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Imaging of protease functions – current guide to spotting cysteine cathepsins in classical and novel scenes of action in mammalian epithelial cells and tissues

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Summary

The human genome encodes some hundreds of proteases. Many of these are well studied and understood with respect to their biochemistry, molecular mechanisms of proteolytic cleavage, expression patterns, molecular structure, substrate preferences and regulatory mechanisms, including their endogenous inhibitors. Moreover, precise determination of protease localisation within subcellular compartments, peri- and extracellular spaces has been extremely useful in elucidating biological functions of peptidases. This can be achieved by refined methodology as will be demonstrated herein for the cysteine cathepsins. Besides localisation, it is now feasible to study in situ enzymatic activity at the various levels of subcellular compartments, cells, tissues, and even whole organisms including mouse.

Key words

Activity based probes, enzyme cytochemistry, green fluorescent protein, immunofluorescence, protein trafficking, endo-lysosomal proteases.

Introduction

Proteases belong to various families, i.e. the aspartic, cysteine, glutamic, metallo-, serine or threonine peptidases (Puente et al., 2003). However, some proteolytic enzymes remain unassigned and unclassified as yet. The most comprehensive overview of our present knowledge about proteases and their inhibitors can be found in the Merops database (Rawlings et al., 2010) at merops.sanger.ac.uk. This website is a valuable tool in approaching investigations on the biochemistry and molecular biology of proteases throughout all kingdoms of living species such as Archaea, Eubacteria and Eukaryota. In addition, viral proteases are included because they have gained increased attention in recent years due to their importance in a variety of infectious diseases. Inhibitors of proteases, the natural counter-parts of proteases, are important in safe-guarding mammalian cells, by regulating proteolytic enzymes in excess or in unwanted locations in diseased cells or tissues.

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This review article will focus on cysteine peptidases. In particular, we will discuss cysteine cathepsins that belong to the C1A subfamily of papain-like enzymes in clan CA (Rawlings et al., 1994; Chapman et al., 1997; Barrett et al., 2001; Reinheckel et al., 2001; Turk et al., 2001; Nagler et al., 2003; Choe et al., 2006; Mohamed et al., 2006; Vasiljeva et al., 2007; Brix et al., 2008). Endogenous inhibitors of mammalian cysteine cathepsins comprise the family of cystatins (Barrett et al., 1981; Barrett et al., 1986; Bode et al., 1988; Abrahamson et al., 1994) and the propeptide-mimicking cytotoxic T-lymphocyte antigens (CTLA) -2a and -2b (Denizot et al., 1989). Cystatins function as intra- and extracellular proteins that integrate into the active site cleft of cysteine cathepsins in a wedge-shaped edge-like fashion (Abrahamson et al., 1987; Stubbs et al., 1990; Jenko et al., 2003). In contrast, the CTLAs bind to the active site of family C1 cysteine peptidases in reverse orientation, similar to their propeptides. This binding involves a chaperoning mechanism, also used during biosynthesis and trafficking of cysteine cathepsins, in order to retain the immature proenzyme in zymogen form until it reaches its target compartment (Guay et al., 2000; Wiederanders et al., 2000). The precise knowledge of the molecular structures of proteolytic enzymes and their endogenous inhibitors, along with the notion of their interactions in forming protease-inhibitor complexes in a reversible fashion, has stimulated increasing interest in using natural (e.g. E64) or synthetic inhibitors and other small molecule derivatives in order to target proteolytic activity (Barrett et al., 1982; Baruch et al., 2004).

Cysteine cathepsins have been considered as useful targets to treat a large diversity of disorders: atherosclerosis, cancer, rheumatoid and osteoarthritis, osteoporosis, neurodegenerative disorders such as Alzheimer’s disease and, more recently, inflammation and obesity (Turk et al., 2006; Vasiljeva et al., 2007; Bromme et al., 2009). This impressive list of cysteine cathepsin involvement in pathological processes highlights their functional versatility, and the strategic move towards better tackling of specific protease inhibition has further entailed the development of invaluable tools to visualize and image proteolytic functions of cysteine cathepsins, through the use of activity based probes (ABPs) (Jeffery et al., 2003; Baruch et al., 2004; Berger et al., 2004; Brix et al., 2005; Fonovic et al., 2007; Blum et al., 2008; Paulick et al., 2008).

