DOI: 10.1002/cbic.201200048

Covalent Tagging of Phosphorylated Peptides by Phosphate-Specific Deoxyribozymes

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Many natural peptides and proteins are phosphorylated on tyrosine or serine residues. Phosphorylated peptides are important in neurochemistry (neuropeptides), immunology (cytokines), and endocrinology (hormones). For such peptides, as well as for larger proteins, side-chain phosphorylation is frequently associated with modulation of biological function.[1] Methods for analysis of phosphopeptides often depend upon their initial chromatographic separation from nonphosphorylated analogues by using support-bound chelators or covalent binders of phosphate groups or products derived from them.[2] Alternatively, phosphotyrosine-specific antibodies can be generated, albeit with the attendant investments in cost and time.[2g,3] Here we describe a proof of principle for an entirely different approach to phosphopeptide analysis in which DNA catalysts (deoxyribozymes) covalently tag the phosphorylated amino acid side chains of peptides. In this approach, it is critical to ensure high selectivity for the modification of phosphorylated amino acid side chains over their nonphosphorylated analogues.

Deoxyribozymes were originally identified for the catalysis of RNA cleavage;[7] however, their use has expanded to encompass a range of chemical reactions.[8] We have reported a variety of deoxyribozymes for different chemical reactions,[9] including the covalent modification of amino acid side chains.[5,10] In particular, we have recently shown that tripeptide substrates can be covalently modified by the attachment of an RNA strand at nonphosphorylated Tyr or Ser.[6] Here we sought to identify deoxyribozymes that covalently modify phosphorylated tyrosine (Tyr^P, Y^P) by in vitro selection, as shown in Figure 1. The hexapeptide substrate $AAAY^PAA$ was connected to a DNA anchor oligonucleotide through either a short or long tether (for structures see the Supporting Information); the short tether connects the hexapeptide directly through its α-amino group to the DNA anchor, whereas the long tether includes an intervening hexa(ethylene glycol) moiety. In vitro selection was used to identify deoxyribozymes that attach a 5′-triphosphorylated RNA tag to Tyr^P, with pyrophosphate as the leaving group.

Two new deoxyribozymes from the selection process, 8VM1 and 8VP1 (one from each of the two selection experiments), were examined in more detail on the basis of their high catalytic activities with the Tyr^P-containing and analogous phosphoserine (Ser^P)-containing hexapeptides. Both deoxyribozymes were highly selective (>200:1) for each phosphorylated peptide over its nonphosphorylated analogue, with no detectable reaction at Tyr^{OH} or Ser^{OH} (<0.5% in 50 h; Figure 2). 8VM1, which was identified by selection with the short tether, favored the Tyr^P peptide over the Ser^P peptide as its substrate by about four- to fivefold. In contrast, 8VP1, which was found by selection with the long tether, functioned equally well with Tyr^P or Ser^P peptides. 8VP1 was also found to accept a range of different amino acid identities—including hydrophobic and charged residues—flanking the Tyr^P that it covalently modifies (Figure 3); this suggests broad generality for different phosphopeptide sequences.

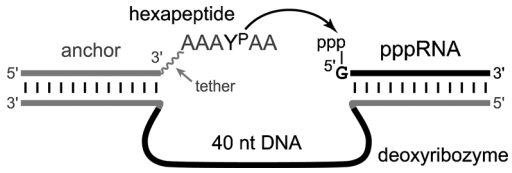

Figure 1. Strategy for selective covalent modification of phosphorylated Tyr (Y^P) within a peptide substrate. In vitro selection identifies deoxyribozymes that function with a Y^P-containing hexapeptide substrate, catalyzing the attachment of 5′-triphosphorylated RNA to the phosphorylated amino acid residue. The hexapeptide substrate is attached to an oligonucleotide anchor through a tether (see text for composition). The product has a pyrophosphate linkage between Tyr and RNA. Further information about the selection procedure, which followed our standard approach,[4–6] is provided in the Supporting Information.

