Regulating cell migration: calpains make the cut

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Summary

The calpain family of proteases has been implicated in cellular processes such as apoptosis, proliferation and cell migration. Calpains are involved in several key aspects of migration, including: adhesion and spreading; detachment of the rear; integrin- and growth-factor-mediated signaling; and membrane protrusion. Our understanding of how calpains are activated and regulated during cell migration has increased as studies have identified roles for calcium and phospholipid binding, autolysis, phosphorylation and inhibition by calpastatin in the modulation of calpain activity. Knockout and knockdown approaches have also contributed significantly to our knowledge of calpain biology, particularly with respect to the specific functions of different calpain isoforms. The mechanisms by which calpain-mediated proteolysis of individual substrates contributes to cell motility have begun to be addressed, and these efforts have revealed roles for proteolysis of specific substrates in integrin activation, adhesion complex turnover and membrane protrusion dynamics. Understanding these mechanisms should provide avenues for novel therapeutic strategies to treat pathological processes such as tumor metastasis and chronic inflammatory disease.

Key words: Calpain, Cell motility, Proteolysis

Introduction

The importance of cell migration is evident from both the physiological processes that depend on the regulated movement of cells, including embryonic development, immune responses and tissue maintenance and repair, and from the disease states driven by aberrant cell motility, such as chronic inflammation, vascular disease and tumor metastasis. Not surprisingly, immense effort has been directed towards furthering our understanding of this complex process. These efforts have provided us with the current concept of cell migration, which comprises a cycle of several highly coordinated and regulated steps (Lauffenburger and Horwitz, 1996; Ridley et al., 2003). In response to various migratory cues, directional movement is initiated by polarization of the cell, as defined by the spatial segregation of molecular machineries that control the different stages of the migratory cycle. At the front of the cell, actin polymerization drives membrane protrusion to form the leading edge. Subsequently, the leading edge is stabilized by attachment to the extracellular matrix (ECM) through integrin-mediated adhesion complexes, which not only link the ECM to the actin cytoskeleton but also function as signal transduction centers that modulate cell migration. Once coupled to adhesion complexes, the actin cytoskeleton can generate the forces necessary to translocate the cell body forward. Finally, adhesive contacts at the rear of the cell must be disassembled to allow detachment of the rear and to complete the migratory cycle.

Because of their involvement in cell motility, integrin-containing adhesion complexes are necessarily dynamic structures that undergo repeated cycles of formation and disassembly (Webb et al., 2002). Likewise, the activities of the actin-based protrusion and contraction machineries must also be continually regulated to ensure proper timing and localization (Rafelski and Theriot, 2004). The calpain family of proteases has been shown to contribute to the control of cell migration through their ability to regulate the dynamics of both integrin-mediated adhesion and actin-based membrane protrusion (Perrin and Huttenlocher, 2002). Although our current understanding of the mechanisms underlying this regulation remains limited, recent studies have begun to shed light on this subject. Here, we discuss recent advances that have provided insight into where calpains fit into the cell migration cycle, how the activities of calpains are modulated, the roles of individual calpain isoforms in motility, and the molecular basis of their effects during directional cell movement.

Calpain family of proteases

The mammalian calpain gene superfamily contains 16 known genes: 14 of these genes encode proteins that contain cysteine protease domains; the other two genes encode smaller regulatory proteins that associate with some of the catalytic subunits to form heterodimeric proteases (Goll et al., 2003; Suzuki et al., 2004). Several calpain isoforms are ubiquitously expressed, whereas many demonstrate tissue-specific expression patterns (Table 1). They are typically thought of as intracellular proteases, although there is some evidence that active calpains are also found in the extracellular space (Nishihara et al., 2001; Xu and Deng, 2004). However, the physiological significance of extracellular calpains is not yet known. Within the cell, the localization patterns of calpains are complex and somewhat variable (Table 2), which means that their subcellular localization might be dynamically regulated

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Table 1. Expression patterns of calpains

Isoform	Pattern	Highest	Lowest	References
Capn1	Ubiquitous	Ac, Dc, Es, Pl, Th, Tr	Te	Farkas et al., 2003
Capn2	Ubiquitous	Ki, Lu, St, Tc, Tr	Bm, Li, Ov	Farkas et al., 2003
Capn3	Ubiquitous	Sm	Ov	Farkas et al., 2003
Capn5	Ubiquitous	Br, Ki, Li, Lu, Te, Tr	He, Sm	Dear et al., 1997; Dear and Boehm, 1999
Capn6	Tissue specific	Pl, Sm	–	Dear et al., 1997; Dear and Boehm, 1999
Capn7	Ubiquitous		–	Franz et al., 1999
Capn8	Tissue specific	Br, Dt, St, Te	He, Ki	Braun et al., 1999; Sorimachi et al., 1993
Capn9	Tissue specific	Dt, He, St	–	Markmann et al., 2005; Sorimachi et al., 1993
Capn10	Ubiquitous	He	–	Horikawa et al., 2000
Capn11	Tissue specific	St, Te	He, Ki	Dear et al., 1999
Capn12	Tissue specific	Hf	–	Dear et al., 2000
Capn13	Tissue specific	Lu, Te	–	Dear and Boehm, 2001
Capn14	Unknown	–	–	Dear and Boehm, 2001
Capn15	Ubiquitous	Br	–	Kamei et al., 1998
CSS1	Ubiquitous	He, Is, Ki, Pa, Pr, Sm, Te	–	Farkas et al., 2003
CSS2	Tissue specific	Bl, Es, Pr, Tr,	–	Farkas et al., 2003; Ma et al., 2004
Calpastatin	Ubiquitous	Is	Ov	Farkas et al., 2003

Abbreviations: Ac, ascending colon; Bl, bladder; Bm, bone marrow; Br, brain; Dc, descending colon; Dt, digestive tract; Es, esophagus; He, heart; Hf, hair follicle; Is, interventricular septum; Ki, kidney; Li, liver; Lu, lung; Ov, ovary; Pa, pancreas; Pl, placenta; Pr, prostate; Sm, skeletal muscle; St, stomach; Tc, transverse colon; Te, testis; Th, thyroid; Tr, trachea.

and constitutes an important factor in the modulation of their functions.

Calpain structure

The enzymatically active (large) calpains each comprise up to four domains (Fig. 1) (Hosfield et al., 1999; Pal et al., 2003; Sorimachi and Suzuki, 2001; Strobl et al., 2000). Domain I is a single α-helix present at the N-terminus of some calpains; it can interact with domain VI of the non-catalytic (small) subunits and may be important for stability. Domain II comprises the protease domain, which contains the active site catalytic triad Cys105, His262 and Asn286. Interestingly, the alignment and spacing of these residues in the inactive molecule is such that catalytic activity is not permitted, indicating that a structural change must take place to activate calpains. Domain III consists of eight β-strands arranged in a β-sandwich configuration similar to that of C2 domains. The C2 domain was first discovered in protein kinase C as a stretch of approximately 130 amino acids that binds phospholipids in a calcium-dependent manner (Newton and Johnson, 1998). Since then, C2-like domains have been identified in nearly 100 proteins, and they are usually involved in binding calcium and phospholipids (Rizo and Sudhof, 1998). Domain III can bind phospholipids in a calcium-dependent fashion (Tompa et al., 2001), which further suggests that it is a C2-like domain. Domains IV and VI in the large and small subunits, respectively, each contain five EF-hand motifs, the fifth EF hand from each subunit interacting with the other to assemble heterodimers (Blanchard et al., 1997; Hosfield et al., 1999; Lin et al., 1997). Domain V of the small subunits appears to have a very flexible structure as a consequence of being glycine rich, which is probably why, unlike the other domains, it remains unresolved by crystallography.