Visualization of proteolysis – a challenging task

Proteolysis is as complex as biology in general. Hence, elucidating expression patterns of proteases and their inhibitors is a basic priority when approaching an understanding of protease functions. A variety of experimental set-ups have been well established in order to investigate the genome, transcriptome and proteome systems, and to compile a comprehensive list of proteases and inhibitors that enable cellular and tissue functions.

Genome sequencing projects have been instrumental in learning more about the so-called degradome, the entire set of proteases expressed in a given organism (Lopez-Otin et al., 2002; Puente et al., 2003; Quesada et al., 2009). Because proteases are regulated by their endogenous inhibitors, these are also covered in The Mammalian Degradome Database, provided at degradome.uniovi.es. Proteolytic enzymes are encoded by 2% of the human genome, a huge proportion of all genes. Thus, it is hardly surprising that almost 90 different hereditary diseases known to date are derived from mutations
Visualization of cathepsins in epithelial cells

in protease genes (Lopez-Otin et al., 2008). Degradomics enable a systems biology analysis of all proteases, protease homologues and protease inhibitors through the use of DNA microarrays (Overall et al., 2006). This approach has been further taken to establish metadegradomics, which includes mass spectrometry for quantitative in vivo degradomics and the introduction of post-translational modifications (Doucet et al., 2008).

The next important step in understanding protease functions is to localize proteolytic enzymes in an in situ context with their natural substrates and their endogenous inhibitors within cells and tissues. While this sounds like a classical domain of cell biologists, it became clear that a wider spectrum of disciplines is needed to provide the tools necessary for grasping the mammalian cell’s repertoire of proteases. With respect to the broader picture, it will be important to keep in mind that proteolysis is the most important post-translational protein modification process, because proteolytic cleavage is irreversible, thus determining the fate of cells in their most critical turning point of deciding between life and death.

The study of protease functions must encompass determination of protease activities, which has often been achieved through the basic use of synthetic substrates in biochemical approaches. However, we are now entering an era where the more complex tasks of monitoring substrate cleavage and tracking protease activities in intact cells (Tab. 1) are not only possible but reveal an overwhelming wealth of data.

**Spotting proteolytic enzymes at high resolution by immunolabeling for light and electron microscopy**

Immunolocalization is the best known and most straightforward experimental approach to detect proteases in subcellular compartments and to localize secreted enzymes in tissues. Most protocols include fixation procedures that are based on formaldehyde fixation for light microscopical detection, or fixation with glutaraldehyde for electron microscopy. While both fixation techniques bear the clear advantage of being very well established and easy-to-use routines, it might be worthwhile considering an alternative fixation strategy, namely alcohol (ethanol or methanol) -based dehydration procedures. For example, it is well known that keratinocytes and oligodendrocytes exhibit a strikingly different membrane lipid composition in comparison to cells such as fibroblasts. Accordingly, our own experience, as summarized in Tab. 2, has convinced us to compare methanol/acetone-based dehydration techniques carried out at low temperatures with paraformaldehyde-based protocols conducted at room temperature or at 37 °C, when visualizing cysteine cathepsins that are thought to reside within endo-lysosomal compartments of mammalian cells (Brix et al., 1996; Tepel et al., 2000; Linke et al., 2002b; Friedrichs et al., 2003; Buth et al., 2007; Jordans et al., 2009; Mayer et al., 2009; Vreemann et al., 2009; Tedelind et al., 2010). For the majority of cell types such as fibroblasts, thyroid epithelial cells, enterocytes or macrophages, our preferred method is chemical cross-linking with mono- or bifunctional aldehydes, whereas keratinocytes are best treated with methanol. Other important measures that improve structural preservation are washing procedures before and after fixation, as well as paying attention to the composition of buffers used in combination with certain fixatives. In our hands, calcium and magnesium-free phosphate buffered saline (CMF-PBS) is best combined with methanol/acetone solutions, where-
as HEPES or PIPES are our preferred biogenic amine-based buffers used in combination with freshly prepared paraformaldehyde for fixation.

