System-Level Analysis of Proteolytic Events in Increased Vascular Permeability and Complement Activation in Skin Inflammation

Science Signaling, 15 January 2013, Vol 6 Issue 258

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+ "Identifying and quantifying proteolytic events and the natural N terminome by terminal amine isotopic labeling of substrates", Nat Protoc., 2011, Sep 22;6(10)
Outline

1. Background

2. Motivation for the study, aims

3. Study design, Models, Methods

4. Results

5. Discussion: Significance and relevance of the results

6. Possible implication in projects of Prof. Dr. Aguzzi
Background

Proteases and their inhibitors (664 genes in mice):
- Kininogen $\rightarrow$ bradykinin
- proChemokines $\rightarrow$ Chemokines

Well studied

Cell migration
Extracellular matrix remodeling
Removal of the debris

Potentially involved

Hard to study proteases: functional information
- Protease abundance and localization
- Protease activity
- Many potential substrates
- Interconnected activity

To study the role of even single protease:

Functional information of a large-scale
Motivation for the study and aims

1. Development of the high-content screening tool: functional information on proteases on a large scale

   Ultimate goal - to generate hypotheses as a basis for more detailed studies that aim to confirm in vivo mechanisms

2. Screen global changes in proteolytic modification of the inflammatory proteome in vivo

   complex protease function in health and disease

3. Dissect the impact of a single protease MMP2 on proteolytic signature of skin inflammation

   MMP2:
   - immunomodulatory, associated with tissue repair, angiogenesis
   - multiple potential substrates

A. Prudova, 2010, Mol&Cell Proteomics
**Study design, Models, Methods**

**CLIP-CHIP: Transcriptome**
All proteases, non-proteolytic homologs, and inhibitors gene transcripts aka DEGRADOME

**In vivo model**
TPA induced skin inflammation 48h
WT and MMP2−/−

Frustrating note on animal model (♀):
2 WT TPA vs 2 WT control
2 KO TPA vs 2 KO control
Only 2 replicates!

«Extracellular fraction» isolation: 100 mM HEPES pH8.0

Taging iTRAQ (isobaric tags for relative and absolute quantification)

Proteome: Shotgun proteomics (HPLC + nanoLC-MS/MS)
N-terminome: iTRAQ-TAILS (iTRAQ-Terminal Amine Isotopic Labeling of Substrates)

Data analysis:
1. Search (mouse International Protein Index database)
2. Evaluation (Trans-Proteomic Pipeline)
3. Quantification
4. Statistical analysis

Validation of the selected events
Study design, Models, Methods

MS/MS

Trypsin digestion

Fragmentation:
- P_EPTIDE
- PE_PTIDE
- PEP_TIDE
- PEPT_IDE
- PEPTI_DE
- PEPTID_E

Sequence → Protein identification

Mixture of proteins: Shotgun proteomics

- tissue lysates are too complex, identification of the most abundant proteins
- «yes/no» information
Study design, Models, Methods

iTRAQ (isobaric Tags for Relative and Absolute Quantification)

Reacts with
N-terminal amino groups
and
amino group of lysine

Proteomics: yes/no

iTRAQ: how much
iTRAQ-TAILS:  
+ reduce sample complexity  
+ identify cleavage spectrum, cleavage sequence  
+ quantify proteolysis of specific target  
+ identify and often quantify N-acetylation

100-300 mkg of tissue
Study design, Models, Methods

Data analysis

- Search (at least 2 databases) **Mascot** and **X! Tandem**
- List of all possible proteins
- Evaluation (Trans-Proteomic Pipeline) + Quantification
- High-confidence identification
- Statistical analysis

\[ \log_2(\text{[treatment]}/[\text{control}]) \]

Results

Degradome

WT<sub>control</sub> vs WT<sub>inflammation</sub> (208 altered significantly)

<table>
<thead>
<tr>
<th>Upwards (↑)</th>
<th>Downwards (↓)</th>
<th>Equilibrium (≡)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteases:</td>
<td>Proteases:</td>
<td>Proteases:</td>
</tr>
<tr>
<td>MMP (3, 8, 13)</td>
<td>MMP9</td>
<td>MMP2</td>
</tr>
<tr>
<td>Cathepsins kallikrein</td>
<td>Serpin</td>
<td></td>
</tr>
</tbody>
</table>

Inhibitors:

- TIMP, Stefin
- Serpin

Tight regulation of increased proteolysis
Results

Proteome

WT<sub>control</sub> vs WT<sub>inflammation</sub>

1147 proteins identified
976 quantified

107 over 2-fold changes

Acute phase proteins
Complement
Coagulation
Proinflammatory S100
Myeloperoxidase (neutrophil influx)

Annexin 8
Cadherin 13
Suprabasin
Filaggrin 2
(hyperproliferative response)

Infiltration of acute phase proteins
Hyperproliferative state
Results

N-terminome (iTRAQ-TAILS)

WT<sub>control</sub>
1813 N-termini, 1104 proteins
N-terminome
457 - Low abundance proteins
647
Proteome
500 -?