Calpain regulation

Calpain activity is highly regulated in vivo by multiple mechanisms (Fig. 2A), although the details are only now beginning to be defined. The best-studied mechanism is activation by calcium – hence the name calpain (Guroff, 1964). In fact, calpain 1 and calpain 2 are commonly referred to by their in vitro requirements for calcium: calpain 1 (μ-calpain) is activated by micromolar calcium concentrations and calpain 2 (m-calpain) requires millimolar concentrations (Suzuki et al., 1981b). Because calpains contain calcium-binding EF-hand motifs in domains IV and VI and, because domain IV of calpain 1 and domain IV of calpain 2 are different, these were originally presumed to be responsible for the calcium-dependent activation of calpains. However, structural data suggest that conformational changes caused by calcium occupancy of the EF hands alone are insufficient to align the active site catalytic residues properly. Furthermore, functional

Table 2. Localization of calpains

Localization	Isoform	References
Adhesion complexes	Capn2	Beckerle et al., 1987
Caveolae	Capn2	Kifor et al., 2003
Diffuse cytoplasmic	Capn1, 2, 5, 7, 10, CSS1	Gafni et al., 2004; Gil-Parrado et al., 2003; Lane et al., 1992; Raynaud et al., 2004
Endoplasmic reticulum	Capn1, 2, CSS1	Hood et al., 2004; Hood et al., 2003; Sakai et al., 1989
Extracellular	Capn1, 2	Nishihara et al., 2001; Raynaud et al., 2004; Xu and Deng, 2004
Golgi	Capn1, 2, CSS1	Hood et al., 2004; Hood et al., 2003
Lipid rafts	Capn2	Morford et al., 2002; P. Nuzzi and A.H., unpublished data
Nuclear	Capn1, 2, 5, 7, 10, CSS1	Gafni et al., 2004; Gil-Parrado et al., 2003; Mellgren and Lu, 1994; Raynaud et al., 2004; Tremper-Wells and Vallano, 2005
Plasma membrane	Capn1, CSS1	Gil-Parrado et al., 2003; Lane et al., 1992; Sakai et al., 1989

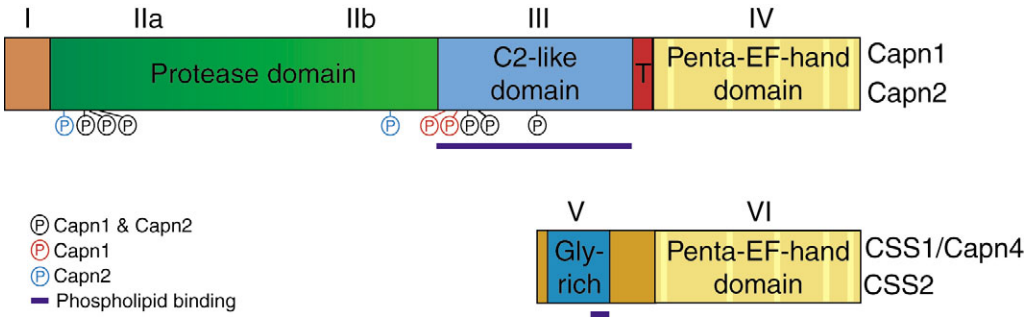

Fig. 1. Schematic representation of the domain architecture of the classical calpains. The 80 kDa large subunits can be divided into four domains, plus a short linker that might be important for transducing conformational changes throughout the molecule upon calcium binding (T). The N-terminal α-helix makes up domain I, which interacts with the small subunits before undergoing intermolecular autolysis on activation. Protease activity is contained within domain II, which is further divided into subdomains (IIa and IIb) that make up the two halves of the active site. Domain III comprises a C2-like domain that harbors sites for phosphorylation and phospholipid binding. Five consecutive EF-hand motifs make up domain IV and contribute to the calcium binding of the large subunits and to dimerization with the small subunits. Domain VI of the small subunits has a similar arrangement; the first four EF hands participate in calcium binding and the last motif interacts with the large subunit. The small subunits also contain a highly flexible, glycine-rich region called domain V. Calpain 1 and calpain 2 large subunits are phosphorylated at several sites in domains I-III; some of these residues are conserved and some are isoform specific.

studies have demonstrated that domain II alone exhibits calcium-dependent protease activity (Hata et al., 2001) and that non-EF-hand calcium-binding sites within the protease domain act as a calcium switch to align the catalytic triad (Hata et al., 2001; Moldoveanu et al., 2002; Moldoveanu et al., 2004).

Except under pathological conditions associated with cell death, such as axonal transection, neurodegeneration and tissue ischemia, the levels of calcium required to activate calpains maximally in vitro do not exist within living cells. This apparent paradox has led researchers towards the idea that other regulatory mechanisms can lower this requirement in vivo. Several different modes of regulation have been identified, although their contributions in vivo have not yet been determined. The large subunits of some calpains are autolyzed on activation, which removes domain I and abolishes the N-terminal link between the large and small subunits, thereby allowing movement of domain II (Baki et al., 1996; Cong et al., 1989; Elce et al., 1997; Guttmann et al., 1997; Imajoh et al., 1986; Molinari et al., 1994; Suzuki and Sorimachi, 1998; Suzuki et al., 1981a). The truncated large subunit is catalytically active and has a lower requirement for calcium (Baki et al., 1996; Imajoh et al., 1986; Suzuki and Sorimachi, 1998; Suzuki et al., 1981b). However, this event is clearly not required for catalytic activity (Cong et al., 1989; Elce et al., 1997; Guttmann et al., 1997; Molinari et al., 1994), which suggests that it functions more in the progression of activation than in its initiation.

The binding of phospholipids also decreases the calcium requirement for calpains in vitro (Arthur and Crawford, 1996; Melloni et al., 1996; Saido et al., 1992; Suzuki et al., 1992; Tompa et al., 2001), but the in vivo relevance of this is unknown. Similarly, regulation of protein-protein interactions changes the calcium requirements of calpains (Melloni et al., 2000a; Melloni et al., 2000b; Melloni et al., 1998; Melloni et al., 2000c; Michetti et al., 1991; Salamino et al., 1993), but their roles in activation are not clear. Finally, calpains are regulated by their best-known interacting partner, the endogenous calpain inhibitor calpastatin (Wendt et al., 2004). Although overexpression of calpastatin in cells can decrease calpain activity, escape from calpastatin is not

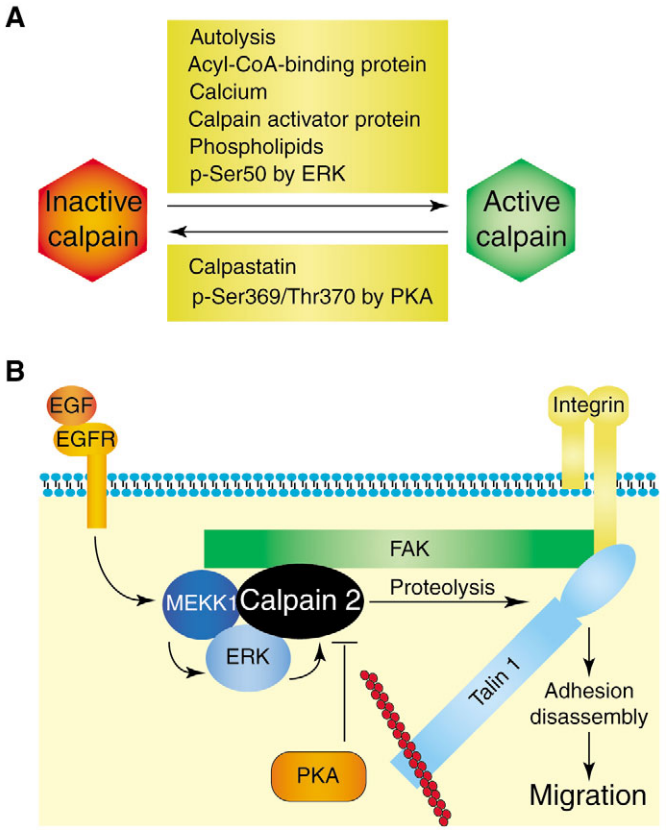

Fig. 2. (A) Some of the mechanisms involved in regulating calpain activity. (B) Possible pathway for growth-factor-induced, calpain-mediated cell migration. Binding of epidermal growth factor (EGF) to its receptor (EGFR) activates a MAP kinase cascade that eventually activates ERK. The scaffolding function of FAK brings ERK and calpain 2 into a complex, resulting in phosphorylation of calpain 2. This ERK-mediated phosphorylation leads to activation of calpain 2, which can be counteracted by phosphorylation of calpain 2 by PKA. Active calpain 2 can then cleave talin 1, leading to adhesion complex turnover and cell migration.

sufficient to activate calpains. Furthermore, structural and biochemical data indicate that calpastatin might bind preferentially to calcium-activated calpains (Barnoy et al., 1999; Tullio et al., 1999), suggesting that this is an attenuation mechanism rather than a preventive one.

Friedrich has recently provided an explanation for this apparent paradox (Friedrich, 2004). He proposes that the calpain system developed this high requirement for calcium during evolution as a safety device to prevent potentially destructive hyperactivity of calpains, and that it is preferable for calpains to work at much less than half-maximal activity. Several pieces of evidence support this idea, including structural considerations, the need for spatial and temporal regulation of calpains and the benefits of a large separation between normal and pathological function.