In this context, it is interesting to note that many researchers appear to be more concerned about the properties of specific antibodies rather than structural preservation of the primary antigen when choosing fixation protocols. Certainly, antibodies that are commercially available often prefer immunoidentification of their antigens in a specific context, such as in formaldehyde-fixed cell or tissue preparations without or after paraffin embedding, or as well exposed antigens and structurally well preserved molecules in frozen tissues. However, we further suggest taking the distinct lipid membrane composition of the cell type of interest into account. For illustration of the results achieved with identical antibodies in different cell types, examples are provided of cysteine cathepsin B localization in keratinocytes after methanol-acetone fixation (Fig. 1A), in thyroid carcinoma cells after fixation with paraformaldehyde (Fig. 1B), and in cryo-sections of mouse intestine tissue (Fig. 1C’). In all cases, the

Table 1 – Sample preparation for various methods of cysteine cathepsin detection.

| 1. Transfection | with vectors encoding fluorescent protein tagged protease |
| 2. Fluid phase | uptake to label endocytic compartments: |
| | wash in PBS, incubate with 1 µM LysoTracker Red DND-99 in culture medium for 5 min (endosomes) or 30 min (lysosomes) at 37°C, chase in complete culture medium for 120 min – 24 h (lysosomes, only) at 37°C, wash in PBS, fix as desired, e.g. 3% PFA in PBS or HEPES for 30 min at 37°C |
| 3. Activity Based Probes | • label pericellular proteases by incubation at 37°C with non-permeable ABP or protease substrate analogs, use inhibitors like E64d or CA-074d as control; |
| | • alternatively, incubate with cell-permeable ABP or protease substrate analogs, use inhibitors as control, incubate at 4°C to block endocytosis, then, wash and perform first minutes of fixation also at 4°C |
| | • peri- and intracellular proteases can be labeled by incubation at 37°C with cell-permeable ABP or protease substrate analogs, incubate with inhibitors as control; ABPs come as non-quenched or quenched probes; principle of labeling: ABPs are cleaved by active protease, releasing quenching group and resulting in covalently labeled protease |
| | – non-quenched ABPs: fluorescence where probe binds, i.e. background could be a problem |
| | – quenched ABPs: fluorescence only where the probe binds specifically to active proteases, i.e. better signal-to-noise ratio is achievable |

Notes:

Protease-specific inhibitors:

- E64 inhibits all cysteine peptidases, use at 1-5 µM; CA-074 inhibits cathepsin B specifically, use at 1-5 µM; Z-FY(t-Bu)-DMK (Z-Phe-Tyr(t-Bu)-diazomethylketone) inhibits cathepsin L specifically, use at 1-5 µM

Activity Based Probes (ABPs):

- NS-173 or NS-196 = rhodamine-conjugated or biotinylated cathepsin B-specific probe (Schaschke et al., 2000), use at 500 nM in culture medium
- DCG-04, e.g. Green-DCG-04, fluorophore-conjugated probes (Greenbaum et al., 2000), use at 500 nM in culture medium
- GB-111 and GB-117, non-quenched and quenched probes (Blum et al., 2005), a variety of next generation ABPs have been synthesized recently (Blum et al., 2009), use at 500 nM in culture medium
### Table 2 – Procedures for indirect immunofluorescence after different fixation procedures.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Methanol/Acetone</th>
<th>Paraformaldehyde (PFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. on cover glasses</td>
<td>Methanol, 8 min, -20°C Acetone, 8 min, -20°C</td>
<td>4 - 8% PFA in 200 mM HEPES, pH 7.4, 30 min, RT</td>
</tr>
<tr>
<td>PBS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>fixation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>---</td>
<td>X</td>
</tr>
<tr>
<td>CMF-PBS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>0.2 - 1% Triton X-100 in CMF-PBS, 5 min, RT</td>
<td>---</td>
<td>optional depending on whether interested in pericellular proteases = w/o TX100, or intracellular proteases = w/ TX100</td>
</tr>
<tr>
<td>block non-specific binding sites</td>
<td>3 mg/ml BSA in CMF-PBS, 30 min, RT</td>
<td>3 mg/ml BSA in CMF-PBS, 30 min, RT</td>
</tr>
<tr>
<td>0.1 mg/ml BSA in CMF-PBS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1° ab in 0.1 mg/ml BSA in CMF-PBS</td>
<td>90 min / over night, 37°C / 4°C</td>
<td>90 min / over night, 37°C / 4°C</td>
</tr>
<tr>
<td>0.1 mg/ml BSA in CMF-PBS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2° ab coupled to FITC / DTAF / Alexa 488 / Alexa 543 / Alexa 633 / Cy2 / Cy3 in 0.1 mg/ml BSA in CMF-PBS</td>
<td>60 min, 37°C</td>
<td>60 min, 37°C</td>
</tr>
<tr>
<td>CMF-PBS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>mounting in 33% glycerol, 14% mowiol in 200 mM Tris (pH 8.5) + 5% 1,4-diazabicyclo(2,2,2) octan</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>view @ excitation as required by fluorophore</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

