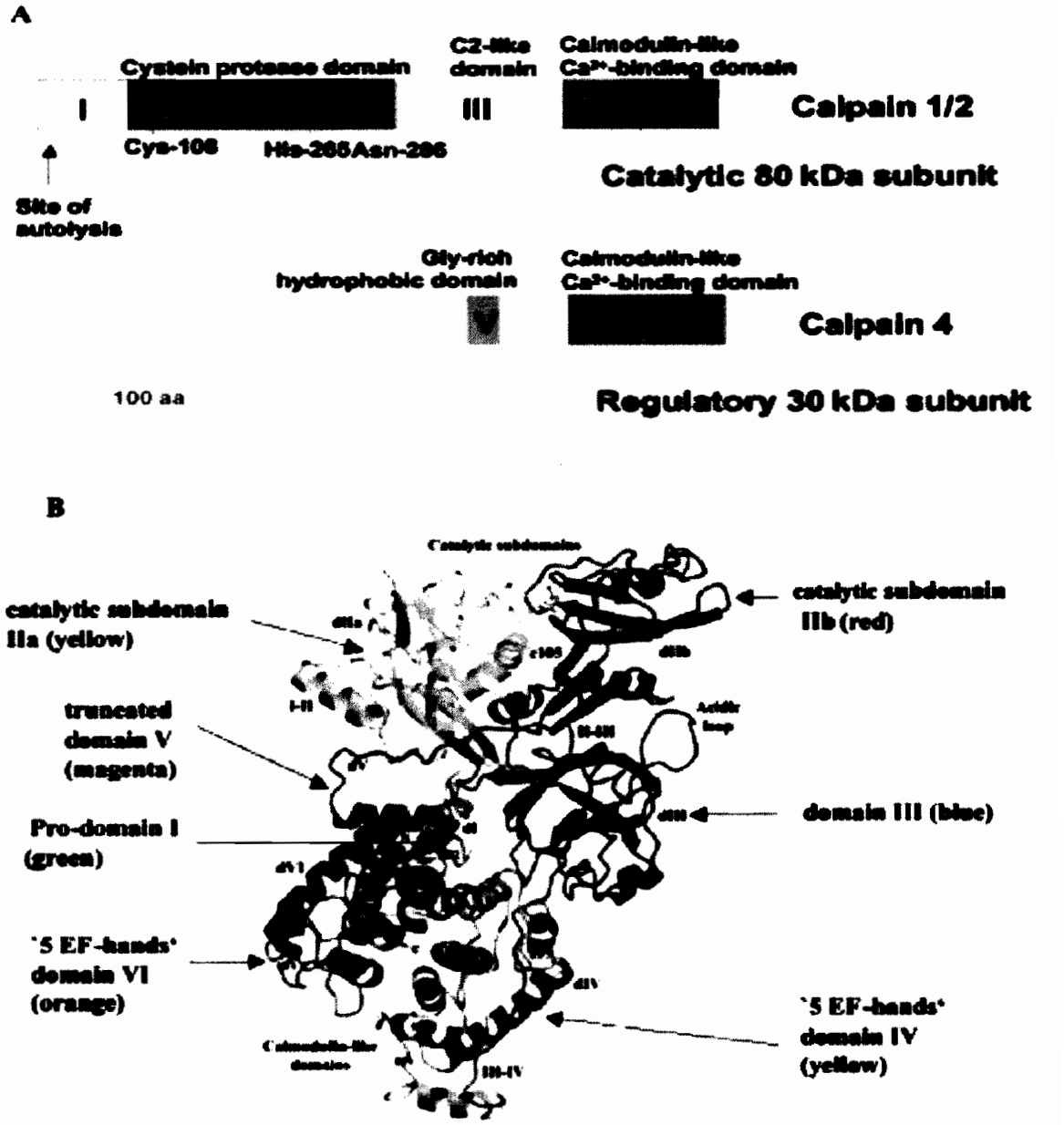


Fig. 2.1. Domain and 3-D structure of human μ/m-calpain. A. Domain structure of μ/m-calpain. Adopted from Carragher and Frame (2002). B. Three-dimensional crystal structure of non-activated human μ/m-calpain heterodimer in the absence of Ca^{2+}. The 80-kDa large subunit starts in the molecular center (green, dI), folds into the surface of the dIIa subdomain (gold, I→II linker), forms the papain-like left-side part of the catalytic domain dIIa (gold) and the right-side barrel-like subdomain dIIb (red), descends through the open II→III loop (red), builds domain dIII (blue), runs down (magenta,

III→IV), and forms the right-side `5 EF-hands'-containing domain dIV (yellow). The 30-kDa small subunit contains the truncated domain V (magenta) and the `5 EF-hands' domain VI (orange) (Strobl et al., 2000).

Besides μ-calpain and m-calpain, several more of the 14 human calpain isoforms are composed of two subunits and share the same domain structure as μ-calpain and m-calpain (Fig. 2.2). Other calpain homologues do not seem to form heterodimers, and some also contain different domains such as NS (N-terminal extension), IS (insertion sequence), and PBH (PalB homologous) and T (tra-3 specific) as additions or substitutions (Diaz et al., 2004; Futai et al., 2001; Mugita et al., 1997). Although each of the 14 mammalian calpains differs in the length of its N-terminal sequence, in regulatory domain structures and in the presence of EF-hands for Ca^{2+} binding, all of them contain the conserved active sites except calpain 6.

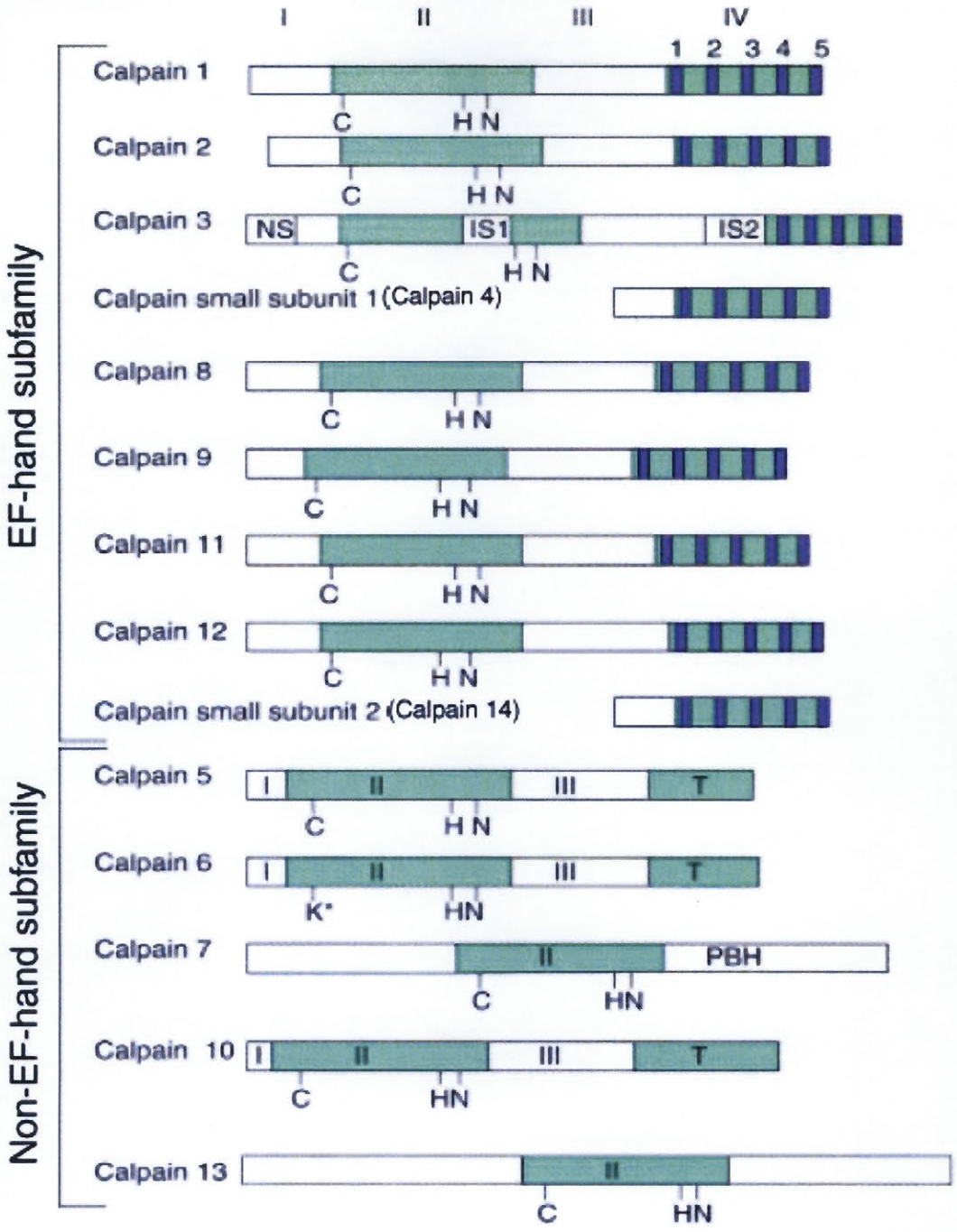


Fig. 2.2. Schematic structures of 14 mammalian calpains aligned based on the presence of EF-hands. C, H, N represent catalytic residues Cys, His and Asn residues. K* represents the unique nonfunctional Lys residue (instead of Cys) in calpain 6 (adopted from Huang and Wang, 2001).

2.2.3 Activation and Regulation of Calpain

The regulation of the general, cytoplasmic calpains is complex. Based on many *in vitro* findings, intricate, multiple and potentially alternative or complementary mechanisms of activation and regulation have been identified for ubiquitous, abundant calpains in the cytoplasm of the cells. These enzymes are capable of cleaving many intracellular signaling and structural proteins. It was presumed at first that calpains were activated by intracellular Ca^{2+} fluxes. The concentrations required for half-maximal activity of calapin (*ka*) measured *in vitro* usually range from 5-50 μM for μ-calpain, and 0.4-1mM for m-calpain (Goll et al., 2003). Nevertheless, both calpains seem to be activated at physiological Ca^{2+} concentrations of 50-500 nM, suggesting that more than Ca^{2+} alone is required for activity *in vivo* (Thompson et al., 2000). Therefore, several mechanisms have been proposed either to increase the Ca^{2+} sensitivity or to substitute for Ca^{2+} altogether. These include autolysis, phospholipid binding, release of calpain from its endogenous inhibitor calpastatin, binding of activator proteins, phosphorylation and regulation of its intracellular distribution *in vivo* (Perrin and Huttenlocher, 2002; Suzuki and Sorimachi, 1998).

Ca^{2+}-dependent calpain activation

Calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increases in the cellular Ca^{2+} level. At the membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Recent x-ray crystallography of Ca^{2+}-free m-calpain has revealed a Ca^{2+}-induced activation mechanism at the molecular level. It has been suggested that there are at least four different Ca^{2+}-binding sites in m-calpain, including a protease domain II, an acidic loop region in the C2-like domain III, and two calmodulin-like domains IV and VI (Moldoveanu et al., 2002). Various acidic-loop mutants of rat m-calpain suggest the particular importance of the loop in domain III in regulating Ca^{2+}-dependent enzyme activity (Alexa et al., 2004).

A proposed mechanism for activity regulation of m-calpain by Ca^{2+} comprises two stages. The first stage is the disruption of the electrostatic interaction between domains. Bindings of Ca^{2+} to domain III, IV and VI release domain I from VI and domain IIb from III, leading to dissociation of calpain large and small subunits, and most

importantly, allowing subdomain IIb to fuse with IIa forming a functional catalytic domain. The second stage is the rearrangement of the active site cleft between IIa and IIb caused by binding of two Ca^{2+} atoms to the protease domain II (one each to the IIa and IIb subdomains). Activation by the second stage occurs only after release of the constraints freed by the first stage (Reverter et al., 2001). In the case of calpains without small subunits (calpain 5,6,7,8,9,10,11,12,13) or without domain III (calpain 13) or IV (calpain 5,6,7,10,13), the first stage of activation might be bypassed and they could be activated directly by the second stage (Sorimachi and Suzuki, 1998). Hence, researchers surmize that the activation of calpain by calcium seems to be dependent on a conformational switch rather than proteolysis of a pro-peptide region that characterizes the activation of other cysteine proteases.

Release of calpain from its endogenous inhibitor calpastatin

Intracellularly, ubiquitous calpain is tightly regulated by calpastatin, a specific endogenous inhibitor. Calpastatin binds to the two calmodulin-like domains IV and VI of calpain and inactivates calpain through each of its four repetitive inhibitory domains of 140 residues. Near the center of each domain there is a conserved 12-amino acid sequence that is essential for inhibitory activity (Tompa et al., 2001). Since the presence of domains IV and VI are necessary for effective inhibition by calpastatin, it appears that calpastatin only functions on calpains associated with the small subunit, such as μ-calpain, m-calpain and calpain 9. Calpain 80 kDa large subunit homologues including autolyzed catalytic subunits of μ- and m- calpains thus escape from the endogenous inhibition. The translocation of calpain from the cytosol to the membrane during the activation process could be a way to segregate the enzyme away from the inhibitor (Goll et al., 2003).

However, the physiological relevance of the association between calpain and calpastatin remains controversial. Studies demonstrate that the Ca^{2+} concentration required for binding of calpastatin to calpain is much higher than the physiological level which is 50-500nM (Table 2). Therefore, it is uncertain whether calpastatin is actually bound to calpain *in vivo*. As well, the calcium requirement for calpastatin binding to calpain is less than that needed to initiate their proteolytic activity based on *in vitro*

experiments. So the simple release of calpain from calpastatin does not appear to be sufficient for calpain activation in living cells (Glading et al., 2002). Nevertheless, overexpression of this molecule can be employed to prevent calpain activity.

Calpastatin is a unique *in vivo* regulator of calpain, but other cellular proteins interacting with calpain, like Gas2, might regulate calpain activity (Benetti et al., 2001). Calpain might also be sensitized to Ca^{2+} by a specific activator protein that affects membrane localization and autolysis of the enzyme (Melloni et al., 1998). Nuclear DNA may be another positive effector promoting the effect of calpain on transcription factors and other nuclear proteins (Mellgren et al., 1993). However, the activation of calpain by these proteins *in vivo,* and the significance of these activators, are still unclear.

Table 2. Estimate of Ca^{2+} concentrations required for different properties of the calpains*.

Calpain Property	Autolyzed μ-calpain	μ-calpain	Autolyzed m-calpain	m-calpain
Proteolytic activity	0.5-2	5-50	50-150	200-1000
Binding to calpastatin	0.042	40	25	250-500
Autolysis without PL		50-150		550-800
Autolysis with PL		0.8-50		90-400

*Numbers are Ca^{2+} concentration calculated in μM required for half-maximal activity, binding, or rate of autolysis and based on the experiments with bovine skeletal calpain, which may slightly differ from other calpain species on Ca^{2+} requirement. PL is phospholipid; numbers are based on experiments using phosphatidylinositol or phosphatidylinositol 4,4-bisphosphate (from Goll et al., 2003).

Intracellular distribution

Studies suggests that upon activation, calpain translocates from cytosol to plasma membrane followed by its autolytic activation, and this membrane localization may be an essential mechanism regulating calpain activity (Mellgren, 1987). One line of evidence is the partitioning of active calpain to membrane/cytoskeletal fractions in ionomycin-stimulated T Cells (Rock et al., 1997). Further biochemical analysis of fibronectin-stimulated T cells demonstrates that m-calpain localizes to T cell focal adhesion-like complexes with ß1-integrin, talin and other membrane associated proteins (Rock et al., 2000). In a recent investigation of calpain activity promoted by the epidermal growth factor receptor (EGFR), calpain activity was found to be triggered only by plasma

membrane-restrictedly activated EGFR, not by internalized EGFR (Glading et al., 2001). Thus, these studies imply that some molecules such as calpain's endogeneous inhibitor calpastatin prevents calpain activation in cytosol, whereas membrane localization evokes this activation, in turn promoting the regulation of membrane-associated proteins by calpain.

Autolysis

It has been hypothesized that μ- and m- calpains dissociate into subunits upon activation by calcium *in vivo*, and the dissociated 80 kDa itself functions as a monomer and becomes an active enzyme through autocatalytic hydrolysis.

