Abstract: Mono-ADP-ribosylation is a dynamic posttranslational modification (PTM) with important roles in signaling. Mammalian proteins that recognize or hydrolyze mono-ADP-ribosylated proteins have been described. We report the synthesis of ADP-ribosylated peptides from the proteins histone H2B, RhoA and, HNP-1. An innovative procedure was applied that makes use of pre-phosphorylated amino acid building blocks. Binding assays revealed that the macrodomains of human MacroD2 and TARG1 exhibit distinct specificities for the different ADP-ribosylated peptides, thus showing that the sequence surrounding ADP-ribosylated residues affects the substrate selectivity of macrodomains.

PTMs play key roles in signaling and protein–ligand interactions. They usually occur through the reversible modification of one or more amino acids. Adenosine diphosphate ribosylation (ADP-ribosylation) is a PTM associated with DNA damage, apoptosis, and gene regulation. ADP-ribosylation involves the enzymatic transfer of ADP-ribose (ADPr) from β-NAD⁺ to the side chain of amino acids. Numerous ADP-ribosyltransferases (ARTs), including bacterial toxins, mono-ADP-ribosylate (MARylate) acceptor proteins. MARylation is also a starting point for poly-ADP-ribosylation (PARylation). Recently, new mono-ARTs have been characterized that function in human diseases.

Mechanisms and functions of MARylation are considerably less well understood compared to poly-ADP-ribosylation. The human MacroD1, MacroD2, and TARG1 proteins contain a macrodomain capable of “reading” and “erasing” MARylation. Currently, the only known substrates are ADP-ribosylated ARTD1 and MARylated GSK3β, ARTD10, or ARTD1E988Q. The basis of substrate selectivity of these mono-ADP-ribosylhydrolases is not known.

To facilitate the analysis of MARylation, we synthesized MARylated peptides (ADPr peptides) and investigated their binding to different macrodomains. Our pioneering studies on the synthesis of ADPr peptides revealed that the “on-resin” formation of a phosphomonoester, which is the precursor of the pyrophosphate, was an inefficient step. Therefore, we decided to develop pre-phosphorylated amino acid building blocks to remove this bottleneck. First, we attempted the synthesis of a histone H2B peptide with ADP-ribosylation of Glu2. Although the synthesis of a phosphoribosylated glutamic acid building block was accomplished, solid-phase synthesis of an N-terminal tetrapeptide of H2B could not be completed owing to side reactions initiated by migration of the O-glutamyl moiety (see the Supporting Information). Therefore, we turned to the H2B N terminus (Scheme 2) MARylated at glutamine, which is resistant to acyl migration and hydrolysis. The MARylation sites from RhoA (Asn41, Scheme 2) and human neutrophil defensin 1 (HNP-1: Arg14, Scheme 2) were also targeted. Citrulline (Cit) was selected as an isostere for arginine (Arg14). We used citrulline since we expected native arginine to be troublesome as a result of the very high basicity of its side chain and the extremely poor solubility of arginine derivatives with a free guanidino group in organic solvents.

First, we synthesized protected ribosylated amino acids (see 4 in Scheme 1). For the synthesis of ribosylated Asn and Cit, we used donor 1 and TBSOTf as an activator (Scheme 1). The ribosylation of Asn proceeded in an α-selective manner (α/β = 97:3, 79%). Ribosylation of the less reactive Cit with donor 1 proceeded less selective (α/β = 78:22, 40%). Next, the 4-methoxybenzyl ethers (PMB) were acetylated (HCl/HFIP). No anomerization was observed for 2 (Asn) but 3 anomerized (α/β = 46:54) during PMB deprotection. Subsequent bis-acetylation yielded compounds 5 and 6 in good yields, and silica gel chromatography enabled separation of the individual anomers. We then cleaved the 5-O-silyl ether and introduced the protected phosphate. The TIPS group in 4 (Gln) was cleaved with Et,N·3HF and the TBDPS group in compounds 5 and 6 was removed using HF-pyridine. Alcohols 7 and 8 were obtained in good yields as pure α-anomers. In contrast, alcohol 9 was obtained as a 95:5 mixture of α/β anomers. The different anomeric ratios and optical purities were determined by NMR.
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Accordingly, resin 16 was shaken for one hour in the presence of one equivalent of HCl in HFIP, followed by a pyridine wash. “On-resin” analysis with 31P-NMR spectroscopy showed almost complete removal of the tBu groups, with trace amounts of mono-tBu-protected phosphate. Successive treatment with HCl/HFIP for 30 minutes yielded intermediate 17. Next, immobilized 5′-phosphoribosylpeptide 17 was reacted with adenosine phosphoramidite 18 with ETT as an activator.14,18 Oxidation of the intermediate PIII-PV species (with CSO) followed by cyanoethyl cleavage (with DBU) afforded protected ADPr peptides 19–22. Next, the Alloc (Arg) and Dmal (Glu and Asp) groups in protected 21 and 22 were removed by using Pd(PPh3)4 and hydrazine, respectively. Finally, ADPr peptides were cleaved from the resin using saturated ammonia in TFE,16 which also removed most of the remaining protecting groups. An NH2/TFE mixture proved superior to methanolic ammonia in terms of yield and provided peptide carboxamide as the only product. Addition of concentrated aqueous ammonia to the mixture ensured benzoyl removal from the adenosine-NH2 to yield crude ADPr peptides 19–22. A combination of reversed-phase (RP)-HPLC and boronate affinity chromatography29 was used to purify the products. Repeated lyophilization afforded pure 19 and 20 in 4% and 5% overall yield, respectively. ADPr peptides 21 and 22 were purified by anion-exchange chromatography to yield the products in 0.5% and 0.2% overall yield. Premature cleavage of the peptide from the solid support during hydrazine treatment is a likely reason for the the lower yield.21 The anomeric purity of the obtained peptides was determined by 1H-NMR. The α-anomeric configuration in peptides 19 and 21 was retained, while for peptide 20, which was treated with TFA, a ratio of 65:35 (α/β)
was observed. Peptide 22 was also obtained as anomeric mixture (α/β = 60:40). The peptides without ADPr modification (19S–22S) were synthesized using standard solid-phase peptide synthesis (SPPS; see the Supporting Information) and used as negative controls in our binding assays.