X, step required; ---, step not required; RT, room temperature; ddH$_2$O, double distillled water; PBS, phosphate-buffered saline; CMF, calcium- and magnesium-free

**Notes:**

DRAQ5™ for counter-staining of nucleic acids can be applied with 2° ab at final concentration of 5 µM, view at 633 nm excitation. Propidium iodide (2 µg/µl in CMF-PBS) is applied to cell cultures before fixation to stain dead cells with ruptured membranes, when applied after fixation and TX100 permeabilisation, it stains all cellular DNA, view at 543 nm excitation.
Figure 1 – Visualization of cathepsin B in cells and tissue using different fixation methods and indirect immunofluorescence.

(A) Human HaCaT keratinocytes were fixed with methanol and acetone for 8 min each, at -20°C before immunolabeling with primary anti-cathepsin B and secondary, fluorophore-conjugated antibodies (green). Nuclei were counter-stained with the nucleic acid-specific dye DRAQ5™ (red). Endolysosomal (arrows), nuclear (arrowheads) and plasma membrane (open arrowheads) localizations of cathepsin B were readily observed. For further details on subnuclear labeling patterns, see (Tedelind et al., 2010).

(B) Human thyroid carcinoma KTC-1 cells were fixed with paraformaldehyde before immunolabeling with primary anti-cathepsin B and secondary fluorophore-conjugated antibodies (red). Nuclei were counter-stained with DRAQ5™ (blue) and cytoskeletal F-actin was visualized by FITC-phalloidin staining (green). Cathepsin B accumulated in the perinuclear Golgi region of thyroid carcinoma cells (broken arrow) and the protease was additionally sorted into vesicular structures spreading from the cell center in a star-like fashion (arrow), characteristic for vesicles transported along microtubules during protease trafficking with destination to endo-lysosomes.

(C and C') Mouse intestine tissue was paraformaldehyde-fixed before cryo-sectioning and immunolabeling with primary anti-cathepsin B and secondary fluorophore-conjugated antibodies (C'). Cathepsin B was detectable within endo-lysosomes of intestine epithelial cells (arrows) and the protease was abundantly immunolabeled within endocytic vesicles of cells in the lamina propria.

In all three examples the same primary anti-cathepsin B-specific antibody was used to detect its differently fixed antigen. Single channel and merged fluorescence micrographs in A and B are shown in false-colors; right hand panels in A and B and pictures in C and C' depict single channel micrographs and phase contrast images as indicated. N, nucleus; scale bars represent 20 µm in A and B, and 50 µm in C.'
Visualization of cathepsins in epithelial cells
cysteine cathepsin-containing structures could be easily visualized by fluorescence microscopy and are compared to well-preserved cellular structures as viewed in the corresponding phase contrast images (Fig. 1).

Since cysteine cathepsins are redundantly expressed in both a ubiquitous and cell type-specific manner (Chapman et al., 1997; Nagler et al., 2003; Brix et al., 2008), it may also become important to simultaneously visualize other closely related enzymes. In such cases, we prefer to immunolabel in a consecutive fashion, meaning first to label the proteases present in small amounts with green fluorophores and then to visualize the more abundant proteins with employment of red fluorophores (Tepel et al., 2000; Jordans et al., 2009). This strategy ensures that the brightness of each fluorophore as measured by its characteristic extinction coefficient and fluorescent quantum yield is applied for the best detection of rare proteins as bright spots by the emitted green fluorescence, on a usually more dimmed background fluorescence derived from labeling abundantly present proteolytic enzymes with red fluorophores.