In addition, phosphorylation at several sites controls the activities of calpains. Calpain 2 is activated by phosphorylation of Ser50 by the ERK mitogen-activated protein (MAP) kinase (Glading et al., 2004) during migration of fibroblasts and in keratinocytes stimulated with epidermal growth factor (EGF; Glading et al., 2000; Satish et al., 2005). Phosphorylation of calpain 2 at this site is particularly interesting since calpain 1, which does not contain a phosphorylatable site in this region, does not seem to play a major role in EGF-mediated motility (Glading et al., 2000; Satish et al., 2005). Instead, calpain 1 is important for IP-9-induced motility, which requires intracellular calcium flux (Satish et al., 2005). By contrast, EGF-mediated activation of calpain 2 by phosphorylation occurs in the absence of increased calcium levels. Furthermore, calpain 3 has a glutamic acid residue at this position that could act as an activating phosphomimetic, which might explain why calpain 3 lacks a requirement for increased calcium levels. Together, these data suggest that calcium and growth-factor-mediated phosphorylation can independently activate calpains in an isoform-specific fashion. Interestingly, only membrane-proximal calpain 2 is activated by ERK-mediated phosphorylation (Glading et al., 2001), which suggests that there are alternative modes of activation for certain calpain 2 subpopulations.

Unsurprisingly, the MAP kinase kinase MEKK1 is required for normal calpain 2 activity (Cuevas et al., 2003). MEKK1 associates with focal adhesion kinase (FAK) in adhesion complexes and appears to act upstream of ERK in the regulation of calpain 2 activation and subsequent detachment of the rear of the cell during migration. Note that the adaptor function of FAK mediates the assembly of an ERK-calpain 2 complex at peripheral adhesion sites (Carragher et al., 2003). The formation of this complex and the activity of ERK are both required for normal calpain 2 activity and for processes dependent on calpain 2 such as adhesion complex turnover, transformation and cell migration. There is thus a novel signaling pathway by which growth factors regulate cell migration via phosphorylation-dependent activation of calpains (Fig. 2B).

Calpain activity can also be inhibited by phosphorylation. Cyclic-AMP-mediated activation of protein kinase A (PKA) can block EGF-induced activation of calpain 2 and fibroblast migration (Shiraha et al., 2002). This appears to occur through phosphorylation of calpain 2 by PKA, which probably restricts calpain 2 to an inactive conformation (Shiraha et al., 2002; Smith et al., 2003). The residues in calpain 2 (Ser369 and Thr370) that appear to be the PKA targets are conserved in other calpains, which suggests that phosphorylation of domain III represents yet another mechanism for regulating calpain activity.

Calpain substrates

Among the >100 proteins identified as calpain substrates are transcription factors, transmembrane receptors, signaling enzymes and cytoskeletal proteins. Although calpains can lead to extensive degradation of some of these substrates, most are cleaved in a limited fashion, resulting in stable protein fragments that can have functions different from those of their intact forms. Such limited proteolysis might be correlated with a highly specific recognition sequence. However, no single consensus sequence has been found to have significant value for predicting whether a protein can be proteolyzed by calpains or even where calpains cleave a known substrate. Instead, recognition and proteolysis seem to be controlled by multiple determinants, including but not limited to secondary structure and PEST score[†] (Tompa et al., 2004). This suggests that calpains cleave their substrates in disordered regions between structured domains. Nevertheless, despite this complex set of determinants, there is a significant preference for particular sequences immediately surrounding the site of proteolysis, and studies that have elucidated these preferences have provided valuable tools with which the calpain systems may be specifically and efficiently manipulated (Tompa et al., 2004).

One obvious clue as to how calpains might affect cell motility comes from the fact that numerous adhesion complex components and migration-related proteins are substrates for calpains (Table 3) (Glading et al., 2002; Goll et al., 2003). Although proteolysis of most of these adhesion-related substrates has been demonstrated in vitro as well as in cell culture, several issues have made it difficult to determine which are relevant to calpain-mediated pathways in vivo. The specificity and extent of proteolysis of adhesion complex components can vary between cell types (S.J.F. and A.H., unpublished). Further complicating the issue is the fact that most of these substrates can be proteolyzed in vitro equally well by either calpain 1 or calpain 2, which can have widely differing subcellular localizations and cell-type-specific expression patterns even in culture.

Recent studies have begun to identify the motility-related substrates that are most readily and consistently cleaved by calpains, as well as the isoforms responsible in living cells. Knockout of calpain small subunit 1 (CSS1 or Capn4) in mice (Arthur et al., 2000) leads to reduced expression and activities of both calpain 1 and calpain 2 (Dourdin et al., 2001). Embryonic fibroblasts isolated from these mice exhibit decreased proteolysis of several reported substrates, including FAK, paxillin, spectrin, cortactin and talin 1. However, others do not appear to be consistently cleaved in mouse embryonic fibroblasts, including vinculin, RhoA, α-actinin and Src (Dourdin et al., 2001) (S.J.F. and A.H., unpublished). The

[†]PEST score is a calculation of the quality of potential PEST motifs [characterized by high local concentrations of the amino acids proline (P), glutamic acid (E), serine (S), threonine (T) and, to a lesser extent, aspartic acid (D)], which can reduce the half-lives of proteins by serving as signals for proteolysis. PEST score=0.55 × DPEST – 0.5 × (10 × Kyte-Doolittle hydropathy index + 45); where DPEST represents the corresponding amino acids expressed in mass % (w/w) and corrected for one equivalent of D or E, one of P and one of S or T.

Table 3. Motility-related calpain substrates

Calpain substrate	In vitro	Cell culture	In vitro isoforms*	Cell culture isoforms	References
α-actinin	No	Yes	–	ND	Goll et al., 1991; Selliah et al., 1996
β integrins	Yes	Yes	Capn2	ND	Du et al., 1995; Pfaff et al., 1999
β-catenin	Yes	Yes	Capn1	Capn2	Rios-Doria et al., 2004
Cadherins	Yes	Yes	Capn1, 2	ND	Covault et al., 1991; Rios-Doria et al., 2003
Cortactin	Yes	Yes	Capn1, 2	Capn2	Huang et al., 1997
EGFR	Yes	Yes	ND	ND	Gates and King, 1983; King and Gates, 1985; Stoscheck et al., 1988
FAK	Yes	Yes	Capn1, 2	Capn2	Carragher et al., 1999; Cooray et al., 1996; Franco et al., 2004a
Filamin	Yes	Yes	Capn1, 2, 3	Capn2, 3	Huff-Lonergan et al., 1996; Kwak et al., 1993; Verhallen et al., 1987
MARCKS	Yes	Yes	Capn2	Capn1, 2	Dedieu et al., 2003; Dulong et al., 2004a
MLCK	Yes	Yes	ND	ND	Kambayashi et al., 1986; Kosaki et al., 1983
Paxillin	Yes	Yes	Capn1, 2	Capn2	Carragher et al., 1999; Franco et al., 2004a
PTP-1B	Yes	Yes	Capn1	ND	Frangioni et al., 1993; Schoenwaelder et al., 1997
RhoA	Yes	Yes	Capn1	ND	Kulkarni et al., 2002
Spectrin	Yes	Yes	Capn1, 2	Capn2	Croall et al., 1986; Fox et al., 1987; Franco et al., 2004a
Src	ND	Yes	ND	ND	Oda et al., 1993
Talin 1	Yes	Yes	Capn1, 2	Capn2	Carragher et al., 1999; Franco et al., 2004a
Vinculin	ND	Yes	ND	ND	Serrano and Devine, 2004

*Proteolysis by unlisted isoforms has not been determined. ND, not determined.

relevance of these findings remains to be determined, but, together with other reports, they indicate that some calpain substrates are more readily cleaved than others in certain cell types. However, this cell line cannot reveal which calpain isoform is responsible in each case, since the activities of both calpain 1 and calpain 2 are reduced in these cells.

Studies of cells isolated from calpain-1-knockout mice reveal that, despite the absence of calpain 1, these cells can proteolyze many substrates normally, including FAK, paxillin, spectrin and talin 1 (Azam et al., 2001). However, other calpain isoforms could be compensating for calpain 1 deficiency in these cells. More recently, RNA interference (RNAi) technology has been employed to knockdown expression of individual calpain isoforms (Franco et al., 2004a). Interestingly, knockdown of calpain 2 results in decreased proteolysis of FAK, paxillin, spectrin, cortactin and talin 1, while knockdown of calpain 1 has no effect on proteolysis of these proteins (Franco et al., 2004a) (B. Perrin and A.H., unpublished). Therefore, it seems that many motility-related proteins require calpain 2 for proteolysis and are either not cleaved by calpain 1 in living cells or are cleaved by compensatory mechanisms in the absence of calpain 1.

