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COMMUNICATIONS

Published on Web 11/10/2004

Microarray Platform for Profiling Enzyme Activities in Complex Proteomes

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The field of proteomics aims to develop methods for the comparative analysis of large numbers of proteins in complex biological samples.[1] Classical proteomic methods measure changes in the expression level of proteins. However, many enzymes are regulated by post-translational events in vivo,[2] meaning that their abundance may not correlate with their activity. A chemical strategy referred to as activity-based protein profiling (ABPP)[3] utilizes active-site-directed probes to profile the functional state of enzymes in proteomes. To date, ABPP probes have been developed for several enzyme classes, including proteases (serine,[4] cysteine,[5] metallo[6]), oxido-reductases,[7] and phosphatases,[8] and have been used to discover enzyme activities upregulated in aggressive cancer cells[9,10] and to evaluate the specificity of inhibitors in cells/tissues.[11]

In typical ABPP experiments, probe-labeled enzymes are detected by in-gel fluorescence scanning,[9] a robust technique that nonetheless exhibits key deficiencies, including limited sensitivity and resolution, as well as ambiguity regarding the identity of the enzymes under investigation. Although independent affinity enrichment and liquid chromatography−mass spectrometry (LC−MS) analysis of probe-labeled enzymes can resolve some of these problems,[9] it does so at the expense of throughput and sample conservation. Here, we report a microarray platform for ABPP that overcomes many of the technical limitations of both gel- and LC−MS-based methods.

A microarray version of ABPP has been previously described that employs small-molecule probes coupled to peptide−nucleic acids;[12] however, in this method, the probe serves as both the protein labeling and capture reagent. As a consequence, this approach mandates that probes show high specificity for individual enzymes and is therefore not suitable for many ABPP experiments, which utilize probes that broadly target large numbers of enzymes in proteomes. We postulated that a more general microarray platform for ABPP could be created by incorporating orthogonal strategies for the labeling and capture of enzyme activities. In this method, proteomes are first treated with fluorescent activity-based probes, and then the labeled enzymes captured and visualized on glass slides displaying anti-enzyme antibodies. Several enzyme activities targeted by a single (or multiple) ABPP probe(s) could thus be profiled in parallel on individual slides by arraying a complementary set of antibodies (Figure 1).

To evaluate antibody microarrays as a platform for ABPP, four commercially available antibodies were selected for analysis: three that recognize serine proteases [prostate-specific antigen (PSA), urokinase (uPA), and tissue plasminogen activator (tPA)], which are labeled by the ABPP probe fluorophosphonate−rhodamine (FP−Rh),[9] and a fourth that targets matrix metalloproteinase 9 (MMP9), which is labeled by the photoreactive probe, hydroxamate−benzophenone Rh (HxBP-Rh).[6] Notably, each of these proteases is upregulated in human cancer,[13,14] and therefore, sensitive methods to measure their activity in proteomes may be of great diagnostic value. Microarrays were printed in the contact mode on NHS-functionalized hydrogel slides with antibody concentrations

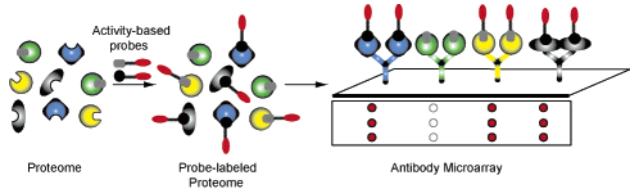

Figure 1. General strategy for antibody-based ABPP microarrays. Proteomes are labeled in solution with fluorescent activity-based probes and captured on glass slides arrayed with enzyme-specific antibodies.

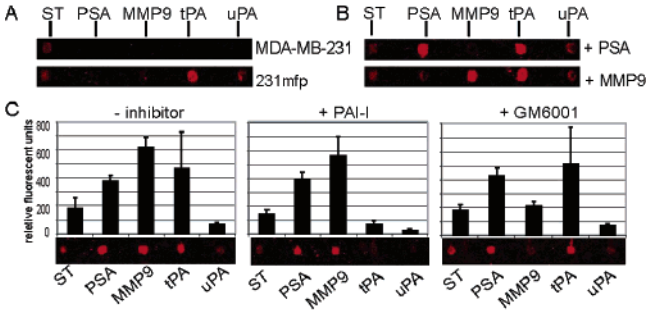

Figure 2. Profiling protease activities in proteomes by ABPP microarrays. (A) Microarrays correctly report that tPA and uPA activities are higher in 213mfp versus MDA-MB-231 proteomes. (B) Addition of PSA (0.6 μg/mL) or MMP9 (2 μg/mL) to the 213mfp proteome results in specific detection of each protease activity on microarrays. (C) Addition of protein (PAI-1) or small-molecule (GM6001) inhibitors reduces signals of their respective protease targets (tPA/uPA and MMP9) (also see Figure S4). ST, fluorescent streptavidin as a control for slide quality.

of 0.5−1.3 mg/mL. In a first set of experiments, the secreted proteomes from the human breast cancer cell lines MDA-MB-231 and 231mfp were analyzed.[15] The 231mfp line exhibits enhanced tumorigenic properties compared to MDA-MB-231 cells that correlate with elevated levels of active tPA and uPA.[9]

Proteomes were treated with FP−Rh under standard labeling conditions,[16] after which excess probe was removed by gel-filtration, the reactions were concentrated by spin filtration, and 3 μL (1.5 μg of protein) was applied to individual microarrays for 2 h in a humidified chamber at room temperature. Slides were washed in PBS/0.5% Tween 20 and analyzed in a fluorescent scanner. All experiments were carried out in triplicate (unless otherwise indicated) on arrays with six replicate spots per antibody. Comparisons between arrays incubated with MDA-MB-231 and 213mfp proteomes revealed stronger fluorescence signals for uPA and tPA activities in the 231mfp proteome (Figure 2A), consistent with previous gel-based ABPP studies.[9]