Autolysis is a proteolytic process in which calpain itself modifies the N-terminal regions of its large subunit, and so becomes active and decreases the Ca^{2+} requirement significantly (Table 2). In this Ca^{2+}-induced calpain autoproteolysis, the 80 kDa inactive catalytic subunit of either μ-calpain or m-calpain convert to an active 76 kDa subunit for μ-calpain or a 78 kDa subunit for m-calpain. The shortened subunits possess catalytic activity and retain their dependency on calcium but with a much lower requirement. However, the Ca^{2+} requirement for initiating autolysis is much higher than would be encountered in living cells, raising the question of whether Ca^{2+} is necessary for autolysis *in vivo* (Goll et al., 2003). Moreover, the physiological significance of autolysis still remains controversial. Some studies including the crystal structure analysis indicate that unautolyzed calpains are active and are not proenzymes that require autolysis for their activation, which happens in many members of the cysteine protease family (Strobl et al., 2000). Some studies also point out that autolysis is more likely a mechanism of attenuation since autolyzed calpain is rapidly degraded (Li et al., 2004) and non-autolyzed intact calpains retain wild-type calpain activity (Suzuki et al., 2004). On the other hand, it has been reported that the peptides released during autolysis of the catalytic subunit of μ- calpain have chemotactic activities on neutrophils and immunocytes (Kunimatsu et al., 1990, 1995). Because autolysis seems to occur consistently under conditions where the calpains are proteolytically active in cells, it is presumed to have some important roles in calpain function.

Phospholipid binding

There has been an effort to identify molecules in cells that could interact with calpain and its autolyzed forms, to permit activity at physiological Ca^{2+} condition. Phospholipids, especially acidic phospholipids, are found to greatly reduce the Ca^{2+} concentration required for autolysis and proteolytic activity of calpain *in vitro* (Table 2). The G17TAMRILGG region in domain V of the m-calpain regulatory subunit has been suggested to be essential for the role of phosphatidylinositol in the decrease of the optimal Ca^{2+} concentration for calpain autolysis, and may be the putative phosphatidylinositol-binding site of this protease (Arthur and Crawford, 1996). However, a more recent study indicates that domain III of m-calpain large subunit can bind phospholipids in a Ca^{2+}-dependent manner and is thought to be responsible for orchestrating Ca^{2+} and lipid activation of the enzyme (Tompa et al., 2001). Therefore the hypothesis is, upon activation, calpain translocates from the cytosol to the membrane followed by binding to phospholipid vesicles, and then activates itself *via* autolysis. The autolyzed form represents the active form of calpain at the membrane level, suggesting that transmembrane proteins could be the preferential substrates of calpain.

Phosphorylation

Phosphorylation of calpain may be another important mechanism for activity regulation and recent research demonstrates that both μ- and m- calpains are phosphorylated at multiple sites *in situ*. Under unstimulated conditions, there are three sites each of tyrosine, serine and threonine phosphorylation, with calpains prepared from a variety of tissues showing varied substoichiometric phosphorylation (Goll et al., 2003).

Phosphorylation of calpain is found to function in both negative and positive control of calpain activity. A study suggests that the interferon inducible chemokine IP-10 may inhibit EGFR-mediated m-calpain activity *via* a protein kinase A (PKA)-dependent phosphorylation pathway (Shiraha et al., 1999). Structural modeling further suggests that phosphorylation of m-calpain at Ser-369 in domain III by PKA restricts domain movement and freezes m-calpain in an inactive state (Shiraha et al., 2002). On the other hand, research points out that m-calpain can be activated by epidermal growth factor (EGF) through the extracellular signal-regulated kinase (ERK) cascade *via* direct

phosphorylation, and this activation may occur in the absence of cytosolic calcium fluxes. The conversion from serine 50 to glutamic acid maintains m-calpain activity *in vivo* (Glading et al., 2004). This study is interesting since muscle-specific calpain 3, which does not require Ca^{2+} for activation, presents a glutamic acid residue at this site (Branca et al., 1999). These findings propose a novel mechanism for calpain regulation by growth factor-induced phosphorylation events, suggesting that phosphorylation and calcium may act to coordinately regulate calpain activity *in vivo* (Perrin and Huttenlocher, 2002).

All of these mechanisms proposed reduce the calcium requirement for calpain *in vitro*, but not to the levels noted in motile cells. This suggests that these mechanisms may contribute to, but may not be solely responsible for, calpain activation *in vivo*. In summary, calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increase in the cellular Ca^{2+} level. At the membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Autolysis of domain I takes place during activation, and dissociation of regulatory subunit from catalytic subunits and autolyzed active forms of large catalytic subunit occur as a result. Activated calpain hydrolyzes substrate proteins at the membrane or in the cytosol after release from the membrane.

2.2.4 Biological Function of Calpain

Calpains have been implicated in a large number of biological processes including cell spreading and migration, myoblast fusion, apoptosis and oncogenic transformation, cell cycle control, and regulation of gene expression by selective proteolysis. Various forms of calpains have also been related to pathological processes such as tumor metastasis, wound healing, ischemia-induced damage, Alzheimer's disease, muscular dystrophy, and, more recently, diabetes (Perrin and Huttenlocher, 2002).

Apoptosis

Apoptosis may involve an activation of calpains that is limited to certain cell types and to specific stimuli, such as neutrophil apoptosis (Squier et al., 1999), and glucocorticoid-induced thymocyte apoptosis (Squier and Cohen, 1997). The activation of

calpain may occur downstream of caspases in a mouse photoreceptor degeneration cell line (Sharma and Rohrer, 2004), but has also been reported to occur upstream of caspases in apoptosis induced by ionizing irradiation (Wang et al., 2001). Moreover, the synergistic effect of caspase and calpain inhibitors in protecting neurons from ischemic damage (Rami et al., 2000) or spinal cord injury (Ray et al., 2003) and oxidative stress-induced apoptosis (Sanvicens et al., 2004), suggests cross-talk between caspases and calpain during apoptosis. However, recent research reveals that β-lapachone-induced, μ-calpain-stimulated apoptosis does not involve any known apoptotic caspase (Tagliarino et al., 2003).

Overall, it is generally accepted that calpains are involved in cell death processes. But their exact role is unknown, and little has been done to sort out their degree of participation in any of the more common apoptotic models.

Myoblast fusion

Myoblasts are differentiated muscle cells which, when fused, become the myotubules that eventually grow into muscle fibers. It has been suggested that two calpain isoforms play an important role in muscle growth by selectively proteolytic processes.

M-calpain activity significantly increases during early myoblast differentiation. It degrades certain proteins, such as desmin, talin, fibronectin (Dourdin et al., 1999), integrin β1 subunit and β-tropomyosin (Barnoy et al., 1998) in the fusing myoblasts, permitting the reorganization of membrane and cytoskeleton for the myoblasts to fuse. Moreover, considerable μ-calpain expression is also detected during the later stages of myogenesis. It has been suggested that μ-calpain may be involved in the myogenesis regulation pathway *via* its action on myogenin, MyoD and Myf5, which are members of the myogenic regulatory factor (MRF) family capable of stimulating or preventing myogenesis (Moyen et al., 2004). These observations regarding the particular activity patterns, as well as the different distributions of μ- and m-calpains in myoblasts (Moraczewski et al., 1996), indicate that these two isoenzymes have different biological functions and thus different contributions in muscular dystrophies.

Regulation of gene expression

The concept that calpains have functions in regulating gene expression is based on the discovery that various calpain substrate proteins are associated with carcinogenesis, including products of oncogenes and tumor suppressor genes, such as several transcription factors c-Jun and c-Fos, p53, pp60src, and the estrogen receptors (ERs).

A recent study demonstrates that the digestive tract-specific calpain gene, calpain 9, is down-regulated in human gastric cancer tissue as compared to normal gastric mucosa, suggesting that calpain 9 might be a new type of tumor suppressor that degrades certain oncogenic products important for carcinogenesis in digestive tracts (Yoshikawa et al., 2000). Calpain plays important roles in the p53 and c-Jun degradative responses by hydrolyzing and hence lowering their *in vivo* levels. Overexpression of calpastatin in NIH3T3 cells decreases and introduction of an anti-sense calpastatin increases the rate of degradation of c-Jun in these cells (Hirai et al., 1991). Although the proteasome is likely the principle route of degradation of these transcription factors in cells, studies point out that c-Fos, c-Jun, and p53 could be degraded *via* several different proteolytic pathways *in vivo* and that one of these pathways may involve the calpains (Pariat et al., 1997, 2000). More recently, nuclear factor-κB (NF-κB), a transcription factor that plays a pivotal role in the induction of genes involved in the response to injury and inflammation, was also found to be calpain-sensitive (Virlos et al., 2004).

Cell cycle control

A number of studies using synthetic inhibitors or calpastatin have suggested that calpain activity is required for cell cycle progression, specifically through the G1 to S transition.

Cyclin D is rate limiting and is involved in progression through G1. Serum starvation of NIH3T3 cells leads to a rapid loss of cyclin D1 that is prevented by overexpression of calpastatin (Zhang et al., 1997). The progression of serum-stimulated WI-38 fibroblasts into S-phase was partially inhibited by E64d, a cell-permeant inhibitor of calpain (Mellgren, 1997). Calpastatin overexpression represses progression of v-Src-transformed cells through the G1 stage of the cell cycle, which correlates with decreased phosphorylation and decreased levels of cyclins A and D and cyclin-dependent kinase 2

(Carragher et al., 2002). More recently, it was reported that calpain plays a role as an effector of the Gq signaling pathway for inhibition of Wnt/beta-catenin-regulated cell proliferation (Li and Iyengar, 2002). By using RNAi technology, cells with a reduced level of m-calpain failed to generate a polar ejection force and consequencely have difficulty in chromosome alignment during mitosis (Honda et al., 2004).

However, synthetic inhibitors may have unexpected effects resulting from nonspecific inhibition. In addition, both the calpastatin-overexpressing EcR-CHO cells and fibroblasts from the calpain 4-deficient (*Capn4*-/-) mice proliferated, *albeit* at a reduced rate, indicating that cells can proliferate in the absence of calpain activity (Xu and Mellgren, 2002). This raises the question as to the roles of calpains in the mitotic cycle.

Oncogenic transformation

The mechanism by which calpain activity is regulated by oncogenes is beginning to be elucidated, and several lines of evidence suggest a role for calpain in oncogene-induced cell transformation.

The transforming viral Src gene (v-*src*) is associated with classic characteristics of oncogenic cell transformation, including deregulated growth control, cell rounding, and substrate detachment resulting from adhesion loss and disruption of the actin cytoskeleton (Fincham and Frame, 1998). Recent studies indicate that synthesis of m-calpain is increased in v-Src-transformed cells, and is accompanied by the proteolysis of focal adhesion kinase (FAK) and by focal adhesion disruption and morphological transformation (Carragher et al., 2001). However, v-Src-induced morphological transformation has less of an effect on fibroblasts from *Capn4*-/- mice, indicating that calpain system is essential for v-Src transformation of cells.

Transformation induced by other oncoproteins such as v-Myc, K-Ras, and v-Fos is also shown to be accompanied by proteolytic cleavage of FAK, implicating the general involvement of calpain in promoting oncogenic transformation. In contrast to these findings, calpain was found to be a suppressor of Ha-*ras*-transformed NIH3T3 through proteolytic cleavage of protein kinase C (PKC)ε but not by that of PKCα (Hiwasa et al.,

2002), suggesting possible substrate specificity and particular oncoprotein induction may determine the role of calpain in individual oncogenic transformation.

Cell migration

Cell migration is a dynamic process involving a repeated cycle of membrane protrusion and attachment, cytoskeletal contraction and rear detachment. This process includes at least four basic steps: extension of the leading edge, formation of new focal adhesions at the leading edge, breaking of adhesions at the trailing edge, and translocation of the cell mass (Lauffenburger and Horwitz, 1996). Intervention of any one of these steps is sufficient to prevent cell motility. However, the molecular and biochemical mechanisms underlying these biophysical processes are unknown. Up to now, few proteins have been characterized as regulators of the adhesion-release process during cell migration.

Recently, calpains, specifically μ- and m- calpains have emerged as the important regulators of the adhesion-release process during cell migration by limited cleavage of several focal adhesion and cytoskeletal proteins (Carragher et al., 2001; Carragher and Frame, 2002). Studies have demonstrated that calpain is associated with cell adhesion complexes (Beckerle et al., 1987; Rock et al., 2000). Furthermore, fibroblasts from *Capn4*$^{-/-}$ mice exhibit reduced migration rates (Dourdin et al., 2001). Overexpression of the calpain inhibitor calpastatin also impairs cell detachment and migration (Temm-Grove et al., 1999).

However, the relevant *in vivo* substrates involved in calpain-regulated cell migration are not well defined. The contribution of specific isoforms to this particular cell response is also not clear. Since calpain has long been tied to pathophysiological issues in wound healing, cancer and the immune and inflammatory response, an understanding of calpain regulation during migration has remained elusive but crucial. In the following sections of the review, I will focus on the discussion concerning the different aspects of calpain in its associated cell migration.

2.2.5 Calpain and Pathological Significance

Muscular dystrophy was identified as a disease associated with inappropriate calpain activity 20 years ago (Ishhiura et al., 1980). Since then, the calpain family has been implicated in a wide range of pathological states in cells or tissues. Some but possibly not all of these calpain-related pathologies are listed in Table 3. With three exceptions, namely limb-girdle muscular dystrophy, gastric cancer and type II diabetes, the common theme for calpain-related genetic diseases is cellular Ca^{2+} overload accompanied by degradation of proteins that are calpain's substrates. Physiologically, the majority of calpain is inactive in a resting cell. As a result of either a transient or localized accumulation of calcium (*e.g.*, next to the cytosolic side of a calcium channel), a small fraction of the calpain is activated to produce selective and limited proteolysis of one or more of its target substrates, fulfilling its role in different kinds of cellular functions. Under pathological conditions, sustained calcium overload can activate a large fraction of calpain leading to uncontrolled and extensive degradation of functional cellular proteins (Fig. 2.3). The compromised cell structure and functions would ultimately result in cell death.

Table 3, Calpain family and various pathological conditions (see Huang and Wang, 2001).

Calpain	Diseases
Calpain 1 (μ-calpain)	Brain trauma, Alzheimer' disease, stroke
Calpain 2 (m-calpain)	Brain trauma, Alzheimer' disease, stroke, cataracts
Calpain 3	Limb-girdle muscular dystrophy, Rheumatoid arthritis
Calpain 9	Gastric cancer
Calpain 8/10	Type 2 diabetes mellitus

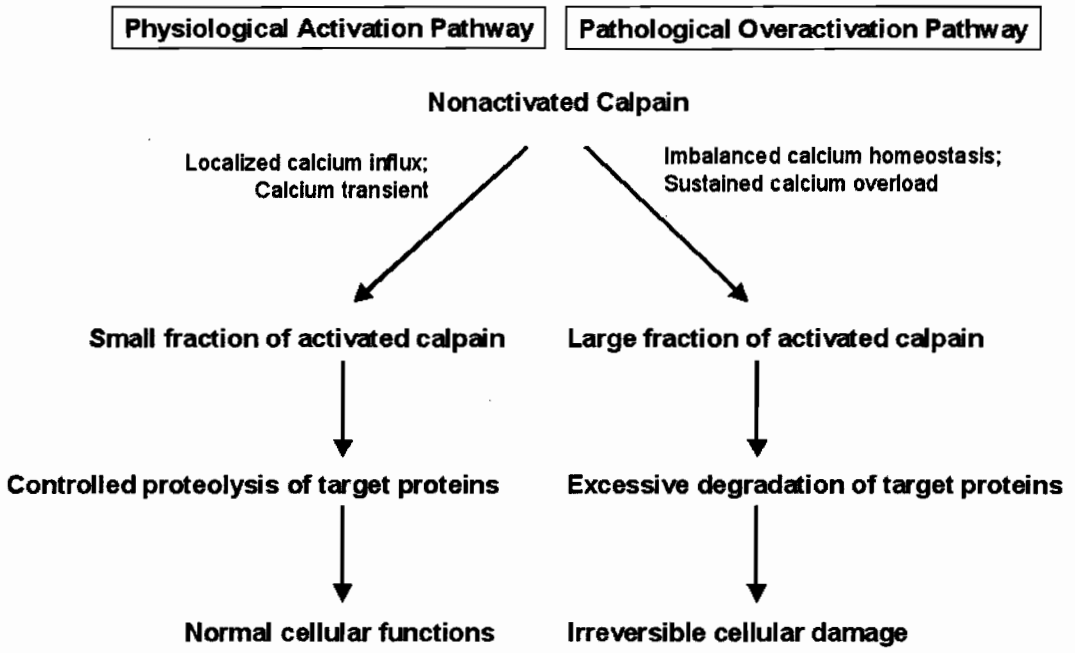


Fig. 2.3. Schematic description of physiological activation and pathological overactivation of calpain (from Wang and Yuen, 1994).