With the target peptides in hand, we established their affinity toward human MacroD2 and TARG1. MacroD2 and TARG1, both MARylation readers and erasers, diverge in sequence. To ensure that the binding results not complicated by possible catalytic events, we used point mutants of MacroD2 and TARG1 [MacroD2G100E/I189R/Y190N (referred to as MacroD2TM) and TARG1D125A]. The catalytic activity of these mutants is impaired, while ADPr and MARylated ARTD10 binding is retained.[7a,c] We realize that the binding affinity of ADPr peptides containing isosteric replacements instead of native amino acids (Glu and Arg) may be different from the native counterparts. However, the difference is likely to be small, since the ADPr moiety recognized by all ADPr-binding macrodomains is identical in all cases. Even if the presence of an amine instead of a carbonyl group was to change the affinity by 0.2–2 kcalmol⁻¹ (due to one more or one fewer hydrogen bonds, for example), then this would be unlikely to create a situation whereby all tested macrodomains have the same affinity and/or selectivity for distinct MARylated synthetic peptides.

Where possible, we derived the thermodynamic parameters of the MacroD2 and TARG1 proteins for compounds 19, 21, 22 and for the negative controls 19S, 21S, and 22S by using isothermal titration calorimetry (Figure 1 and the Supporting Information). MacroD2TM binds peptide 19 with a KD of 2.8 ± 0.8 μm, while TARG1D125A shows no binding (Figure 1). This was confirmed using biotinylated ADPr H2B peptide 20 in a streptavidin pull-down (Figure S2 in the Supporting Information). In contrast, peptide 21 binds both macrodomains with similar affinity (180 ± 80 nM for MacroD2TM and 550 ± 90 nM for TARG1D125A; Figure 1). Strikingly, the ADPr HNP-1 peptide 22 binds much better to TARG1D125A compared to MacroD2TM. The TARG1D125A-derived KD value for peptide 22 is 150 ± 20 nM, whereas the affinity for MacroD2TM is about 16-fold lower at 2.4 ± 0.4 μm, and ADPr binds TARG1D125A with an approximately 20-fold lower affinity at 2.6 μm (Figure 1).[7b] Our results reveal distinct selectivities of MacroD2TM and TARG1D125A toward ADPr peptides. Indeed, MacroD2TM binds all three tested peptides, irrespective of peptide sequence, length, and nature of the modified amino acid. This indicates that MacroD2TM tolerates diverse sequence contexts surrounding the ADP-ribosylated amino acid. MacroD2 thus either exhibits promiscuous binding toward ADPr substrates, or other mechanisms confer binding specificity on MacroD2 in vivo. In contrast, the amino acid context surrounding the ADPr amino acid strongly influences the binding of TARG1D125A toward ADPr peptides, thus suggesting a more specific target range for TARG1 that will have to be tested in vivo. Our data reveal that macrodomains bind synthesized mono-ADP-ribosylated peptides and that the local sequence context surrounding MARylation sites greatly affects the affinity of a given macrodomain for their PTM-modified target. The synthetic
method, which uses phosphoribosylated amino acids, gives entry to pure, well-defined ADPr peptides of biological relevance in amounts sufficient to study the emerging roles of MARylation in biological recognition and regulation.

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[21] This is because deprotection and cleavage using 0.1 M NaOH in dioxane/MeOH for compound 21 resulted in a significantly higher yield (6%). However, the glycosidic linkage was also affected when using this method, resulting in a complex mixture of products, so this approach was dismissed.

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Synthesis and Macrodomain Binding of Mono-ADP-Ribosylated Peptides

Ties that bind: The synthesis of a number of mono-ADP ribosylated peptides is described. Binding studies of these peptides with different macrodomains showed that the peptide fragment surrounding the ADPr modification influences the binding properties.