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Chemical Proteomic Platform To Identify Citrullinated Proteins

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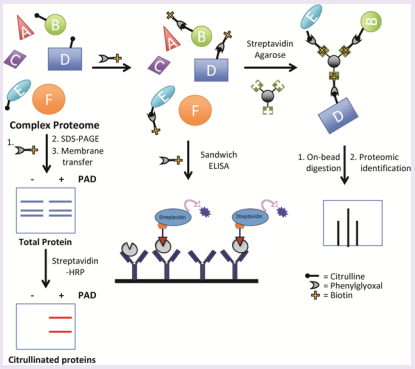
Supporting Information

ABSTRACT: Anti-citrullinated protein antibodies (ACPAs) are a hallmark of rheumatoid arthritis (RA) and are routinely used for disease diagnosis. Protein citrullination is also increased in cancer and other autoimmune disorders, suggesting that citrullinated proteins may serve as biomarkers for diseases beyond RA. To identify these citrullinated proteins, we developed biotin-conjugated phenylglyoxal (biotin-PG). Using this probe and our platform technology, we identified >50 intracellular citrullinated proteins. More than 20 of these are involved in RNA splicing, suggesting, for the first time, that citrullination modulates RNA biology. Overall, this chemical proteomic platform will play a key role in furthering our understanding of protein citrullination in rheumatoid arthritis and potentially a wider spectrum of inflammatory diseases.



Although the importance of protein citrullination to human pathology was first recognized in RA, more recent studies indicate that dysregulated citrullination is a general feature of autoimmunity and cancer.[1−8] This post-translational modification (PTM) is catalyzed by the protein arginine deiminases (PADs), a small family of calcium-dependent enzymes that hydrolyze the side chain guanidinium of arginine residues to form the noncoded amino acid citrulline. How PADs contribute to such a diverse set of pathologies is unclear, but one common feature of this enzyme family is their ability to citrullinate histones. Histone citrullination is known to modulate the chromatin architecture with consequent downstream effects on gene transcription, differentiation, and pluripotency.[9−14] For example, PAD4 citrullinates histones H3 and H4, and this activity is generally associated with increased expression of growth-promoting genes and decreased expression of growth-inhibiting genes.[12,15] PAD2 also citrullinates histone H3 at R26, and this modification is associated with the increased expression of HER2 and more than 200 genes under the control of the estrogen receptor (ER).[5] Indeed, RNAi knockdown of PAD2 decreases ER target gene expression and citrullination of histone H3R26, suggesting that modification of this site promotes an open chromatin state that is conducive to the expression of ER target genes.[4,5] Additionally, PAD2 levels are highly correlated with HER2 expression in both HER2+ breast tumors and HER2 breast cancer cell lines, suggesting that PAD2 plays a key role in breast cancer biology via its involvement in both ER- and HER2-mediated gene transcription.[4,16]

In addition to modulating gene expression, the histone modifying activity of PADs is required for the formation of neutrophil and macrophage extracellular traps (NETs and METs).[1,17−19] For example, in response to stimuli of bacterial or immunological origin, neutrophils decondense and externalize their chromatin to form web-like structures to capture pathogens. PAD4 activity appears to be critical for this process, as PAD4$^{-/-}$ knockout mice do not form NETs and PAD inhibitors, e.g., Cl-amidine and BB-Cl-amidine,[20,21] block this pro-inflammatory form of programmed cell death. Although NET formation is a normal and essential component of the innate immune response,[22,23] aberrantly increased NET formation is a hallmark of RA,[24] lupus,[25,26] colitis,[27] atherosclerosis,[26] and a variety of cancers.[28] As such, aberrant NET formation is thought to be a key driver of these diseases.

Given these disease links, there is keen interest in developing PAD inhibitors as therapeutics; however, we are only beginning

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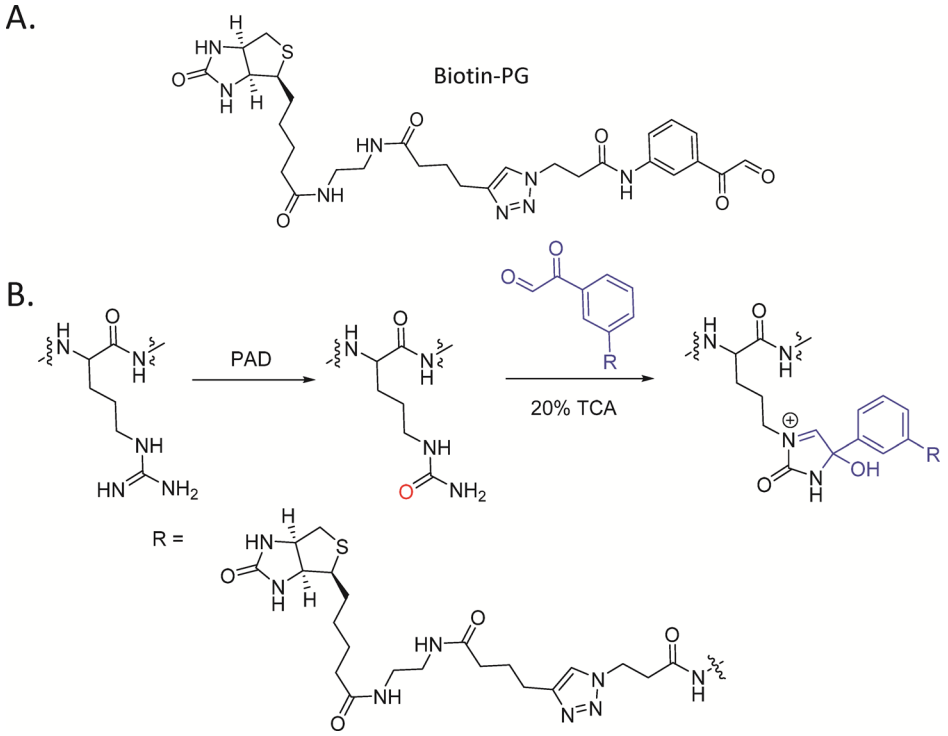

