Combining affinity purification by ADP-ribose-binding *macro* domains with mass spectrometry to define the mammalian ADP-ribosyl proteome

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Mono-ADP-ribosylation is a reversible posttranslational modification that modulates the function of target proteins. The enzymes that catalyze this reaction in mammalian cells are either bacterial pathogenic toxins or endogenous cellular ADP-ribosyltransferases. For the latter, both the enzymes and their targets have largely remained elusive, mainly due to the lack of specific techniques to study this reaction. The recent discovery of the *macro* domain, a protein module that interacts selectively with ADP-ribose, prompted us to investigate whether this interaction can be extended to the identification of ADP-ribosylated proteins. Here, we report that *macro* domains can indeed be used as selective baits for high-affinity purification of mono-ADP-ribosylated proteins, which can then be identified by mass spectrometry. Using this approach, we have identified a series of cellular targets of ADP-ribosylation reactions catalyzed by cellular ADP-ribosyltransferases and toxins. These proteins include most of the known targets of ADP-ribosylation, indicating the validity of this method, and a large number of other proteins, which now need to be individually validated. This represents an important step toward the discovery of new ADP-ribosyltransferase targets and an understanding of the physiological role and the pharmacological potential of this protein modification.

ADP-ribosylation | ADP-ribosyltransferase | NAD | toxin | posttranslational modification

Mono-ADP-ribosylation is a posttranslational modification that is catalyzed by bacterial toxins and eukaryotic enzymes that transfer the ADP-ribose moiety from β-NAD$^+$ to various cellular acceptor proteins (1–3). Several bacterial ADP-ribosyltransferases (ARTs) are potent virulence factors. After translocation into the host cell, they modify eukaryotic proteins with high specificity and affect their biological functions (4). *Diphtheria* toxin (DT), produced by *Corynebacterium diphtheriae*, was the first bacterial ART family member to be identified (5–7). Along with the related exotoxin A, which is produced by *Pseudomonas aeruginosa*, DT inhibits protein synthesis in intoxicated cells by catalyzing the ADP-ribosylation of diphthamide of elongation factor 2 (eEF2) (5–7). Cholera toxin and pertussis toxin (PT) are two other well-characterized bacterial ARTs that are produced by *Vibrio cholerae* and *Bordetella pertussis*, respectively, and that ADP-ribosylate the α subunits of the heterotrimeric G proteins (4, 8, 9). Other toxins disrupt the cytoskeleton of mammalian cells by ADP-ribosylating either monomeric GTP-binding proteins of the Rho family or actin (4). In addition to these, it is possible that many more ADP-ribosylating toxins and targets remain to be discovered (10–12).

Importantly, endogenous, toxin-related ARTs have also been found in mammals, where they appear to mediate both intracellular and extracellular reactions (1–3). The latter are due to glycosylphosphatidylinositol-anchored or secreted enzymes that can modify either soluble or plasma-membrane-associated protein targets, including the P_2X_7 purinergic receptor, human neutrophil protein 1, and α_7 integrin (13–15). Intracellular, enzymatic mono-ADP-ribosylation modifies proteins with roles in cell signaling and metabolism, such as the chaperone GRP78/BiP, the β subunit of the heterotrimeric G proteins, and mitochondrial glutamate dehydrogenase (GDH) (16–19).

Despite its potential to have crucial roles in the regulation of complex biological processes, including cell signaling and metabolism, membrane trafficking, apoptosis, and gene regulation (2, 3, 20), protein mono-ADP-ribosylation in intact cells remains little explored. This is mainly because of the difficulties in the isolation of the mono-ADP-ribosylated target proteins, due to a lack of specific tools. To date, the antibodies produced in different laboratories can detect only specific proteins that are ADP-ribosylated *in vitro* by either cellular enzymes or toxins (21–24). Unfortunately, these antibodies do not have a wide application, because they do not recognize some of the well-characterized mono-ADP-ribosylated proteins. This may arise from recognition of the ADP-ribose environment by their specific recognition epitopes, thus limiting their use to their specific targets and their use for the detection of previously unknown ADP-ribosylated target proteins.

Recently, a protein module known as the "*macro* domain" was described that recognizes monomeric, polymeric, or both forms of ADP-ribose (25, 26). After determination of the crystal structure of the ADP-ribose–*macro*-domain complex (25, 26), we have explored the possibility that selected *macro* domains can interact not only with free ADP-ribose-like molecules but also with ADP-ribosylated proteins. We now demonstrate that the *macro* domain is a powerful tool that can specifically recognize this posttranslational modification on cellular proteins. By combining the use of *macro* domains with mass spectrometry, we show that this method can be applied to the identification of the protein targets within a host cell that are ADP-ribosylated by bacterial toxins and by endogenous ARTs. This new methodology has the potential for defining both the endogenous and the infection-induced ADP-ribosyl proteomes in mammalian and other cell systems.