Curse of all proteomics: high abundant proteins

Origin of 1813 N-termini

N-terminal after cleavage «real» N-terminal

High level of background proteolysis
Results

N-terminome (iTRAQ-TAILS)

WT_{control} vs WT_{inflammation}

18 proteins appear (local synthesis or import)

1. Acute phase proteins from serum
2. Produced in epidermal cells (MMP3)
3. Released from resident/infiltrated inflammatory cells

Degradation processes

1. N-terminal ragging of antithrombin III
2. Enzyme activation: plasminogen → plasmin
3. Precursor processing: kininogen → bradykinin
5. Release of cryptic growth factors from ECM: collagen IV → arrestin
6. S100 protein cleavage (proinflammatory)
7. Proteolytic maturation by profilaggrin endoproteinase

Increased rate of proteolysis
Results

\[ \text{WT}_{\text{control}} \text{ vs } \text{WT}_{\text{inflammation}} \]

CLIP-CHIP

Proteome

N-terminome

Altered mRNA of several proteases and inhibitors: tight regulation of increased proteolysis

Infiltration of acute phase proteins

Hyperprolyferative state

Increased proteolysis rate

Infiltration of serum/inflammatory cells proteins

Detection of specific cleavage processes

In some cases divergent abundance in mRNA and protein level
**Results**

- WT\_control vs KO\_control
- WT\_inflammation vs KO\_inflammation

KO = MMP2\(^{-/-}\)

WT\_control vs KO\_control (29 altered significantly)
WT\_inflammation vs KO\_inflammation (82 altered significantly)

**Inflammation:**
- KO\(\uparrow\) WT\(\equiv\) MMP11, MMP28, CatL, KLK9
- WT\(\uparrow\) KO\(\equiv\) CatJ

MMP2 regulation of gene expression indirect? compensation?
Results

WT\textsubscript{control} vs Ko\textsubscript{control}
WT\textsubscript{inflammation} vs KO\textsubscript{inflammation}

Proteome

No differences in the inflammatory cell infiltrate or epidermal hyperproliferation

Hematoxylin eosin staining.

Decrease:
Reduction in the extent of exudation of acute-phase proteins, including complement factors and general plasma proteins

Increase:
cell shape, adhesion, junction

No Haptoglobin changes in liver

Decrease:
Reduction in the extent of exudation of acute-phase proteins, including complement factors and general plasma proteins

Increase:
cell shape, adhesion, junction
Results

Hypothesis

WT inflammation:

- Acute phase proteins
- Complement
- Coagulation
- Proinflammatory S100
- Myeloperoxidase (neutrophil influx)

WT vs KO inflammation:

- Decrease: Reduction in the extent of exudation of acute-phase proteins, including complement factors and general plasma proteins.

In Mmp2\(^{-/-}\) mice there was less of an increase in vascular permeability in inflammation compared to that in wild-type mice, and hence, there was less exudation of serum and acute-phase proteins into the tissue.
Results

Digging the N-terminome

MMP2 and vascular permeability

C1 inh – Serpin
KLKB1 – plasma kallikerin
KNG1 – kininogen
BDK – bradykinin (vascular permeability)
Results

MMP2 and vascular permeability

validation

\[ \text{log}_2 \text{ratio} = \log_2(\text{wt-TPA/ko-TPA}) \]

\[ \text{m/z} = 1060.57 \]

\[ \text{C1 Inh fragment} \quad 4 \text{kD} \]

\[ \text{C1 Inh} \quad \text{KNG1} \quad \text{KNG1a} \]

\[ \text{BDK} \]

\[ \text{log}_2 \text{ratio} = 1.02; \text{QCF} 2.21 \]

\[ \text{log}_2 \text{ratio} = 2.01; \text{QCF} 1.72 \]

\[ B \]

\[ BDK \quad \text{Standard} \]

\[ - \text{C1 Inh} + \text{MMP2} \]

\[ - \text{C1 Inh} \]

\[ BDK \]

\[ + \text{C1 Inh} \]

\[ + \text{C1 Inh} + \text{MMP2} \]

\[ \text{Rel EBDK generation} \]

\[ \% \]

\[ 1060 \quad 1296 \quad m/z \]

\[ \text{in vitro cleavage of C1 inh by MMP2} \]

marimastat – MMP inhibitor

\[ \text{in vitro cleavage of KNG1 by KLKB} \]

\[ \text{in vivo Miles assay using Evans Blue} \]

vascular permeability
Results

MMP2 and complement activation

MMP2 cleaves C1inh and enhances complement activation

Complement factor I (IF) cleaves:
- C4b to C4d'
- C3b to C3dg
inactivating action

MMP KO mice – less C4b and C3b (and their inactivation products)
Results

MMP2 and complement activation, validation

ex vivo hemolysis
(membrane attack complex)
1. Developed tool to read proteolytic signature of the inflamed tissue

2. Compared and quantified proteolytic signature of the inflamed tissue in WT and MMP2 KO
   - important and background proteolytic events

3. Identified proteins origin (newly imported in to the inflamed region)

4. Reconstructed the relevant proteolytic function of MMP2:
   - regulation of vascular permeability in inflammation (bradykinin release)
   - regulation of complement activation (C1 complex formation)
Possible implication


“prion neurotoxicity is calpain-dependent but caspase-independent in CGCs”

fodrin cleavage and...?