The results of such co-localization studies demonstrate that it is now possible to visualize even small amounts of rare nuclear cysteine cathepsin variants, which maximally constitute 5-10% of all cysteine peptidases in e.g. thyroid carcinoma cells (Tedeilind et al., 2010), and which have escaped immunodetection for many decades. Nonetheless, with improved detection methods it is now possible to also study such rare proteases that are likely to be crucial for cell differentiation and cell fate determinations.

Likewise, co-localization studies have allowed the investigation of protein composition of specific vesicles of endocytic compartments and have revealed the existence of different vesicle populations, such as in thyroid epithelial cells that are loaded with distinct sets of proteases (Tepel et al., 2000; Jordans et al., 2009; Tedeilind et al., 2011). Such results are not at all trivial since they raise important questions; for example, how loading of vesicles with soluble enzyme cargo is achieved in mammalian cells. Clearly, thyroid epithelial cells are able to selectively recruit specific proteases that are present in different vesicle populations in particular biological contexts, for example in resting or thyroid stimulating hormone (TSH) -stimulated conditions (Brix et al., 1996; Brix et al., 2001; Linke et al., 2002a; Linke et al., 2002b). This enables cells to fine-tune their protease cocktails in a subcellular compartment or even subcompartment, so that they can cope with the necessity of degrading a huge variety of internalized molecules that are likely to differ in resting or stimulated conditions.

In order to detect cysteine cathepsins with high spatial resolution in various cellular compartments, immunoelectron microscopy is a useful tool. Visualization of cysteine cathepsins with specific antibodies has been performed by classical approaches that involve post-Epon-embedding-labeling techniques or cryo-immuno-electron microscopy procedures as well as by rather unusual approaches which include immunolabeling of whole-mount preparations (Brix et al., 1996).

Following protease tracks using fluorescent protein tagging experiments

Cysteine cathepsins are not exclusively present within endo-lysosomes; they are detectable in all compartments of the secretory route and in vesicles targeted to reach the compartments of the endocytic pathway. In addition, cysteine cathepsins are often actively secreted from cells in processes such as cell migration (Buth et al., 2004), tis-
sue repair (Buth et al., 2007) or invasion of carcinoma cells (Mohamed and Sloane, 2006), where the secreted enzymes are thought to be involved in ECM remodeling processes. Secreted cysteine cathepsins can further accumulate in structures such as caveolae (Cavallo-Medved et al., 2009) and they may also re-associate with the cell surface as shown in Fig. 1A. In peri-cellular locations such as in the vicinity of the apical plasma membrane of epithelial cells, cysteine cathepsins have been shown to contribute to limited proteolysis of molecules en-route to endosomes and lysosomes (Brix et al., 1996; Brix et al., 2001; Friedrichs et al., 2003; Buth et al., 2004; Mayer et al., 2009; Vreemann et al., 2009; Dauth et al., 2011) and hence, act in the direct pericellular environment. In addition, rare variants of cysteine cathepsins have been detected which appear to serve key functions in unusual locations such as the mitochondrial matrix (Muntener et al., 2004) or the nucleus (Goulet et al., 2004; Ong et al., 2007; Duncan et al., 2008; Maubach et al., 2008; Tedelind et al., 2010).

