A Ribonucleopeptide Receptor Targets Phosphotyrosine

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Tools for selective recognition and sensing of kinase-catalyzed phosphorylation at tyrosine or serine/threonine residues located on the protein surface are essential for understanding signal transduction cascades of living cell. A stable complex of RNA and peptide (ribonucleopeptide) provides a convenient approach to tailor a receptor for small molecules. In vitro selection of an RNA-derived pool of ribonucleopeptide afforded a ribonucleopeptide receptor specific for phosphotyrosine. The phosphotyrosine-binding ribonucleopeptide receptor discriminated phosphotyrosine against tyrosine, phosphoserine and phosphothreonine. [DOI: 10.1380/ejssnt.2005.33]

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I. INTRODUCTION

A great advance in molecular cell biology and biochemistry unveiled the fact that kinase-catalyzed phosphorylation of Tyr or Ser/Thr located on a protein surface is an essential event for switching enzymatic activities and/or regulation of protein-protein interactions in signal transduction cascades of living cell [1, 2]. The selective recognition and sensing of a phosphorylated protein surface is required not only for elucidation of protein-protein recognition at the molecular level, but also for regulation of signal transduction through the surface of proteins. In order to understand such complicated signal transduction schemes, it is necessary to develop versatile methods and/or molecular probes that can selectively recognize phosphorylated proteins [3–5]. Generally the specificity of protein kinase is dominated by acidic, basic, or hydrophobic residues adjacent to the phosphorylated residue [6]. The large variations in the protein-phosphorylation sites make it difficult to inspect protein sequences manually and to predict the location of biologically active sites.

Receptors tailor-made for a specific ligand are an ideal tool for targeting the complex protein surface. Selection of functional RNAs from randomized pool of RNA molecules successfully affords RNA aptamers that specifically bind to small molecules, and that have catalytic activities [7–10]. Recent structural analyses of ribosomal RNA complex [11, 12] revealed a remarkable diversity of the RNA-protein complexes, and suggested that its minuteness, a ribonucleopeptide (RNP), would serve as an excellent framework for the design of tailor-made receptors and enzymes. We have designed an ATP receptor that consists of an RNA subunit and a peptide subunit by means of structure-based design approach and successive in vitro selection method [13, 14]. The RNA subunit is designed to consist of two functional domains; an ATP binding domain with randomized nucleotides and an adjacent stem region that serves as a binding site for the RNA-binding peptide (Fig. 1). The randomized nucleotide region was placed next to the HIV-1 Rev response element to enable the formation of RNP pools in the presence of the Rev peptide. In vitro selection of RNA from the randomized pool afforded an RNP-receptor specific for ATP. The ATP-binding RNP did not share the known consensus nucleotide sequence for ATP aptamers [15]. The ATP-binding ribonucleopeptide receptors have high sensitivity to the functional group of the substrate, such as the nucleic acid bases, the number of phosphate groups, and the hydroxyl group of the sugar moiety. We describe here a strategy for molecular recognition of the phosphorylated protein surface. As the first step, O-phospho-L-tyrosine (pTyr) was chosen for the target of ribonucleopeptide receptors.

II. EXPERIMENTAL

A. Materials

pTyr-agarose (immobilized on cross-linked 4% beaded agarose, 13.4 µ mol pTyr/ml packed gel) was purchased from Sigma-Aldrich. N-α-Fmoc-protected amino acids and HATU were from Watanabe Chemicals and Applied Biosystems, respectively.