Figure 1. Structure of biotin-PG and citrulline-specific labeling chemistry. (A) Structure of biotin-PG. (B) Schematic depicting the labeling chemistry for using biotin-PG as a citrulline specific probe.

to understand the biological processes impacted by this PTM. In fact, the specific substrates targeted by PADs remain mostly unknown in the aforementioned diseases, making their discovery of upmost importance. Identifying these proteins will not only further our understanding of how PADs contribute to disease pathology but also lay the foundation for identifying novel biomarkers to expedite disease diagnosis and treatment, thereby improving therapeutic outcomes. Although a number of citrulline-specific antibodies and proteomic methods have been described,[29−34] these methods suffer from a number of limitations, most especially the need to chemically derivatize citrullinated proteins after transfer to a membrane in western blotting applications or post-tryptic digestion for proteomic detection, which necessitates protein identifications based on a single peptide. By contrast, a key strength of our probe, along with our methodology, is that it identifies intact proteins without the need for up-front processing.

Building on our recent development of a fluorescent citrulline-specific probe (i.e., rhodamine-conjugated phenylglyoxal, Rh-PG) that is used to visualize protein citrullination,[35] we report herein the design, synthesis, and use of biotin-conjugated phenylglyoxal (biotin-PG, Figure 1A). Specifically, we demonstrate its use in three distinct platforms: (i) as an antibody surrogate for western blotting, (ii) as a chemical handle to enrich and isolate PAD substrates from complex mixtures for mass spectrometry identification, and (iii) as a detection element to qualitatively and quantitatively analyze the levels of citrullinated proteins. Utilizing these three platforms, we used biotin-PG to identify more than 50 proteins that are citrullinated in cells. Enriched among these proteins are several mRNA splicing and processing proteins, suggesting, for the first time, that PAD activity modulates RNA biology. On the basis of our data, biotin-PG and the methodology described in this article will play a key role in furthering our understanding of PAD biology.

RESULTS

Probe Design. We previously reported the development of a rhodamine-tagged phenylglyoxal derivative (Rh-PG) and used it to visualize the citrullination of both purified proteins and proteins present in complex mixtures such as serum.[35] We additionally demonstrated that Rh-PG could detect differences in the levels of citrullinated proteins present in serum samples obtained from a preclinical study investigating the efficacy of the pan-PAD inhibitor Cl-amidine in ulcerative colitis.[35] Importantly, the levels of several citrullinated proteins showed strong correlations with disease severity, suggesting that citrullinated proteins can be used as disease-specific biomarkers. While Rh-PG is extremely useful for quantifying differences in the levels of citrullinated proteins, it cannot be readily used to identify proteins. To remedy this limitation, we envisioned a biotinylated version of the probe, biotin-PG (Figure 1A), that would readily isolate citrullinated proteins directly from complex mixtures. A key strength of our probe, along with our methodology (Figure 1B), is that it identifies intact proteins without the need for up-front proteolytic processing. We also envisioned that such a probe could be used as a probe to visualize protein citrullination in western blotting applications. To this end, we synthesized biotin-PG (Figures 1 and S1). Briefly, this compound was accessed from azido-phenylglyoxal by coupling to biotin-yne (50% yield) using the Huisgen copper catalyzed azide−alkyne cycloaddition reaction, which exclusively generates the 1,4-disubstituted 1,2,3-triazole depicted in Figure 1.[36,37]

Use of Biotin-PG as a Surrogate Antibody. Historically, protein citrullination has been detected using an antibody that recognizes citrullinated residues that have been chemically

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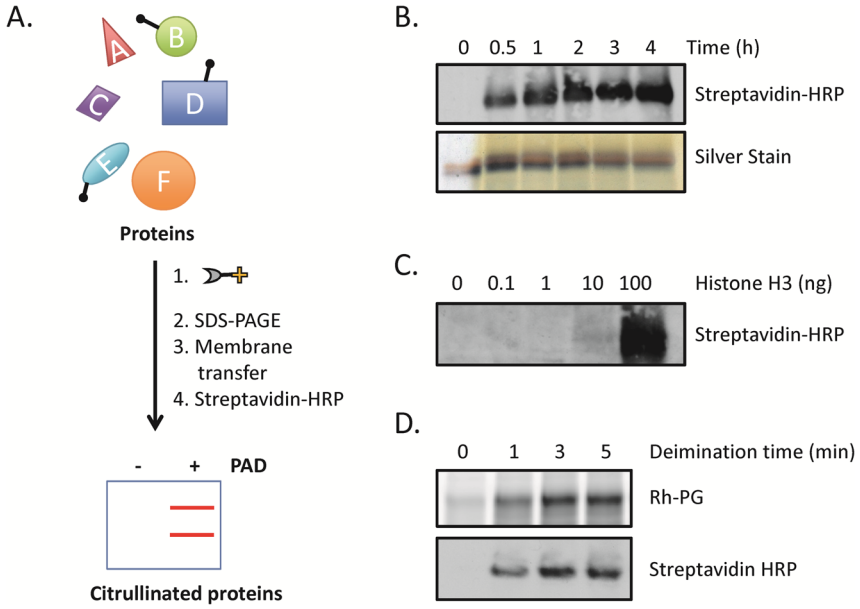