These observations illustrate that trafficking studies are important in elucidating the transport pathways of proteases within cells, which in turn provide clues to their function. Such studies are best performed by fluorescent protein tagging, which allows tracking of protease transport, from the rough endoplasmic reticulum (rER) where de novo-biosynthesis of the full-length forms occurs, to their main cellular destination within endo-lysosomes (Mach et al., 1994; Mort et al., 1997; Linke et al., 2002a; Brix et al., 2008). One must bear in mind to tag the proteases in such a way that intrinsic protein sequences, essential for protein targeting and correct transport, will not become affected by introduction of the fluorescent protein-encoding sequences. Cysteine cathepsins can be tagged easily and without obvious mistargeting or loss-of-function effects when the green fluorescent protein (GFP) or other fluorescent proteins are attached to the C-terminus via a spacer peptide (Linke et al., 2002a; Katayama et al., 2008; Mayer et al., 2008). Cathepsin B has for example been localized to endo-lysosomes through visualization of the eGFP-tagged chimeric protein (Linke et al., 2002a) in thyroid carcinoma KTC-1 cells (Tedeñind et al., 2011), in the absence or presence of LysoTracker® that accumulates in endo-lysosomal compartments (Fig. 2). A variety of fluorescent proteins are available, some of which might also be useful in studying the precise timing of transport reactions, while others might be instrumental in determining interactions of proteases with substrates or other partners, such as split GFP (Waldron et al., 2008) or fluorescence resonance energy transfer (FRET) approaches, which are achievable and in use (Chudakov et al., 2010).

However, fluorescent protein tagging can be used as well to study secretion pathways of the zymogen forms along the secretory route in particular cells, e.g. cancer cells (Moin et al., 2000; Sameni et al., 2001; Mohamed and Sloane, 2006), or to analyze the strategies that epithelial cells use to recruit mature cysteine cathepsins out of late endosomes/lysosomes for retrograde trafficking and secretion into the extracellular space (Linke et al., 2002a; Linke et al., 2002b; Brix et al., 2008; Dauth et al., 2011). Interestingly, many cell types have been recognized as being competent in secretion of endo-lysosomal enzymes (Brix et al., 1994; Andrews et al., 2000; Brix et al., 2008). However, the precise transport pathways are not always studied in sufficient detail. Macrophages and dendritic cells are especially exceptional in the transport of proteases because they need to adapt quickly to situations such as infection and inflammation, where they serve as first-line defence and antigen-presenting cells respectively (Bogyo and Ploegh, 1998; Honey and Rudensky, 2003; Beers et al., 2005; Hsing et al., 2005).
Moreover, fluorescent protein-tagged cysteine cathepsins might become useful in determining the exact timing and to quantify the extent of lysosomal membrane rupture. That is, for instance, essential for release of mature cathepsin B into the cytosol which eventually leads to induction of apoptotic cell death by an alternative intrinsic induction pathway due to Bid-cleavage (Houseweart et al., 2003; Turk et al., 2009) and may be involved in killing of tumor cells by irradiation to induce lysosomal burst (Kroemer and Jaattela, 2005).

**Caught in the act – monitoring protease activities and substrate cleavage in cells and tissues**

From the above, it becomes clear that proteases are a group of extremely versatile enzymes that function in all possible locations within and outside cells. The classical view of cysteine cathepsins purely as lysosomal enzymes is now clearly outdated. Therefore, strategies are needed to visualize and image proteases in sites of action (Baruch et al., 2004; Blum et al., 2005; Brix and Jordans, 2005; Brix et al., 2008; Blum et al., 2009).

A variety of such strategies can include enzyme cyto- and histochemistry encompassing various photo conversion methods (Spiess et al., 1994). However, the principal possibility of combining immunolabeling with enzyme cytochemistry has
rarely been realized (Brix et al., 1996). The procedures that may be used to precisely determine the locations of cysteine cathepsin actions are summarized in Fig. 3. The protocols for detection of cysteine cathepsins are detailed in Tabs 1 to 3, and can be varied such that specific substrates in combination with enzyme-specific inhibitors may be used for ultrastructural detection, which are then verified by post-embedding immunolabeling procedures (Brix et al., 1996; Brix et al., 2008). Furthermore, a variety of antibodies can be tested by immunogold labeling techniques. However, those protocols bear the obvious disadvantage of inducing alterations of protease activities that may be caused by harsh fixation methods and other drastic chemical treatments of cells.