Since calpain is most active during pathological events, it appears to be an ideal pharmaceutical target. The current challenge is to identify cell permeable and selective calpain inhibitors for evaluation in various *in vivo* disease models

2.3 The Role of Calpain in Cell Motility

It has been suggested that focal adhesion complexes mediate the stability of the actin cytoskeleton and the adhesive properties of cells (Burridge and Chrzanowska-Wodnicka, 1996). Cells utilize a complicated, temporally and spatially regulated mechanism of focal adhesion assembly at the leading edge, coordinated with focal adhesion disassembly at the cell rear to promote cell migration (Lauffenburger and Horwitz, 1996). The mechanisms regulating turnover of focal adhesions are not well understood. However, studies indicate that two calpain isoforms, m-calpain and μ-calpain, are linked to cell migration and adhesion. Colocalization of m-calpain and talin at sites of cell adhesion (Beckerle et al., 1987) and the identification of several focal

adhesion proteins as calpain substrates *in vitro* (Cooray et al., 1996; Yamaguchi et al., 1994; Yoshida et al., 1984) imply that calpains are functional at focal adhesion sites. Calpain inhibitor studies further suggest that calpain inhibition modulates cell migration by stabilizing cytoskeletal linkages and decreasing the rate of retraction of the cell's rear (Huttenlocher et al., 1997). However, the molecular mechanism by which calpain regulates cell migration and the relevant *in vivo* substrates are not clear.

2.3.1 Calpain and Cell Adhesion and Spreading

Calpain was reported to play an important role in mediating cell adhesion, initially from the study of platelets. Calpain has been found to associate with focal adhesion proteins in platelets (Fox et al., 1993). Reorganization of the actin cytoskeleton is required for a number of platelet activation processes, including shape change, aggregation and spreading. Therefore cleavage of cytoskeletal proteins by calpain in activated platelets suggests a regulatory role for calpain in early cytoskeletal remodeling as the result of platelet activation (Saido et al., 1993). Calpain has also been found to modulate the late events of platelet-mediated fibrin clot retraction. Proteolysis of focal adhesion proteins and signaling molecules promotes dissociation of talin, $pp60^{c\text{-}src}$, and integrin $\alpha_{IIb}\beta3$ from the contractile cytoskeleton, as well as the tyrosine dephosphorylation of multiple cytoskeletal proteins, leading to relaxed fibrin clot retraction (Schoenwaelder et al., 1997). More recently, μ-calpain null platelets were found to exhibit impaired tyrosine phosphorylation of several proteins including the β3 subunit of integrin $\alpha_{IIb}\beta3$, correlating with the agonist-induced reduction in platelet aggregation and clot retraction (Azam et al., 2001).

T cells also use calpain to regulate assembly of cell adhesions required for attachment, where integrin and ligand interaction activates calpain to promote integrin diffusion, assembly of focal complex and T cell adhesion and cell spreading on fibronectin (Rock et al., 2000).

Other studies show that calpain regulates the actin remodeling events of endothelial wound healing and fibroblast spreading, including lamellipodial and filopodial protrusion formation (Potter et al., 1998; Shuster and Herman, 1995). Calpain has been reported to facilitate the spreading of the lammelipod in NIH-3T3 cells (Potter

et al., 1998). The use of NIH-3T3 cells that overexpress the biological inhibitor calpastatin, as well as the use of other pharmacological inhibitors of calpain, allowed examination of the dependence of cell spreading and actin modification on calpain. Inhibition of calpain suppressed early filopodal and lammelipodal protrusion formation and eliminated the appearance of staining for F-actin at the lamellar border. These effects may have resulted from malformation of the actin network in the forming lammelipod, perhaps due to inhibition of calpain cleavage of ezrin, an actin-binding protein (Potter et al., 1998; Wang and Yuen, 1997).

In addition to physically altering focal adhesion complexes to promote cell spreading, evidence indicates that calpain also plays an important role in regulating the formation of focal adhesions and cell protrusion by acting on a signaling intermediate upstream of the Rho family of GTPase (Perrin and Huttenlocher, 2002). Rac1 and RhoA are the best-characterised Rho family members in human cells. Each controls the formation of a distinct cytoskeletal element in mammalian cells, and coordinates regulation of cell adhesion and migration by selective activation of one GTPase over the other (Wherlock and Mellor, 2002). Investigations demonstrate that during the early stage of integrin-mediated cell spreading, signals transmitted across integrins following integrin-ligand interactions result in the integrin acting to cluster and assemble complexes of signaling molecules. These transiently formed integrin clusters contain calpain, cleaved talin, β3-integrin and spectrin. Recruitment of Rac to these clusters leads to the activation of Rac and the formation of Rac-induced focal clusters. The calpain in the integrin clusters initially inactivates RhoA allowing for the formation of lamellipodia (Kulkarni et al., 2002). The subsequent activation of newly synthesized RhoA transforms these clusters into focal adhesion complexes and the formation of contractile actin-myosin stress fibers to generate mature adhesions (Kulkarni et al., 1999). However, a contrasting finding suggests that formation of focal complexes and focal adhesions in the same endothelial cells by constitutively active Rac or Rho occurs even in the presence of calpain inhibitors (Bialkowska et al., 2000). Hence, the way that calpain modulates the activity of RhoA and the subsequent formation of focal adhesion complexes needs to be further investigated.

2.3.2 Calpain and Integrin-Mediated Motility (Haptokinetic Motility)

Integrin receptors, which are αβ heterodimers present on the cell surface, play an important role during cell migration by mediating interactions and transmitting forces between the extracellular matrix (ECM) and the actin cytoskeleton (Hynes, 1992). Studies indicate that haptokinetic motility mediated primarily by integrins was calpain dependent (Palecek et al., 1998). When treated with calpain inhibitors, migration rates on fibronectin of Chinese hamster ovary cells transfected with both β1 and β3 integrins was decreased. This occurred along with an increase in tail length, a minimal retraction of the rear of cells and a reduced appearance of patches of integrins on the substrate as cells move forward. These results indicate that cleavage of the intracellular portion of integrins by calpain is important in regulating rear detachment in migrating cells. Calpain inhibition stabilizes the integrin-cytoskeletal linkage and thus increases both the likelihood of integrin detachment from the extracellular matrix and the stability of peripheral focal adhesions, thus causing a decrease in cell speed during migration (Huttenlocher et al., 1997).

Evidence has gradually produced a rough model of the regulation of calpain on haptokinetic motility, which supports a front-versus-rear asymmetry. Several possible asymmetric systems have been identified during the past years. The spatial asymmetries in the distribution of adhesion receptors (integrin receptors) or adhesion receptor-cytoskeleton linkage avidity in the cell may lead to a higher adhesiveness at the cell front than the cell rear (Schmidt et al., 1993). The asymmetry in affinity between the adhesion receptor and extracellular matrix ligand or myosin light chain kinase (MLCK)-dependent contraction could produce an adhesive gradient within the cell (Huttenlocher et al., 1998; Klemke et al., 1997). In particular, intracellular Ca^{2+} transients at the rear may activate calpain for action (Maxfield, 1993). Although the exact system for the regulation of calpain on haptokinetic motility is still undetermined, all possibilities bring out one consideration that such mechanical stretching of the membrane might result in activation of stretch-activated Ca^{2+} channels leading to calpain activation (Palecek et al., 1998).

Asymmetric systems also may explain the function of calpain on regulation of both adhesion formation in the leading edge and adhesion disruption at the cell rear

(Lauffenburger and Horwitz, 1996). Together with spatial asymmetry of calpain activation, asymmetric localization of calpain isoforms or calpain targets on the membrane could contribute the asymmetric action of calpain in a front-versus-rear manner.

2.3.3 Calpain and Growth Factor Induced Cell Deadhesion (Chemokinetic Motility)

EGFR signaling stimulates cell motility in adherent cells such as endothelial cells, fibroblasts and epithelial cells. Required downstream signaling pathways include one *via* PLCγ causing calcium mobilization and another *via* MAP kinase leading to focal adhesion disassembly and decreased adhesiveness (Ware et al., 1998). Recently studies focus on the linkage between calpain and EGF-induced cell motility.

Although it is still difficult to fully define the mechanism by which calpains regulate cell migration, evidence shows that calpain activity is needed for EGFR-mediated motility. Inhibition of calpain by either calpeptin or calpain inhibitor I prevents EGF-induced cell de-adhesion in fibroblasts (Shiraha et al., 1999). Calpain activation in response to EGF was inhibited by disruption of the Ras-ERK signaling pathway by the MEK inhibitor PD98059. Evidence supports that the extracellular signal-regulated kinase/mitogen-activated protein (ERK/MAP) kinase pathway, and not the calcium-mobilizing PLCγ pathway, links EGFR signaling to calpain activation (Glading et al., 2001). Hence, EGFR signaling acutely induces calpain activity downstream of ERK/MAP kinase and this activation is required for both EGFR-mediated fibroblast de-adhesion and motility. A very recent report further shows that direct phosphorylation of protein kinase A (PKA) inhibits EGF-induced calpain activation, which in turn results in reduced migration rates (Shiraha et al., 2002). Together, these findings may provide a possible regulatory mechanism for control of calpain activity required for EGF-stimulated cell motility.

Interestingly, the link between ERK/MAP kinase signaling and cell motility requires only the m- but not μ-calpain, based on specific antisense-mediated down-regulation. Furthermore, other evidence indicates that EGF induces calpain activity only when both EGFR and ERK are associated with the plasma membrane (Glading et al.,

2000, 2001). This may imply that m-calpain translocates to the membrane upon EGF-induced activation and proteolyses putative targets in the adhension complex.

However, two questions may need further investigation. The m-calpain isoform needs a higher concentration of calcium for activation, and the PLCγ signaling pathway was thought to trigger intracellular calcium mobilization, so whether calcium fluxes are required in this m-calpain regulated and PLCγ signaling pathway-independent cell migration process are still uncertain. If so, where do these fluxes originate? The second question is how the ERK/MAP kinase physiologically activates calpain, even though EGFR signaling does activate calpain *via* the ERK/MAP kinase cascade. Some possibilites are direct phosphorylation of calpain by ERK/MAP kinases; other downstream effectors mediating the ERK/MAP kinase-calpain interaction; conformational change of molecular association without phosphorylation by ERK/MAP kinase; negative regulation of its endogenous inhibitor, calpastatin; or affecting gene transcription of calpain or calpastatin (Glading et al., 2000). Overall, the precise linkage between ERK and calpain still remains unclear.

In conclusion, calpain performs vital functions in regulating cell motility both by stabilizing cell-substratum adhesions to allow forward extension and rear detachment, and by possibly acting as a positive signal to modulate functioning of the RhoGTPases. Despite lack of a detailed mechanism, almost all evidence supports the function of calpain in regulating cell detachment and focal adhesion disassembly. Two possible systems are generally simplified here to help better understand the clearly complicated role of calpain in cell spreading and migration (Perrin and Huttenlocher, 2002) (Fig. 2.4).

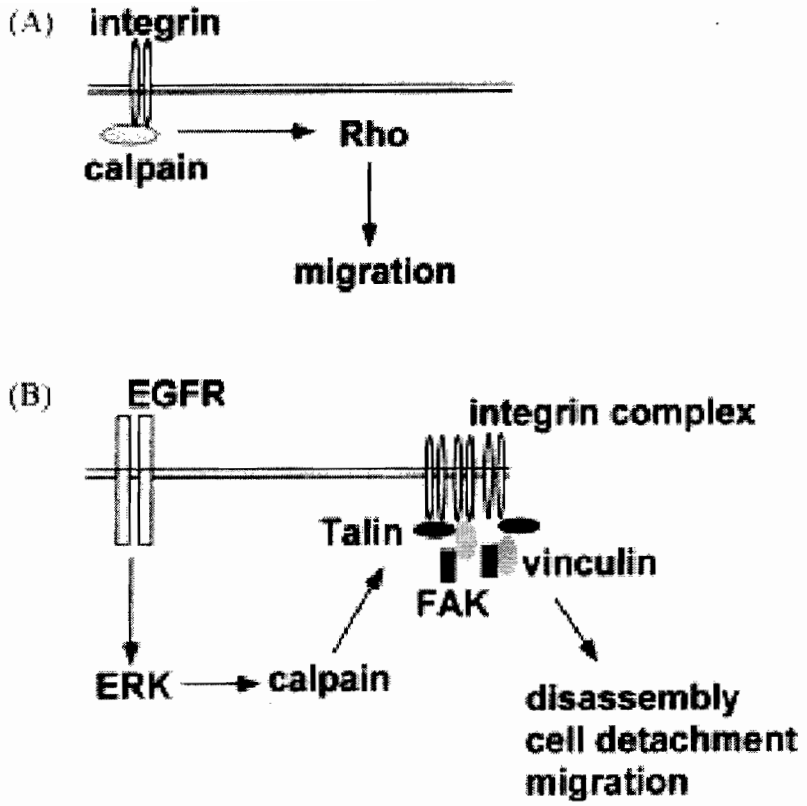


Fig. 2.4. Schematic summarization of two putative mechanisms of calpain action during migration. (A) Calpain has been implicated in cell spreading upstream of Rho-mediated signaling. (B) EGFR signaling regulates calpain activity *via* an ERK/MAPK pathway (from Perrin and Huttenlocher, 2002).

2.3.4 Calpain Targets Involved in Motility and Adhesion

The ubiquitous calpain isoforms, m- and μ-calpain, are found in various animal tissues and cells, but the physiological substrates of calpain are not well known. However, a number of *in vitro* and *in vivo* substrates provide excellent candidates relating to calpain-regulated cell migration. These calpain target molecules are either present in adhesion complexes or in the cytosol as shown in Table 4. By proteolysis of focal adhesion proteins, calpains release the link between the integrin-dependent focal adhesion complex and the actin cytoskeleton, which allows proper cell migration (Shiraha et al., 2002). Despite the precise molecular basis for calpain-mediated de-adhesion being uncertain, it has been demonstrated at least one of these adhesion proteins, the cytoplasmic domain of β-integrins, FAK, and paxillin, or talin is required to be

involved in calpain-regulated detachment (Huttenlocher et al., 1997; Palecek et al., 1998; Shiraha et al., 1999).

Table 4. Calpain targets - the proteins associated with cell migration and adhesion (Glading et al., 2002).

Target	Cellular locale	*In vivo*	*In vitro*
α-Actin	Plasma membrane	Yes	No
EGFR	Adhesion complex	Yes	Yes
Ezrin	Adhesion complex	Yes	Yes
FAK	Adhesion complex	Yes	Yes
Filamin	Adhesion complex	-	Yes
Intergrin β1	Adhesion complex	Yes	Yes
Intergrin β3	Adhesion complex	Yes	Yes
Intergrin β4	Adhesion complex	Yes	-
MAP2	Adhesion complex	-	Yes
MARCKS	Adhesion complex	Yes	-
MLCK	Pan-cellular	-	Yes
Paxillin	Adhesion complex	Yes	-
PKC	Pan-cellular	Yes	Yes
pp60Src	Adhesion complex	Yes	-
RhoA	Pan-cellular	Yes	-
Spectrin	Adhesion complex	-	Yes
Talin	Adhesion complex	Yes	Yes
Tau	Pan-cellular	Yes	Yes
Vinculin	Adhesion complex	Yes	-

Recently, it has been proposed that calpain functions to regulate adhesion turnover, rather than adhesion breakdown, because calpain-mediated proteolysis of its substrates is limited rather than complete (Glading et al., 2002). Moreover, cleavage of these proteins often results in active molecules with perhaps altered binding affinities. Proteolysis of talin, for example, generates a 190-kDa C-terminal fragment that retains its protein binding sites but loses its membrane association domain (Muguruma et al., 1995). Although the biophysical consequences of this cleavage have not been directly determined, it is possible that this cleavage acts to facilitate the dissociation of talin from the focal adhesion, along with its companion molecules (Glading et al., 2000). On the other hand, investigations point out that the calpain cleaved N-terminal 47-kDa talin head domain has a 6-fold higher binding affinity than intact talin for the cytoplasmic tail of β3-

integrin and this fragment subsequently regulates integrin activation (Calderwood et al., 1999; Yan et al., 2001). Nevertheless, these observations would argue a possible mechanism whereby calpain could regulate focal adhesion disassembly by limited cleavage of focal adhesion substrates.