Figure 2. Using biotin-PG to visualize protein citrullination. (A) Schematic depicting the experimental approach for using biotin-PG to visualize protein citrullination on a membrane. (B) Time dependence of biotin-PG labeling. Histone H3 was citrullinated by PAD2 for 5 min and incubated with TCA and 0.1 mM biotin-PG at 37 °C for 0, 0.5, 1, 2, 3, or 4 h. Proteins were then TCA precipitated, resolubilized, and subjected to SDS-PAGE, followed by electroblotting and detecting labeled proteins with streptavidin-HRP. (C) Decreasing amounts of citrullinated histone H3 were labeled with 0.1 mM biotin-PG at 37 °C for 30 min and analyzed by blotting with streptavidin-HRP. (D) Time dependence of histone H3 citrullination. Histone H3 was citrullinated for various lengths of time (0 to 5 min), and then the aliquots were labeled with biotin-PG (0.1 mM) or Rh-PG (0.1 mM). Proteins were then separated by SDS-PAGE and either visualized directly (Rh-PG samples) or the proteins were first transferred to nitrocellulose and citrullinated proteins were then detected with streptavidin-HRP.

modified by diacetylmonooxime and antipyrine under strongly acidic conditions.[38] Despite the widespread use of this technique, the availability of this antimodified citrulline antibody has been inconsistent due to lot-to-lot variations. Since chemical derivatization occurs on the membrane after protein transfer, we first tested whether biotin-PG could be used to directly label citrullinated proteins on a membrane. Unfortunately, the results of these experiments were inconsistent and difficult to replicate, in contrast to a recent report suggesting the feasibility of such an approach.[39]

Given the above, we next identified the conditions necessary to label citrullinated proteins *in vitro* and then detect their abundance after SDS-PAGE and electroblotting using streptavidin-HRP (Figure 2A). As a first test of this modality, a fixed amount of citrullinated histone H3 was labeled with biotin-PG for various lengths of time (Figure 2B). The results indicate that 30 min provides a good balance between assay throughput and overall yield (Figure 2B). The limit of detection was ~10 ng or ~700 fmol of citrullinated histone H3 (Figure 2C). This value is similar to that obtained with Rh-PG,[35] indicating that the two methods provide comparable results. We next citrullinated histone H3 for various lengths of time (0 to 5 min) and then labeled the aliquots with biotin-PG (0.1 mM) or Rh-PG (0.1 mM) for 30 min at 37 °C. Proteins were then separated by SDS-PAGE and either visualized directly (Rh-PG samples) or the proteins were first transferred to nitrocellulose and citrullinated proteins were then detected with streptavidin-HRP. These studies revealed that biotin-PG enables the quantitative assessment of the levels of a citrullinated protein similarly to our previous results with Rh-PG (Figure 2D). Notably, this approach is also less reagent intensive than derivatizing citrullinated proteins on the membrane.

To more stringently test this detection platform, we next determined whether biotin-PG could detect changes in the citrullination status of a complex proteome. Since PADs are calcium-dependent enzymes, we induced PAD activity in a stable PAD2 overexpressing cell line (HEK293T·PAD2) by treatment with ionomycin in the absence and presence of increasing amounts of extracellular calcium (Figure 3A). As a control, we used the parent HEK293T cell line, which express very low levels of PADs (Figure 3A). As expected, these studies showed that calcium influx triggers PAD2 activation in a calcium-dependent manner in only the PAD2 overexpressing cells and that global proteome-wide citrullination is readily detected after biotin-PG labeling and detection with streptavidin-HRP. Notably, we also see increased labeling of bands in the 17 kDa range, the approximate molecular weight of histone H3. Importantly, these conditions also led to the robust citrullination of histone H3, as detected with an antibody targeting histone H3 citrullinated at arginines 2, 8, and 17 (Figure 3A, lower panel).

Having demonstrated that biotin-PG readily detects cellular protein citrullination, we next evaluated whether this detection modality could be used to monitor changes in PAD activity as a function of added inhibitor. Toward this goal, our stable PAD2 overexpressing cell line (HEK293T·PAD2) was incubated in the absence or presence of ionomycin to induce proteome-wide citrullination (Figure 3B). In the presence of biphenylbenzimidazole Cl-amidine (BB-Cl-amidine; Figure S2), however, citrullination is markedly reduced in a dose-dependent manner; BB-Cl-amidine is a next-generation pan-PAD inhibitor that possesses enhanced cellular uptake and potency relative to that of the parent compound Cl-amidine.[40] Consistent with our prior studies, this compound showed enhanced efficacy relative to that of Cl-amidine, even when Cl-amidine is used at a 20-

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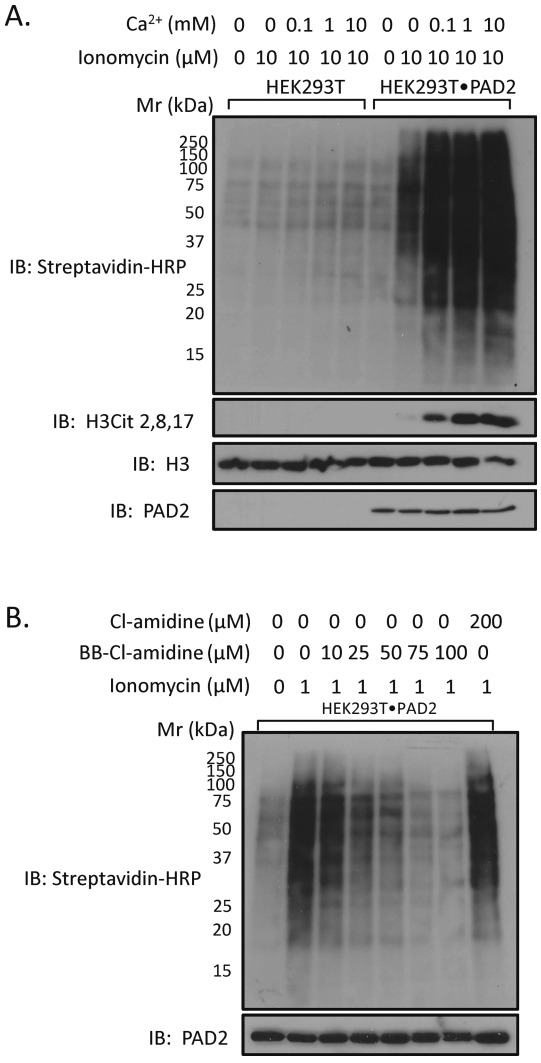