Calpains and cell motility

Calpains were first implicated in cell migration by studies showing that pharmacological inhibition of calpains results in reduced integrin-mediated cell migration (Huttenlocher et al., 1997; Palecek et al., 1998). This inhibition leads to stabilization of adhesion complexes and therefore an increase in adhesiveness, thus reducing the rate of detachment of the rear of the cell and decreasing cell migration. Knockout studies support these findings: embryonic fibroblasts from CSS1-deficient mice display a similar reduction in integrin-mediated motility (Dourdin et al., 2001). Since inhibition of calpains by several means results in formation of large peripheral adhesion complexes, calpains were initially thought to regulate cell motility primarily by destabilizing adhesion to the ECM and promoting rear detachment. However, subsequent studies have demonstrated roles for calpains in many aspects of migration, such as cell spreading, membrane protrusion, chemotaxis, and adhesion complex formation and turnover (Fig. 3).

Cell spreading

Cell spreading on ECM components is a complex process involving dynamic reorganization of the actin cytoskeleton in response to integrin-mediated signaling through various pathways. Roles for calpains during cell spreading have been demonstrated in several different systems, but these studies do not reveal one clear function for calpains during this process. Inhibition of the primary calpain in platelets, calpain 1, reduces the ability of these cells to spread, possibly by decreasing proteolysis of adhesion complex proteins (Croce et al., 1999). Inhibition of calpains also reduces spreading in T cells, vascular smooth muscle cells and pancreatic β cells (Parnaud et al., 2005; Paulhe et al., 2001; Rock et al., 2000).

In fibroblasts, overexpression of calpastatin leads to decreased levels of calpain 2 and a decrease in cell spreading and spreading-related actin rearrangements (Potter et al., 1998). This might be caused by an increase in the steady-state levels of the ERM protein ezrin; calpains can proteolyze ezrin and regulate its mRNA levels by an unknown mechanism (Potter et al., 1998). By contrast, spreading of bovine aortic endothelial (BAE) cells is reported to depend specifically on calpain 1, since overexpression of calpain 1 leads to increased cell spreading and a dominant-negative calpain 1 reduces spreading (Kulkarni et al., 1999). However, in the same cell type, calpain 1 can proteolyze RhoA, thereby generating a dominant-negative fragment that inhibits cell spreading (Kulkarni et al., 2002). Studies showing that calpain 1 is important for the formation of early clusters of adhesion molecules that might be sites of Rac1 activation in the early stages of spreading in BAE cells support the idea that calpain 1 positively regulates spreading (Bialkowska et al., 2000). By contrast, knockdown of calpain 1 in several fibroblast cell lines does not affect the ability of these cells to spread (Franco et al., 2004a). Further complicating the issue is the fact that inhibition of calpains in neutrophils might increase spreading of these cells (Lokuta et al., 2003).

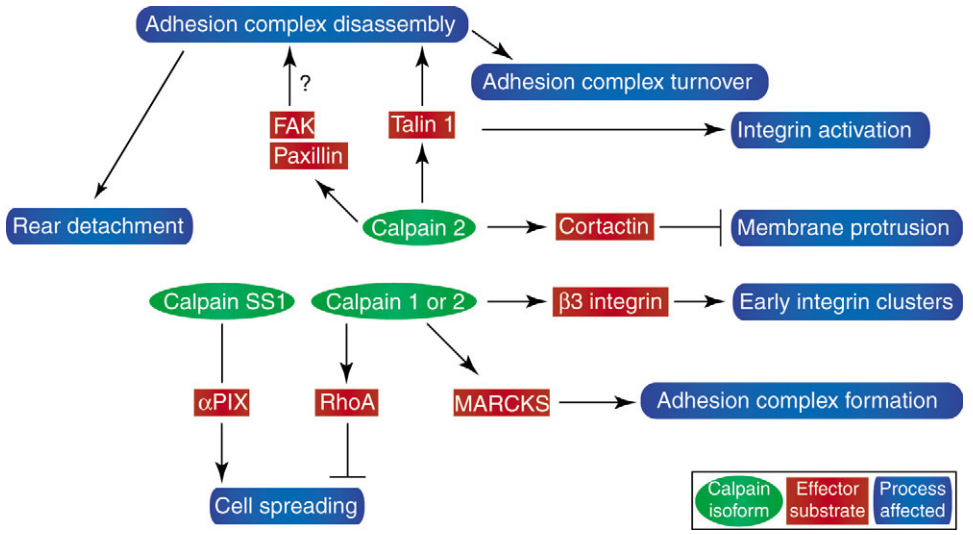

Fig. 3. Motility-related processes known to be affected by calpains and the substrates or binding partners acting as effectors. Calpain 2 can cleave adhesion complex proteins such as FAK, paxillin and talin 1, possibly resulting in integrin activation, adhesion complex turnover or detachment of the cell rear. Proteolysis of the actin-regulating protein cortactin might lead to inhibition of membrane protrusion. Cleavage of integrin β-tails might be important for the formation of small integrin clusters during the early stages of cell spreading, whereas proteolysis of the small GTPase RhoA negatively regulates cell spreading. Interaction of αPIX with calpain small subunit 1 (CSS1) can also mediate cell spreading. Proteolysis of the adaptor protein MARCKS might also regulate cell migration in myoblasts, possibly by promoting adhesion formation. The isoforms required for proteolysis of integrins, RhoA and MARCKS remain to be determined, as do the processes affected by proteolysis of nearly 100 other calpain substrates.

CSS1 also appears to play a role in cell spreading through its interaction with the guanine nucleotide exchange factor (GEF) αPIX (Rosenberger et al., 2005). αPIX binds to and colocalizes with calpains in small integrin-containing clusters during the early stages of cell spreading in CHO-K1 cells. Treatment of these cells with calpain inhibitors reduces spreading, which can be overcome by overexpression of αPIX. Interestingly, an αPIX mutant that cannot bind CSS1 does not rescue the spreading defect, but a GEF-deficient αPIX mutant does. αPIX therefore appears to have a GEF-independent role in cell spreading downstream of calpains.

Membrane protrusion

Many studies of calpains and cell spreading also suggest that calpains regulate actin-based mechanisms involved in membrane protrusion. Inhibition of calpains by calpastatin or pharmacological inhibitors leads to formation of abnormal lamellipodia and filopodia (Potter et al., 1998). Likewise, embryonic fibroblasts from CSS1-knockout mice exhibit altered morphologies, displaying thin membrane projections (Dourdin et al., 2001). These cells also exhibit global increases in transient membrane protrusiveness and faster and more-frequent, but less-stable, leading edge protrusions (Franco et al., 2004a). Calpain 2 appears to be the isoform responsible, since knocking it down reproduces the protrusion defects of CSS1-deficient cells. The actin-regulatory protein cortactin is a calpain substrate and probably an important downstream target of calpain 2 in the regulation of membrane protrusions (B. Perrin and A.H., unpublished), because expression of a calpain-resistant form of cortactin leads to membrane defects similar to those seen in calpain-2-knockdown cells. Further support for calpains negatively regulating membrane protrusion comes from studies showing that calcium transients in filopodia of neuronal growth cones act through calpains to reduce lamellipodial protrusion (Robles et al., 2003).

Chemotaxis

Calpains also negatively regulate membrane protrusion in neutrophils. High levels of calpain activity exist in resting neutrophils, and inhibition of these enzymes promotes membrane protrusion and rapid chemokinesis (Lokuta et al., 2003). This contrasts with most other cell types, in which calpain inhibition reduces cell migration. In cell types in which calpains inhibit cell migration, the underlying mechanism might involve negative regulation of the Rho GTPases Cdc42 and Rac1, since calpain inhibition promotes activation of Cdc42 and Rac in neutrophils. The effects are comparable with treatment with chemoattractants such as *N*-formyl-methionyl-luecyl-phenylalanine (fMLP), which increase chemokinesis (Lokuta et al., 2003). Interestingly, whereas inhibition of calpains promotes random migration of neutrophils, it reduces the directional migration of neutrophils up a gradient of chemoattractant (Lokuta et al., 2003). Spatial regulation of calpain activity might therefore be required for optimum chemotaxis of neutrophils, and calpains could play a role in directional sensing or cell polarization during directed cell migration.

