Development of activity-based probes for trypsin-family serine proteases

Zhengying Pan,* Douglas A. Jeffery,† Kareem Chehade, Jerlyn Beltman,‡ James M. Clark, Paul Grothaus, Matthew Bogyo*§ and Amos Baruch*

Celera Genomics, 180 Kimball Way, South San Francisco, CA 94080, USA

Received 2 February 2006; revised 28 February 2006; accepted 2 March 2006
Available online 22 March 2006

Abstract—A series of diphenylphosphonate-based probes were developed for the trypsin-like serine proteases. These probes selectively target serine proteases rather than general serine hydrolases that are targets for fluorophosphonate-based probes. This increased selectivity allows detection of low abundance serine proteases in complex proteomes using simple SDS–PAGE methods. We present here the application of multiple probes in enzyme activity profiling of intact mast cells, a type of inflammatory cell implicated in allergy and autoimmune diseases.

© 2006 Elsevier Ltd. All rights reserved.

The primary goal of proteomics is to assign functions to all proteins in a given cell, tissue or organism.1 The challenges implicit in this field have generated novel approaches to functionally dissect the proteome. Chemical proteomics or activity-based protein profiling (ABPP) makes use of small molecule probes that form covalent complexes with their targets using an activity-dependent chemical reaction. These activity-based probes (ABPs) can be used to profile enzymatic activities in complex proteomes and provide information on protein targets at the functional, rather than the expression level.2 Moreover, due to its use of small molecules, this approach naturally focuses on ‘druggable’ enzymes that show specific ligand binding.

A number of classes of ABPs have successfully been designed and applied to serine hydrolases3 and cysteine proteases.4 Recently the scope of this approach has expanded with the development of chemical probes that target additional important enzyme families including phosphatases5 and kinases.6 Many approaches have focused on broad-spectrum probes that target multiple related enzyme family members.7 Thus, there is a great need to develop new chemical probes to selectively target sub-classes of enzymes to study their roles in biological processes.

Serine hydrolases represent a large family of enzymes, members of which participate in many crucial biological processes. One of the most intriguing sub-classes of this family is the trypsin-like serine proteases. This sub-group is comprised of 65 enzymes in humans with unique cellular and physiological regulatory roles in health and disease.8 Several enzymes within this group, such as thrombin, factor VIIa, factor Xa, and trypsin, are being extensively pursued as drug targets in cardiovascular and inflammatory indications. A fluorophosphonate (FP) probe, similar to probe 1 (BioFP), has been previously reported to target the serine hydrolase family (Ref. 3). The broad reactivity of probe 1 enables simultaneous labeling of a large number of enzymes including esterases, proteases, lipases, and amidases. In many cases, this probe generates a highly complex activity profile that prevents its usage for studying low-abundance, specific enzymes using simple analytical methods such as SDS–PAGE. To

Keywords: Activity-based probes; Tryptase; Chemical proteomics.
*Corresponding authors; e-mail addresses: zypan@yahoo.com; zhengying.pan@celera.com; mbogyo@stanford.edu; amos.baruch@celera.com
†Present address: Novartis Institute of Biomedical Research, Boston, MA 02139, USA.
‡Present address: Chiron Corporation, Emeryville, CA 94608, USA.
§Present address: Department of Pathology, School of Medicine, Stanford University, 300 Pasteur Drive, Stanford, CA 94305, USA.
circumvent this issue, we developed a series of selective probes that specifically target the trypsin-like serine proteases (see Fig. 1).

To achieve selectivity toward serine proteases, we focused our efforts on phosphonate-based probes. Initial attempts to use an α-amino fluorophosphonate reactive group failed due to its short half-life in an aqueous environment. The more stable reactive group, diphenylphosphonate, has previously been used to generate potent irreversible serine protease inhibitors. Here, we describe our efforts in developing diphenylphosphonate (DPP)-derived probes for activity profiling of trypsin-like serine proteases (see Scheme 1).

Based on previous studies of substrate specificity, a lysine residue was incorporated at the P1 site with either a proline or asparagine at the P2 position to generate a general trypsin-family protease probe 4 (Bio-PK-DPP) and a β-trypsin-selective probe 5 (Bio-NK-DPP), respectively. Lysine-based DPPs can be made through the Oleksyszyn 3-component reaction using either conventional heating or microwave-assisted agitation. Intermediate 2 was obtained through three steps of protecting group manipulations. A biotinylated probe 3 (Bio-K-DPP) was made by coupling 2 with a commercially available biotinylation reagent NHS-(PEG)$_4$-biotin. Following addition of an amino acid residue at the P$_2$ position, similar procedures produced biotinylated probes 4 and 5.

Apparent inhibition constants [$K_{app}$] were obtained for these probes against four trypsin-like serine proteases (Table 1). The addition of a P$_2$ proline residue increased the overall reactivity of probe 4 toward trypsin-like serine proteases. Incorporation of an asparagine residue at P$_2$ yielded a 23-fold selectivity increase for probe 5 favoring trypsinase over its closely related family member trypsin.

To confirm that the potency and selectivity observed in kinetic assays were reflected in enzyme labeling profiles, probes 1, 4, and 5 were used for activity-based labeling of a panel of recombinant enzymes from different serine hydrolase families (Fig. 2). The extent of covalent labeling was determined by Western blotting with streptavidin detection of the biotin reporter group. Probe 1 labeled all recombinant enzymes tested including butyrylcholine esterase (BCE), a hydrolase enzyme. In contrast, the lysine-DPP-based probes were completely inactive against both chymotrypsin and BCE. The general trypsin-family probe 4 efficiently labeled all trypsin-like proteases.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Bio-FP 1</th>
<th>Bio-PK-DPP 4</th>
<th>Bio-NK-DPP 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Trypsin</td>
<td>30</td>
<td>6.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Trypsin</td>
<td>16.5</td>
<td>0.57</td>
<td>57.3</td>
</tr>
<tr>
<td>Thrombin</td>
<td>7.9</td>
<td>1.07</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Plasmin</td>
<td>&gt;100</td>
<td>2.9</td>
<td>4.03</td>
</tr>
</tbody>
</table>

Table 1. Apparent inhibition constants [$K_{app}$] μM of probes 1, 4, and 5 for trypsin-like serine proteases following 30 min of incubation.