To examine the applicability of deoxyribozymes for analyzing mixtures of phosphorylated and nonphosphorylated peptides, such a mixture (each peptide attached through a disulfide to the DNA anchor) was tagged with RNA by the 8VP1 deoxyribozyme. Analysis of the product mixture by MALDI mass spectrometry [after dithiothreitol (DTT) cleavage of the DNA anchor] revealed selective RNA tagging of only the phosphopeptides, despite the presence of a large amount of nonphosphorylated peptide (Figure 4).

In summary, we have demonstrated a proof of principle that DNA can catalyze highly selective covalent modification of phosphorylated Tyr or Ser residues in phosphopeptides by attaching an RNA tag at those positions. To our knowledge, this is the first report of any chemical approach for covalent, specific tagging of phosphopeptide side chains. In downstream applications, this RNA tag should be useful to report upon the amount of phosphorylated peptides present in a sample, for example, by RT-PCR; this might help to avoid the issues encountered during mass spectrometric analysis of peptide phosphorylation.[11] A wide range of peptide sequence contexts are accepted by the investigated deoxyribozymes, thus suggesting that this general approach could be made competitive with

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201200048.

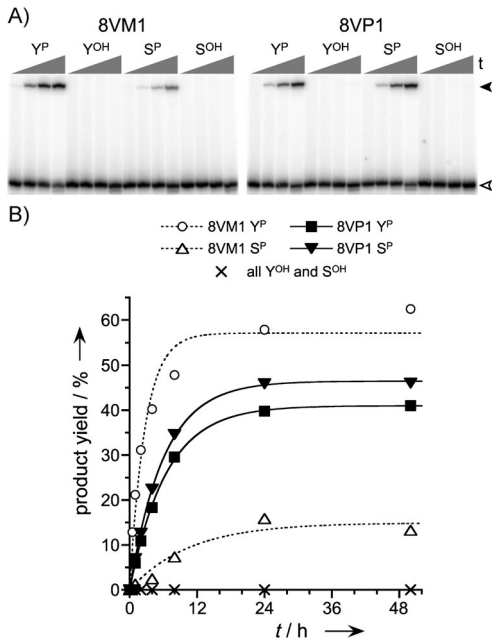

Figure 2. The 8VM1 and 8VP1 deoxyribozymes covalently modify phosphotyrosine and phosphoserine. A) PAGE image showing high selectivity for Y^P over Y^{OH} and for S^P over S^{OH} (50 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM $MnCl_2$, 150 mM NaCl, 2 mM KCl, 37 °C; single-turnover assays). Representative time points at 0, 0.5, 4, 24 h. Open arrowhead: 3′-^{32}P-radiolabeled RNA substrate. Filled arrowhead: DNA-anchored hexapeptide attached to RNA. Each peptide was attached to the oligonucleotide anchor through its amino terminus and a short (8VM1) or long (8VP1) tether (see the Supporting Information). Each product identity was verified by MALDI mass spectrometry (see the Experimental Section). B) Kinetic data for the tagging reactions. k_{obs} (top to bottom for plots) = 0.37, 0.17, 0.15, 0.095 h^{-1}. For 8VP1, but not 8VM1, 2–4% product in 50 h is observed when the Y^P or S^P hexapeptide is unattached to the DNA anchor, that is, a free hexapeptide substrate (data not shown).

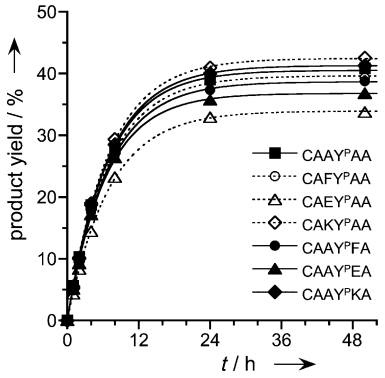

Figure 3. Sequence generality for the phosphopeptide substrate. Covalent modification by 8VP1 was examined with DNA-anchored hexapeptide substrate CAAY^{P}AA and several illustrated sequence variants, for which one amino acid adjacent to Y^P (on either side) was changed to one of F (hydrophobic), E (negatively charged), or K (positively charged). Experiments were performed as in Figure 2. Each peptide was attached to the DNA anchor through the N-terminal cysteine side chain, which enables inclusion of K within the sequence and also allows cleavage of the peptide from the anchor by DTT reduction.