Adhesion complex regulation

Because dynamic regulation of adhesion to the ECM is required for cell migration, the mechanisms by which adhesion complexes are formed and subsequently disassembled are key to cell motility. For some time, inhibition of calpains has been known to alter the morphology and stability of adhesion

complexes, but only now are we beginning to elucidate the details of calpain-mediated regulation of adhesion complexes. Although a role for calpains in the disassembly of adhesion sites has been well documented, whether calpains are important for the formation of adhesion complexes remains unclear. As previously mentioned, calpains appear to be important for induction of small, integrin-containing protein clusters at the early stages of spreading (Bialkowska et al., 2000; Bialkowska et al., 2005). However, these clusters do not seem to be precursors of typical adhesion complexes; so their significance is not known. Calpain-mediated proteolysis of talin 1 might be involved in assembling adhesion complexes, since proteolysis of talin 1 by calpains promotes its binding to integrin β-tails, which is known to be crucial for inside-out activation of integrins (Calderwood, 2004; Calderwood et al., 2002; Calderwood et al., 1999; Yan et al., 2001). Proteolysis of the actin-binding protein myristoylated alanine-rich protein kinase C substrate (MARCKS) might also play a role in the formation of adhesion complexes, since inhibition of calpains in myoblasts leads to defects in new adhesion formation and migration coincident with an accumulation of MARCKS (Dedieu et al., 2004). However, several lines of evidence indicate that adhesion complexes can form normally when calpain activity is reduced; so calpains do not appear to be required for assembly of adhesion complexes in most cell types.

As discussed above, calpains can cleave many adhesion complex proteins and downregulation of calpain activity results in large adhesion complexes and inhibits cell detachment. Calpains could therefore be important for destabilization/disassembly of adhesion complexes. Inhibition of calpains by calpastatin or pharmacological agents blocks microtubule-mediated turnover of adhesion complexes after nocodazole washout. This suggests that calpains act downstream of microtubules to mediate adhesion complex disassembly (Bhatt et al., 2002). Knockdown of calpain 2 by RNAi slows the rate at which adhesion complexes disassemble, leading to formation of large, elongated adhesion complexes (Franco et al., 2004b). Furthermore, expression of a calpain-resistant talin 1 mutant in talin-1-null cells also decreases adhesion complex disassembly rates. This indicates that calpain-2-mediated proteolysis of talin 1 regulates adhesion turnover. How talin 1 proteolysis results in adhesion disassembly is not known, but it is likely that this affects both the structural and signaling functions of talin 1 within adhesion complexes. Since talin 1 is cleaved more readily than most other calpain 2 substrates (S.J.F. and A.H., unpublished), its proteolysis might represent the major mode of calpain-2-mediated adhesion disassembly. Future studies will have to determine whether proteolysis of other substrates is also involved.

Calpains in human disease

Calpains have been connected to a variety of pathological conditions (Zatz and Starling, 2005), including stroke and ischemia (Vanderklish and Bahr, 2000), susceptibility to non-insulin diabetes mellitus (Horikawa et al., 2000) and in the pathogenesis of muscular dystrophies (Ono et al., 1998; Sorimachi et al., 2000). Calpain activity appears to play a central role in the movement of immune cells (Lokuta et al., 2003; Stewart et al., 1998), thereby participating in the development of inflammation in normal and pathological conditions such as chronic inflammatory disease (Cuzzocrea et al., 2000; Shields and Banik, 1998; Shields et al., 1998). Furthermore, calpain 2 expression is upregulated in some cancers and has recently been associated with disease progression in patients with breast cancer (Rios-Doria et al., 2003; Wang et al., 2005; Carragher et al., 2004; Huber et al., 2004). The coordinate regulation of adhesion structures by calpains and Src tyrosine kinases places calpains in a crucial role at the interface of kinase and protease cascades that regulate migration of tumor cells and their invasive properties (Carragher et al., 2001; Carragher and Frame, 2002; Carragher et al., 2002; Mamoune et al., 2003). Pathways involving calpains might thus represent an attractive therapeutic target. Future investigations should delineate whether information about the roles of calpains in motility can facilitate development of drugs to treat a variety of human diseases, including cancer and chronic inflammatory disease.

Conclusions and perspectives

Limited proteolysis by calpains has emerged as a key signal-transducing mechanism that probably functions at the interface of integrin- and growth-factor-mediated signaling to regulate cell migration. The regulation of calpains by calcium, phosphoinositides, and phosphorylation by MAP kinase and PKA pathways places calpains at the center of different signaling pathways controlling many basic cellular processes in addition to cell motility. Crucial to calpain function is the tight regulation of its proteolytic activity, which must be both temporally and spatially controlled during cell migration. Substantial evidence suggests that calpains are activated in a highly localized manner and can be targeted to discrete regions within the cell. However, despite recent progress, our understanding of calpain function during cell migration remains limited. This might be partly attributed to the number and diversity of calpain isoforms; of the 16 known calpain isoforms, only calpain 1, calpain 2 and CSS1 have been studied with respect to migration. Furthermore, there are >100 substrates that can be cleaved by calpains, which makes it difficult to dissect how calpains orchestrate their effects during cell migration. Generation of calpain-resistant substrates will provide important mechanistic clues, but it is important to consider that calpains, like Src-mediated signaling pathways, probably operate by targeting multiple substrates to modify specific stages of the cell motility cycle. Establishing the details involved will require the combination of sophisticated imaging and proteomics-based approaches in both in vitro and in vivo systems.

References

Arthur, J. S. and Crawford, C. (1996). Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. *Biochim. Biophys. Acta* **1293**, 201-206.

Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K. and Greer, P. A. (2000). Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol. Cell Biol.* **20**, 4474-4481.

Azam, M., Andrabi, S. S., Sahr, K. E., Kamath, L., Kuliopulos, A. and Chishti, A. H. (2001). Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol. Cell Biol.* **21**, 2213-2220.

Baki, A., Tompa, P., Alexa, A., Molnar, O. and Friedrich, P. (1996). Autolysis parallels activation of mu-calpain. *Biochem. J.* **318**, 897-901.

Barnoy, S., Zipser, Y., Glaser, T., Grimberg, Y. and Kosower, N. S. (1999). Association of calpain (Ca(2+)-dependent thiol protease) with its endogenous inhibitor calpastatin in myoblasts. *J. Cell Biochem.* **74**, 522-531.

Beckerle, M. C., Burridge, K., DeMartino, G. N. and Croall, D. E. (1987). Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* **51**, 569-577.

Bhatt, A., Kaverina, I., Otey, C. and Huttenlocher, A. (2002). Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J. Cell Sci.* **115**, 3415-3425.

Bialkowska, K., Kulkarni, S., Du, X., Goll, D. E., Saido, T. C. and Fox, J. E. (2000). Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J. Cell Biol.* **151**, 685-696.

Bialkowska, K., Saido, T. C. and Fox, J. E. (2005). SH3 domain of spectrin participates in the activation of Rac in specialized calpain-induced integrin signaling complexes. *J. Cell Sci.* **118**, 381-395.

Blanchard, H., Grochulski, P., Li, Y., Arthur, J. S., Davies, P. L., Elce, J. S. and Cygler, M. (1997). Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. *Nat. Struct. Biol.* **4**, 532-538.

Braun, C., Engel, M., Theisinger, B., Welter, C. and Seifert, M. (1999). CAPN **8**, isolation of a new mouse calpain-isoenzyme. *Biochem. Biophys. Res. Commun.* **260**, 671-675.

Calderwood, D. A. (2004). Integrin activation. *J. Cell Sci.* **117**, 657-666.

Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999). The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* **274**, 28071-28074.

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C. and Ginsberg, M. H. (2002). The phosphotyrosine binding-like domain of talin activates integrins. *J. Biol. Chem.* **277**, 21749-21758.

Carragher, N. O. and Frame, M. C. (2002). Calpain: a role in cell transformation and migration. *Int. J. Biochem. Cell Biol.* **34**, 1539-1543.

Carragher, N. O., Levkau, B., Ross, R. and Raines, E. W. (1999). Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J. Cell Biol.* **147**, 619-630.

Carragher, N. O., Fincham, V. J., Riley, D. and Frame, M. C. (2001). Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. *J. Biol. Chem.* **276**, 4270-4275.

Carragher, N. O., Westhoff, M. A., Riley, D., Potter, D. A., Dutt, P., Elce, J. S., Greer, P. A. and Frame, M. C. (2002). v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. *Mol. Cell Biol.* **22**, 257-269.

Carragher, N. O., Westhoff, M. A., Fincham, V. J., Schaller, M. D. and Frame, M. C. (2003). A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Curr. Biol.* **13**, 1442-1450.

Carragher, N. O., Fonseca, B. D. and Frame, M. C. (2004). Calpain activity is generally elevated during transformation but has oncogene-specific biological functions. *Neoplasia* **6**, 53-73.