Figure 3. Using biotin-PG for visualizing citrullination in complex proteomes. (A) HEK293T or HEK293T cells overexpressing PAD2 were treated with or without ionomycin in the presence of increasing concentrations of calcium. The lysates were labeled with biotin-PG and probed with streptavidin-HRP (top) or antibodies specific for histone H3 Cit 2, 8, 17; histone H3; and PAD2 (bottom panels). (B) HEK293T cells overexpressing PAD2 were treated with or without ionomycin in the presence of increasing concentrations of BB-Cl-amidine or Cl-amidine. The lysates were labeled with biotin-PG and probed with streptavidin-HRP (top). The PAD2 western blot (bottom) serves as a loading control.

fold higher concentration (compare the 10 μM BB-Cl-amidine lane to the 200 μM Cl-amidine in Figure 3B). Overall, these data demonstrate that biotin-PG enables both the robust detection of protein citrullination and can be used to evaluate the cellular efficacy of PAD inhibitors.

Use of Biotin-PG in a Sandwich ELISA Platform. Given that our long-term goal is the discovery of citrullinated protein biomarkers, we envisioned that biotin-PG could be used in an ELISA-based platform to quantitatively analyze the citrullination levels of distinct proteins (Figure 4A). In this assay format, a citrullinated protein, or mixture of proteins, is labeled with biotin-PG and then the resolubilized protein is incubated with antibody-coated microwell plates to bind the protein of interest. Streptavidin-HRP is then added to the wells, and citrullinated protein levels are quantified by adding a fluorescent HRP substrate. To demonstrate the utility of this approach, we began by evaluating the levels of citrullinated apolipoprotein A1 (ApoA1). We focused on ApoA1 because this protein is citrullinated in serum samples obtained from a mouse ulcerative colitis study that investigated the efficacy of the PAD inhibitor Cl-amidine.[41] We began by labeling citrullinated ApoA1 with biotin-PG and serially diluting it before incubation with the plate. The plates were then washed, streptavidin-HRP was added, and the amount of bound protein was quantified by the addition of a fluorescent HRP substrate. Using this method, the limit of detection is <100 nM (Figure 4B), and the assay shows excellent day-to-day reproducibility (Figure 4C), thereby indicating that this assay format can be used to reliably measure the citrullination state of specific proteins.

Isolation of Citrullinated Proteins. Since a key motivation for developing biotin-PG was its ability to identify novel citrullinated proteins, we next evaluated this application (Figure 5A). Initially, we optimized methods to isolate proteins citrullinated *in vitro*. First, histone H3 was citrullinated for various lengths of time (0, 1, and 3 min), and then those samples were treated with biotin-PG. After quenching the labeling reaction with L-citrulline and TCA precipitating the protein, the precipitates were resuspended at neutral pH and incubated with streptavidin-agarose to isolate the citrullinated fraction. Bound proteins were eluted, separated by SDS-PAGE, and electrotransferred to nitrocellulose for detection by streptavidin-HRP (Figure 5B). These results show that citrullinated histone H3 is readily isolated by this approach (Figures 5B, left panel, and S3). As a more stringent test of this platform, citrullinated histone H3 was added to MCF7 whole cell extracts, labeled, and isolated using the methodology described above. Similar to our results with purified proteins, citrullinated histone H3 was successfully isolated from this complex cellular milieu (Figures 5B, right panel, and S3). Importantly, silver staining shows equal protein amounts in control samples, but the amount of histone H3 isolated by biotin-PG increases as a function of citrullination time, thereby indicating that this method facilitates the isolation of citrullinated proteins in a quantitative manner. In total, these results demonstrate the robustness of our citrullinated protein isolation platform and further demonstrate, for the first time, that it is possible to use this technique to isolate intact citrullinated proteins.

Characterization of the PAD2 Citrullinome. To test this platform in a more physiologically relevant system and identify novel PAD substrates, PAD activity was stimulated by the addition of ionomycin to our stable PAD2 overexpressing cell line (HEK293T·PAD2) as well as the parent HEK293T cell line, which was used as a control. After lysis, the soluble protein fraction was labeled with biotin-PG using the methodology outlined above. Citrullinated proteins were then isolated on streptavidin-agarose. After thorough washing, bound proteins were subjected to on-bead tryptic digestion and subsequently analyzed by LC-MS/MS. Using this streamlined workflow, we identified more than 50 citrullinated proteins that were significantly enriched by at least 2-fold in the PAD2 overexpressing cell line versus the controls (Tables 1 and S1). A 2-fold cutoff was chosen because this is an acceptable fold-change that can be quantified through spectral counting.[42] Notably, we isolated PAD2, which is known to autocitrullinate, from the overexpressing cell line but not control HEK293T cells, thereby confirming the selectivity of our methodology.