B. Nucleic Acids Preparation

The original double-stranded DNA pools were constructed by Klenow polymerase (New England Biolabs) reaction from a synthesized oligonucleotide containing 30 random nucleotides (5’-GGATAGGTCTGGGCGCA-N30-TGACGGTACAGGCCGAAAG-3’) and a 3’-DNA primer (5’-CTTTTCGCGCCTGTACCGTCA-3’), followed by PCR amplification to add the promoter for T7 RNA polymerase using Pyrobest DNA polymerase (TaKaRa) with the 3’-DNA and a 5’-DNA primer (5’-TCTTAATACGACTCACTATAGG-3’ and 5’-GGAATAGGTCTGGGCGCA-N30-TGACGGTACAGGCCGAAAG-3’). Reaction from a synthesized oligonucleotide containing 30 random nucleotides (5’-GGATAGGTCTGGGCGCA-N30-TGACGGTACAGGCCGAAAG-3’) and a 3’-DNA primer (5’-CTTTTCGCGCCTGTACCGTCA-3’), followed by PCR amplification to add the promoter for T7 RNA polymerase using Pyrobest DNA polymerase (TaKaRa) with the 3’-DNA and a 5’-DNA primer (5’-TCTTAATACGACTCACTATAGG-3’ and 5’-GGAATAGGTCTGGGCGCA-N30-TGACGGTACAGGCCGAAAG-3’). RNA transcription was performed using AmpliScriptT7 Kit (Epiconcentre) for 3 h at 37°C according to a supplier’s recommended protocols. The resulting RNA was phenol/chloroform extracted, precipitated with ethanol, and pelleted by centrifugation. The RNA was suspended in water and passed through an NAP-5 column (Amersham Pharmacia Biotech) to remove unincorporated nucleotides and recovered. Concentrations of RNAs were quantitated by UV-spectroscopy.
C. In Vitro Selection Procedure

RNPs that bound pTyr were selected as follows: RNA was heated at 80°C for 3 min and cooled to room temperature for 2 h for proper secondary structure. A binding buffer (100 µL) containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 1 µM RNA, 1.5 µM Ac-Rev, and 100 µL of pTyr-agarose was incubated to allow the formation of a specific ribonucleoprotein complex for 30 min on ice. RNP-pTyr resin complexes were washed three times with 300 µL (3 volumes of resin) of binding buffer to remove unbound RNPs and eluted three times with 150 µL (1.5 volumes of resin) of binding buffer containing 1 mM pTyr. Recovered RNPs were precipitated with ethanol and resuspended in TE buffer. After reverse transcription with AMV reverse transcriptase (Promega) of the selected RNA using the 3’-DNA primer used in PCR amplification and successive PCR amplification (RT-PCR) using the 5’- and 3’-DNA primers, the DNA templates were transcribed, and the resulting RNAs were subjected to the next round of selection.

D. Sequencing Analysis of Selected RNA

Selected RNA pools were converted to DNA and PCR-amplified to introduce BamHI, EcoRI restriction sites by using primers 5’-GGCGGATCCCTTTCGGCTGACGTCA-3’ and 5’-GGGAAATTCGATCCACTCTATAGGG-3’. After enzymatic digestion (New England Biolabs), DNAs were cloned into the pUC19 vector using Ligation Kit Ver. 2 (TaKaRa) and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with a model 377 DNA sequencer (Applied Biosystems).

E. pTyr-Binding Assay

Double-stranded DNA templates were prepared by PCR amplification from individual clones by using primers 5’-GAATTCGCTGGGAGCAGCTCTCCTACGCTCGTACGTCAA-3’ and 5’-GGGAATTCGATCCACTCTATAGGG-3’, and these templates were transcribed as described above. Individual RNAs were labeled at the 5’-terminus with T4 Polynucleotide kinase (New England Biolabs) and [γ-32P] ATP (Amersham Pharmacia Biotech). The pTyr-binding assay was performed as follows. A binding buffer (20 µL) containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 1 µM RNA, 1 µM Ac-Rev, and a 20 µL volume of pTyr-agarose was incubated for 30 min on ice. The resulting RNP-pTyr complexes were washed three times with 150 µL (7.5 volumes of resin) of binding buffer to remove unbound RNPs and eluted three times with 100 µL (5 volumes of resin) of binding buffer containing 1 mM pTyr. The fractions of RNA bound to the pTyr resin were quantitated by Cherenkov counting in a scintillation counter.