Cong, J., Goll, D. E., Peterson, A. M. and Kapprell, H. P. (1989). The role of autolysis in activity of the Ca^{2+}-dependent proteinases (mu-calpain and m-calpain). *J. Biol. Chem.* **264**, 10096-10103.

Cooray, P., Yuan, Y., Schoenwaelder, S. M., Mitchell, C. A., Salem, H. H. and Jackson, S. P. (1996). Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochem. J.* **318**, 41-47.

Covault, J., Liu, Q. Y. and el-Deeb, S. (1991). Calcium-activated proteolysis of intracellular domains in the cell adhesion molecules NCAM and N-cadherin. *Brian Res. Mol. Brain Res.* **11**, 11-16.

Croall, D. E., Morrow, J. S. and DeMartino, G. N. (1986). Limited proteolysis of the erythrocyte membrane skeleton by calcium-dependent proteinases. *Biochim. Biophys. Acta* **882**, 287-296.

Croce, K., Flaumenhaft, R., Rivers, M., Furie, B., Furie, B. C., Herman, I. M. and Potter, D. A. (1999). Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J. Biol. Chem.* **274**, 36321-36327.

Cuevas, B. D., Abell, A. N., Witowsky, J. A., Yujiri, T., Johnson, N. L., Kesavan, K., Ware, M., Jones, P. L., Weed, S. A., DeBiasi, R. L. et al. (2003). MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. *EMBO J.* **22**, 3346-3355.

Cuzzocrea, S., McDonald, M. C., Mazzon, E., Siriwardena, D., Serraino, I., Dugo, L., Britti, D., Mazzullo, G., Caputi, A. P. and Thiemermann, C. (2000). Calpain inhibitor I reduces the development of acute and chronic inflammation. *Am. J. Pathol.* **157**, 2065-2079.

Dear, T. N. and Boehm, T. (1999). Diverse mRNA expression patterns of the mouse calpain genes Capn5, Capn6 and Capn11 during development. *Mech. Dev.* **89**, 201-209.

Dear, T. N. and Boehm, T. (2001). Identification and characterization of two novel calpain large subunit genes. *Gene* **274**, 245-252.

Dear, N., Matena, K., Vingron, M. and Boehm, T. (1997). A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. *Genomics* **45**, 175-184.

Dear, T. N., Moller, A. and Boehm, T. (1999). CAPN11: a calpain with high mRNA levels in testis and located on chromosome 6. *Genomics* **59**, 243-247.

Dear, T. N., Meier, N. T., Hunn, M. and Boehm, T. (2000). Gene structure, chromosomal localization, and expression pattern of Capn12, a new member of the calpain large subunit gene family. *Genomics* **68**, 152-160.

Dedieu, S., Mazeres, G., Poussard, S., Brustis, J. J. and Cottin, P. (2003). Myoblast migration is prevented by a calpain-dependent accumulation of MARCKS. *Biol. Cell* **95**, 615-623.

Dourdin, N., Bhatt, A. K., Dutt, P., Greer, P. A., Arthur, J. S., Elce, J. S. and Huttenlocher, A. (2001). Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J. Biol. Chem.* **276**, 48382-48388.

Du, X., Saido, T. C., Tsubuki, S., Indig, F. E., Williams, M. J. and Ginsberg, M. H. (1995). Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. *J. Biol. Chem.* **270**, 26146-26151.

Dulong, S., Goudenege, S., Vuillier-Devillers, K., Manenti, S., Poussard, S. and Cottin, P. (2004). Myristoylated alanine-rich C kinase substrate (MARCKS) is involved in myoblast fusion through its regulation by protein kinase Calpha and calpain proteolytic cleavage. *Biochem. J.* **382**, 1015-1023.

Elce, J. S., Hegadorn, C. and Arthur, J. S. (1997). Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. *J. Biol. Chem.* **272**, 11268-11275.

Farkas, A., Tompa, P. and Friedrich, P. (2003). Revisiting ubiquity and tissue specificity of human calpains. *Biol. Chem.* **384**, 945-949.

Fox, J. E., Reynolds, C. C., Morrow, J. S. and Phillips, D. R. (1987). Spectrin is associated with membrane-bound actin filaments in platelets and is hydrolyzed by the Ca^{2+}-dependent protease during platelet activation. *Blood* **69**, 537-545.

Franco, S., Perrin, B. and Huttenlocher, A. (2004a). Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp. Cell. Res.* **299**, 179-187.

Franco, S. J., Rodgers, M. A., Perrin, B. J., Han, J., Bennin, D. A., Critchley, D. R. and Huttenlocher, A. (2004b). Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat. Cell. Biol.* **6**, 977-983.

Frangioni, J. V., Oda, A., Smith, M., Salzman, E. W. and Neel, B. G. (1993). Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. *EMBO J.* **12**, 4843-4856.

Franz, T., Vingron, M., Boehm, T. and Dear, T. N. (1999). Capn7: a highly divergent vertebrate calpain with a novel C-terminal domain. *Mamm. Genome* **10**, 318-321.

Friedrich, P. (2004). The intriguing Ca^{2+} requirement of calpain activation. *Biochem. Biophys. Res. Commun.* **323**, 1131-1133.

Gafni, J., Hermel, E., Young, J. E., Wellington, C. L., Hayden, M. R. and Ellerby, L. M. (2004). Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. *J. Biol. Chem.* **279**, 20211-20220.

Gates, R. E. and King, L. E., Jr (1983). Proteolysis of the epidermal growth factor receptor by endogenous calcium-activated neutral protease from rat liver. *Biochem. Biophys. Res. Commun.* **113**, 255-261.

Gil-Parrado, S., Popp, O., Knoch, T. A., Zahler, S., Bestvater, F., Felgentrager, M., Holloschi, A., Fernandez-Montalvan, A., Auerswald, E. A., Fritz, H. et al. (2003). Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain. *J. Biol. Chem.* **278**, 16336-16346.

Glading, A., Chang, P., Lauffenburger, D. A. and Wells, A. (2000). Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J. Biol. Chem.* **275**, 2390-2398.

Glading, A., Uberall, F., Keyse, S. M., Lauffenburger, D. A. and Wells, A. (2001). Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J. Biol. Chem.* **276**, 23341-23348.

Glading, A., Lauffenburger, D. A. and Wells, A. (2002). Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol.* **12**, 46-54.

Glading, A., Bodnar, R. J., Reynolds, I. J., Shiraha, H., Satish, L., Potter, D. A., Blair, H. C. and Wells, A. (2004). Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol. Cell Biol.* **24**, 2499-2512.

Goll, D. E., Dayton, W. R., Singh, I. and Robson, R. M. (1991). Studies of the alpha-actinin/actin interaction in the Z-disk by using calpain. *J. Biol. Chem.* **266**, 8501-8510.

Goll, D. E., Thompson, V. F., Li, H., Wei, W. and Cong, J. (2003). The calpain system. *Physiol. Rev.* **83**, 731-801.

Guroff, G. (1964). A neutral, calcium-activated proteinase from the soluble fraction of rat brain. *J. Biol. Chem.* **239**, 149-155.

Guttmann, R. P., Elce, J. S., Bell, P. D., Isbell, J. C. and Johnson, G. V. (1997). Oxidation inhibits substrate proteolysis by calpain I but not autolysis. *J. Biol. Chem.* **272**, 2005-2012.

Hata, S., Sorimachi, H., Nakagawa, K., Maeda, T., Abe, K. and Suzuki, K. (2001). Domain II of m-calpain is a Ca(2+)-dependent cysteine protease. *FEBS Lett.* **501**, 111-114.

Hood, J. L., Logan, B. B., Sinai, A. P., Brooks, W. H. and Roszman, T. L. (2003). Association of the calpain/calpastatin network with subcellular organelles. *Biochem. Biophys. Res. Commun.* **310**, 1200-1212.

Hood, J. L., Brooks, W. H. and Roszman, T. L. (2004). Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus. *J. Biol. Chem.* **279**, 43126-43135.

Horikawa, Y., Oda, N., Cox, N. J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T. H., Mashima, H., Schwarz, P. E. et al. (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat. Genet.* **26**, 163-175.

Hosfield, C. M., Elce, J. S., Davies, P. L. and Jia, Z. (1999). Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *EMBO J.* **18**, 6880-6889.

Huang, C., Tandon, N. N., Greco, N. J., Ni, Y., Wang, T. and Zhan, X. (1997). Proteolysis of platelet cortactin by calpain. *J. Biol. Chem.* **272**, 19248-19252.