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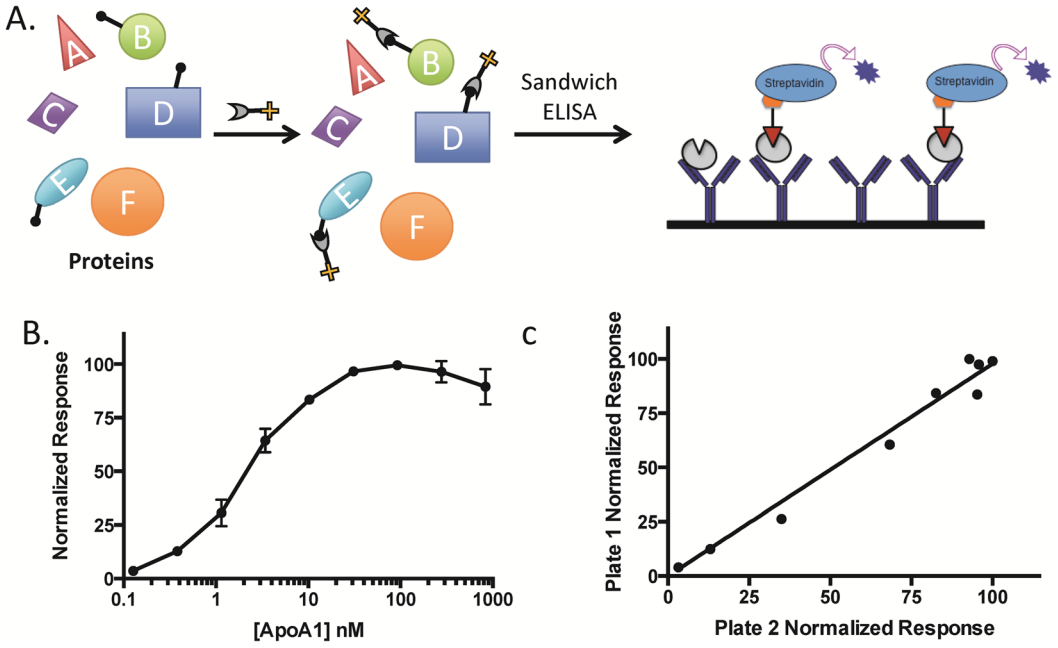

Figure 4. Using biotin-PG in an ELISA-based assay. (A) Schematic depicting the experimental approach for using biotin-PG in a sandwich ELISA-based assay. (B) ApoA1 was citrullinated by PAD1, serially diluted, and incubated in an α-ApoA1-coated microwell plate. After thorough washing, the bound citrullinated ApoA1 was detected with streptavidin-HRP. (C) Correlation plot from two different days of the ApoA1 ELISA assay shows excellent reproducibility.

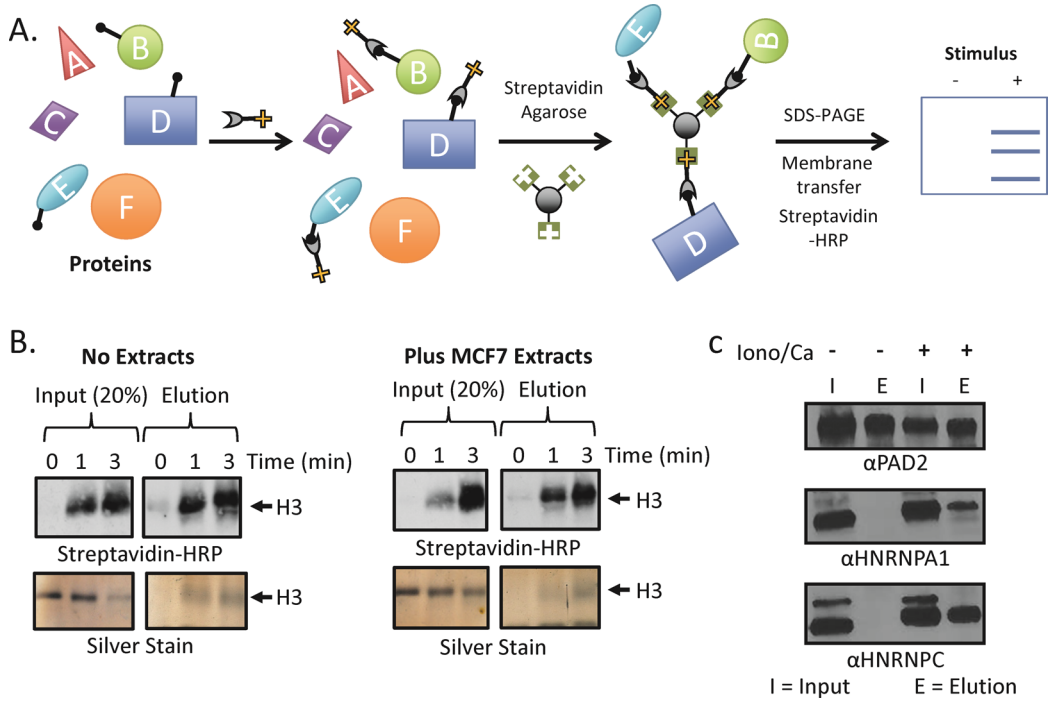

Figure 5. Use of biotin-PG to isolate citrullinated proteins. (A) Schematic depicting the use of biotin-PG to isolate citrullinated proteins. (B) Histone H3, citrullinated by PAD2 for various times (i.e., 0, 1, or 3 min), was treated with biotin-PG in the absence or presence of MCF7 whole cell extracts. Citrullinated proteins were isolated using streptavidin-agarose, and the supernatant from the resin was analyzed by streptavidin-HRP western blot and silver staining. Figures depicting the entire blot are provide in Figure S3. (C) HEK293T cells overexpressing PAD2 were treated plus or minus calcium and ionomycin. The cell lysates were labeled with biotin-PG (I), and the labeled proteins were isolated with streptavidin-agarose. The inputs and eluents (E) were probed by western blotting using antibodies against PAD2, HNRNPA1, and HNRNPC.