F. Determination of the Equilibrium Dissociation Constants

The affinity of the RNPs for pTyr was determined by measuring the fraction of RNP bound to pTyr-agarose at a range of immobilized ligand concentrations in a binding buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 20 mM MgCl₂ as described previously. For binding studies, the fraction of the RNP specifically eluted as a function of immobilized pTyr concentration was plotted and fitted by nonlinear regression to a function of the form:
FIG. 2: Nucleotide sequences in the random region of the RNA subunit from the pool obtained after the 6th round of in vitro selection. Numbers in parentheses represent the number of colonies with the same sequence. A possible consensus sequence UGC- GGUAGAA is highlighted in blue letters.

\[ f = \frac{([\text{RNP}] + [\text{pTyr}] + K_D) - \{( [\text{RNP}] + [\text{pTyr}] + K_D)^2 - 4 [\text{RNP}][\text{pTyr}] \}^{1/2}}{2[RNP]} \]

where \( f \) is the fraction of bound RNP to input RNP, \([\text{RNP}]\) is the concentration of ribonucleopeptide, and \( K_D \) is the dissociation constant of RNP for pTyr.

G. pTyr-Binding Competition Assay

The competition assay was performed as follows. A binding buffer (20 \( \mu \text{L} \)) containing 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 20 mM MgCl\(_2\), 1 \( \mu \text{M} \) RNA, 1 \( \mu \text{M} \) Ac-Rev, and a 2 \( \mu \text{L} \) volume of pTyr-agarose was incubated for 30 min on ice in the presence of competitive ligand. RNP-pTyr complexes were washed three times with 150 \( \mu \text{L} \) (7.5 volumes of resin) of binding buffer to remove unbound RNPs and eluted three times with 100 \( \mu \text{L} \) (5 volumes of resin) of binding buffer containing 1 mM pTyr. The fractions of RNA bound to the pTyr resin are quantitated by Cherenkov counting in a scintillation counter.

III. RESULTS AND DISCUSSION

A. Isolation of ribonucleopeptide complexes that bound to pTyr

Ribonucleopeptide complex was designed in a structure-based manner by using the three-dimensional structure of the Rev Response Element (RRE) RNA and HIV-1 Rev peptide complex [16]. An RNA subunit, referred to as RRE30N, was designed to consist of two functionally separated regions, a possible ligand-binding region with 30 randomized nucleotides, and an adjacent stem region that served as a binding site for the Rev peptide (Fig. 1). Ribonucleopeptide receptors for pTyr were isolated from a pool of RNA sequences \((4^{30})\) by the in vitro selection method. In each round of selection RNA pools were incubated with immobilized pTyr in the presence of the Rev peptide (Ac-Rev), the unbound sequences were washed away, and then bound RNP was eluted with free pTyr. The eluted fractions were collected, reverse transcribed, and applied to successive RT-PCR amplification to generate new DNA pools. DNA templates were transcribed and the resulting RNA pools were subjected to the next round of selection. After six rounds of selective amplification in the presence of Ac-Rev, the fraction of RRE30N/Ac-Rev complex eluting with free pTyr increased to 50%.

Analysis of the nucleotide sequences of clones revealed distinct consensus sequences (Fig. 2). Among the 29 clones, 9 revealed the same nucleotide sequence pY03. A 10-nucleotides consensus sequence 5’-UGC—GGUAGAA-3’ was thoroughly observed for other clones.
B. Characteristics of pTyr-binding of RNP

Individual RNA clones of 17 different nucleotide sequences were transcribed from the respective DNA sequences, and tested for the pTyr-binding activity. The fraction of RNP bound pTyr-resin was determined in the presence or absence of Ac-Rev (Fig. 3). All the RNP complexes showed pTyr-binding activity, while RNPs of pY04, pY06 and pY25 did not show obvious binding activity. Unlike the case of ATP-binding RNP, most of the pTyr-binding RNPs show comparable affinity in the presence or absence of Ac-Rev [13, 14].

The stability of the pTyr complex of RNP was analyzed by measuring the fraction of RNP bound to pTyr-agarose at a range of immobilized pTyr concentrations by using pY03, pY09, and pY32. A $^{32}$P-end-labeled pTyr-binding RNP was applied to a pTyr-agarose resin, and unbound RNP was removed by washing with the binding buffer, and specifically bound RNP was competitively eluted off the immobilized ligand with a buffer containing pTyr. The fraction of RNP specifically eluted at each pTyr concentration yields a binding saturation curve as shown in Fig. 4. Equilibrium dissociation constants for the complexes of RNP03, RNP09, and RNP32 to pTyr were determined from the saturation curves by non-linear least-square analysis using the equation as described in Experimental section. RNP of the most tightly conserved pY03 formed a pTyr complex with $K_D$ of 376 µM. pY09 with a consensus 5’-UGC—GGUAGAA-3’ sequence formed RNP that bound pTyr with $K_D$ of 754 µM, while pY32 with a consensus 5’-GGUAGAA—UGC-3’ bound pTyr with $K_D$ of 1.4 mM.