2.3.5 Pathological Conditions and Therapeutical Inhibitors

Calpain-regulated cell migration plays an important role in many physiological events including embryonic development, wound healing and especially tumor invasion (Glading et al., 2002). It is one of the defining characteristics of invasive tumors, enabling tumors to migrate into adjacent tissues or transmigrate limiting basement membranes and extracellular matrices (Wells et al., 2002).

Three separate studies point to the importance of calpain activity during tumour development and invasion. In human renal cell carcinomas, there is a correlation of higher μ-calpain expression with increased malignancy, with calpain expression showing much higher levels in tumours that metastasised to peripheral lymph nodes relative to tumours that had not metastasized (Braun et al., 1999). Activities of calpain were significantly higher in breast cancer tissues relative to normal breast tissues (Shiba et al., 1996). Calpain-mediated proteolysis of the tumour suppressor protein neurofibromatosis type 2 (NF2), is associated with the development of schwannomas and meningiomas (Kimura et al., 2000). Fibroblasts derived from calpain 4 knockout embryos display impaired cell migration rates and abnormal organization of the actin cytoskeleton with a loss of central stress fibers (Dourdin et al., 2001). The recently determined crystal structure of m-calpain shed light on our understanding of the physiological roles of calpain and their mechanism of activation (Strobl et al., 2000). This information will facilitate the design of improved specific inhibitors of calpain activity that may present useful therapeutic approaches for the treatment of calpain-associated pathological disorders.

The searching for calpain inhibitors has not stopped since calpain was discovered. Early protease inhibitors were Ca^{2+} chelators, such as EDTA and EGTA, or sulfhydryl group-specific reagents, most of which are neither selective nor potent and thus not suitable to use. Current knowledge about calpain substrate specificity and the nature of

the active site of the enzyme makes it possible to design more specific and powerful inhibitors for the study of calpain-mediated disorders. To date, all inhibitors derived from either natural source or chemical syntheses can be grouped into three major categories as shown in Table 5. The most widely used are, leupeptin, E64d, calpeptin, and calpain inhibitor I, MDL28170, with the latter four having demonstrated relative cell permeability. Unfortunately, these compounds will also inhibit other cysteine proteases.

Table 5. Summary of calpain inhibitors.

Category		Name	Specifity	Example (s)
Protein inhibitors	Biological inhibitor	Calpastatin	Both μ-calpain and m calpain	Whole molecule
		Calpastat		27-mer Calpastatin peptide
	Others	Heavy chains of L-Kininogen and H- Kininogen	Calpain, papain and cathepsin L	GLN-Val-Val-Ala-Gly-NH_2
Peptide inhibitors	Irreversible inhibitors	Peptidyl chloromethanes	Cysteine and serine proteases	Dansyl-Leu-Leu-Phe-CH_2Cl
		Peptidyl monofluoromethanes	Favor cysteine proteases than serine proteases	Z-Phe-AlaCH_2F, Ala-Phe-Lys-CH_2F
		Peptidyl haloacetyl hydrazines	Cysteine proteases, favor cathepsin B than calpain	1-Peptidyl-2-haloacetyl hydrazines
		Peptidyl diazomethanes	Cysteine and serine proteases	Z-Leu-Leu-Tyr-CHN_2
		Peptidyl arylacyloxyketones	Cysteine proteases	Z-$_D$-Ala-Leu-Phe-OCO-2,6-F_2-Ph
		Peptidyl epoxides	Cysteine proteases	E64, E64c, **E64d**
		Peptidyl sulfonium methyl ketones	Favor calpain	Z-Leu-Leu-Phe-$CH_2S^+(CH_3)\cdot Br^-$
		Peptide disulfides	Favor calpain, no inhibition on serine and aspartic proteases	H-Leu-Leu-Cys(Npys)-NH_2
	Reversible inhibitors	Peptidyl aldehydes (first generation)	Calpain and serine proteases	**Leupetin**, Antipain, Staccopins P1 and P2
		Peptidyl aldehydes (second generation)	Calpain, cathepsin L and B	**Calpeptin, Calpain inhibitor I and II, MDL28170**
		Peptidyl α-keto esters	Cysteine and serine proteases	Z-Leu-Phe-COOH, Z-Leu-Abu-CONH-*i*-Bu
Nonpeptide inhibitors		ATA	Calpain, endonucleases, DNA polymerases	
		α-mercaptoacrulic acid PD150606	Both μ-calpain and m -calpain	
		α-mercaptoacrulic acid PD151746	Favor μ-calpain than m -calpain	

*Bold are the widely used inhibitors for calpains.

Calpain participates in various calcium-regulated cellular functions, but its precise physiological functions are still not clear. To analyze the physiological functions of calpain, inhibitors specific for calpain, especially those specific for each calpain homologue, are essential. At present, various calpain inhibitors have been developed, but none of them are truly specific for calpain (Donkor, 2000; Wang and Yuen, 1997), particularly for the two calpain isoforms. However, RNA interference, also known as Post-Transcriptional Gene Silencing (PTGS), referring to a sequence specific degradation of target messenger RNA (mRNA), may help to solve this problem.

2.4 RNA Interference Techniques

2.4.1 Brief Overview of *In Vivo* and *In Vitro* Methodology

RNA intereference (RNAi) refers to the introduction of homologous double-stranded RNA (dsRNA) into a cell to specifically target a gene's product, where it induces the degradation of the complementary mRNA, and thereby suppresses gene expression and results in a null or hypomorphic phenotype (Fig. 2.5). RNAi has proven to be a powerful tool in the elucidation of gene function in a diversity of organisms ranging from worms (Fire et al., 1998; Pothof et al., 2003), to *Trypanosoma brucei* (Ngo et al., 1998), to plants and fruit flies (Cottrell and Doering, 2003; Kennerdell and Carthew, 1998; Liu et al., 2002), and even to mammalian cells (Elbashir et al., 2001a; Hannon, 2002; Wianny and Zernicka-Goetz, 2000), as shown in Table 6.

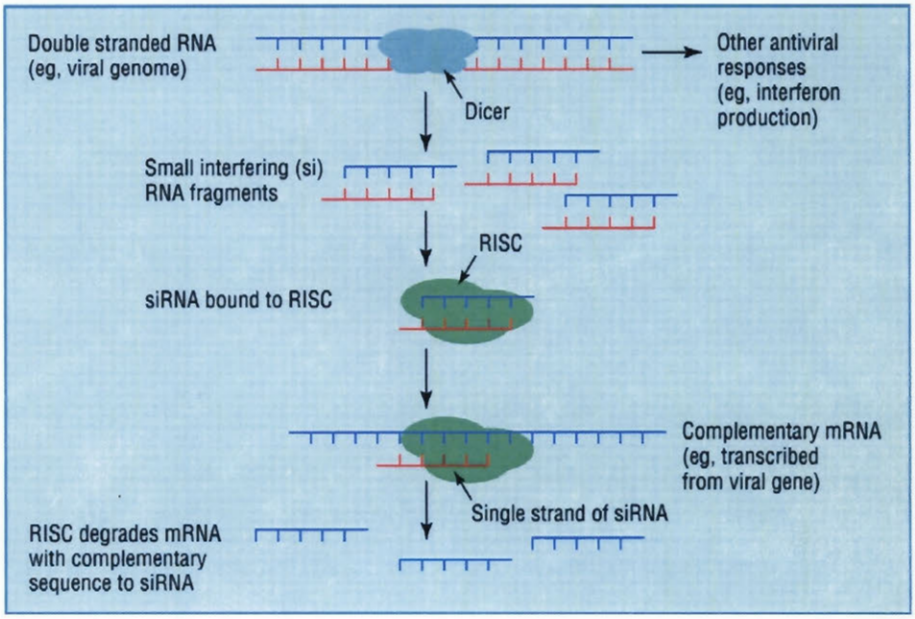


Fig. 2.5. Schematics of RNA interference. Double stranded RNA (dsRNA) triggers an RNA interference response. The dsRNA is cut into 20 bp or so in length, known as small interfering RNAs (siRNAs), through a cellular enzyme Dicer. The siRNAs bind a cellular enzyme complex, RNA induced silencing complex (RISC), which uses one strand of the siRNA to bind single stranded mRNA. Then RISC degrades the target mRNA, and thus silences gene expression. In mammals, long dsRNA also causes interferon response (adopted from Downward, 2004).

Table 6. Eukaryotic organisms exhibiting RNAi-related phenomena [adopted from Agrawal (Agrawal et al., 2003)]*.

Kingdom	Species	Delivery method
Protozoans	*Trypanosoma brucei*	Transfection
	Plasmodium falciparum	Electroporation and soaking
	Toxoplasma gondii	Transfection
	Paramecium	Transfection and feeding
	Leishmania donovanii	Tried but not working
	*Entamoeba histolytica**	Soaking
Invertebrates	*Caenorhabditis elegans*	Transfection, feeding bacteria carrying dsRNA, soaking
	Caenorhabditis briggsae	Injection
	Brugia malayi (filarial worm)	Soaking
	Schistosoma mansoni	Soaking
	Hydra	Delivered by micropipette
	Planaria	Soaking
	Lymnea stagnalis (snail)	Injection
	Drosophila melanogaster	Injection for adult and embryonic stages, soaking and transfection for cell lines
	*Anopheles gambiae**	Transfection
	*Haemaphysalis longicornis**	Injection
	Cyclorrphan (fly)	Injection
	Milkweed bug	Injection
	Beetle	Injection
	Cockroach	Injection
	Spodoptera frugiperda	Injection and soaking

Vertebrates	Zebra fish	Microinjection
	Xenopus laevis	Injection
	Mice	Injection
	Humans	Transfection
Plants	Monocots/dicots	Particle bombardment with siRNA/transgenics
Fungi	*Neurospora crassa*	Transfection
	Schizosaccharomyces pombe	Transgene
	Dictyostelium discoideum	Transgene
	*Cryptococcus neoformans**	Transgene
	*Histoplasma capsulatum**	Transgene
Algae	*Chlamydomonas reinhardtii*	Transfection

* The list has been updated since then. The relevant references are shown below:

1. *Cryptococcus neoformans* (Liu et al., 2002)
2. *Anopheles gambiae* (Hoa et al., 2003)
3. *Histoplasma capsulatum* (Rappleye et al., 2004)
4. *Haemaphysalis longicornis* (Miyoshi et al., 2004)
5. *Entamoeba histolytica* (Vayssie et al., 2004)

2.4.2 Forms of dsRNA and Delivery Systems.

Basically, there are three forms of dsRNA that are used for RNAi: long hairpin RNA (lhRNA), short hairpin RNA (shRNA), and small interference RNA (siRNA). Long hairpin RNA can be easily generated through *in vitro* transcription of long dsRNA (up to 1-2 kb), which is then cleaved by Dicer-like enzymes *in vivo* into functional siRNAs. However, this simple approach cannot be used in mammalian cells, because introduction of long dsRNAs elicit a strong antiviral response obscuring any gene-specific silencing effect (Stark et al., 1998). On the other hand, introduction of 21-23 nt siRNAs with 2 nt 3' overhangs can overcome this problem (Brummelkamp et al., 2002a; Elbashir et al., 2001a; Sui et al., 2002). Technically, shRNA is the precursor to siRNA in vector systems. After a vector is transfected or transformed into cells, shRNA will be transcribed and finally processed into functional siRNA. The siRNA molecules along can be prepared by

chemical synthesis or *in vitro* transcription of longer dsRNA and then chopped *in vitro* by a Dicer-like enzyme. All the three forms of dsRNAs were practically dependent on their relevant delivery systems.

Physical or chemical transfection

Application of siRNA duplexes to interfere with the expression of a specific gene requires highly effective delivery of siRNAs into target cells. *In vitro* synthesized siRNAs can be easily delivered through normal cell transfection, microinjection, soaking (Tabara et al., 1998), or even ingestion (Timmons et al., 2001). However, the interference effectiveness may depend on the transfection efficiency, and long-term siRNA expression is required for some applications. Hence, effective strategies to deliver siRNAs are needed, especially to mammalian cells.

Plasmid-based siRNA delivery systems

Since long dsRNAs (usually greater than 30 nucleotides in length) trigger the interferon pathway, thus activating protein kinase R and 2',5'-oligoadenylate synthetase in mammalian cells (Elbashir et al., 2001a), this pathway can lead to global gene downregulation and RNA degradation. However, shRNAs exogenously introduced into mammalian cells have been reported to bypass the interferon pathway, although this may not always be the case (Sledz et al., 2003). This system was mainly based on various *Pol* III promoter expression cassettes. The H1 promoter has been used in mammalian cells to drive the constant expression of shRNA (Brummelkamp et al., 2002a). In some case, the U6 promoter also can be used for this purpose (Paddison et al., 2002a; Sui et al., 2002). All these systems have been commercialized through Ambion (http://www.ambion.com/tech lib/tn/95/952. html), and other companies as well.

Viral vector-based systems for RNAi

Retroviruses (including lentiviruses), and adenoviruses are two major types of viruses in the center of interest for gene therapy. Although the viruses are obligate intra-cellular parasites, and therefore elicit a host immune system response to some extent, they are very efficient at transfecting their own DNA into a particular host cell type with

great specificity. In recent years, recombinant viral vectors have been used to introduce siRNAs into cells for potential RNAi in functional genomics and in gene therapy. The reports up to now include the application of different viruses for RNAi delivery, such as retrovirus and lentivirus (Liu et al., 2004), moloney leukemia virus in primary cells (Stewart et al., 2003) , and adenovirus (Shen et al., 2003; Xia et al., 2002; Zhao et al., 2003). The major advantages are that the system can lead to inducible/repressible tissue-specific or cell-specific expression of siRNAs by using *Pol* II promoters (Calegari et al., 2004).

2.4.3 Computer-Aid Design of Functional siRNAs

A functional siRNA must fulfill certain properties including sequence length, GC-content, nucleotide composition, and RNA structure. In addition, to avoid off-target effects, the cross-silencing capability of siRNAs for other genes must be evaluated. Therefore, the selection of siRNAs for a target gene is a crucial step in siRNA-mediated gene silencing.

Different criteria have been set up for the selection of siRNAs. The most common rules used up to now are Tuschl's rules (Elbashir et al., 2002) and rational rules (Ding et al., 2004; Reynolds et al., 2004; Ui-Tei et al., 2004). Although these rules help to improve the siRNA design, none of these rules can guarantee that all the selected siRNAs are functional given that siRNAs are quite sequence-dependent. Recently, major efforts have been made to predict the possible siRNAs using web-based computer-aided systems (Table 7). Among the various rules for design of a functional siRNA, overall internal stability of the antisense RNA and the second structure of the mRNA target are recently reported to be more important than the number of mismatches in the siRNAs (Saxena et al., 2003). Therefore, in general, 3-8 siRNAs have been proposed to be designed to target one gene, regardless of the rules chosen to apply.