Among the various other citrullinated proteins enriched using our methodology were several chromatin binding proteins, ribosomal proteins, and lamin B1. Additionally, almost half of the isolated proteins are components of the mRNA splicing and processing machinery. These proteins include several heterogeneous nuclear ribonucleoproteins (e.g., hnRNPs C, A3, and AB), RNA helicases (e.g., DDX5 and DDX21), the nucleolar protein nucleophosmin (NPM1), and SNRNP200, an essential component of the U5 spliceosome complex. To validate our findings, our stable PAD2 over-

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Table 1. Subset of Citrullinated Proteins Enriched in Stimulated PAD2 Overexpressing Cells Relative to HEK293T Cells[a]

protein	fold increase	function
SNRNP200	≫25	spliceosome component/RNA helicase
PAD2	≫25	histone modifying enzyme
LMNB1	≫25	component of nuclear matrix
MCM2	≫25	DNA replication licensing factor
PDIA6	≫25	protein disulfide isomerase
G3BP2	≫25	Ras GTPase-activating protein-binding protein
DNAJB1	≫25	chaperone
EIF4H	≫25	translation
U2AF2	≫25	RNA splicing
SRSF7	≫25	RNA splicing
RBMX	≫25	RNA binding protein
RBM39	≫25	RNA binding protein
HNRNPAB	≫25	mRNA splicing
HNRNPH3	≫25	mRNA splicing
CPSF6	≫25	polyadenylation processing
PTBP1	≫25	mRNA splicing
DDX21	≫25	RNA helicase
U2AF2	≫25	mRNA splicing factor
HMGB1	≫25	chromatin binding protein
RPS10	≫25	ribosome component
MCM3	≫25	DNA replication licensing factor
SRSF3	4.8	RNA splicing
HNRNPA3	3.7	cytoplasmic trafficking of mRNA
SFPQ	3.6	DNA/RNA binding protein
Vimentin	3.6	cytoskeletal component
HNRNPA1	3.3	mRNA processing and transport
HNRNPC	3.0	RNA splicing
HNRNPH1	2.9	mRNA splicing
NPM1	2.4	nucleolar protein/ribosome biogenesis
DDX5	2.4	RNA-dependent helicase
NONO	2.3	RNA splicing
HNRNPA2B1	2.1	mRNA splicing factor
DDX17	2.1	RNA helicase

[a]The complete data set is provided in Table S1.

expressing cell line was treated in the absence and presence of ionomycin, and the cell lysates thus obtained were labeled with biotin-PG. Subsequently, the biotin-PG tagged proteins were isolated on streptavidin-agarose, and then the inputs and eluents were probed for PAD2, HNRNPA1, and HNRNPC. The results of these studies confirmed that all three proteins were enriched in the ionomycin treated cells, thereby confirming the mass spectrometry data showing that these proteins are citrullinated *in vivo* (Figure 5C). While further work is needed to determine how citrullination affects RNA splicing, it is noteworthy that arginine methylation of a similar set of mRNA processing factors can modulate spliceosome activity.[43] Since citrullination antagonizes arginine methylation,[10,44,45] these results suggest that the effects of citrullination, particularly when dysregulated, may act beyond the level of regulating the chromatin architecture and also impact mRNA splicing.

DISCUSSION

An increasing body of work links aberrant protein citrullination to human diseases such as RA, colitis, lupus, and several cancers.[1−8] Despite these links, our general understanding of PAD biology remains quite limited, especially as it relates to the full spectrum of cellular processes regulated by these enzymes and how their dysregulation contributes to disease pathogenesis. Therefore, a better understanding of the full substrate scope of the enzymes is needed. Toward that end, we synthesized a suite of phenylglyoxal-based probes and developed the enabling methodology to detect, enrich, and quantify protein citrullination. Using these new tools and methods, we demonstrate here that biotin-PG can be used to detect cellular protein citrullination as well as monitor the efficacy of BB-Cl-amidine, a second-generation PAD inhibitor that shows a greater than 20-fold improvement in cellular bioactivity.

We additionally used biotin-PG to enrich for the citrullinated fraction of the proteome, i.e., the citrullinome, and show for the first time *in vivo* that components of the RNA splicing apparatus are PAD substrates. Notably, an overlapping set of proteins was identified by Fast and colleagues *in vitro* using high-density protein arrays to identify potential PAD4 substrates.[46] Arginine methylation has long been known to influence RNA splicing, and citrullination can antagonize arginine methylation (by modifying the substrate guanidinium). Indeed, these findings are significant because they open up an entirely new avenue of research and additionally suggest that the PADs may play a role in spliceopathies, i.e., RNA splicing diseases.

Key advantages of these probes, as well as our optimized work flow, include the fact that by enriching intact proteins we enhance peptide coverage, thereby giving higher confidence that the proteins isolated are truly citrullinated. It is also important to recognize that derivatization occurs under denaturing conditions (20% TCA), which likely increases access to otherwise buried citrullines. Also, since the protein-bound streptavidin-agarose beads are treated with highly denaturing conditions of 6 M urea, which should disrupt any noncovalent protein−protein complexes, it is unlikely that this procedure will detect proteins that are not directly associated with the streptavidin-agarose beads. Since our work flow utilizes an on-bead trypsin digest that releases only the unmodified (noncitrullinated) peptides from the beads, one limitation is that the citrullinated peptides remain bound to the bead, and therefore are not present in the peptide mixture that is analyzed by MS. A cleavable biotin linker is needed to selectively release the citrullinated peptides, and we are currently optimizing this approach and will report on our progress in a future publication. A second limitation is that we cannot readily distinguish whether the elevation in citrullinated proteins is due to more protein or to higher citrullination of the same amount of the protein. Ratiometric methods will be required to address this issue. Regardless of the source, however, elevated citrullinated protein levels still means that PAD activity is increased and can be detected using our assay.

Overall, this single diagnostic platform has the potential to revolutionize our understanding of PAD biology by uncovering the full scope of the substrates modified by these enzymes in response to a variety of cell signaling paradigms. Additionally, extension of this methodology to diseases in which PAD activity is dysregulated promises to uncover biomarkers associated with a wide range of human ailments.