Results

Macro-Domain Interactions with ADP-Ribosylated Proteins. Until recently, no specific protein domains that recognize ADP-ribose had been described. The discovery of the *macro* domain as an ADP-ribose-binding module (25) prompted us to determine whether these domains can also recognize mono-ADP-ribosylated proteins. The *macro* domains from both the human poly(ADP-ribose)-polymerase (PARP9) enzyme BAL (*m*BAL) and the ar-

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chaeobacterial protein Af1521 from *Archaeoglobus fulgidus* (*m*Af1521) recognize free ADP-ribose with high affinities and specificities (e.g., they bind NAD$^+$ and adenosine with a >1,000-fold lower affinity) (25). Despite their specificities, the *macro* domains of BAL9 and Af1521 also recognize the poly(ADP-ribose) polymer *in vitro*, consistent with a binding pocket that can accommodate additional chemical groups beyond the core ADP-ribose (25). We therefore surmised that the *macro*-domain module can also selectively bind mono-ADP-ribosylated proteins.

To examine whether we could reveal mono-ADP-ribosylated proteins by using the *macro*-domain module, we first used a pull-down assay based on the binding between the Ni^{2+}-nitrilotriacetic acid (Ni-NTA)-resin-bound His-tagged *m*Af1521 (His$_6$-*m*Af1521) and the mono-ADP-ribosylated β subunit of G proteins (2, 17), a well-characterized model target protein for ADP-ribosylation. Plasma membranes were isolated from Chinese hamster ovary (CHO) cells and used as the source of the endogenously ADP-ribosylated $\beta\gamma$ dimer (19). His$_6$-*m*Af1521 was incubated with radioimmunoprecipitation-assay-buffer-solubilized plasma membranes from CHO cells in which the endogenous β subunit had been [^{32}P]-ADP-ribosylated, as described in ref. 19, and its binding with the β subunit was assessed (Fig. 1). His$_6$-*m*Af1521 quantitatively retained the [^{32}P]-ADP-ribosylated β subunit, whereas the Ni-NTA resin did not. In a control experiment, an unrelated GST-tagged hTaf250 double bromodomain module that does not bind ADP-ribose (25, 27) did not bind detectable levels of the [^{32}P]-ADP-ribosylated β subunit (Fig. 1*A*; unbound protein in Fig. 1*B*). When the amount of the [^{32}P]-ADP-ribosylated β subunit to be loaded onto the resin was increased by adding purified $\beta\gamma$ dimer to the ADP-ribosylation assay [a condition in which $\beta\gamma$ is known to associate with the plasma membrane and to be ADP-ribosylated (17, 28)], it was again quantitatively retained by His$_6$-*m*Af1521 (Fig. 1*C*). Finally, to ascertain that these interactions represented specific binding between *m*Af1521 and the mono-ADP-ribosylated form of the β subunit, the pull-down assay was carried out with plasma membranes that had been incubated with α-NAD$^+$ [which, unlike β-NAD$^+$, cannot sustain enzymatic mono-ADP-ribosylation (28)]. Under these conditions, His$_6$-*m*Af1521 did not retain the β subunit, as expected (Fig. 1*D*).

To ascertain a specific role of the ADP-ribose binding pocket in the binding reaction, we also used a site-specific mutant (His$_6$-*m*Af1521/G42E) as a further control. This mutation, from Gly to Glu, is sufficient to abrogate the ADP-ribose binding of Af1521 (25). As expected, this *m*Af1521 mutant failed to pull down the [^{32}P]-ADP-ribosylated β subunit, confirming that the ADP-ribose binding pocket is essential for the interaction of *m*Af1521 with mono-ADP-ribosylated proteins (Fig. 1*A*).

Taken together, these data demonstrate that the ADP-ribose-binding *m*Af1521 interacts with the mono-ADP-ribosylated β subunit in a specific manner that depends on the β subunit ADP-ribosylation state. Thus, *macro* modules can, in principle, be exploited to bind mono-ADP-ribosylated proteins, indicating that they represent valuable tools for the study of endogenous mono-ADP-ribosylation of proteins.