Table 7. Available websites for the siRNA design

Basics of RNAi

The RNA interference resource:
http://www.ambion.com/techlib/resources/RNAi/
A compendium of reviews and original articles on RNA interference:
http://www.nature.com/focus/rnai/library/news_views.html
The siRNA user guide:
http://www.rockefeller.edu/labheads/tuschl/sirna.html
Rules of siRNA design for RNAi:
http://www.protocol-online.org/prot/Detailed/3210.html

siRNA design

Ambion siRNA Target Finder:
http://www.ambion.com/techlib/misc/siRNA_finder.html
BLOCK-iT™ RNAi Designer:
https://rnaidesigner.invitrogen.com/sirna/
DEQOR, a web-based tool for the Design and Quality Control of siRNAs:
http://cluster-1.mpi-cbg.de/Deqor/deqor.html
Dharmacon siDESIGN Center:
http://design.dharmacon.com/rnadesign/default.aspx?SID=691011983
Gene-specific siRNA selector:
http://bioinfo.wistar.upenn.edu/siRNA/backup/siRNA.htm
Jack Lin's siRNA Sequence Finder:
http://www.sinc.sunysb.edu/Stu/shilin/rnai.html
OptiRNAi ---- An RNAi Design Tool:
http://bioit.dbi.udel.edu/rnai/
siDirect:
http://design.RNAi.jp/
siRNA Design Service:
http://www.proligo.com/pro_primprobes/PP_07-1_DS-siRNA.html
siRNA Selection Program White-head:
http://jura.wi.mit.edu/pubint/http://iona.wi.mit.edu/siRNAext/
siRNA Sequence Selector:
http://bioinfo2.clontech.com/rnaidesigner/sirnaSequenceDesign.do
siRNA Target Designer Promega:
http://www.promega.com/siRNADesigner/default.htm
siRNA Target Finder Genscript:
https://www.genscript.com/ssl-bin/app/rnai
Rational siRNA Design:
http://boz094.ust.hk/RNAi/siRNA
T7 RNAi Oligo Designer
http://websoft2.unige.ch/sciences/biologie/bicel/RNAi.html
Target accessibility prediction and RNA duplex thermodynamics for rational siRNA design:
http://sfold.wadsworth.org/sirna.pl

The siRNA design tool EMBOSS:
http://www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/sirna.html
The siRNA design tool Qiagen:
http://python.penguindreams.net/Xeragon_Order_Entry/jsp/Index.jsp

RNA structure
Mfold (version 3.1):
http://www.bioinfo.rpi.edu/applications/mfold/old/rna/

2.4.4 Applications of RNAi

RNAi has been widely used for basic research in mammalian cells, and now been hold promise as a potential therapy for many human diseases related to the elevated gene expression.

Targeting individual genes and functional dissection of gene isoforms.

RNAi is normally used to study the effect of a given gene through blocking its expression. Initially, long dsRNAs were used in plants, *C. elegans*, *Drosophila* and other species. Since the long dsRNAs (>30 nt) can cause a sequence-nonspecific interferon response in mammals, which in turn results in global inhibition of mRNA translation, siRNAs are instead widely adopted by large numbers of groups to study an ever increasing number of genes in a variety of mammalian cell lines (Elbashir et al., 2001a; McManus and Sharp, 2002; Tuschl and Borkhardt, 2002).

One of the particular applications is to study the functions of a specific protein isoform. Many eukaryotic genes are expressed as multiple isoforms through the differential utilization of transcription/translation initiation sites or alternative splicing. The common method to resolve this problem is to express this isoform in clean backgrounds without the presence of other isoforms, or to use isoform-specific inhibitors. These methods are either time-consuming or not available because of the lack of isoform-specific inhibitors. Taking advantage of the RNAi technology, the human adaptor protein ShcA has been investigated in an isoform-specific manner through suppression of other non-target isoforms using siRNA (Kisielow et al., 2002). In the very similar way, five isoforms of vascular endothelial growth factor (VEGF) have been studied (Zhang et al., 2003). Scientists have already succeeded in using RNAi to suppress dominant disease

genes *in vitro*; in some cases, this suppression has been allele-specific to silence the disease-causing allele while maintaining expression of the normal allele, such as selective silencing of the alleles that cause amyotrophic lateral sclerosis without affecting the wild-type alleles (Ding et al., 2003; Gonzalez-Alegre et al., 2003). The challenge now is to bring this powerful technology *in vivo* to animal models to suppress disease genes and correct disease phenotypes (Miller et al., 2003). This strategy provides a novel tool to study the function of various gene isoforms and may contribute to gene isoform-specific treatment in human diseases and/or disorders.

I will take this approach to exploit the function of human μ-calpain (see below), which is closely related to its isoform, m-calpain, in sequence similarity and perhaps in function as well.

Genome-widely dissecting gene functions

To extend the power of RNAi, genome-wide studies have been reported from *Caenorhabditis elegans* (Fraser et al., 2000; Keating et al., 2003) and *Drosophila* cells (Boutros et al., 2004; Lum et al., 2003; Simmer et al., 2003). The application of RNAi has been comparable to some traditional gene knockout strategies and to some yet-applied new powerful technologies for the systematic genome-wide functional screens (Carpenter and Sabatini, 2004). Theoretically, application of siRNA in genome-wide studies can provide and, in practice, have provided, a unique high-throughput approach for simultaneously discovering multiple genes that control almost any biological process or cellular response. These processes include signal transduction, cell cycle regulation, growth and differentiation, macromolecular synthesis and degradation, cell motility and cell death, disease pathogenesis, and drug resistance. At present, human siRNA libraries based on Lentiviral expression vectors have been commercially available for this purpose from some companies, such as System Biosciences (http://www.systembio.com /sirna_tech.htm) and Ambion (http://www. ambion.com/about/pr_032403.html). High throughput gene functional analysis with these siRNA libraries remains to be tested, but it is predicted to provide researchers with insight into the mechanisms controlling functional pathways and speed up the discovery of new potential targets for diagnostics and therapeutics.

Viral invasion/replication and other infectious diseases

The sequence-selective specificity and robust capacity of RNAi to inactivate gene expression in eukaryotes was recorded in *in vitro* and *in vivo* models of viral infection. For instance, RNAi has been used to inhibit viral-induced liver-cell inflammation and replication, and the replication of several other viruses, including HIV, in mammalian cells (Novina et al., 2002; Radhakrishnan et al., 2004). Of course, this ought to include viral-induced cancers (*e.g.* cervical carcinoma, hepatocellular carcinomas and haematopoietic and lymphoid malignancies) (Milner, 2003). Although some counter-defenses against RNAi have been found from some viruses, which may involve mutation and other escape mechanisms, it can be at least one part of a combined therapeutic approach to combat the virus.

In a similar fashion, RNAi can also be applied to other infectious diseases, such as human pathogenic fungi (Cottrell and Doering, 2003) and parasites (Aboobaker and Blaxter, 2004; Malhotra et al., 2002). It can be predicted that RNAi could repress essential genes in eukaryotic human pathogens or viruses that are dissimilar from any human genes. However, there is some evidence suggesting that RNAi may not work in some organisms, such as baker's yeast *Saccharomyces cerevisiae* (Paddison et al., 2002b) and the protozoan parasite *Leishmania* (Robinson and Beverley, 2003). This is because RNAi is an ancient antiviral and/or anti-transposon system to protect cells from invasion of other DNA's and/or RNA's, and which may have lost its function during evolution in these organisms and become naturally deficient for its activity.

Cancer and other human diseases

RNAi is arguably one of the hottest topics in science, and a large number of papers published on RNAi over the last few years have described successful suppression of some cancer-related genes, such as oncogenic K-rasv12 allele-induced tumorigenesis (Brummelkamp et al., 2002b; Duursma and Agami, 2003). It is obvious that RNAi could be harnessed successfully to treat a wide variety of diseases with high specificity. It is no wonder that the journal Science proclaimed it the biggest scientific breakthrough of the year 2002 (Couzin, 2002).

siRNAs have also been found promising in treating life-threatening conditions other than cancers, such as neurodegenerative disorders (Miller et al., 2004; Wood et al., 2003). Currently untreatable neurological diseases are especially attractive targets for RNAi, and RNAi is also proposed to be used for cardiovascular and cerebrovascular diseases (Milhavet et al., 2003). Finally, RNAi can be used to treat any disease that is related to elevated gene expression.

2.4.5 Challenge and Solutions

RNAi is a powerful tool used to manipulate gene expression or determine gene function. One technique of expressing the short double-stranded (ds) RNA intermediates required for interference in the mammalian system is the introduction of short-interfering (si) RNAs. Although RNAi strategies are reliant on a high degree of specificity, little attention has been given to the potential non-specific effects that might be induced, *i.e.* the antiviral response of mammalian cells to dsRNA or off-target of siRNAs.

Interferon

The use of RNAi in mammals has been complicated by the antiviral response of mammalian cells to dsRNA. The presence of foreign dsRNA in a mammalian cell initiates the so-called "interferon response", the non-specific degradation of mRNA, and ensuing death of the cell (Stark et al., 1998; Williams, 1999).

Mammalian RNAi researchers have undertaken a few different routes to avoid eliciting the interferon response. The most successful one is to introduce a siRNA, which is less than 30 nucleotide pairs long, into the cells (Elbashir et al., 2001b; Elbashir et al., 2001a). However, even these siRNAs may cause the similar interferon response (Bridge et al., 2003; Sledz et al., 2003). In recent reports, the results show that the side effects of siRNA may be caused by selection of target sequence or structure, or by preserving the wild-type sequence around the transcription start site, in particular a C/G sequence at positions -1/+1, and a simple cloning strategy using the Gateway recombination system has been proposed to facilitate this task (Pebernard and Iggo, 2004).

In addition, in order to use RNAi in a tissue-specific manner, and at the same time to overcome the interferon response elicited by long dsRNA, a valuable new experimental system has been developed for tissue-specific RNAi knockdown in mammalian cells and organisms (Shinagawa and Ishii, 2003). In the system, an engineered vector was used to transcribe dsRNA that lacks the sequences needed to export it from the nucleus into the cytosol so as to avoid the interferon response. The expressed dsRNA is sequestered in the nucleus, where it is processed into small interfering RNAs. These siRNAs are then released into the cytosol, where they direct the degradation of target mRNA without eliciting the interferon response. Moreover, in this system, the RNA polymerase II promoter is utilized to express hairpin-type dsRNAs and then to generate the tissue-specific knockdown mice, which couldn't be achieived through expression of siRNA using the RNA polymerase III promoter or the virus promoter (Shinagawa and Ishii, 2003).

Choice of cell lines that lack the interferon response, such as nondifferentiated mouse embryonic cancer cell lines (Billy et al., 2001), and mouse oocytes and preimplantation embryo (Wianny and Zernicka-Goetz, 2000), will be an another option to avoid the cytotoxic response. Thus RNAi can undoubtedly offer the opportunity to study mammalian development and gene regulation in some cell lines with long dsRNA, but with cautious concern about its side effects.

Non-specific targets (off target)

In order to accurately infer gene function, it is essential to determine the specificity of siRNA-mediated RNAi. siRNAs can induce nonspecific effects on protein levels that are siRNA sequence dependent, and partial complementary sequence matches to off-target genes is proposed to result in a micro-RNA-like inhibition of translation (Scacheri et al., 2004). This is consistent with a previous report based on gene expression profiling, showing that siRNAs may cross-react with targets of limited sequence similarity (Jackson et al., 2003). However, a contrasting result showed that siRNA is a highly specific tool for targeted gene knockdown by DNA microarrays to generate gene expression signatures (Semizarov et al., 2003). The main trick may lay in the siRNA design, optimized transfection conditions, and stringent homology searches. Therefore,

the design and selection of an ideal siRNA as the first step of RNAi appears to be critical for RNAi to achieve the necessary specificity and efficiency in mammals.

Practically it might be impossible to assess the specificity of each siRNA by using microarrays, but it would be wise to confirm the phenotype caused by an siRNA using a second, independent siRNA that is against the same target, and/or by rescuing the loss-of-function phenotype through expressing a modified version of the target gene that has silent mutations in the siRNA-target region (as an example of this, such as human calpain and RNAi, I will give some details in the next section of my thesis, see below).

In conclusions, RNAi has been a revolutionary tool in analysis of gene function through the targeting of any transcript. However, there is still a long way to go before the RNAi technology can be applied for medical or therapeutic purposes. Nevertheless, the RNAi field has been developing at a high pace so that it will not be surprising if this issue could be successfully addressed within the near future. There is no doubt that RNAi can be an invaluable research tool to tackle some problems or questions in basic medical research.

III. Functional Dissection of Human Protease μ-Calpain in Cell Migration Using RNAi

3.1 Introduction

Among calpains, a large family of calcium-dependent cysteine proteases, two major isoforms, μ-calpain (calpain 1, CAPN1) and m-calpain (calpain 2, CAPN2), are considered to play a vital role in most of important biological processes as shown in previous literature review. Calpains are involved mainly with cell migration through cleavage of several components of focal adhesions, such as talin, paxillin and FAK (Rock et al., 2000; Carragher et al., 2001; Carragher and Frame, 2002). However, the relevant *in vivo* substrates for calpain's effects on cell motility have not been well defined, even though calpains have many target molecules found in adhesion complex (Glading et al., 2002; Carragher et al., 1999). In addition, many functional studies are based on combined effects of μ- calpain and m-calpain, the specific contribution of μ- calpain isoform to focal adhesion dissociation and cell migration remains poorly understood. Inhibition of calpain activity by different pharmacological inhibitors (Dedieu et al., 2004), and calpastatin, the endogenous inhibitor of calpains (Balcerzak et al., 1998; Dedieu et al., 2004; Temm-Grove et al., 1999) or antisense oligodeoxyribonucleotides (Balcerzak et al., 1995) argues for the isoform-specific involvement of calpains. Because μ- and m-calpains seem to have different distribution patterns in multiple subcellular locations (Lane et al., 1992), they might contribute to cell migration in their distinct extents. One major hindrance is the shortage of commonly used isoform-specific inhibitors for calpains (Donkor, 2000; Wang and Yuen, 1994; Wang and Yuen, 1997).

Therefore, it is necessary to further understand the roles of each calpain in cell motility, especially μ-calpain, whose requirement of Ca^{2+} concentration for activation by its autolyzed active form is much closer to the physiological condition than that of m-calpain on the basis of *in vitro* studies (Goll et al., 2003). To understand the isoform-specific contribution of calpains to cell mobility, I mainly focused on the dissection of function of μ-calpain using RNAi technology, which has proven to be of great potential in discerning distinct functions of each member, especially a particular isoform, in a closely related gene family (Miller et al., 2004; Zhang et al., 2003).

3.2 Experimental Procedures

3.2.1 Materials and Cell Lines

Mouse anti-μ-Calpain monoclonal antibody was from Affinity BioReagents (Golden, CO). Rabbit anti-m-Calpain (Domain III), mouse anti-Vinculin (clone VIN-11-5), mouse anti-Talin (clone 8D4) and mouse anti-Actin (clone AC-74) antibodies were purchased from Sigma (St. Louis, MO). Mouse anti-Filamin A monoclonal antibody (clone TI10, PM6/317) was obtained from Chemicon (Temecula, CA). Mouse and rabbit anti-IgG-horseradish peroxidase conjugated polyclonal antibodies were obtained from Bio-Rad (Hercules, CA). HRP-labeled proteins were visualized using ECL chemiluminescence reagent from PerkinElmer Life Science Inc (Boston, MA) according to the manufacturer's instructions. Protease inhibitor cocktail without EDTA was purchased from Roche Diagnostics (Indianapolis, IN). MCF-7 cells (Human breast cancer cells) and Hela cells (Human cervical cancer cell line) were from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37° C in a 5% CO_2 atmosphere. Fibronectin from human plasma was purchased from Sigma.

3.2.2 Plasmid Construction for Protein Expression in Mammalian Cells

Full-length cDNAs for silent mutants of μ-calpain carrying six mutant sites within the siRNA targeting sequence were generated by PCR-based site-directed mutagenesis from a human brain cDNA library using Pfu DNA polymerase. The DNA fragments were then cloned into the mammalian expression vector pCDNA3.1 (Invitrogen) at the NheI and XhoI sites to yield pCDNA3.1/μCalpain-re.

3.2.3 RNA Interference

The vector-based RNAi constructs were built in the plasmid pSilencer™ 3.1-H1 (Ambion, Austin, TX). Four siRNAs were designed using the siRNA target finder program from the Ambion Inc web page (www.ambion.com/techlib/misc/siRNA_finder.html) and compared using BLAST to the appropriate human genome database to eliminate from consideration any target sequence with more than 16 contiguous base pairs of homology to other coding sequences. SiRNAs were then

based on internal stability profile (Khvorova et al., 2003; Reynolds et al., 2004). For the RNAi experiments, two complementary oligonucleotides encode a hairpin structure with two 19-mer-reverse and complementary stems separated by a 9-mer loop. The 5'-end of each oligonucleotide forms BamHI and HindIII restriction site overhangs to facilitate directional cloning into the expression vector. siRNA templates were then annealed and ligated into pSilencer™ 3.1-H1 at the HindIII and BamHI sites using a pSilencer™ siRNA Construction Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Generated constructs of pSilencer™ 3.1-H1/siRNA2 and control pSilencer™ 3.1 H1/siRNA4 were then transfected into MCF-7 cells and selected.