Figure 1. A general serine hydrolase probe 1.

Scheme 1. Synthesis of ABPs with the diphenylphosphonate reactive group. Reagents and conditions: (a) HOAC as solvent, microwave, 150 °C, 5–10 min or oil bath, 70 °C, 1–3 h; (b) hydrazine: Boc$_2$O, H$_2$, Pd–C; (c) NHS-(PEG)$_4$-biotin, triethylamine; then TFA; (d) Cbz-Pro-OH, EDCI, HOBT, H$_2$, Pd–C; (e) Cbz-Asn(Trt)-OH, EDCI, HOBT; H$_2$, Pd–C.
enzymes were serine hydrolases that were not proteases. In addition, several identified serine hydrolases were abundant housekeeping enzymes such as acyl-transferase, lysophospholipase, and acyl-CoA thioester hydrolase. In sharp contrast, the trypsin-family-selective probe 3 selectively labeled β-tryptase (Fig. 3, right panel). Further analysis of the labeling with probe 1 revealed that acyl-transferase and β-tryptase co-migrate on the gel, thus making it difficult to distinguish activity-profiles for these two enzymes in HMC-1 cells. Since no labeling of acyl-transferase was observed with probe 3, β-tryptase activity could be directly evaluated in the mast cell proteome without a need for any further purification steps. This exemplifies the utility of protease-selective probes for profiling trypsin-like proteases within a complex proteome.

HMC-1 cells are immature cells that do not fully reflect properties of mature mast cells. A prominent feature of mast cell is the presence of granules that store preformed mediators including proteases such as tryptases. Upon activation, the content of the granules is released into the extracellular milieu to promote local wound healing and inflammatory responses. We characterized serine hydrolase and protease activities in more physiologically relevant, mature mast cells that were derived from CD34+ hematopoietic progenitors. These cells were stimulated to degranulate via activation of the high affinity receptor for IgE (FcεRI) (Fig. 4). Following activation, intact cells were incubated with probes for 1 h, conditioned medium was collected, and labeled enzymes were purified and identified. Probe 1 yielded a complex labeling pattern regardless of the mast cell activation state (Fig. 4, lanes 1 and 2). The trypsin-selective probe 5 predominantly labeled β-tryptase (Fig. 4, lanes 3 and 4). Notably, when the general trypsin-family probe 4 was used for labeling, both trypsin and cathepsin G were labeled (Fig. 4, lanes 5 and 6). The observed reactivity of probe 4 is consistent with the known substrate specificity of these two enzymes, with trypsin exhibiting trypsin-like activity and cathepsin G having both trypsin and chymotryptic activities. Labeling with probes 4 and 5 showed clear up-regulation in the activity of these enzymes following IgE stimulation (Fig. 4, lane 3 vs lane 4 and lane 5 vs lane 6). This demonstrates that probes 4...
and 5 can be used to study the cellular β-tryptase's activity upon biological stimulation.

In summary, we have demonstrated the design, synthesis, and application of selective trypsin-family protease-selective probes 3, 4, and 5. These probes are capable of specifically labeling trypsin-like serine proteases either in their pure forms or as components of complex proteomes. Thus, these probes represent powerful biochemical tools for monitoring the activity of proteases in their natural environment. Attachment of other reporter groups such as fluorophores may also allow imaging of protease activity in situ as recently described for cysteine proteases.16 Finally, probes that target other sub-families of serine proteases can be designed using a similar approach. Increasing the number and type of chemical proteomics probes targeting serine proteases will significantly enhance our ability to understand the activity, regulation, and function of these important enzymes.

References and notes


13. Inhibition profiles for the serine proteases were generated by incubating each enzyme in the presence of probes (various concentrations) or 10% DMSO (vehicle control) for 30 min in 96-well clear polystyrene plates at room temperature prior to the addition of substrate. Enzyme activity was measured by monitoring the hydrolysis of the synthetic substrate tosyl-Gly-Pro-Lys-nitroanilide at 405 nM over 5 min using a UV/MAX kinetic plate reader (Molecular Devices). The apparent inhibition constants [K_{iapp}] were calculated from the progress curves using the software package Batch K (BioKin, Ltd). In each case, the substrate concentration was at or below the K_{m} for the enzyme.


15. The following hydrolases were identified by LC/MS/MS analysis (Q-Star) (serine protease in bold). Secreted serine hydrolases: dipeptidyl peptidase 7 (54 kDa); esterase 10 (37 kDa); tryptase β1 (34 kDa); lysophospholipase 2 (24 kDa); platelet-activating factor acetylhydrolase (26 kDa). Membrane serine hydrolases: oxidized protein hydrolase (82 kDa); prolyl endopeptidase (81 kDa); angiotensinase C/propylcarboxypeptidase (56 kDa); retinoid-inducible serine carboxypeptidase (51 kDa); KIAA1363/carboxy esterase (48 kDa); peroxisomal acyl-coenzyme A thioesterhydrolase 2a (46 kDa); aldolase A (39 kDa); protein FLJ11342/lipase (32 kDa); similar to hypothetical protein 4833421E05Rik/lipase (28kDa); OVCA2/tumor suppressor in ovarian cancer (25 kDa).