Efficiency and Reversibility of the Binding of ADP-Ribosylated Proteins to *m*Af1521. Affinity purification procedures ideally require that the binding between the proteins to be isolated and the affinity resin be both efficient and reversible. To investigate these characteristics in our system, CHO cell plasma membranes enriched with purified $\beta\gamma$ dimer were first ADP-ribosylated in the presence of [^{32}P]-NAD$^+$ (see *Materials and Methods* and refs. 19 and 31). The amount of [^{32}P]-ADP-ribosylated β subunit that was pulled down using increasing concentrations of His$_6$-*m*Af1521 was then evaluated by autoradiography and Western blot analysis (Fig. 2 *A* and *B*; unbound fractions shown as control in Fig. 2*B*, lower panel). Approximately a ≈25-fold molar excess of resin-bound His$_6$-
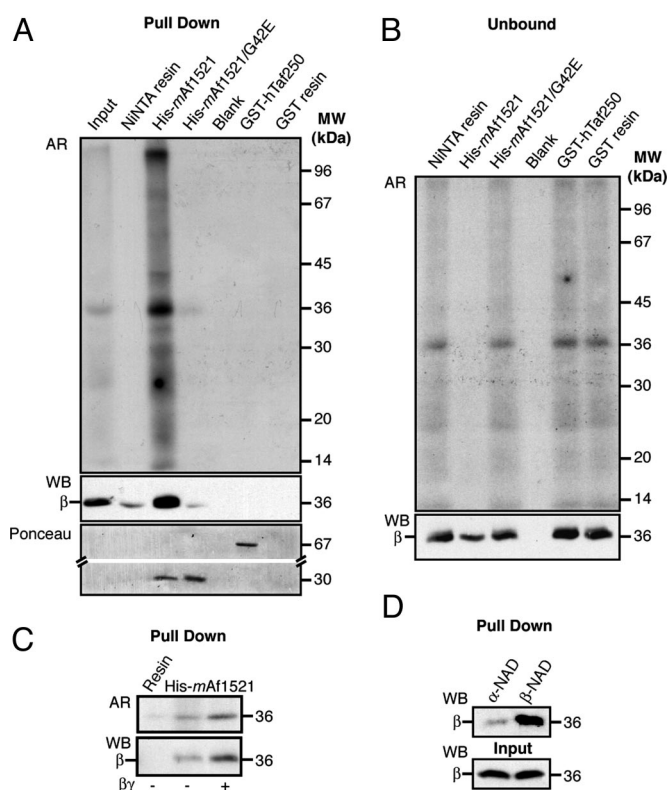

Fig. 1. *Macro* module pull-down assay of mono-ADP-ribosylated G protein β subunit from CHO cells. (*A–C*) Plasma membrane proteins (10 μg) were ADP-ribosylated with [^{32}P]-NAD$^+$ (1 h at 37°C) in the absence (*A–C*) or presence (*C*) of the purified $\beta\gamma$ dimer (250 ng) (see *Materials and Methods*), and solubilized and pulled down with 200 pmol of the indicated *macro* modules. The pulled-down samples (*A*, *C*) and the unbound fractions (*B*; one-eighth of the total volume) were analyzed by autoradiography (AR) and Western blot analysis (WB) with an anti-β subunit antibody. The Ponceau red staining (*A*, *lower*) of the pulled-down samples shows that equivalent amounts of the different *macro* modules were used. (*D*) Plasma membrane proteins (10 μg) were incubated with unlabeled β-NAD$^+$ or α-NAD$^+$ (12 h, 30°C). His$_6$-*m*Af1521-pulled-down proteins and the corresponding input material were analyzed by Western blot with an antibody to the β subunit. When β-NAD$^+$ was replaced with α-NAD$^+$, the residual labeling of substrates can be ascribed to the contaminating β-NAD$^+$ present in the commercial α-NAD$^+$ preparations. The data shown are representative of two to six experiments.

*m*Af1521 was required to nearly completely (≈85%) adsorb the ADP-ribosylated $\beta\gamma$ (Fig. 2*A*).

We also examined the reversibility of the binding of the [^{32}P]-ADP-ribosylated β subunit to His$_6$-*m*Af1521. Here, a range of ADP-ribose concentrations (1 nM to 10 mM) and 30 pmol of His$_6$-*m*Af1521 were used. In the "competition" experiments (Fig. 2*C*), the His$_6$-*m*Af1521 and ADP-ribose were added simultaneously to the labeled, solubilized, CHO plasma membranes. Here, the ADP-ribose competes with the [^{32}P]-ADP-ribosylated β subunit for binding to the resin-bound His$_6$-*m*Af1521. In the "displacement" experiments (Fig. 2*D*), the [^{32}P]-ADP-ribosylated β subunit was initially pulled down with the resin-bound His$_6$-*m*Af1521, and then ADP-ribose was added to displace the [^{32}P]-ADP-ribosylated β subunit from the His$_6$-*m*Af1521 (see *Materials and Methods* for further details). These assays demonstrated that free ADP-ribose can displace the [^{32}P]-ADP-ribosylated β subunit from the resin with EC$_{50}$ values of 5 and 10 μM, respectively. This indicates that our *macro*-domain-based procedure can yield highly pure and intact ADP-ribosylated proteins in solution.