3.2.4 Transfection and Immunoblot Analysis

Transient and/or stable transfections of cells with DNAs were carried out with an optimal ratio of DNAs and liposome using Lipofectamine 2000 (Life Technologies, Inc.) as described in the manufacturer's protocol. For stable cell line constructions, cell lines were isolated with 300 μg /ml of hygromycin selection for three weeks after transfection. For immunoblot analysis, monolayer cells were washed twice with cold phosphate-buffered saline (PBS), and lysed in lysis buffer (50 mM Hepes, pH7.4, 150 mM sodium chloride and 1% Triton X-100) containing an EDTA-free protease inhibitor cocktail. The samples were centrifuged at 13,000 rpm for 5 min at 4°C to remove insoluble materials. Total protein was quantified using the Bio-Rad Protein Assay kit. The proteins were blotted onto nitrocellulose membranes after separation by SDS-PAGE gel. The membranes were blocked overnight with 5% milk in Tris-buffered saline (TBS) and incubated for 2 h at room temperature with primary antibody. After washing four times with TBS containing 0.05% Tween-20 (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and then washed four times with TBS-T. The blots were developed using the ECL chemiluminescence reagent kit according to the manufacturer's instructions.

3.2.5 RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted using RNeasy Midi Kit (Qiagen). Thirty micrograms of total RNA from each cell line were electrophoresed in a 1.2%

agarose/formaldehyde gel, transferred to a nylon membrane by upward capillary transfer, and crosslinked by a UV Stratalinker 2400 (Stratagene). The membrane was hybridized overnight at 42° with a cDNA probe that was labeled with ^{32}P by random priming using Ready·To·Go™ DNA Labeling Beads (Amersham Biosciences), in ULTRAhyb™ Hybridization Buffer (Ambion) according to the manufacturer's specifications. The membrane was washed for 2 x 5 min in 2 x SSC- 0.1% SDS at 42°C, then washed for 2 x 15 min in 0.1 x SSC-0.1% SDS at 42°C and autoradiographed.

3.2.6 Casein Zymography

μ-Calpain activity in cell extracts was analyzed by casein zymography in a standard Tris-glycine system (Arthur and Mykles, 2000). Briefly, cultured cells were washed and scraped into lysis buffer (50 mM Hepes, pH 7.6, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 10 mM β-mercaptoethanol, 100 μM PMSF, 10 μg/ml leupeptin). After centrifugation at 12,000g for 5 min at 4°C to remove any insoluble material, 30 μg of protein was run on a non-denaturing acrylamide gel containing casein (0.2%, w/v) at 125 v for 3 h. The gel was then removed and incubated overnight at room temperature with 5 mM Ca^{2+} and 10 mM β-mercaptoethanol in 25 mM Tris-HCl (pH 7.6), followed by fixation and staining with Coomassie Brilliant Blue R250. As a control, an identical gel was treated with incubation buffer containing 5 mM EDTA in place of Ca^{2+}. Protease activity was detected as a clear band in the dark blue background after destaining the gel.

3.2.7 Cell Growth Assay

Cell counting was carried out by plating cells at a density of 8×10^4 cells per 100-mm plate. At indicated times, cells were trypsinized and counted by a hemocytometer using trypan blue staining. At least three counts were taken for each sample and the average was applied for calculating the cell number.

3.2.8 Wound Healing Assay

The ability of cells to migrate in monolayer cultures was assessed by a wound healing assay. Prior to the assay, 6-well dishes were pre-coated with 1.3 μg/ml of

fibronectin in PBS overnight at 4°C. Cells were seeded and cultured to 90% confluency. On the day of the assay, a line was drawn with a marker on the bottom of the dish. Two separate wounds were scratched with a sterile yellow pipette tip through the cells moving perpendicular to the line drawn. The cells were then rinsed once with PBS to remove any cellular debris and replaced with new medium containing 10% of FBS. Photographs were taken just above and just below each line marker immediately after wounding and at intervals up to 36 h. The experiments were repeated in duplicate wells at least three times.

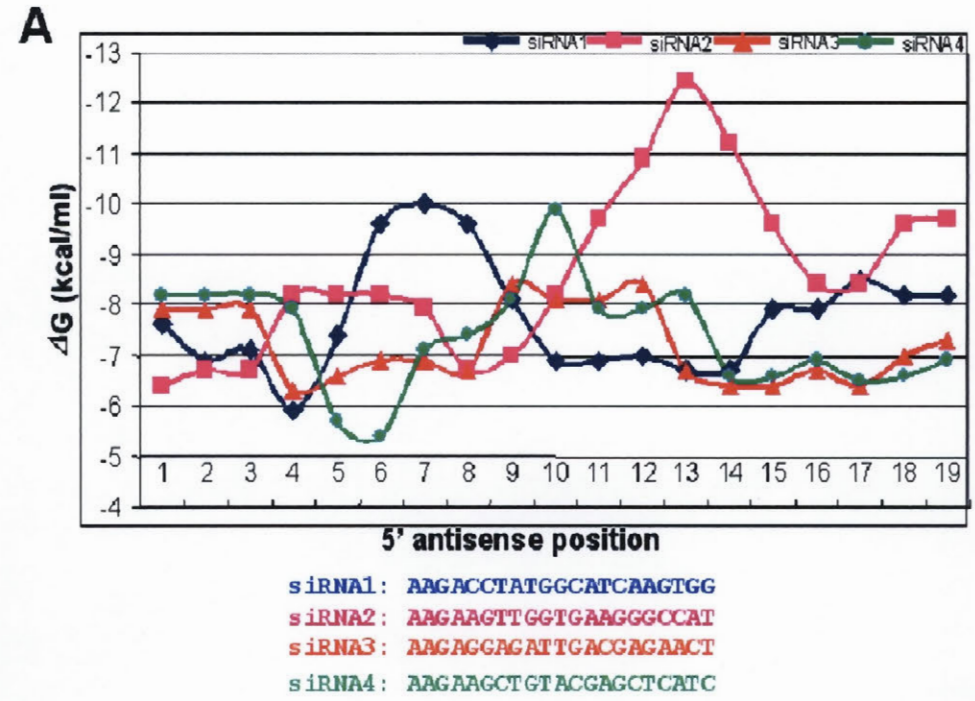
3.2.9 Time Lapse Videomicroscopy

Cells were plated at 15% density onto a glass Delta T dish (0.15 mm; Bioptechs, Inc. Butler, PA) coated with fibronectin (1.3 μg/ml) and allowed to culture overnight at 37 °C under 5% CO_2. The dish was partitioned into three compartments to test three different conditions. On the day of the assay, the cell dish was placed directly onto a heated stage after replacing the medium with CO_2-independent L15 medium. A Leica DMIRB inverted microscope with motorized Ludl hardware was used to capture individual focused images for 10 h at 10-min intervals on a CCD camera (Qimaging.) attached to a PowerMac G5 computer (Apple, Cuperttino, CA). Image capture and processing were done using Openlab Software Version 3 (Improvision). Cell motility was assessed by drawing a rectangular Region Of Interest (ROI) encompassing each cell at time 0. The time lapse movie for each stage position was played and each cell was scored for motility according to an arbitrary criterion: if most of a cell's area exited the ROI at any time, the cell was scored as motile.

3.3 Results

3.3.1 Screening of Functional siRNAs for Knockdown of Human μ-Calpain

In order to dissect the particular role of μ-calpain in cell migration, I used small interfering RNAs to disrupt μ-calpain expression. The designed siRNAs were screened by thermodynamic profiling (Khvorova et al., 2003) (Fig. 3.1A) and verified by transient transfection of siRNAs into Hela cells followed by Western blot analysis (Fig. 3.2B). The results showed that two of these siRNAs, siRNA1 and siRNA2, knocked down about 60% of μ-calpain expression. The specificity of knockdown of μ-calpain was also examined and showed the level of m-calpain remains constant. siRNA2 displayed a lower internal strand stability (approximately –6.2 kcal/mol) at the 5'-anti-sense (5'-AS) terminus, while siRNA1 was enriched with molecules that had lower internal strand stability (approximately –7.6 kcal/mol) at position 9-14 (counting from the 5'-AS end). siRNA3 and siRNA4 did not meet these two criteria and had no effect on silencing of μ-calpain. siRNA2 was selected for later study and named μ-calpain siRNA, whereas non-functional siRNA4 was used as negative control siRNA.



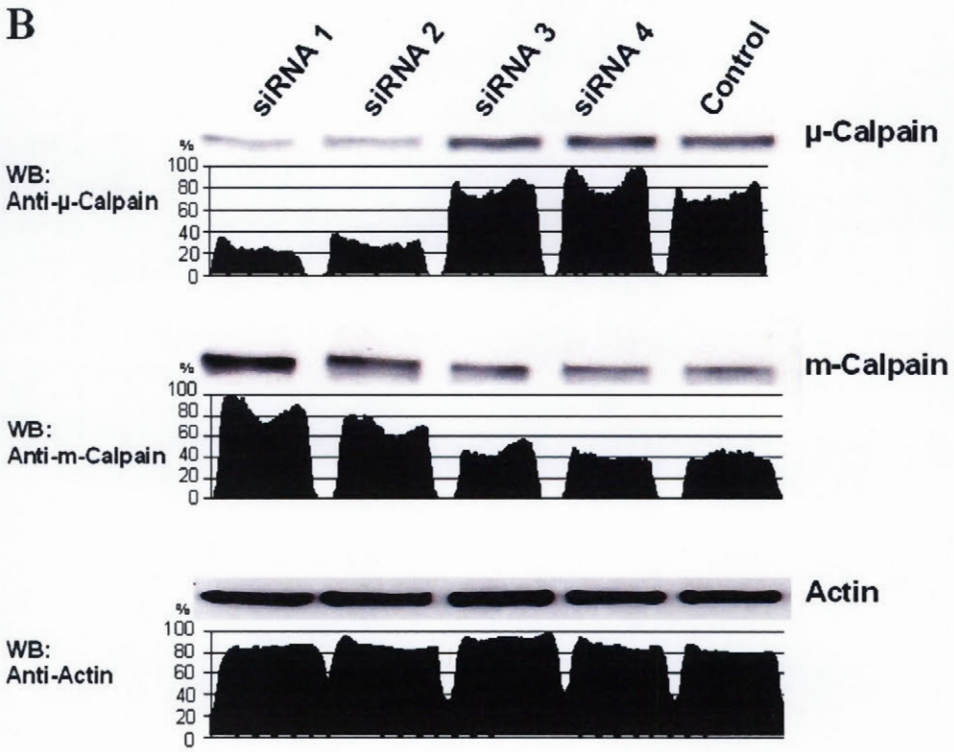


Fig. 3.1. Screening of functional siRNAs for knockdown of human μ-calpain. (A) Characterization of four siRNA duplexes targeting human μ-calpain using oligo internal stability profile. Internal strand thermodynamic stabilities ⊿G (-kcal/mol) at the 5'-AS (anti-sense) end were calculated using the free energy values established by Freier et al.

and according to the nearest neighbor method (Freier et al., 1986). To simplify calculations, the average internal stability values of the terminal four bases on the 3'-end of the molecule are not presented here. Shown are siRNA1 (♦), siRNA2 (■), siRNA3 (▲), siRNA4 (•). siRNA2 was chosen for the later study. siRNA4 was used as a non-functional control. (B) Western blot analysis of siRNA-transfected Hela cells. The siRNAs were synthesized *in vitro* by T7 polymerase (Ambion Inc.) and transiently transfected into Hela cells. 48 h after transfection, cells were lysed and whole cell lysates were subjected to Western blot with anti-μ-Calpain, anti-m-Calpain and anti-Actin antibodies, respectively. Quantitation of each band was performed by densitometry analysis with Scion Image software.

3.3.2 Activity of μ-Calpain is Decreased in MCF-7 Cells Constitutivly Expressing μ-Calpain siRNA

Previous research indicated that μ-calpain is a long-lived protein with a metabolic half-life of approximately 5 days (Zhang et al., 1996). In order to maximize the knockdown efficiency for functional assays of μ-calpain on cell migration, stable cell lines persistently expressing siRNAs were made in MCF-7 cells that have a significant level of μ-calpain (Tagliarino et al., 2003). The p*Silencer*™ 3.1-H1 vector with a hygromycin selection marker facilitated the establishing of a μ-calpain silencing cell line by constitutive expression of hairpin siRNAs (Fig. 3.2A).

The effectiveness of selected sequence-specific hairpin siRNAs was examined for steady knockdown of μ-calpain by Western blot analysis. μ-Calpain expression in MCF-7 cells stably transfected with the μ-calpain siRNA-expressing plasmid was significantly inhibited to 70-80% measured densitometrically compared to that in cells transfected with the control siRNA expressing plasmid (Fig. 3.2B). Northern blot analysis further verified the disruption of μ-calpain mRNA as the result of RNA interference (Fig. 3.2C). Casein zymography demonstrated that μ-calpain activity was lower in the μ-calpain siRNA-expressing stable cell line in comparison with the control siRNA-expressing stable cell line and the wildtype MCF cells (Fig. 3.2D). Thus, silencing of μ-calpain leads to substantial inhibition of its expression and activity in the MCF-7 stable cell line.

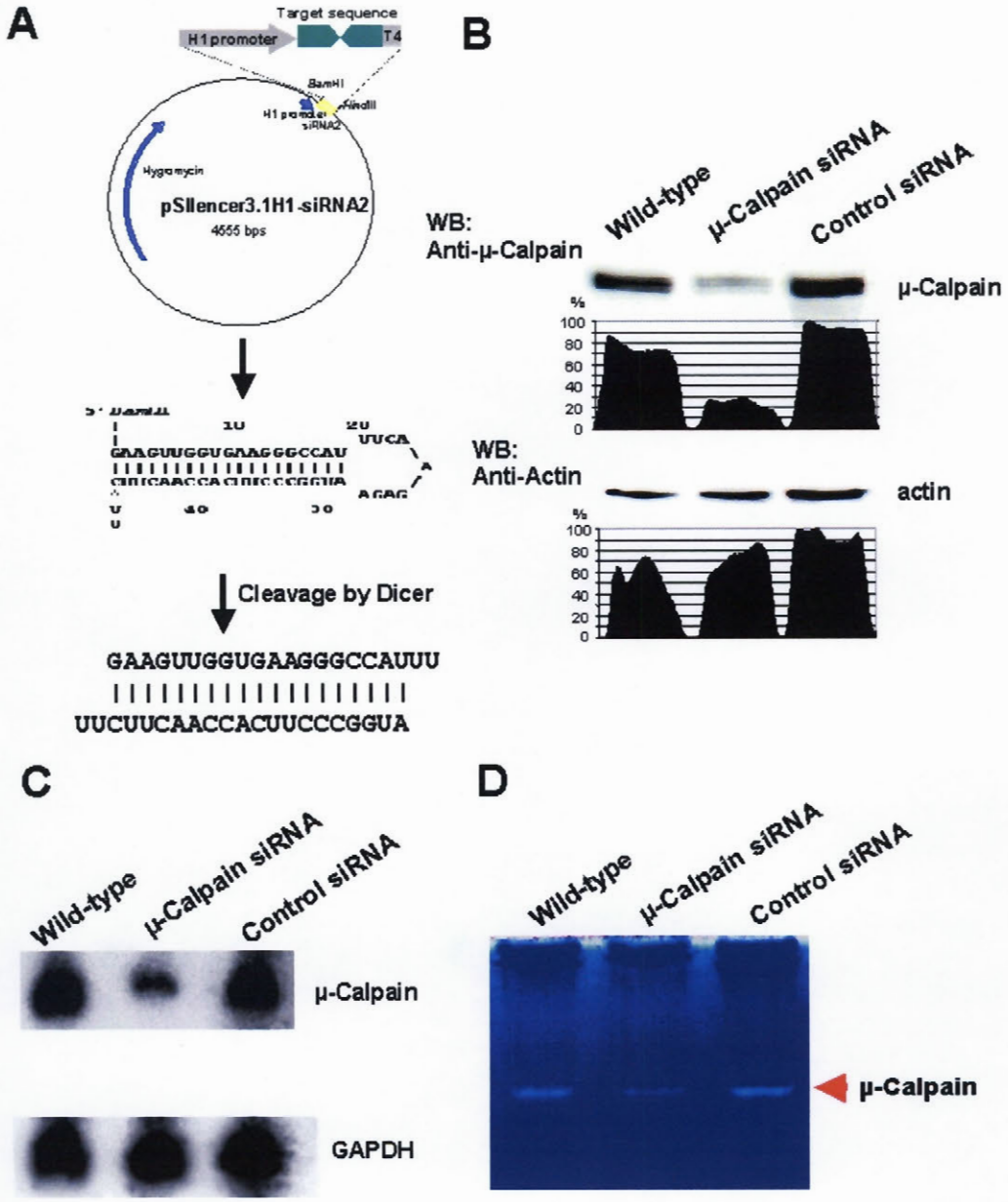


Fig. 3.2. Sequence-specific hairpin siRNA knocks down μ-calpain expression in stably transfected MCF-7 cells. (A) Schematic representation of μ-calpain siRNA2 expression from p*Silencer*™ 3.1-H1 vector. *BamH*I and *Hind*III sites were designed for cloning of hairpin siRNA templates. The H1 promoter drives the transcription of short hairpin RNAs, which, in turn, were recognized by an RNase III family nuclease, Dicer, to process into a 21-nt siRNA2 within the cells. Control hairpin siRNA4 was processed in the same way. (B) Western blot analysis of siRNA-stably transfected MCF-7 cells. Total proteins extracted from wild-type MCF-7 cells and transfectants that were stably transfected with p*Silencer*™ 3.1-H1/siRNA2 (μ-calpain siRNA-expressing stable cell line) or p*Silencer*™ 3.1-H1/siRNA4 (control siRNA-expressing stable cell line) were