To test whether ABPP microarrays could profile several enzyme activities in parallel, two additional proteases, PSA and MMP9, were added to the 213mfp proteome, and the sample was treated sequentially with HxBP−Rh and FP−Rh.[17] The ABPP microarrays detected signals for PSA and MMP9 specifically in proteomes to which these proteases were added (Figure 2B). Notably, inclusion

10.1021/ja044286+ CCC: $27.50

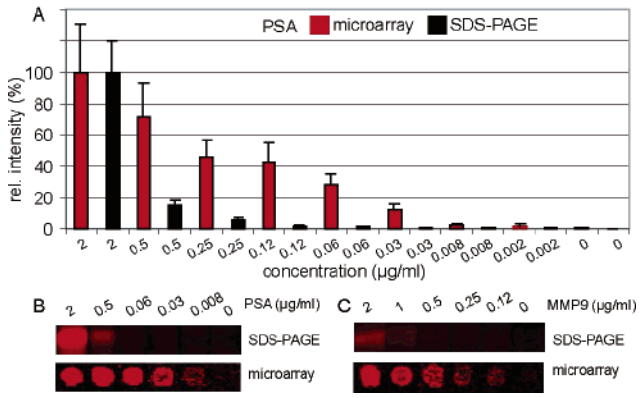

Figure 3. Testing the sensitivity of ABPP microarrays. (A,B) ABPP microarrays exhibit ~30-fold greater sensitivity for the detection of PSA activity compared to gel-based ABPP (sensitivity limits: 0.002−0.008 and 0.12−0.25 ng/mL, respectively). (C) ABPP microarrays also display greater sensitivity for the detection of MMP9 (also see Figure S6).

of the MMP9 inhibitor GM6001 or the uPA/tPA inhibitor PAI-1 to proteomes strongly reduced the signals for their respective targets, indicating that ABPP microarrays provide a bona fide readout of enzyme activity in whole proteomes (Figures 2C, S3, and S4).

We next compared the sensitivity of ABPP microarrays to gel-based methods. PSA (400 ng) was added to the 213mfp proteome (100 μg protein, 0.5 μg/μL) and, following treatment with FP−Rh, this reaction was serially diluted into FP−Rh-treated 213mfp proteome lacking PSA. These samples were then analyzed by 1D SDS−PAGE or antibody microarrays. The detection limits for PSA were ~125−250 and 2−8 μg/mL for gel- and microarray-based ABPP, respectively (Figure 3A,B). Qualitatively similar results were obtained for MMP9, which was also detected with greater sensitivity by microarrays (Figures 3C, S6). The enhanced sensitivity of ABPP microarrays appears to be due in part to superior resolving power. For example, at lower concentrations, strong signals for PSA were measured on microarrays, whereas the detection of this protease on gels was obscured by co-migrating enzymes (Figure S5). Finally, ABPP microarrays could measure PSA activity over a linear range of ~2 orders of magnitude (0.002−0.125 μg/mL), above which signals saturated (Figures 3A, S7).

In summary, we have described an antibody-based microarray platform for ABPP that consolidates into a single step the isolation, detection, and identification of probe-labeled enzymes. By implementing orthogonal strategies for the labeling and capture of enzyme activities, ABPP microarrays enable the proteomic analysis of many enzymes in parallel with a sensitivity and resolving power that greatly exceed those of gel-based methods. Additionally, ABPP microarrays consume less proteomic material than gels (1.5 versus 15 μg/experiment, respectively), an important advantage for samples of limited quantity (e.g., tumor biopsies). ABPP microarrays also address some key limitations of conventional antibody microarrays.[18] For example, ABPP microarrays eliminate the need for random protein labeling and/or secondary antibodies that are required for antigen detection in typical microarray experiments.

Here, we have profiled a panel of serine proteases and metalloproteases using ABPP microarrays. The concurrent analysis of many more enzyme activities should be possible, given the availability of high-quality antibodies. In this regard, we found that the sensitivity of ABPP microarrays varied from protease to protease, due at least in part to the specific antibodies employed. These results highlight the need for additional high-quality antibodies, which are currently available for only a very small percentage of the proteome.[18] Nonetheless, for proteases such as PSA, for which excellent antibodies are available, ABPP microarrays exhibited a detection limit of 2−8 ng/mL, which is in the range of endogenous serum levels for this protease.[14] PSA is a clinical biomarker for prostate cancer, and there is considerable interest in measuring free (active) versus total PSA to assist in the diagnosis of this disease.[14] ABPP microarrays offer an attractive strategy for making such measurements. More generally, ABPP microarrays should enhance the information content achieved in functional proteomics experiments by permitting the parallel analysis of numerous enzyme activities with exceptional resolution and sensitivity. We anticipate that ABPP microarrays will facilitate the characterization of new markers and targets for the diagnosis and treatment of human disease.

Acknowledgment. We thank J. Chambers and M. Humphrey for technical assistance and members of the Cravatt laboratory for discussions. This work was supported by the NCI (CA087660), the California Breast Cancer Research Foundation, The Skaggs Institute for Chemical Biology, and a DFG Emmy Noether postdoctoral fellowship (S.A.S.).

Supporting Information Available: Experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) These lines correspond to parental 231 cells and 231 cells isolated from the mammary fat pad of xenograft tumors, respectively (see ref 9b).
(16) Conditions: 1 μM FP−Rh, 50 mM Tris, pH 8.0, 1 h at room temperature.
(17) A UV cross-linking step (365 nm on ice for 1 h) was included between the addition of HxBP−Rh (200 nM) and FP−Rh (1 μM).
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JA044286+