Hence, protease activities are best investigated by fluorescence microscopy methods that are optimal for exclusive use in living cells. In former times, fluorescently labelled extracellular matrices have been used to track substrate cleavage during processes such as cell migration (Friedl et al., 2000; Jedeszko et al., 2008; Cavallo-Medved

| Table 3 – Comparison of live and fixed cell procedures for enzyme cytochemistry procedures. |
|-----------------------------------------------|----------------|
| live cells (pericellular activity) | fixed cells |
| cell culture, e.g. on cover glasses | X | X |
| PBS | X | X |
| fixation: 1% PFA in PBS, 20 min, RT | --- | X |
| PBS | --- | X |
| reactivation: | | |
| reaction buffer (RB = 0.2 M ammonium acetate, 0.125 mM β-mercaptoethanol, 0.1 mM EDTA-Na₂, pH 6.2 or pH 7.2 with 0.4 M Na₂HPO₄), 5 min, 40°C without O₂ | X | X |
| reaction at 40°C without O₂ for 15 - 60 min: | | |
| 1 mM protease-specific substrate coupled to 4-methoxy-β-naphthylamine in RB + 0.5 – 1 mM 2-hydroxy-5-nitrosalicylicdehyde (NSA); controls: 1 mM E64 in RB, or omit substrate from RB + NSA | X | X |
| PBS or CMF-PBS | X | X |
| fixation: 1% PFA in PBS, 20 min, RT | X | --- |
| PBS | X | --- |
| ddH₂O | X | X |
| mounting in 33% glycerol, 14% mowiol in 200 mM Tris (pH 8.5) | X | X |
| view at excitation 488 nm | X | X |

X, step required; ---, step not required; RT, room temperature; O₂, oxygen; ddH₂O, double distilled water

Protease-specific substrates
  - cathepsin B: 0.5 – 1 μM Z-Arg-Arg-4MßNA ± CA-074 (pH 6.0 – optimum); controls with E64
  - cathepsin K: 0.5 – 1 μM Z-Gly-Pro-Arg-4MßNA ± 10 μM CA-074; controls with E64
  - cathepsin L: 0.5 – 1 μM Z-Phe-Arg-4MßNA ± 1.5 μM CA-074 (pH 5.5 – optimum); controls with CA-074 + Z-FY(t-Bu)-DMK
et al., 2009). A number of small molecules are now available that have accelerated the field of cysteine cathepsin biology significantly in recent years (Greenbaum et al., 2000; Greenbaum et al., 2002; Blum, 2008; Blum et al., 2009). The basic idea of activity-based probes (ABPs) is the synthesis of small molecules that can easily penetrate biological membranes (see, Tab. 1), therefore reaching all potential locations of proteolytic enzymes within cells and tissues.

In general, ABPs bear a so-called warhead, i.e. they are functionalized to bind to only one group of proteases, because the mechanism of action is specific targeting of ABPs to one family of enzymes (Liu et al., 1999; Kidd et al., 2001; Baruch et al., 2004; Kato et al., 2005; Sieber et al., 2006; Sexton et al., 2007; Wright et al., 2009; Yang et al., 2009). Upon binding, ABPs are cleaved since most of them represent substrate analogs, thereby attaching themselves covalently to the targeted proteases. ABPs are additionally functionalized with fluorophores, iodinatable groups, biotin moieties