subjected to Western blotting with anti-μ-Calpain and anti-Actin antibodies, respectively. Quantitation of each band was performed by densitometry analysis with Scion Image software. (C) Northern blot analysis of total RNAs extracted from MCF-7 cells, μ-calpain siRNA-expressing and control siRNA-expressing stable cell lines. The DNA probes for μ-calpain and GAPDH control were prepared as described under "Experimental Procedures". (D) Casein zymogram. 30 μg of cell lysates from wildtype, μ-calpain siRNA-expressing or control siRNA–expressing stable cell lines were run on a native acrylamide gel containing casein, followed by incubation, fixation and staining as described under "Experimental Procedures".

3.3.3 Knockdown of μ-Calpain Reduces Cell Migration and Alters Cell Morphology

To determine whether specific silencing of μ-calpain is able to affect normal cell migration, I examined the morphology and migration by a wound healing assay and light microscopy analysis on 1.3 μg/ml of fibronectin that confers an intermediate cell-substratum adhesiveness to maximize cell migration (Huttenlocher et al., 1996; Palecek et al., 1997). As shown in Fig. 3.3A, μ-calpain siRNA-expressing stable cells displayed a 45-50% reduced migration rate in comparison with that of control siRNA-expressing stable cells. In order to exclude the possibility that different cell growth rates might contribute to the observed phenotype, I counted cell numbers at individual time points under normal cell culture conditions. The result showed that the growth rates of these two stable cells were basically the same (Fig. 3.3B). Moreover, compared to the control, 8-fold more of the μ-calpain siRNA-expressing stable cells displayed filopodial projections and a highly elongated tail that seemed to prevent cell spreading and migration with reduced rear detachment ability (Fig. 3.3C and data not shown). Together, the results revealed that decreased μ-calpain activity is related to a reduced migration rate and altered morphology of MCF-7 cells.

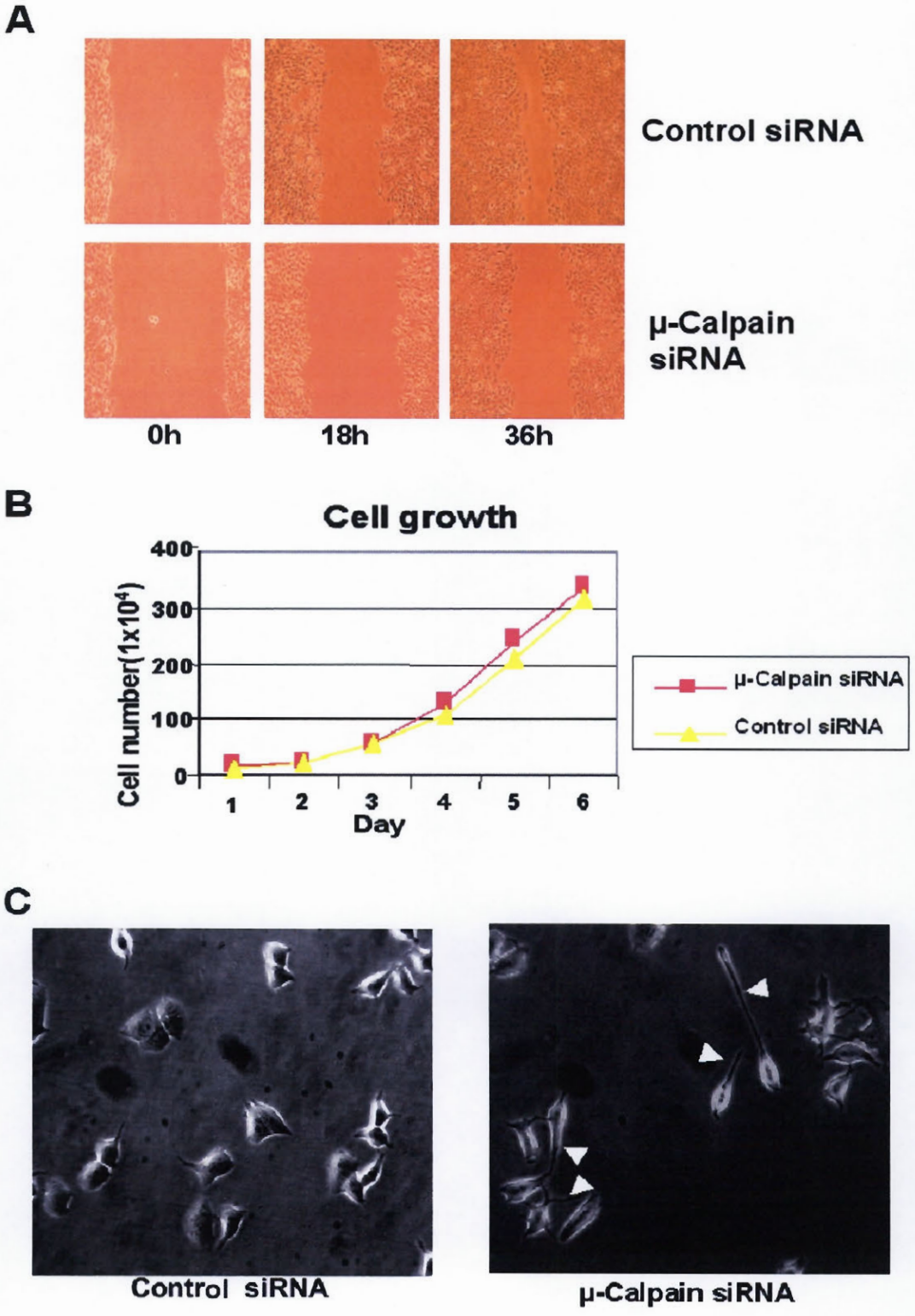


Fig. 3.3. Knockdown of μ-calpain reduces cell migration and causes cell morphological changes in stably transfected MCF-7 cells. (A) μ-Calpain siRNA-expresing stable cell line and control siRNA-expressing stable cell line were seeded on

six-well tissue culture dishes and cultured to confluent cell monolayers, which were then wounded as described under "Experimental Procedures" and photographed at indicated intervals. (B) μ-Calpain siRNA-expressing stable cell line and control siRNA-expressing stable cell line were seeded on 100-mm dishes at a density of 8 $x10^4$ cells/dish. Viable cells were then counted at the time indicated. (C) μ-Calpain siRNA-expressing and control siRNA-expressing stable cell lines were plated on 1.3 μg/ml fibronectin at 10% density and incubated at 37 °C under 5% CO_2. Phase contrast images were acquired 16 h later using a 32x objective. The arrows highlight extended filopodia and/or elongated tail.

3.3.4 Rescue of μ-Calpain Restores its Expression and Activity Resulting in Normal Cell Motility and Morphology

To confirm that the phenotypes observed in the μ-calpain siRNA-expressing stable cell line are the direct consequence of knockdown of targeted μ-calpain, I carried out a rescue experiment by overexpressing wild-type μ-calpain. In order to avoid the overexpressed μ-calpain being targeted by the existing siRNA in the established μ-calpain siRNA-expressing stable cell line, a six-site silent mutation was made at the siRNA targeting sequence of μCalpain-wildtype (μCalpain-*wt*) to generate μCalpain-rescue (μCalpain-*re*) (Fig. 3.3A). Since the changed codons were selected based on the maximum frequency of codon usage, I anticipated a successful rescue of μ-calpain that could function normally. As expected, a highly elevated expression level of μ-calpain was achieved by ectopic transfection of pCDNA3.1/μCalpain-*re* into the μ-calpain siRNA-expressing stable cell line, with an empty vector transfection as the control (Fig. 3.4B). The restoration of μ-calpain activity was also verified by casein zymography, showing that μ-calpain activity was fully recovered and even higher than that in wildtype MCF-7 cells (Fig. 3.4C). Taken together, rescue of μ-calpain restores a normal level of activity and can be further applied to analyze reverted phenotypes.

To test whether the revertant could reinstate μ-calpain-related migration and morphology, pCDNA3.1/μCalpain-*re* and the empty vector were transfected into μ-calpain siRNA-expressing stable cell lines that were then replated on fibronectin at a low density to facilitate cell spreading and migration. Only approximately 20-25% of pCDNA3.1/ μCalpain-*re*-transfected cells retained an elongated morphology with delayed retraction of membrane projections, whereas the phenotype in the control vector transfection was essentially the same as that of μ-calpain siRNA-expressing stable cell

line displayed in Fig.3.3C (Fig. 3.4D and data not shown). The increase of migration rate as a direct consequence of μ-calpain replacement was demonstrated by the wound healing assay when pCDNA3.1/μCalpain-*re* was co-transfected with pFRED/GFP into confluent μ-calpain siRNA-expressing stable cells (Fig. 3.4E). The motile/static assay by time lapse videomicroscopy also demonstrated an ~75 % increase of migration rate in pCDNA3.1/μCalpain-*re*-transfected cells compared to the control (Fig. 3.4F). Therefore, MCF-7 cells lacking μ-calpain show a notable reduction of the ability in cell migration and translocation, and the restored expression of μ-calpain re-establishes the activity.

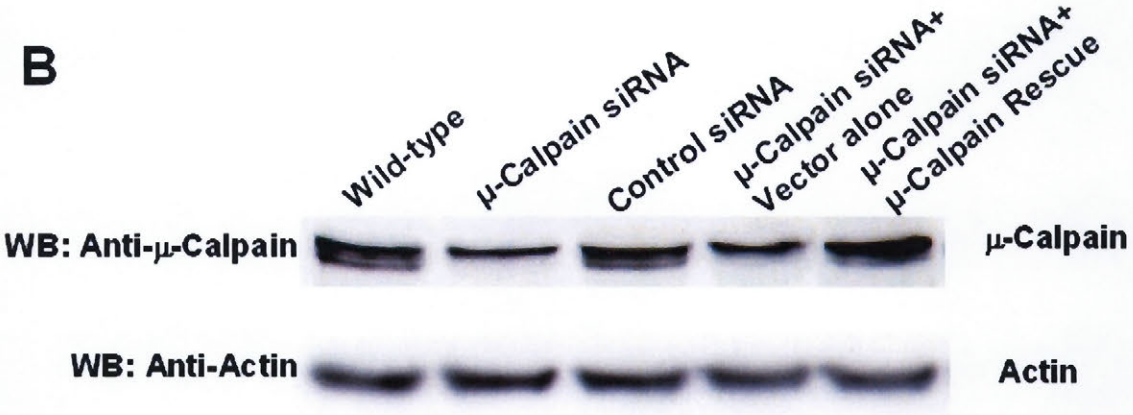
3.3.5 Knockdown of μ-Calpain Increases the Stability of Intracellular Filamin and Talin

It has been demonstrated that integrin-mediated signals target calpain to focal adhesion complexes, in which some components have been identified as possible calpain substrates *in vitro*. However, the relevant substrates *in vivo* for calpain-dependent adhesion turnover, cell rear detachment and subsequent cell movement remain unclear.

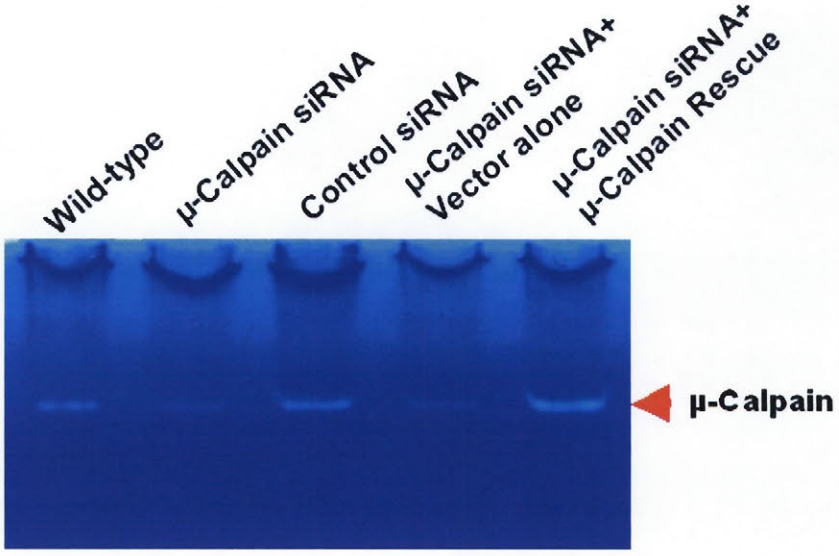
A


μCalpain-wt : GAAGTTGGTGAAGGGCCAT
μCalpain-re : GAAACTGGTTAAAGGTCAC
Lys Leu Val Lys Gly His

B


Wild-type
μ-Calpain siRNA
Control siRNA
μ-Calpain siRNA+ Vector alone
μ-Calpain siRNA+ μ-Calpain Rescue
WB: Anti-μ-Calpain
μ-Calpain
WB: Anti-Actin
Actin

C


Wild-type
μ-Calpain siRNA
Control siRNA
μ-Calpain siRNA+ Vector alone
μ-Calpain siRNA+ μ-Calpain Rescue
μ-Calpain