Use of the *Macro* Module To Identify Substrates of Bacterial ART Toxins from Intact Cells. We next investigated whether this method can be used to identify substrates of bacterial toxins that display
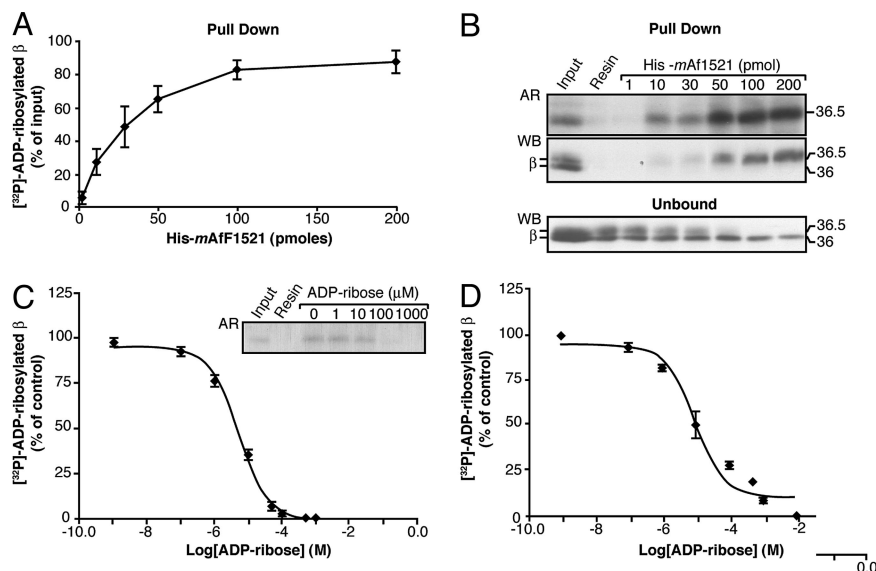
Fig. 2. Efficiency and reversibility of binding to His$_6$-*m*Af1521 of the ADP-ribosylated $\beta\gamma$ dimer. CHO plasma membrane proteins (10 μg) and 250 ng of purified $\beta\gamma$ dimer were ADP-ribosylated in the presence of [^{32}P]-NAD$^+$ for 12 h at 30°C. After solubilization, they were pulled down with His$_6$-*m*Af1521. (*A*) Increasing concentrations of His$_6$-*m*Af1521 in the [^{32}P]-ADP-ribosylated β subunit pull-down assay, shown as percentage of the input of [^{32}P]-ADP-ribosylated β subunit ($\approx$4 pmol). Data are means $\pm$ SD of five independent experiments. (*B*) As for (*A*), showing pulled-down samples and unbound fractions (one-eighth of the total volume) from a representative experiment, as analyzed by autoradiography (AR) and by Western blotting (WB) with an antibody against the β subunit that recognizes both the unmodified (36 kDa) and the ADP-ribosylated (36.5 kDa) β subunit. The disappearance of the [^{32}P]-ADP-ribosylated β subunit from the unbound fraction was also followed in parallel (*B, lower*). (*C*) Pull-down of the [^{32}P]-ADP-ribosylated β subunit after its incubation with 30 pmol of His$_6$-*m*Af1521 in the presence of increasing concentrations of ADP-ribose (1 nM to 1 mM) ("competition" conditions). Data are expressed as percentages of [^{32}P]-ADP-ribosylated β subunit pulled down in the absence of ADP-ribose and are means $\pm$ SD from three independent experiments. *Inset*: Autoradiography of the pulled-down samples from a representative experiment. (*D*) As for (*C*), but with the addition of ADP-ribose after the pull-down assay of the [^{32}P]-ADP-ribosylated β subunit by 30 pmol of His$_6$-*m*Af1521 ("displacement" conditions). At 100 μM ADP-ribose ($\approx$400 pmol under these incubation conditions), all of the His$_6$-*m*Af1521-bound [^{32}P]-ADP-ribosylated β subunit ($\approx$4 pmol) was displaced. A $\approx$100-fold excess of ADP-ribose can displace the immobilized ADP-ribosylated β subunit from the His$_6$-*m*Af1521-resin complex. Data are means $\pm$ SD from three independent experiments. Curve fitting analyses in (*C*) and (*D*) were performed with GraphPad PRISM, giving ADP-ribose EC$_{50}$ values of 5 and 10 μM, respectively.