C. Selectivity of pTyr-binding RNP

To investigate the pTyr-recognition mode of RNP receptors, competitive binding assays were performed by using ligands structurally related to pTyr by using RNP of pY03 (Fig. 5). RNP03 preferentially bound pTyr over other pTyr derivatives $O$-phospho-L-serine (pSer), Gly-Tyr, and Leu-pTyr. A pTyr-containing dipeptide Gly-pTyr bound RNP03 with a similar affinity to pTyr,
demonstrating that the pTyr moiety actually participated in the selective binding. The phosphate charge of pTyr contributed to the specific binding complex formation of RNP03 as the non-phosphorylated dipeptide Gly-Tyr did not bind RNP03. The fact that pSer did not bind RNP03 indicates that the aromatic ring of pTyr is also a key determinant for the selective binding of RNP03. Interestingly, a pTyr-containing dipeptide Leu-pTyr showed almost no affinity to RNP03. It is likely that the steric hindrance at the N-terminal portion of pTyr group strongly prohibits the binding of pTyr group to the binding pocket of RNP03.

IV. CONCLUSIONS

The results presented here show that the RNP receptor is tailored for pTyr. Among the pTyr-binding RNP obtained, RNP03 discriminates an amino acid residue at the N-terminal of pTyr residue. The RNP complex reported here consists of two subunits, the RNA subunit and the Rev peptide. The pTyr-binding pocket is mainly consisted of the ribonucleotides of the RNA subunit. The peptide subunit can also contribute to the formation of a ligand-binding pocket within RNP. A stepwise molding of the ligand-binding surface would afford a pTyr-binding RNP that has a specific affinity to a pTyr residue of defined amino acid sequence on the protein surface.

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[1] L. N. Johnson, and R. J. Lewis, Chem. Rev. 101, 2209 (2001).
[2] Zhu Chen, T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, Bing-e Xu, A. Wright, C. Vandebilt, and M. H. Cobb, Chem. Rev. 101, 2449 (2001).
[3] M. D. Jackson, and J. M. Denu, Chem. Rev. 101, 2313 (2001).
[4] A. Ojida, Y. Mito-oka, M. Inoue, and I. Hamachi, J. Am. Chem. Soc. 124, 6256 (2002).
[5] A. Ojida, Y. Mito-oka, K. Sada, and I. Hamachi, J. Am. Chem. Soc. 126, 2454 (2004).
[6] N. Blom, S. Gammeltoft, S. Brunak, J. Mol. Biol. 294, 1351 (1999).
[7] D. S. Wilson and J. W. Szostak, Annu. Rev. Biochem. 68, 611 (1999).
[8] R. R. Breaker, Chem. Rev. 97, 371 (1997).
[9] S. E. Osborne and A. D. Ellington, Chem. Rev. 97, 349 (1997).
[10] G. F. Joyce, Annu. Rev. Biochem. 73, 791 (2004).
[11] N. Ban, P. Nissen, J. Hansen, P. B. Moore, and T. A. Steitz, Science 289, 905 (2000).
[12] P. Nissen, J. Hansen, N. Ban, P. B. Moore, and T. A. Steitz, Science 289, 920 (2000).
[13] T. Morii, M. Hagihara, S. Sato, and K. Makino, J. Am. Chem. Soc. 124, 4617 (2002).
[14] S. Sato, M. Fukuda, M. Hagihara, Y. Tanabe, K. Ohkubo, T. Morii, J. Am. Chem. Soc. 127, 30 (2005).
[15] M. Sassanfar, J. W. Szostak, Nature 364, 550 (1993).
[16] J. L. Battiste, H. Mao, N. S. Rao, R. Tan, D. R. Muhandiram, L. E. Kay, A. D. Frankel, and J. R. Williamson, Science 273, 1547 (1996).