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METHODS

Chemicals and Proteins. MCF7 cells were purchased from the ATCC. DMEM, trypsin−EDTA, and FBS were purchased from Corning. Rh-PG, Histone H3, and PADs1−4 were prepared as previously described.[35,47,48] Trypsin was purchased from Promega. Antibodies were purchased from Abcam (histone H3 Cit26 (cat. no. ab19847) and histone H3 Cit2,8,17 (cat. no. ab5103)), Novus Biologicals (ApoA1 (cat. no. 102134-360)), Cell Signaling (Histone H3 (cat. no. 9715S), anti-rabbit IgG-HRP (cat. no. 7074S)), and ProteinTech (PAD2 (cat. no. 12110-1-AP)). Streptavidin-HRP (cat. no. 434323) was purchased from Invitrogen.

Time Dependence of Biotin-PG Labeling. Histone H3 (10 μM) was citrullinated by PAD2 (0.2 μM) for 5 min and then incubated with TCA and 0.1 mM biotin-PG at 37 °C for various lengths of time (i.e., 0, 0.5, 1, 2, 3, and 4 h). Briefly, citrullinated histones (20 μL) were incubated with 20% trichloroacetic acid (TCA; 5 μL of 100% TCA) and 0.1 mM biotin-PG (0.5 μL of a 5 mM stock) for 30 min at 37 °C. Solutions were quenched by the addition of citrulline to the acidic solution (5 μL of a 500 mM stock, 100 mM final). The sample was then cooled on ice for 30 min and centrifuged at 13 200 g for 15 min at 4 °C to TCA precipitate the protein. The supernatant was removed, and precipitates were washed with cold acetone and dried. Proteins were resuspended in a neutral resuspension buffer (50 mM HEPES, pH 8.0, containing 100 mM arginine, 20 μL), boiled with 6× SDS loading dye, and sonicated in a bath sonicator for 2−5 s. The proteins were separated by SDS-PAGE and transferred to nitrocellulose (Towins buffer; 80 V; 60 min). The membrane was blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at rt before incubation with streptavidin-HRP (0.5 μL; 1:20000) in 5% BSA in PBS for 10 min at rt. The blot was washed 3× with PBS (5 min) and 1× with water (5 min) and visualized by ECL. The adduct thus formed is stable during the course of the experiment.

Time-Dependent Citrullination of Histone H3. Histone H3 (10 μM) was treated with PAD2 (0.2 μM) in reaction buffer at 37 °C for various lengths of time (i.e., 0, 1, 3, and 5 min). PAD activity was then quenched with 50 mM EDTA. The aliquot was divided evenly, and one-half was analyzed with Rh-PG, as previously reported.[35] The other half was treated with biotin-PG using the methodology described above.

Limit of Detection Studies. Citrullinated histone H3, prepared as described above, was diluted into 50 mM HEPES, pH 7.6, to final concentrations of 0.22 μM, 22 nM, 2.2 nM, and 0.22 nM H3 (20 μL total). These samples were then treated with biotin-PG as above. Proteins were resuspended in resuspension buffer (10 μL) and boiled with 6× SDS loading dye, and various amounts of protein (i.e., 100, 10, 1, 0.1, and 0 ng) were loaded onto an SDS-PAGE gel. Separated proteins were transferred to nitrocellulose and analyzed as above.

Labeling Citrullinated Proteins in Cell Lysates. HEK293T and HEK293T cells stably expressing human PAD2 (HEK293T·PAD2) were cultured as previously described.[49] Cells were grown to ∼80% confluence (8×10^6 cells), trypsinized, and quenched with complete media. The cells were harvested by centrifugation at 800g for 2 min and washed 4× with HBS. Cells were resuspended in HBS at 8×10^6 cells/mL, and 4×10^5 cells were added to 0.65 mL tubes for subsequent assays. Ionomycin (10 μM) and $CaCl_2$ (0−10 mM) were added to the cells and incubated at 37 °C for 60 min before quenching with EDTA (10 mM). Protease inhibitor cocktail (Roche) was added before addition of Triton X-100 (2% final) and incubated on ice for 30 min. Lysates were cleared by centrifugation at 21 000g for 10 min, and soluble proteins were removed and quantified by DC assay (Bio-Rad). Lysate (10 μg, 20 μL total) was labeled with biotin-PG and resolubilized as described above. The resolubilized protein was separated by SDS-PAGE (12.5% gel) and transferred to PVDF membranes (Biorad) at 80 V for 60 min. The membranes were analyzed with streptavidin-HRP as described above.

Western Blot Detection of PAD2, Histone H3, and Citrullinated Histones. Twenty micrograms of total protein was separated by SDS-PAGE (12.5% gel) and electrotransferred as described above. Membranes were blocked with TBST and BSA (5%) for 1 h at 23 °C. Blocked membranes were incubated with antibodies for PAD2 (1:2000), histone H3 (1:2000), or histone H3 Cit 2,8,17 (1:1000) in TBS-T with 5% BSA for 12 h at 4 °C. Membranes were then washed with TBS-T (6×) and incubated with anti-rabbit IgG HRP conjugate (1:5000) for 1 h at 23 °C. Membranes were washed with TBST (6×) and developed and imaged as described above.

Inhibition of Cellular Citrullination. HEK293T and HEK293T cells stably expressing human PAD2 (HEK293T·PAD2) were cultured as previously described.[49] Cells were grown to ∼80% confluence (8×10^6 cells), trypsinized and quenched with complete media. The cells were harvested by centrifugation at 800g for 2 min and washed 4× with HBS. Cells were resuspended in HBS at 8×10^6 cells/mL and 4×10^5 cells were added to 0.65 mL tubes for subsequent assays. Cells were incubated with the indicated amount of BB-Cl-amidine or Cl-amidine for 30 min at 37 °C for 30 min prior to adding ionomycin (1 μM) to activate PAD2 activity. The final concentration of DMSO was 1% in each sample. Cells were then incubated at 37 °C for 3 h. Cells were lysed, labeled with biotin-PG and analyzed with streptavidin-HRP as described above.