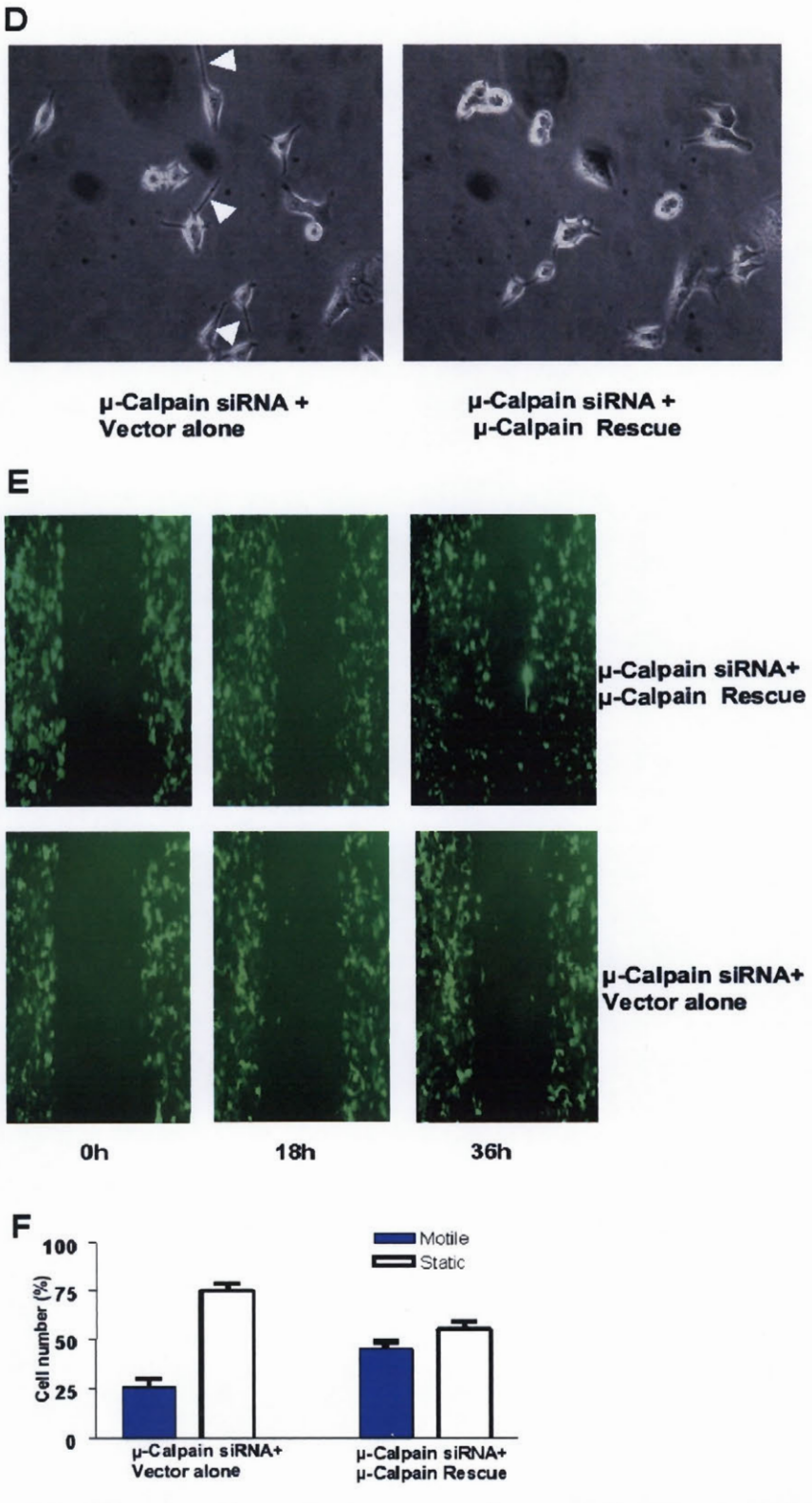


Fig. 3.4. Rescue of μ-calpain expression results in normal cell motility and morphology. (A) A six-site silent mutation of nucleotides was made in the siRNA targeting sequence of μ-calpain-wildtype (μCalpain-*wt*) to generate μ-calpain-rescue

(μCalpain-*re*). (B) μ-Calpain siRNA-expressing stable cell line was transiently transfected with pCDNA3.1 and pCDNA3.1/μCalpain-*re* respectively. Cells were lysed three days after transfection and 30 μg of total protein extract was then subjected to Western blotting with anti-μ-Calpain antibody and anti-Actin antibody respectively. (C) Casein zymogram. μ-Calpain siRNA-expressing stable cell line was transiently transfected with pCDNA3.1 and pCDNA3.1/μCalpain-*re* respectively. 72 h post transfection, 30 μg of cell lysates from each cell line were subjected to a native acrylamide gel containing casein, followed by incubation, fixation and staining as described under "Experimental Procedures". (D) μ-Calpain siRNA-expressing stable cells transfected with pCDNA3.1/μCalpain-*re* or empty vector were plated on 1.3 μg/ml of fibronectin at 10% density and incubated at 37°C under 5% CO_2. Phase contrast images were acquired 16 h later using a 32x objective. The arrows highlight extended filopodia and/or elongated tail. (E) Confluent μ-calpain siRNA-expressing stable cells co-transfected with pFRED/GFP and pCDNA3.1/μCalpain-*re* at 90% transfection efficiency, were carefully scratched 24 h post-transfection in six-well culture dishes. 0, 18, 36 h after wounding, green fluorescent photographs were taken at the same spot. μ-Calpain siRNA-expressing stable cell line co-transfected with pFRED/GFP and pCDNA3.1 was the control. (F) Prior to videotaping by time lapse videomicroscope, pCDNA3.1/μCalpain-*re*-transfected μ-calpain siRNA-expressing stable cell line and pCDNA3.1vector-transfected one were allowed to adhere on dishes coated with 1.3 μg/ml fibronectin for overnight to 20% confluency at 37 °C under 5% CO_2. Cell dishes were then placed directly onto a heated stage and supplemented with 5% CO_2 to maintain the pH. At least 105 cells in each sample were subjected to the motile and static assay. The filled columns represent the percentage of motile cells, and the open columns represent the percentage of static cells. Each column with an error bar represents the mean ± S.D.

In an attempt to clarify potential substrates involved in μ-calpain-regulated migration, the expression level and proteolysis of several focal adhesion components including filamin A, talin and vinculin were studied. As shown in Fig. 3.5, native filamin A (280 kDa) was cleaved giving rise to a 190-kDa fragment (N-terminus) and a 90-kDa fragment (C-terminus) in a control siRNA-expressing stable cell line but to a lesser extent for the 190-kDa fragment and hardly at all for the 90-kDa fragment in μ-calpain siRNA-expressing stable cell line. Similarly, intact talin (230 kDa) also appeared to be proteolyzed producing a 190-kDa fragment that was observed in the control siRNA-expressing stable cell line and less detected in μ-calpain siRNA-expressing stable cell line. Both the 190-kDa and 90-kDa cleavage fragments of filamin A and the 190-kDa proteolytic product of talin are identical in size to the previously identified calpain-mediated cleavage products of filamin (Fox et al., 1985) and talin *in vitro* (Schoenwaelder et al., 1997; Tranqui and Block, 1995).

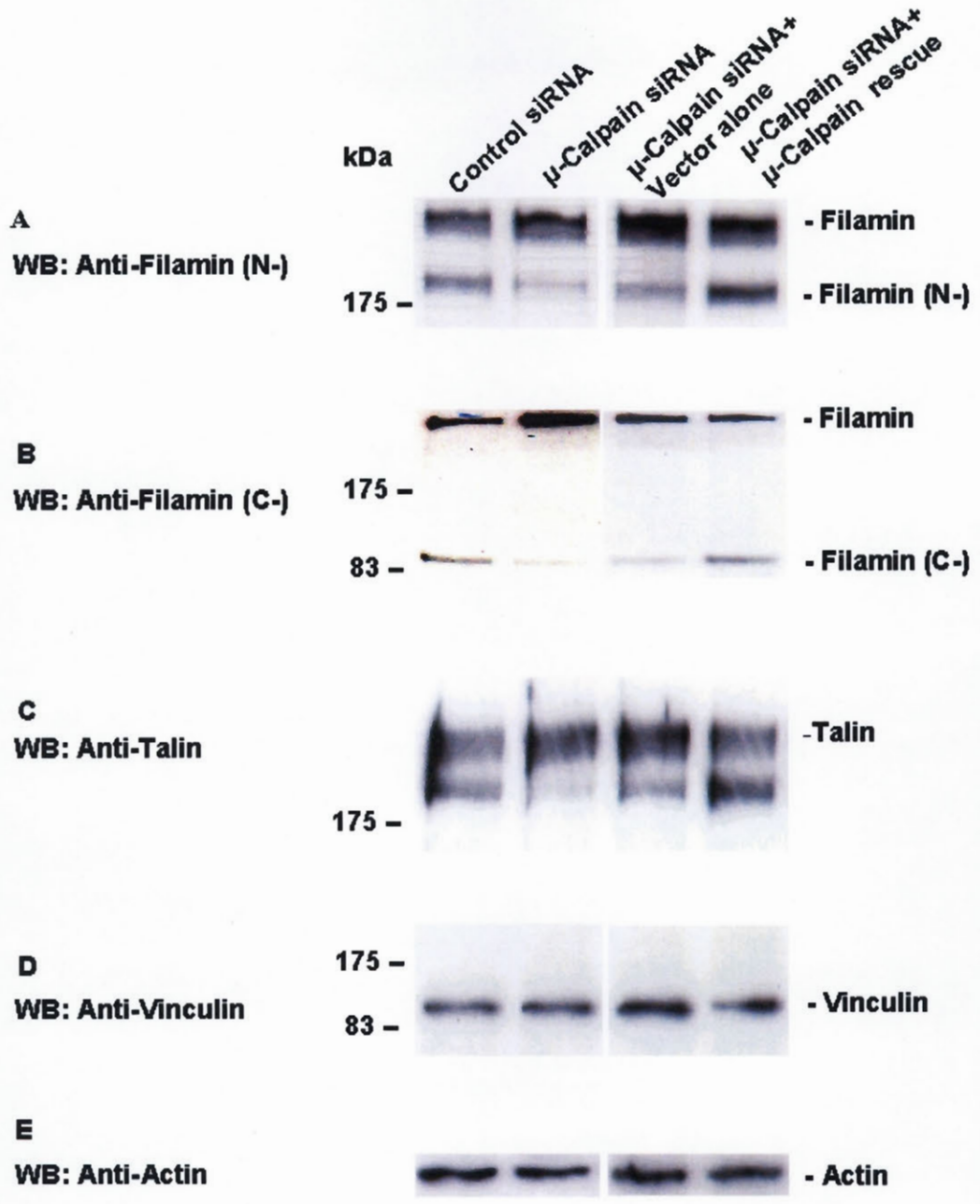


Fig. 3.5. Knockdown of μ-calpain increases the stability of intracellular filamin and talin. μ-Calpain siRNA-expressing cell line was transiently transfected with pCDNA3.1/μCalpain-*re* or vector pCDNA3.1. 24 h post-transfection, cells were detached and replated at a low density on dishes coated with 1.3 μg/ml of fibronectin. After an additional 24 h incubation, cells were processed for Western blot analysis with anti-Filamin A (N-terminal) (A), anti-Filamin A (C-terminal) (B), and anti-Talin (C), anti-Vinculin (D), anti-Actin (E) antibodies, respectively. Molecular mass (kDa) is shown on the left of the gels.

In accordance with the full recovery of μ-calpain activity, improved cleavage of filamin and talin were seen in the μ-calpain rescue cells, indicating these cells have regained a high level of μ-calpain activity. In either cell type the level of vinculin was unchanged. Thus, these results suggest that knockdown of μ-calpain increases the intracellular stability of filamin and talin. Cleavage of these two adhesion components *in vivo* by μ-calpain may represent the central mechanism by which μ-calpain regulates cell motility.

3.4 Discussion

The major finding of this study is that I functionally dissected the particular role of μ-calpain in cell motility and cytoskeleton remodeling by employing RNAi technology. Four observations have been achieved in my study. First, RNAi technology allowed us to sequence-specifically silence μ-calpain without applying the conventional calpain inhibitors that give non-specificity to m- and μ-calpain, these two major isoforms of human calpain family. Second, knockdown of μ-calpain significantly reduced cell migration on 1.3ug/ml of fibronectin, an intermediate cell-substratum that permits maximal cell migration. Third, a cell morphological change with a high frequency of lamellipodal extension and retraction in the μ-calpain knockdown cell line suggested the decreased detachment rate on the cell rear correlated with less movement of the cell body. Finally, μ-calpain played an important role in focal adhesion disassembly and reorganization of the actin cytoskeleton that is a requisite for integrin-mediated cell motility. This occurred through the cleavage of filamin A and talin in migrating MCF-7 cells. To my knowledge, this is the first report demonstrating that proteolysis of filamin *in vivo* is involved in calpain-regulated cell locomotion, although reports indicated previously that cleavage of filamin by calpain *in vivo* is associated with blood coagulation and platelet physiology (Azam et al., 2001; Fox et al., 1985; Glading et al., 2002).

Based on previous *in vitro* experiments, it is anticipated that calpains are activated by intracellular Ca^{2+} fluxes with 5-50 μM for μ-calpain and 0.2-1mM for m-calpain (Strobl et al., 2000). But these Ca^{2+}-release events are physiologically unachieved under normal cell homeostasis where free Ca^{2+} concentrations range from 50-500 nM (Thompson et al., 2000), except some exclusive conditions such as cell death (Lemasters et al., 1998; Thomas et al., 1996). So the *in vitro* findings according to the variation on the activation requirement between these two isoforms could be significantly different from what their actual behavior and function *in vivo*. On the other hand, although they cleave most substrates in a similar manner, it may also be possible that the activation process and favorable substrates *in vivo* are different since the major distinction between μ- and m- calpains is their Ca^{2+} sensitivity *in vitro*.

Spatial patterns of the ubiquitous isoforms are probably divergent in multiple subcellular locations (Lane et al., 1992; Moraczewski et al., 1996). This brings out the consideration on the spatial nature of calpain activation, which may implicate different cellular response and molecular regulation on cell migration by two isoforms (Glading et al., 2002). Moreover, m- and μ-calpain are found to affect cell motility in an opposing fashion showing inhibition of m-calpain in fibroblats and activation of μ-calpain in undifferentiated keratinocytes, in a single wound healing event (Satish et al., 2003). Since calpain-regulated cell adhesion and migration has long linked to pathophysiological issues in tumor metastasis, wound healing and the immune and inflammatory responses, functional dissection of individual isoforms and specific therapeutic treatments are even more crucial.

In addition, many human disorders are caused by overactivation of a single isoform of calpain. Lens m-calpain is thought to be over-activated leading to crystalline precipitation and, ultimately, lens opacity (cataract) (Wang and Yuen, 1997; Yoshida et al., 1984). M-calpain can be targeted for limiting prostate cancer invasion (Mamoune et al., 2003). Excessive amounts of m-calpain are also found in arthritic knee joints of mice (Szomor et al., 1995). μ-Calpain overexpression is associated with increased malignancy in human renal cell carcinoma (Braun et al., 1999). However, the lack of potent, selective and cell-permeable calpain inhibitors, at present, makes it impossible to control these situations.

In conclusion, I demonstrate that vector-based RNAi can be a powerful tool to functionally dissect the role of μ-calpain in cell migration. Most importantly, this strategy helps me to better understand the relevant pathological response in a calpain isoform-specific manner, and may provide us an attractive therapeutical approach in treatment of various pathological disorders associated with over-activation of μ- and/or m-calpain, or other particular calpain family members.

IV. Conclusions and Summary

Calpains, calcium-dependent cytosolic cysteine proteases, are implicated in a multitude of cellular functions. Two isoforms, m-calpain and μ-calpain, have been associated with various aspects of carcinogenesis, including cell proliferation, apoptosis and metastasis. However, their particular physiological contributions in cell motility are not well studied. This is partially because of the shortage of isoform-specific inhibitors. On the other hand, RNAi has the potential to allow a sequence-specific destruction of target RNA for functional assays of genes of interest.

Through the study of human μ-calpain using RNAi technology, I found that RNAi has a great potential to replace traditional protease inhibitors in a fashion of isoform-specific or on a therapeutic purpose. Based on the results of μ-calpain silencing by the target-specific siRNA, I observed that μ-calpain alone was able to regulate intergin-mediated cell migration possibly through the proteolytic process of focal adhesion molecules.

In the study of my master research project, I designed four human μ-calpain-specific small interference RNAs (siRNAs) on the basis of empirical rules, and screened for functional siRNAs judged by their internal stability and protein expression profiling. Two out of four *in vitro* synthesized siRNAs were able to significantly knock down μ-calpain expression. Accordingly, I established stable MCF-7 cell line expressing the functional μ-calpain siRNAs. Casein zymography revealed that the enzymatic activity of μ-calpain was reduced in comparison with that of the control siRNA-expressing cells. The cell line with 70-80 % knock down of μ-calpain displayed reduced cell mobility and obvious morphology changes. In addition, knockdown of μ-calpain decreased the proteolytic products of filamin and talin, suggesting that their proteolysis could be one of the key mechanisms by which μ-calpain regulates cell migration.

In conclusion, siRNAs can be used as calpain isoform-specific inhibitors for studying the functions of calcium-dependent calpain and its related intracellular signaling in carcinogenesis, and may have potential for therapeutic use.

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