ART activity. We focused our attention on the cellular substrates of PT, a cysteine-specific ART. For this, we used CHO cells that were either left untreated (control) or were intoxicated with PT. First, to verify that the α_i subunit of the heterotrimeric G proteins was indeed fully modified by the treatment of intact cells with PT, membranes from control and intoxicated cells were analyzed in an *in vitro* ADP-ribosylation assay using PT and radiolabeled NAD, according to established procedures (see *Materials and Methods* and ref. 34). Although in control membranes α_i was radiolabeled by PT, in the intoxicated membranes this G$_i$ subunit was not, confirming that it had already been fully modified *in situ* by the PT pretreatment and was therefore not available for further *in vitro* labeling (Fig. 3*A*).

These PT-treated membranes were next subjected to the *macro*-domain pull-down protocol with GST-*m*Af1521. A band in the PT-treated samples with a molecular mass of 40 kDa, as revealed by silver staining (Fig. 3*B*), was indeed identified by MALDI-TOF-MS analysis as the G protein α_{i2} subunit (Table 1).

Thus, the cellular targets of bacterial toxins can be identified using this *macro* domain, further supporting the potential of this method to identify mono-ADP-ribosylated proteins in intact cells.

Purification and Identification of Substrates of Intracellular ART Activities by *Macro* Domains. Intracellular mono-ADP-ribosylation in mammals modifies proteins that have important roles in cell signaling and metabolism, including, among those best characterized, the chaperone GRP78/BiP, the β subunit of G proteins, and GDH (16–19, 29).

We evaluated whether *macro*-domain-based pull-down assays coupled to MALDI-TOF-MS analysis can be used to identify such proteins that carry an endogenous ADP-ribosyl modification under

resting conditions in an intact-cell model. Postnuclear and membrane fractions isolated from CHO and HL60 cells were used in parallel, where they were first subjected to a nonspecific pull-down step using the His$_6$-tagged *m*Af1521/G42E mutant; this thus initially eliminated proteins that bound to the resin or to the *macro* domain in an ADP-ribose-independent manner (Fig. 3 *C* and *D*). Next, the unbound material underwent a second pull-down assay with wild-type His$_6$-*m*Af1521 to specifically retain the ADP-ribosylated proteins (Fig. 3 *C* and *D*).

This two-step procedure resulted in the separation of >20 visible bands in well-resolved gels, all representing potential ADP-ribosylated substrates (Fig. 3*D*). Among the 10 major bands from CHO cells, four were identified by MALDI-TOF-MS analysis as GRP78/BiP, vimentin, GDH, and the elongation factor α1/2 (Fig. 3*D* and Table 1). Moreover, the endogenously ADP-ribosylated β subunit from HL60 membranes, which was at too low a level to be revealed by colloidal Coomassie blue staining, was readily identified by a specific antibody against the β subunit (Fig. 3*C*). These proteins are well-known substrates of enzymatic ADP-ribosylation, and so they provide the "proof of principle" that our two-step procedure can successfully isolate different ADP-ribosylated proteins. Importantly, previously unknown putative substrates for endogenous ART activity were also identified. One of these is the intermediate filament protein vimentin (Fig. 3*D*), which has not been demonstrated to be a substrate of endogenous mono-ADP-ribosylation, although it has been described as a cellular target of the bacterial toxin exoenzyme S (ExoS) from *P. aeruginosa* (30).