more traditional chromatographic separations of phosphopeptides.[2] The phosphopeptide analytical approach outlined here is distinct from methods that depend upon engineering individual kinases to accept modified ATP substrates.[12] Our findings also expand the repertoire of DNA catalysis to include covalent modification of phosphorylated amino acid side chains. Independently, we have shown that RNA-tagging deoxyribozymes can discriminate *against* phosphorylated residues in favor of their nonphosphorylated analogues with promising selectivity (>20:1; data not shown). That observation along with this work suggests the viability of ratiometric analyses in which both phosphorylated and nonphosphorylated peptides are covalently modified with different tags in the same sample.

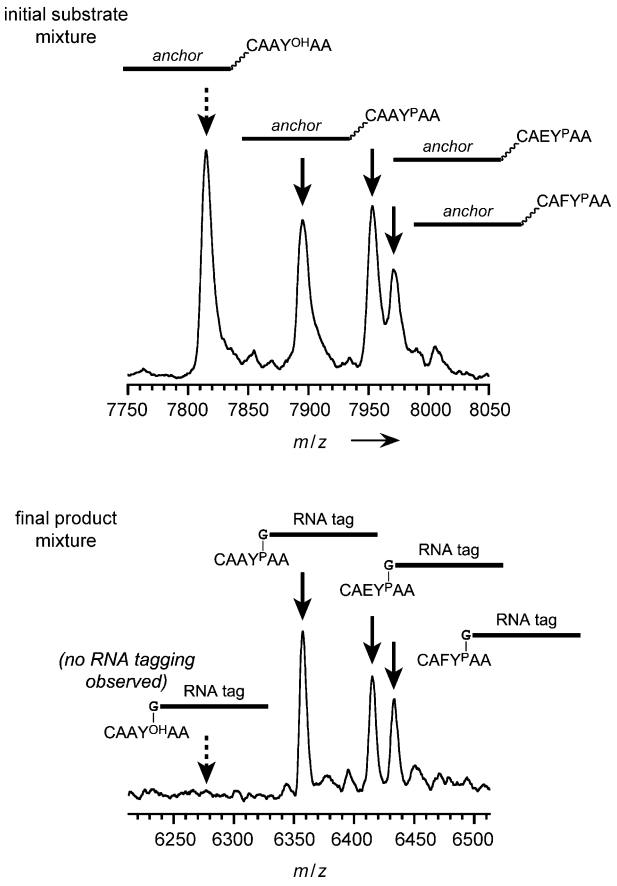

Figure 4. Analysis of a peptide mixture by mass spectrometry using the DNA-catalyzed tagging approach. (For a diagram of this experiment, see the Supporting Information.) Each of a mixture of nonphosphorylated (100 pmol) and three phosphorylated (33 pmol each) peptides was attached through a HEG tether to a common DNA oligonucleotide anchor, and the mixture was tagged with RNA by 8VP1. After PAGE separation of RNA-tagged peptides and removal of the DNA anchors by DTT reduction, analysis by MALDI MS revealed that only the phosphorylated peptides were covalently modified with the RNA tag, as desired. No tagging of the nonphosphorylated peptide was observed (lower spectrum, left side; compare with substantial nonphosphorylated peptide signal in the upper spectrum).

Several important issues must be addressed in the future development of this approach. We will seek DNA catalysts that tag specific sequences of phosphopeptides, rather than accepting a broad range of peptide sequences. The approach must also be developed to work with free, rather than oligonucleotide-anchored, peptide substrates as well as with large phosphorylated proteins, ideally in complex mixtures such as cell lysates. Towards this goal, we have recently demonstrated

the first steps towards DNA-catalyzed reactivity of free peptides;[6,13] such efforts must be merged with the present work to establish a useful analytical method.