Figure 3 – Schematic representation of experimental strategies for imaging of cysteine cathepsin functions. An appropriate choice from the selection of detection methods available for cysteine peptidase localization is dependent on factors such as the nature of the experimental question, type of specimen and specimen preparation. Most established protocols of indirect immunofluorescence employ the use of antibodies which can effectively detect both zymogens (A and F) and active cathepsins (B – E and G – K). Cathepsin activity can be visualized through the use of substrate analogs that are cleaved upon encountering active protease with the leaving groups precipitating at the spot of enzymatic activity (B and G). Leaving groups may be visualized through signal enhancement by incubation with nitrosalicylaldehyde in fluorescence microscopy, or pararosanilin and OsO4 in electron microscopy (Brix et al., 1996). Active cysteine cathepsins in complex with their endogenous inhibitors such as the cystatins, can be detected using cystatin-specific antibodies (C and H). A very valuable tool for detecting active cathepsins at their sites of action is the use of quenched (D and J) or non-quenched activity-based probes (ABP; E and K). Quenched ABPs are designed to mimic the natural substrate and covalently interact with the protease in its active site, whereby the fluorescent signal is generated upon proteolytic cleavage. Non-quenched ABPs are fluorescent before interacting covalently with the active protease in a 1-to-1 ratio, thereby fluorescently highlighting enzymatic activities on the spot (E and K; Brix and Jordans, 2005). Another useful approach of studying cathepsins in living cells is the use of GFP-tagged chimeras, which constitutively track the position of the proteins regardless of whether immature (F) or enzymatically active (G – K) proteases are tagged with GFP (Brix et al., 2008). GFP fusion at the C-terminus of cysteine cathepsin B has been shown not to interfere with protease trafficking. GFP tagging of proteases can be combined with immunofluorescence or enzyme cytochemical or ABP labelings of all kinds (F – K).
etc., which are attached to the opposite end of the small molecule backbone. Hence, ABPs target protease families rather than individual enzymes as shown for the membrane permeable and fluorophore-conjugated DCG-04 that visualizes active cysteine peptidases in human keratinocytes (Fig. 4B). An alternative approach combines...
propeptide-like with substrate-like binding properties in a synthetic small molecule (Schaschke et al., 2000). These probes are suitable in targeting individual enzymes in larger families of related proteases. For instance, a cathepsin B-specific probe has been designed which is able to clearly distinguish between the most abundantly expressed cathepsins B and L by an impressive factor of 2,000 (Schaschke et al., 2000; Schaschke et al., 2002). The fluorophore-conjugated ABP specific for cathepsin B, NS173, enabled the visualization of active, plasma membrane associated cathepsin B in human keratinocytes (Fig. 4A), and it can be used additionally for visualization of active cathepsin B in situ in mouse intestine (Fig. 4C) while pre-incubation with the cysteine cathepsin inhibitor E64 serves as a control for probe selectivity (Fig. 4C).

Quenched ABPs provide better signal-to-noise ratios than non-quenched probes because they become fluorescent only upon cleavage and covalent attachment to the target protease (Blum et al., 2005; Brix and Jordans, 2005; Blum et al., 2007; Blum et al., 2009). This elegant approach has now reached as far as the design of ABPs for diagnostic use in mouse (Blum et al., 2007; Blum et al., 2009). The long-term goal is to utilize them in translational approaches of rational drug design as therapeutics.

These most promising features of ABPs point to their future applications as useful tools to visualize excess protease activities and to even treat diseases like cancer at the molecular level. However, the molecular features of ABPs and their mechanism of action also bear a serious disadvantage to this approach. Namely, ABPs act as inhibitors of the targeted proteases. ABPs bind and covalently attach to their target proteases in a one-to-one fashion. Hence, for therapy they must be used in extremely high concentrations, thereby increasing the risk of toxicity and induction of off-target effects. On one hand, since they are protease inhibitors, treatment of a cell with ABPs may elegantly target protease activities; on the other hand, a pharmacological knock-down can be easily produced.

In future, ABPs and related molecules could become as useful as gene knock-down or knock-out strategies in altering the composition of proteolytic networks on a long-term basis. It would then also be possible to challenge protease activities and actions by using animal models of various diseases. The goal is to target, inhibit or initiate protease activities, in order to learn more about their true biological significance as enzymes mediating quick cuts that irreversibly change the fate of proteins.

**Conclusions and Perspectives**

Tracking and localizing proteases in living cells and co-localizing proteases with their natural substrates in an entire organism is our ultimate objective in specifying the significance of proteolysis in vivo (see, Fig. 3). This aim is not far away. Numerous experimental achievements have demonstrated that pharmacological inhibition, gene targeting through knock-outs, knock-ins, transgenic over-expression or knock-downs, and combinations with imaging studies of protease functions in living cells have been conducive in answering several important questions. These include, but are not limited to, issues on the in vivo importance of a certain protease in any given biological context. To this end, we will continue in our aim of clarifying how post-translational proteolytic modifications are involved in biological processes during development, adulthood and in ageing. Proteases encoding two percent of the entire human genome must be expressed for fun-
damentally crucial reasons - to enable the molecular basis of decision-making processes of cells in tissues and in all conditions of life, disease, repair and death.

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References


Visualizaion of cathepsins in epithelial cells