An additional interesting feature of the *macro*-domain recognition of ADP-ribosylated substrates is its independence from the nature of the modified amino acid. The *m*Af1521 not only binds the G protein β subunit that we have shown to be modified on arginine
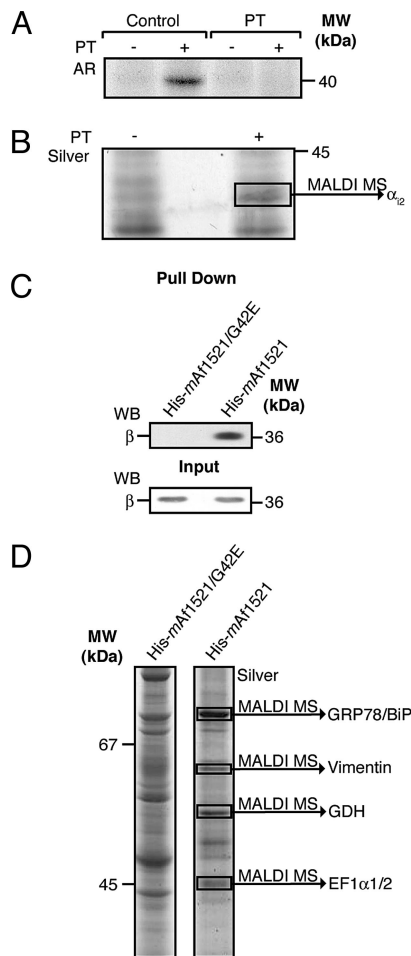
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Fig. 3. (*A*, *B*) GST-*m*Af1521 pull-down assay of proteins from CHO cell membranes that are either ADP-ribosylated by PT or endogenously ADP-ribosylated. (*A*) Cells were either untreated (control) or intoxicated with 5 nM PT for 15 h (PT), and then their solubilized membrane proteins (25 μg) were ADP-ribosylated with [^{32}P]-NAD$^+$ and in the absence (−) or presence of PT (+) (see *Materials and Methods*). The samples were analyzed by SDS/PAGE and visualized by autoradiography (AR). (*B*) Cells were untreated (−) or intoxicated with PT (+), and their solubilized membrane proteins (2 mg) were pulled down with 2 nmol of GST-*m*Af1521. The samples were analyzed by SDS/PAGE and visualized by silver staining. The indicated band was identified by MALDI-TOF-MS (see *Materials and Methods* and Table 1). (*C*, *D*) His$_6$-*m*Af1521 pull-down assay of endogenous ADP-ribosyltransferase substrates. (*C*) Solubilized HL60 cell membrane proteins (2 mg) were pulled down with 2 nmol of His$_6$-*m*Af1521. The samples were analyzed by SDS/PAGE followed by Western blot analysis (WB) with an anti-β subunit antibody. The β subunit relative content in the input was revealed by Western blot analysis (*C, lower*, WB). (*D*) Solubilized CHO postnuclear membrane proteins (5 mg) were pulled down with 2 nmol of His$_6$-*m*Af1521, resolved by SDS/PAGE, and visualized by silver staining. The indicated bands were identified by MALDI-TOF-MS (see *Materials and Methods* and Table 1). The data shown are representative of two to six experiments.

(17) but also GDH that is modified on cysteine (18), eEF2 on diphthamide (7, 31), and GRP78/BiP on an unidentified amino acid, clearly indicating that the recognition of the ADP-ribose is independent of the modified amino acid. This highlights the potential of our methodology and its relevance for the definition of the role of the ADP-ribosylation reaction.

To analyze in more detail the total mammalian ADP-ribosylation proteome, we introduced some further modifications to this method. The pattern of endogenously ADP-ribosylated proteins was determined by examining HL60 cells (Fig. 4). To minimize nonspecific resin interaction with proteins, we used GST-*m*Af1521 and GST-*m*Af1521/G42E cross-linked to the Sepharose resin, instead of His$_6$-*m*Af1521 and His$_6$-*m*Af1521/G42E. The HL60 cell postnuclear fractions were thus first incubated with glutathione-Sepharose resin to remove endogenous GST. Then the unbound proteins were subjected to the two-step procedure, with the first pull-down assay with the GST-tagged mAf1521/G42E mutant as above, to initially eliminate proteins that bound to the *macro* domain in an ADP-ribose-independent manner. The unbound material was then divided into two aliquots for the second pull-down assay, one using GST-*m*Af1521/G42E (control) and the other wild-type GST-*m*Af1521 (sample), to specifically retain the ADP-ribosylated proteins. Finally, to add further specificity to this method, the proteins bound to both the control and the sample columns were eluted with ADP-ribose, and the eluates were separated by SDS/PAGE. The corresponding bands from both the control and the sample lanes were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), and only the proteins specific to the sample were considered ADP-ribosylated [Fig. 4 and scheme in supporting information (SI) Fig. S1].

Some of the main bands identified with this two-step procedure revealed PARP1, GRP78/BiP, protein disulfide isomerase (PDI), T-complex protein 1 subunit zeta (CCT-zeta), tubulin (both α and β), and GAPDH (Fig. 4 and Table 1). In the case of tubulin, this was initially proposed to be regulated by mono-ADP-ribosylation by cellular enzymes, although only in an *in vitro* analysis (32). More recently, evidence of poly-ADP-ribosylation of this protein has also been reported in mouse brain homogenates (33). However, consistent with the single narrow band seen around the expected molecular mass (Fig. 4), this tubulin modification should involve a single ADP-ribose molecule, suggesting that tubulin is indeed a cellular target of mono-ADP-ribosylation in intact human cells.

Finally, of particular note, this procedure also identified proteins that have not been associated with either bacterial toxin or endogenous ADP-ribosylation, such as heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) (Table 1).

In summary, these data show that a two-step procedure with first the mutant and then the wild-type *m*Af1521 provides a very effective method for the isolation and identification of substrates of endogenous ART activities.