Isolation of Citrullinated Histone H3. Histone H3 (10 μM) was treated with PAD2 (0.2 μM) in reaction buffer. Aliquots were removed at various times (i.e., 0, 1, and 3 min), quenched with 50 mM EDTA, and then divided evenly into two parts. To one set was added MCF7 whole cell extracts (1 mg mL^{-1}). The other samples were added to buffer. All samples were then treated with 20% TCA and 0.1 mM biotin-PG for 30 min at 37 °C before quenching with citrulline, cooling, centrifuging, washing, and drying as described above. Proteins were resuspended in resuspension buffer containing 0.1% SDS (50 μL), boiled for 10 min, and sonicated in a bath sonicator for 2−5 s. A small aliquot was removed to serve as a loading control. The remaining sample was added to 50 μL of high-capacity streptavidin-agarose (Thermo Fisher Scientific Inc.), equilibrated in PBS, and tumbled gently overnight at 4 °C. Samples were then centrifuged at 500g for 2 min at 4 °C, and the supernatant was removed. Samples were then washed with 0.2% SDS in PBS for 10 min at rt, 3× with PBS, and 3× with water. Proteins were eluted from the resin in freshly prepared elution buffer (50 μL; 6 M urea, 2 M thiourea, 30 mM biotin, and 2% SDS) at 42 °C for 1 h. After a brief centrifugation, the supernatant was then transferred to a 10 kDa microconcentrator, the resin was washed 1× with water (100 μL) and centrifuged again, and the water was combined with the previous eluent in the microconcentrator. To remove excess chaotropic agents, the microconcentrators were centrifuged at 16 000g for 10 min, and the sample was diluted with water (100 μL) and centrifuged twice more. Proteins were collected from the microconcentrator and boiled with 6× SDS 10 min. The samples were analyzed as above.

Isolation of Citrullinated Proteins from PAD2 Overexpressing Cells. HEK293T and HEK293T cells stably expressing human PAD2 (HEK293T·PAD2) were cultured as previously described.[49] Cells were grown to ∼80% confluence (8×10^6 cells), trypsinized, and quenched with complete media. The cells were harvested by centrifugation at 800g for 2 min and washed 4× with HBS. Cells were resuspended in HBS at 2×10^7 cells/mL (8×10^6 cells in 0.4 mL in a 1.5 mL tube) and 1 mM $CaCl_2$ and 10 μM ionomycin were added for 1 h at 37 °C with frequent gentle mixing. Cells were lysed as above, and 10 μg of protein was labeled as above except that after the acetone was removed the protein pellet was air-dried for subsequent shotgun proteomics. MS experiments were performed on three biological replicates.

Shotgun Proteomics. Biotin-PG labeled samples in the form of an acetone-washed pellet were solubilized in PBS containing 1.2% SDS via sonication and heating (5 min, 80 °C). The SDS-solubilized samples were diluted with PBS (5 mL) for a final SDS concentration of 0.2%. The solutions were incubated with 100 μL of streptavidin-agarose beads (Thermo Scientific) at 4 °C for 16 h. The solutions were then incubated at rt for 3 h. The beads were washed with 0.2% SDS/PBS (5 mL), PBS (3 × 5 mL), and water (3 × 5 mL). The beads were pelleted by centrifugation (1400g, 3 min) between washes. The washed beads were suspended in 6 M urea in PBS (500 μL) and 10

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mM dithiothreitol (from 20× stock in water) and placed in a 65 °C heat block for 15 min. Iodoacetamide (20 mM, from 20× stock in water) was then added to the samples and allowed to react at 37 °C for 30 min. Following reduction and alkylation, the beads were pelleted by centrifugation (1400*g*, 3 min) and resuspended in a premixed solution of 2 M urea in PBS (200 μL), 100 mM $CaCl_2$ in water (2 μL), and trypsin (4 μL of 20 mg reconstituted in 40 μL of trypsin buffer). The digestion was allowed to proceed overnight at 37 °C. The digested peptides were separated from the beads using a Micro Bio-Spin column (Bio-Rad), and the beads were washed twice with 50 μL of H_2O. Formic acid (15 μL) was added to the samples, and the samples were stored at −20 °C until MS analysis. LC-MS/MS analysis was performed on an LTQ Orbitrap Discovery mass spectrometer (ThermoFisher) coupled to an Agilent 1200 series HPLC. Digests were pressure loaded onto a 250 μm fused silica desalting column packed with 4 cm of Aqua C18 reverse-phase resin (Phenomenex). The peptides were eluted onto a biphasic column (100 μm fused silica with a 5 μm tip, packed with 10 cm C18 and 3 cm Partisphere strong cation exchange resin (SCX, Whatman)) using a gradient of 5−100% buffer B in buffer A (buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The peptides were eluted from the SCX onto the C18 resin and into the mass spectrometer following the four salt steps outlined in Weerapana et al.[50] The flow rate through the column was set to ∼0.25 μL/min, and the spray voltage was set to 2.75 kV. One full MS scan (400−1800 MW) was followed by eight data-dependent scans of the *n*th most intense ions with dynamic exclusion enabled.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00438.

Synthesis of Biotin-PG probe (Figure S1); structures of Cl-amidine and BB-Cl-amidine (Figure S2); analysis of the supernatant from Biotin-PG labeling and isolation of citrullinated H3 from MCF7 whole cell extracts (PDF). Complete set of citrullinated proteins enriched in stimulated PAD2 overexpressing cells relative to HEK293T cells (XLSX).

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Notes

The authors declare the following competing financial interest(s): P.R.T. is a cofounder of and consultant to Padlock Therapeutics.

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