Experimental Section

General procedures: Hexapeptides were prepared by solid-phase synthesis on Fmoc Rink amide MBHA resin and attached to DNA anchor oligonucleotides as described in the Supporting Information. DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument by using reagents from Glen Research (Sterling, VA). 5′-Triphosphorylated RNA oligonucleotides were prepared by in vitro transcription using synthetic DNA templates and T7 RNA polymerase.[14] All oligonucleotides were purified by denaturing PAGE with running buffer 1×TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3) as described previously.[4,15] The in vitro selection procedure is described in the Supporting Information. Deoxyribozyme sequences (catalytic regions underlined): 8VM1, 5′-CCGTC GCCAT CTCTT CGACT GCGGG AGCGG TGAGC GGGTA GGTCT ACATG AGGGC TATAG TGAGT CGTAT TATCC-3′; 8VP1, 5′-CCGTC GCCAT CTCTT CGGAC ACGAT GAGTG ACTAA GTGGA ATGAG GAAAG CACGA GATAG TGAGT CGTAT TATCC-3′.

Kinetic assays: Single-turnover kinetic assays of the 8VM1 and 8VP1 deoxyribozymes with various substrates (Figures 2 and 3) were performed according to the following procedure. A sample (14 μL) containing 3′-^{32}P-radiolabeled 5′-triphosphorylated RNA substrate (1 pmol; radiolabel attached with ^{32}P-pCp and T4 RNA ligase), deoxyribozyme (5 pmol), and DNA-anchored hexapeptide (10 pmol) was annealed in HEPES (5 mM, pH 7.5), NaCl (15 mM), and EDTA (0.1 mM) by heating it at 95 °C for 3 min and cooling it on ice for 5 min. The DNA-catalyzed tagging reaction was initiated by bringing the sample to 20 μL total volume with HEPES (50 mM, pH 7.5), $MgCl_2$ (40 mM), $MnCl_2$ (20 mM), NaCl (150 mM), and KCl (2 mM) and incubating it at 37 °C. At appropriate times, aliquots (2 μL) were quenched with stop solution (5 μL; 80% formamide, 1×TBE (89 mM each Tris and boric acid, 2 mM EDTA, pH 8.3), 50 mM EDTA, and 0.025% each bromophenol blue and xylene cyanol). Before PAGE, a "decoy oligonucleotide" (50 pmol) was added to each sample; it was a 60-mer complementary to the deoxyribozyme's 40 nt enzyme region along with 10 nt of binding arm on each side (added to displace the deoxyribozyme from the substrates and ligation product). Samples were separated by 20% PAGE and quantified with a PhosphorImager. k_{obs} values were obtained by fitting the yield versus time data directly to first-order kinetics.

DNA-catalyzed RNA tagging and mass spectrometry of phosphopeptides: MALDI mass spectrometry was used to verify the identities of the products of DNA-catalyzed RNA tagging of DNA-anchored phosphopeptides. Products were prepared according to the following procedure. A sample (15 μL) containing DNA-anchored hexapeptide substrate (300 pmol), deoxyribozyme (330 pmol), and 5′-triphosphorylated RNA substrate (360 pmol) was annealed in HEPES (5 mM, pH 7.5), NaCl (15 mM), and EDTA (0.1 mM) by heating it at 95 °C for 3 min and cooling it on ice for 5 min. The reaction was initiated by bringing the sample to 30 μL total volume with HEPES (50 mM, pH 7.5), $MgCl_2$ (40 mM), $MnCl_2$ (20 mM), NaCl (150 mM), and KCl (2 mM) and incubating it at 37 °C for 14 h. The product was precipitated with ethanol, separated by 20% PAGE, extracted from the polyacrylamide gel in TEN buffer (10 mM Tris, pH 8.0, 300 mM NaCl, and 1 mM EDTA), and precipitated with ethanol. The sample was dissolved in H_2O (20 μL); a portion (10 μL) was desalted by C_{18} ZipTip and used for mass spectrometry. All observed mass values were in agreement with expectations (for spectra see the Supporting Information). 8VM1 product with DNA-AAAY^{P}AA substrate: *m/z* calcd: 12372.1, found: 12381.9 (Δ = +0.08%). 8VM1 product with DNA-AAAS^{P}AA substrate: *m/z* calcd: 12296.0, found: 12299.3 (Δ = −0.03%). 8VP1 product with DNA-HEG-AAAY^{P}AA substrate: *m/z* calcd: 12719.4, found: 12721.0 (Δ = +0.01%). 8VP1 product with DNA-HEG-AAAS^{P}AA substrate: *m/z* calcd: 12643.3, found: 12645.9 (Δ = +0.02%).