Discussion

In this study, we report on an affinity-based method for the analysis of endogenous and toxin-induced ADP-ribosylation of proteins in intact cells. The method combines the use of a specific *macro*-domain module, *m*Af1521 (which binds ADP-ribosylated proteins potently and selectively), with multistage mass spectrometry analysis (see scheme in Fig. S1). It should be noted that among the different *macro* domains that we have tested so far His$_6$-*m*Af1521 is the most efficient for pulling down ADP-ribosylated protein σ from the mammalian cells analyzed; however, the procedure might require further adjustments according to the *macro* domain used (e.g., detergents or incubation times), when different cells (e.g., from plants) or tissue extracts are to be analyzed, or both. Nevertheless, the methods can be considered to be of wide applicability to a large number of biological samples, which can all be analyzed following the principle of the protocol schematically summarized in Fig. S1. This should lead to the identification of a number of unidentified proteins that can be regulated by ADP-ribosylation, which will help us to define the various functional roles of this posttranslational modification in cellular processes and organelles and in the whole organism.

Here, we have explored the use of this methodology in more than one way. The first was to purify known and previously unknown ADP-ribosylated proteins and to separate modified from unmodified substrates. This is clearly shown for the mono-ADP-ribosylated β subunit, which can be eluted from resin-bound His$_6$-*m*Af1521 by some 100-fold excess of ADP-ribose, whereas the

Table 1. Protein identification

	NCBI accession no.	Name	Score*	Matched peptide, no.	Sequence coverage, %	Molecular mass, kDa
Maldi-TOF-MS	77386129	Gαi2	112	10	27	41
	121570	GRP78	101	12	22	70
	1220484	Elongation factor-1α	109	13	37	50
	6680027	GDH1	134	18	35	62
	62414289	Vimentin	138	19	40	54
	48145673	HNRPH1[†]	106	11	30	49
	130781	PARP1	1135	23	31	113
LC-MS/MS	14916999	GRP78/BiP	436	72	18	72
	56757569	Tubulin	265	5	22	50
	120649	GAPDH	835	8	65	41
	2507460	PDI	238	4	15	57
	730922	CCT-zeta	219	4	12	58

*Protein scores $\approx$65 are significant ($P < 0.05$). See *Methods* for details.
[†]Identified from A375 cells (melanoma cell line).

unmodified protein can easily be collected in the unbound fraction. The ability to separate the modified from the unmodified protein represents a significant advantage in studies of the function of mono-ADP-ribosylated substrates, and it solves a technical aspect that to date could not be addressed by classical chromatography or immunoprecipitation.

The other specific use of the *macro*-domain method that we have investigated is in the field of bacterial toxins. There is significant interest in the role of mono-ADP-ribosylation in human pathology, because many bacterial toxins are ARTs that disrupt cellular functions and affect human health (4, 7). The number of known toxins with ART activity is growing (10–12), and the new candidates require the characterization of their cellular targets. With the tools that have been available to date, this has been far from straightforward [e.g., the characterization of the mammalian proteins that are ADP-ribosylated by the *P. aeruginosa* toxin ExoT required the collection of silver-stained spots from 12 two-dimensional gels for MALDI-TOF-MS analysis (34)]. This technical complexity in part explains why it has not been possible so far to define a compendium of all of the bacterial ART targets in mammalian host cells. This *macro*-domain method will greatly accelerate the analysis of this important class of toxins.

Fig. 4. GST-*m*Af1521 pull-down assay and protein identification from HL60 cells. Solubilized HL60 cell proteins (20 mg) were pulled down with resin-bound GST-tagged *m*Af1521/G42E or *m*Af1521, as indicated, eluted with 20 mM ADP-ribose, then resolved by SDS/PAGE, and visualized by Coomassie blue staining. The indicated bands (in the sample and in the control lanes) were identified by LC-MS/MS analyses (see *Materials and Methods* and Table 1). The data shown are representative of two experiments.

Finally, this new strategy has been used to identify ART targets in mammalian cells. The main proteins to be ADP-ribosylated under basal conditions are cytoskeletal proteins, including actin and tubulin, and other proteins involved in cell metabolism, including GAPDH and GDH (e.g., see Figs. 3 and 4). Of further interest, although the same major bands can be identified with the His and GST *m*Af1521 systems (e.g., GRP78/BiP), the patterns of the major protein bands seen upon ADP-ribosylation are indeed different (e.g., compare Figs. 3*D* and 4). This will reflect functional differences across cellular systems and the specific patterns of protein expression in any given cell type.

These data therefore represent the beginning of the long list of proteins that can now be identified, with analyses not only including different organelles and tissues but also evaluating the changes occurring in the ADP-ribosyl proteome under different experimental conditions (e.g., stimuli versus basal or pathological versus normal). This also becomes more evident considering the various patterns of ADP-ribosylation that we have seen across different cell systems.