Huber, E., Vlasny, D., Jeckel, S., Stubenrauch, F. and Iftner, T. (2004). Gene profiling of cottontail rabbit papillomavirus-induced carcinomas identifies upregulated genes directly involved in stroma invasion as shown by small interfering RNA-mediated gene silencing. *J. Virol.* **78**, 7478-7489.

Huff-Lonergan, E., Mitsuhashi, T., Beekman, D. D., Parrish, F. C., Jr, Olson, D. G. and Robson, R. M. (1996). Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *J. Anim. Sci.* **74**, 993-1008.

Huttenlocher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A., Ginsberg, M. H. and Horwitz, A. F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* **272**, 32719-32722.

Imajoh, S., Kawasaki, H. and Suzuki, K. (1986). Limited autolysis of calcium-activated neutral protease (CANP): reduction of the Ca^{2+}-requirement is due to the NH2-terminal processing of the large subunit. *J. Biochem. (Tokyo)* **100**, 633-642.

Kambayashi, J., Kajiwara, Y., Sakon, M., Ohshiro, T. and Mori, T. (1986). Possible participation of calpain in myosin light chain phosphorylation of human platelets. *Biochem. Int.* **13**, 571-578.

Kamei, M., Webb, G. C., Young, I. G. and Campbell, H. D. (1998). SOLH, a human homologue of the *Drosophila melanogaster* small optic lobes gene is a member of the calpain and zinc-finger gene families and maps to human chromosome 16p13.3 near CATM (cataract with microphthalmia). *Genomics* **51**, 197-206.

Kifor, O., Kifor, I., Moore, F. D., Jr, Butters, R. R., Jr and Brown, E. M. (2003). m-Calpain colocalizes with the calcium-sensing receptor (CaR) in caveolae in parathyroid cells and participates in degradation of the CaR. *J. Biol. Chem.* **278**, 31167-31176.

King, L. E., Jr and Gates, R. E. (1985). Calcium-activated neutral protease purified from beef lung: properties and use in defining structure of epidermal growth factor receptors. *Arch. Biochem. Biophys.* **242**, 146-156.

Kosaki, G., Tsujinaka, T., Kambayashi, J., Morimoto, K., Yamamoto, K., Yamagami, K., Sobue, K. and Kakiuchi, S. (1983). Specific cleavage of calmodulin-binding proteins by low Ca^{2+}-requiring form of Ca^{2+}-activated neutral protease in human platelets. *Biochem. Int.* **6**, 767-775.

Kulkarni, S., Saido, T. C., Suzuki, K. and Fox, J. E. (1999). Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J. Biol. Chem.* **274**, 21265-21275.

Kulkarni, S., Goll, D. E. and Fox, J. E. (2002). Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. *J. Biol. Chem.* **277**, 24435-24441.

Kwak, K. B., Chung, S. S., Kim, O. M., Kang, M. S., Ha, D. B. and Chung, C. H. (1993). Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim. Biophys. Acta* **1175**, 243-249.

Lane, R. D., Allan, D. M. and Mellgren, R. L. (1992). A comparison of the intracellular distribution of mu-calpain, m-calpain, and calpastatin in proliferating human A431 cells. *Exp. Cell Res.* **203**, 5-16.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Lin, G. D., Chattopadhyay, D., Maki, M., Wang, K. K., Carson, M., Jin, L., Yuen, P. W., Takano, E., Hatanaka, M., DeLucas, L. J. et al. (1997). Crystal structure of calcium bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Struct. Biol.* **4**, 539-547.

Lokuta, M. A., Nuzzi, P. A. and Huttenlocher, A. (2003). Calpain regulates neutrophil chemotaxis. *Proc. Natl. Acad. Sci. USA* **100**, 4006-4011.

Ma, H., Nakajima, E., Shih, M., Azuma, M. and Shearer, T. R. (2004). Expression of calpain small subunit 2 in mammalian tissues. *Curr. Eye Res.* **29**, 337-347.

Mamoune, A., Luo, J. H., Lauffenburger, D. A. and Wells, A. (2003). Calpain-2 as a target for limiting prostate cancer invasion. *Cancer Res.* **63**, 4632-4640.

Markmann, A., Schafer, S., Linz, W., Lohn, M., Busch, A. E. and Wohlfart, P. (2005). Down-regulation of calpain 9 is linked to hypertensive heart and kidney. *Cell Physiol. Biochem.* **15**, 109-116.

Mellgren, R. L. and Lu, Q. (1994). Selective nuclear transport of mu-calpain. *Biochem. Biophys. Res. Commun.* **204**, 544-550.

Melloni, E., Michetti, M., Salamino, F., Minafra, R. and Pontremoli, S. (1996). Modulation of the calpain autoproteolysis by calpastatin and phospholipids. *Biochem. Biophys. Res. Commun.* **229**, 193-197.

Melloni, E., Michetti, M., Salamino, F. and Pontremoli, S. (1998). Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *J. Biol. Chem.* **273**, 12827-12831.

Melloni, E., Averna, M., Salamino, F., Sparatore, B., Minafra, R. and Pontremoli, S. (2000a). Acyl-CoA-binding protein is a potent m-calpain activator. *J. Biol. Chem.* **275**, 82-86.

Melloni, E., Michetti, M., Salamino, F., Minafra, R., Sparatore, B. and Pontremoli, S. (2000b). Isolation and characterization of calpain activator protein from bovine brain. *Methods Mol. Biol.* **144**, 99-105.

Melloni, E., Minafra, R., Salamino, F. and Pontremoli, S. (2000c). Properties and intracellular localization of calpain activator protein. *Biochem. Biophys. Res. Commun.* **272**, 472-476.

Michetti, M., Viotti, P. L., Melloni, E. and Pontremoli, S. (1991). Mechanism of action of the calpain activator protein in rat skeletal muscle. *Eur. J. Biochem.* **202**, 1177-1180.

Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z. and Davies, P. L. (2002). A Ca(2+) switch aligns the active site of calpain. *Cell* **108**, 649-660.

Moldoveanu, T., Jia, Z. and Davies, P. L. (2004). Calpain activation by cooperative Ca^{2+} binding at two non-EF-hand sites. *J. Biol. Chem.* **279**, 6106-6114.

Molinari, M., Anagli, J. and Carafoli, E. (1994). Ca(2+)-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. *J. Biol. Chem.* **269**, 27992-27995.

Morford, L. A., Forrest, K., Logan, B., Overstreet, L. K., Goebel, J., Brooks, W. H. and Roszman, T. L. (2002). Calpain II colocalizes with detergent-insoluble rafts on human and Jurkat T-cells. *Biochem. Biophys. Res. Commun.* **295**, 540-546.

Newton, A. C. and Johnson, J. E. (1998). Protein kinase C: a paradigm for regulation of protein function by two membrane-targeting modules. *Biochim. Biophys. Acta* **1376**, 155-172.

Nishihara, H., Nakagawa, Y., Ishikawa, H., Ohba, M., Shimizu, K. and Nakamura, T. (2001). Matrix vesicles and media vesicles as nonclassical pathways for the secretion of m-Calpain from MC3T3-E1 cells. *Biochem. Biophys. Res. Commun.* **285**, 845-853.

Oda, A., Druker, B. J., Ariyoshi, H., Smith, M. and Salzman, E. W. (1993). pp60src is an endogenous substrate for calpain in human blood platelets. *J. Biol. Chem.* **268**, 12603-12608.

Ono, Y., Shimada, H., Sorimachi, H., Richard, I., Saido, T. C., Beckmann, J. S., Ishiura, S. and Suzuki, K. (1998). Functional defects of a muscle-specific calpain, p94, caused by mutations associated with limb-girdle muscular dystrophy type 2A. *J. Biol. Chem.* **273**, 17073-17078.

Pal, G. P., De Veyra, T., Elce, J. S. and Jia, Z. (2003). Crystal structure of a micro-like calpain reveals a partially activated conformation with low Ca^{2+} requirement. *Structure (Camb.)* **11**, 1521-1526.

Palecek, S. P., Huttenlocher, A., Horwitz, A. F. and Lauffenburger, D. A.

(1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J. Cell Sci.* **111**, 929-940.

Parnaud, G., Hammar, E., Rouiller, D. G. and Bosco, D. (2005). Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion. *Am. J. Physiol. Endocrinol. Metab.* **289**, E313-E321.

Paulhe, F., Bogyo, A., Chap, H., Perret, B. and Racaud-Sultan, C. (2001). Vascular smooth muscle cell spreading onto fibrinogen is regulated by calpains and phospholipase C. *Biochem. Biophys. Res. Commun.* **288**, 875-881.