To apply deoxyribozymes to RNA tagging of a mixture of phosphorylated and nonphosphorylated peptides (Figure 4; for a diagram of the experiment see the Supporting Information), the following procedure was used. First, one nonphosphorylated hexapeptide (CAAY^{OH}AA) and three phosphorylated peptides (CAAY^{P}AA, CAEY^{P}AA, and CAFY^{P}AA), each of them HEG-tethered through a disulfide to the same DNA anchor sequence as described above, were mixed in the ratio 100:33:33:33 pmol in H_2O (30 μL). This sample was desalted by using three C_{18} ZipTips and analyzed by MALDI mass spectrometry; the spectrum is shown at the top of Figure 4. All observed mass values were in agreement with expectations. DNA-HEG-CAAY^{OH}AA substrate: *m/z* calcd: 7816.5, found: 7815.6 (Δ = −0.01%). DNA-HEG-CAAY^{P}AA substrate: *m/z* calcd: 7896.5, found: 7896.0 (Δ = −0.006%). DNA-HEG-CAEY^{P}AA substrate: *m/z* calcd: 7954.5, found: 7953.8 (Δ = −0.009%). DNA-HEG-CAFY^{P}AA substrate: *m/z* calcd: 7972.6, found; 7971.8 (Δ = −0.01%). Second, the 8VP1 deoxyribozyme-catalyzed RNA tagging reaction was performed on the same peptide mixture. A sample (20 μL) containing the same four peptides (100:33:33:33 pmol), 8VP1 deoxyribozyme (220 pmol), and 5′-triphosphorylated RNA substrate (240 pmol) was annealed in HEPES (5 mM, pH 7.5), NaCl (15 mM), and EDTA (0.1 mM) by heating it at 95 °C for 3 min and cooling it on ice for 5 min. The DNA-catalyzed tagging reaction was initiated by bringing the sample to 40 μL total volume with HEPES (50 mM, pH 7.5), $MgCl_2$ (40 mM), $MnCl_2$ (20 mM), NaCl (150 mM), and KCl (2 mM) and incubating it at 37 °C for 24 h. The sample was purified by 20% denaturing PAGE; the band corresponding to the mixture of RNA-tagged anchored peptides was extracted with TEN and precipitated with ethanol. To remove the DNA anchor by disulfide reduction, the sample was redissolved in HEPES (50 μL, 50 mM, pH 7.5), and DTT (50 mM) and incubated at 37 °C for 2 h. The sample was again precipitated with ethanol, redissolved in H_2O (30 μL), desalted using three C_{18} ZipTips, and analyzed by MALDI mass spectrometry, with the spectrum shown at the bottom of Figure 4. All observed mass values were in agreement with expectations. As expected, no peak was observed for tagging of the nonphosphorylated CAAY^{OH}AA peptide (*m/z* calcd: 6279.2). CAAY^{P}AA-RNA product: *m/z* calcd: 6359.2, found 6357.7 (Δ = −0.02%). CAEY^{P}AA-RNA product: *m/z* calcd: 6417.4, found 6415.5 (Δ = −0.03%). CAFY^{P}AA-RNA product: *m/z* calcd: 6435.2, found 6433.6 (Δ = −0.02%).

Acknowledgements

This research was supported by grants to S.K.S. from the National Institutes of Health (GM065966), the Defense Threat Reduction Agency (HDTRA1-09-1-0011), and the National Science Foundation (0842534). A.S. was partially supported by NIH T32 GM070421.

Keywords: deoxyribozymes · DNA · in vitro selection · peptides · phosphopeptides

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Received: January 19, 2012
Published online on February 7, 2012