Among the targets for which the specific mono-ADP-ribosylation remains to be confirmed, further molecular chaperones have been identified, including PDI and CCT-zeta, and further cytoskeletal proteins, including vimentin (Table 1). Importantly, in addition to these targets that belong to protein families that include members that are already known to be mono-ADP-ribosylated, we have also identified completely new protein family members, for example, the ubiquitously expressed HNRNPH1. The HNRNPs appear to shuttle between the nucleus, where they are associated with pre-mRNAs, and the cytoplasm; they also appear to have a role in pre-mRNA processing and other aspects of mRNA metabolism and transport.

Clearly, an extensive characterization of targets of mono-ADP-ribosylation is still required for each of these proteins to determine whether they are indeed modified themselves or rather are parts of complexes that include at least one modified protein. The latter could indeed be the case for the different molecular chaperones, because an interaction between GRP78/BiP and PDI has been reported (35). In contrast, the confirmation of HNRNPH1 as a target of mono-ADP-ribosylation will open a new field of investigation, because it is possible that this posttranslational modification of HNRNPH1 affects its ability to bind to RNA and thus to regulate mRNA metabolism and transport.

In conclusion here, with this method now available, there are sure to be discoveries of numerous previously unknown targets of this posttranslational modification, which becomes even more important now that previously unknown ART enzymes have been identified.
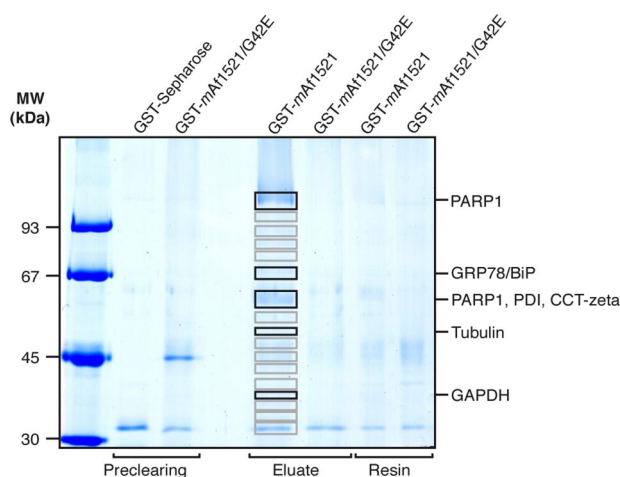
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Indeed, although endogenous mono-ADP-ribosylation is believed to have important roles in cellular signaling and regulation, the enzymatic activities responsible for this intracellular mono-ADP-ribosylation are only now beginning to be identified. The human genome, for example, encodes 17 so-called poly(ADP-ribose)-polymerase-like genes. Many of these are, however, unlikely to carry out genuine enzymatic ADP-ribose polymer formation, and although some have been proposed to act as cellular mono-ADP-ribosyltransferases (20, 36, 37), for PARP10 this has indeed been demonstrated (38). However, their cellular functions and targets remain unknown.

Another NAD$^+$-using family of proteins, the sirtuins, are enzymes that can deacetylate protein substrates, such as acetylated histones (39, 40). The yeast member, silent information regulator 2 (Sir2), catalyzes mono-ADP-ribosylation of the removed acetyl group (39); moreover, SirT6 has been shown to have a mono-ADP-ribosyltransferase activity directed toward itself (41), and SirT4 mono-ADP-ribosylates mitochondrial GDH, thus repressing its activity (19) and resulting in the regulation of insulin secretion in pancreatic β cells. Moreover, to add to this complexity, several enzymes that catalyze the reverse reaction, the ADP-ribosyl hydrolases, have also been reported, although their specific roles and substrates also remain unknown. These examples give us a glimpse of the type of physiologically relevant information that can be gathered by fully exploring the endogenous mono-ADP-ribosylation machinery. Over the next few years, this *macro*-domain-based methodology should allow a systematic study of the ADP-ribosyl proteome in mammalian systems and should lead to the definition of the ADP-ribosylation-dependent pathways in physiology and pathology.

Materials and Methods

Detailed methods for CHO and HL60 cell culture and fractionation, ADP-ribosylation and immunoblot analysis, and expression and purification of *macro* modules are provided as *SI Materials and Methods*.

Pull-Down Assay. Plasma membranes, total membranes, or postnuclear material were solubilized, incubated with the *macro* modules, and pulled down with Ni-NTA or glutathione-Sepharose resins. Detailed procedures, also for the competition, displacement, and affinity purification assays are in *SI Materials and Methods*.

Protein Identification by MALDI-TOF-MS and LC-MS/MS Analyses. The protein bands of interest were isolated by SDS/PAGE and analyzed as detailed in *SI Materials and Methods*.

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