Perrin, B. J. and Huttenlocher, A. (2002). Calpain. *Int. J. Biochem. Cell Biol.* **34**, 722-725.

Pfaff, M., Du, X. and Ginsberg, M. H. (1999). Calpain cleavage of integrin beta cytoplasmic domains. *FEBS Lett.* **460**, 17-22.

Potter, D. A., Tirnauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco, K. A., Mier, J. W., Maki, M. and Herman, I. M. (1998). Calpain regulates actin remodeling during cell spreading. *J. Cell Biol.* **141**, 647-662.

Rafelski, S. M. and Theriot, J. A. (2004). Crawling toward a unified model of cell mobility: spatial and temporal regulation of actin dynamics. *Annu. Rev. Biochem.* **73**, 209-239.

Raynaud, F., Carnac, G., Marcilhac, A. and Benyamin, Y. (2004). m-Calpain implication in cell cycle during muscle precursor cell activation. *Exp. Cell Res.* **298**, 48-57.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.

Rios-Doria, J., Day, K. C., Kuefer, R., Rashid, M. G., Chinnaiyan, A. M., Rubin, M. A. and Day, M. L. (2003). The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. *J. Biol. Chem.* **278**, 1372-1379.

Rios-Doria, J., Kuefer, R., Ethier, S. P. and Day, M. L. (2004). Cleavage of beta-catenin by calpain in prostate and mammary tumor cells. *Cancer Res.* **64**, 7237-7240.

Rizo, J. and Sudhof, T. C. (1998). C2-domains, structure and function of a universal Ca^{2+}-binding domain. *J. Biol. Chem.* **273**, 15879-15882.

Robles, E., Huttenlocher, A. and Gomez, T. M. (2003). Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron* **38**, 597-609.

Rock, M. T., Dix, A. R., Brooks, W. H. and Roszman, T. L. (2000). Beta1 integrin-mediated T cell adhesion and cell spreading are regulated by calpain. *Exp. Cell Res.* **261**, 260-270.

Rosenberger, G., Gal, A. and Kutsche, K. (2005). AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. *J. Biol. Chem.* **280**, 6879-6889.

Saido, T. C., Shibata, M., Takenawa, T., Murofushi, H. and Suzuki, K. (1992). Positive regulation of mu-calpain action by polyphosphoinositides. *J. Biol. Chem.* **267**, 24585-24590.

Sakai, K., Hayashi, M., Kawashima, S. and Akanuma, H. (1989). Calcium-induced localization of calcium-activated neutral proteinase on plasma membranes. *Biochim. Biophys. Acta* **985**, 51-54.

Salamino, F., De Tullio, R., Mengotti, P., Viotti, P. L., Melloni, E. and Pontremoli, S. (1993). Site-directed activation of calpain is promoted by a membrane-associated natural activator protein. *Biochem. J.* **290**, 191-197.

Satish, L., Blair, H. C., Glading, A. and Wells, A. (2005). Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of mu-calpain. *Mol. Cell Biol.* **25**, 1922-1941.

Schoenwaelder, S. M., Kulkarni, S., Salem, H. H., Imajoh-Ohmi, S., Yamao-Harigaya, W., Saido, T. C. and Jackson, S. P. (1997). Distinct substrate specificities and functional roles for the 78- and 76-kDa forms of mu-calpain in human platelets. *J. Biol. Chem.* **272**, 24876-24884.

Selliah, N., Brooks, W. H. and Roszman, T. L. (1996). Proteolytic cleavage of alpha-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. *J. Immunol.* **156**, 3215-3221.

Serrano, K. and Devine, D. V. (2004). Vinculin is proteolyzed by calpain during platelet aggregation: 95 kDa cleavage fragment associates with the platelet cytoskeleton. *Cell Motil. Cytoskeleton* **58**, 242-252.

Shields, D. C., Tyor, W. R., Deibler, G. E., Hogan, E. L. and Banik, N. L. (1998). Increased calpain expression in activated glial and inflammatory cells in experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA* **95**, 5768-5772.

Shields, D. C. and Banik, N. L. (1998). Upregulation of calpain activity and expression in experimental allergic encephalomyelitis: a putative role for calpain in demyelination. *Brain Res.* **794**, 68-74.

Shiraha, H., Glading, A., Chou, J., Jia, Z. and Wells, A. (2002). Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol. Cell Biol.* **22**, 2716-2727.

Smith, S. D., Jia, Z., Huynh, K. K., Wells, A. and Elce, J. S. (2003). Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation. *FEBS Lett.* **542**, 115-118.

Sorimachi, H. and Suzuki, K. (2001). The structure of calpain. *J. Biochem. (Tokyo)* **129**, 653-664.

Sorimachi, H., Ishiura, S. and Suzuki, K. (1993). A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca(2+)-binding domain. *J. Biol. Chem.* **268**, 19476-19482.

Sorimachi, H., Ono, Y. and Suzuki, K. (2000). Molecular analysis of p94 and its application to diagnosis of limb girdle muscular dystrophy type 2A. *Methods Mol. Biol.* **144**, 75-84.

Stewart, M. P., McDowall, A. and Hogg, N. (1998). LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. *J. Cell Biol.* **140**, 699-707.

Stoscheck, C. M., Gates, R. E. and King, L. E., Jr (1988). A search for EGF-elicited degradation products of the EGF receptor. *J. Cell Biochem.* **38**, 51-63.

Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H. et al. (2000). The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl. Acad. Sci. USA* **97**, 588-592.

Suzuki, K. and Sorimachi, H. (1998). A novel aspect of calpain activation. *FEBS Lett.* **433**, 1-4.

Suzuki, K., Tsuji, S., Ishiura, S., Kimura, Y., Kubota, S. and Imahori, K. (1981a). Autolysis of calcium-activated neutral protease of chicken skeletal muscle. *J. Biochem. (Tokyo)* **90**, 1787-1793.

Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y. and Imahori, K. (1981b). Limited autolysis of Ca^{2+}-activated neutral protease (CANP) changes its sensitivity to Ca^{2+} ions. *J. Biochem. (Tokyo)* **90**, 275-278.

Suzuki, K., Saido, T. C. and Hirai, S. (1992). Modulation of cellular signals by calpain. *Ann. New York Acad. Sci.* **674**, 218-227.

Suzuki, K., Hata, S., Kawabata, Y. and Sorimachi, H. (2004). Structure, activation, and biology of calpain. *Diabetes* **53**, S12-S18.

Tompa, P., Emori, Y., Sorimachi, H., Suzuki, K. and Friedrich, P. (2001). Domain III of calpain is a Ca^{2+}-regulated phospholipid-binding domain. *Biochem. Biophys. Res. Commun.* **280**, 1333-1339.

Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilagyi, A., Banoczi, Z., Hudecz, F. and Friedrich, P. (2004). On the sequential determinants of calpain cleavage. *J. Biol. Chem.* **279**, 20775-20785.

Tremper-Wells, B. and Vallano, M. L. (2005). Nuclear calpain regulates Ca^{2+}-dependent signaling via proteolysis of nuclear Ca^{2+}/calmodulin-dependent protein kinase type IV in cultured neurons. *J. Biol. Chem.* **280**, 2165-2175.

Tullio, R. D., Passalacqua, M., Averna, M., Salamino, F., Melloni, E. and Pontremoli, S. (1999). Changes in intracellular localization of calpastatin during calpain activation. *Biochem. J.* **343**, 467-472.

Vanderklish, P. W. and Bahr, B. A. (2000). The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. *Int. J. Exp. Pathol.* **81**, 323-339.

Verhallen, P. F., Bevers, E. M., Comfurius, P. and Zwaal, R. F. (1987). Correlation between calpain-mediated cytoskeletal degradation and expression of platelet procoagulant activity. A role for the platelet membrane-skeleton in the regulation of membrane lipid asymmetry? *Biochim. Biophys. Acta* **903**, 206-217.

Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J. et al. (2005). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-679.

Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nat. Cell. Biol.* **4**, E97-E100.

Wendt, A., Thompson, V. F. and Goll, D. E. (2004). Interaction of calpastatin with calpain: a review. *Biol. Chem.* **385**, 465-472.

Xu, L. and Deng, X. (2004). Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induces phosphorylation of mu- and m-calpain in association with increased secretion, cell migration, and invasion. *J. Biol. Chem.* **279**, 53683-53690.

Yan, B., Calderwood, D. A., Yaspan, B. and Ginsberg, M. H. (2001). Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. *J. Biol. Chem.* **276**, 28164-28170.

Zatz, M. and Starling, A. (2005). Calpains and disease. *New Engl. J. Med.* **352**, 2413-2423.