Arginine-specific mono(ADP-ribosyl)transferase activity on the surface of human polymorphonuclear neutrophil leucocytes

Louise E. DONNELLY, Nigel B. RENDELL, Stephen MURRAY, Jennifer R. ALLPORT, Gar LO, Panagiotis KEFALAS, Graham W. TAYLOR and John MacDERMOT*

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN, U.K.

An Arg-specific mono(ADP-ribosyl)transferase activity on the surface of human polymorphonuclear neutrophil leucocytes (PMNs) was confirmed by the use of diethylamino-(benzylidineamino)guanidine (DEA-BAG) as an ADP-ribose acceptor. Two separate HPLC systems were used to separate ADP-ribosyl-DEA-BAG from reaction mixtures, and its presence was confirmed by electrospray mass spectrometry. ADP-ribosyl-DEA-BAG was produced in the presence of PMNs, but not in their absence. Incubation of DEA-BAG with ADP-ribose (0.1–10 mM) did not yield ADP-ribosyl-DEA-BAG, which indicates that ADP-ribosyl-DEA-BAG formed in the presence of PMNs was not simply a product of a reaction between DEA-BAG and free ADP-ribose, due possibly to the hydrolysis of NAD$^+$ by an NAD$^+$ glycohydrolase. The assay of mono(ADP-ribosyl)transferase with agmatine as a substrate was modified for intact PMNs, and the activity was found to be approx. 50-fold lower than that in rabbit cardiac membranes. The K_m of the enzyme for NAD$^+$ was $100.1 \pm 30.4\,\mu$M and the V_{max} 1.4 ± 0.2 pmol of ADP-ribosylagmatine/h per 10^6 cells. The enzyme is likely to be linked to the cell surface via a glycosylphosphatidylinositol anchor, since incubation of intact PMNs with phosphoinositol-specific phospholipase C (PI-PLC) led to a 98 % decrease in mono(ADP-ribosyl)transferase activity in the cells. Cell surface proteins were labelled after exposure of intact PMNs to [^{32}P]NAD$^+$. Their molecular masses were 79, 67, 46, 36 and 26 kDa. The time course for labelling was non-linear under these conditions over a period of 4 h. The labelled products were identified as mono(ADP-ribosyl)ated proteins by hydrolysis with snake venom phosphodiesterase to yield 5′-AMP.

INTRODUCTION

Mono(ADP-ribosyl)transferases (EC 2.4.2.31) catalyse the cleavage of NAD$^+$ to yield free nicotinamide and transfer ADP-ribose on to an amino acid acceptor through an S- or N-glycosidic linkage. The most extensively characterized forms of this enzyme are the prokaryotic mono(ADP-ribosyl)transferases. Numerous bacteria, including *Vibrio cholerae*, *Bordetella pertussis*, *Escherichia coli* and *Clostridium botulinum*, produce mono(ADP-ribosyl)transferases as exotoxins which ADP-ribosylate specific G-protein subunits and modify their function [1,2].

Eukaryotic mono(ADP-ribosyl)transferases were identified some years ago [3], although little is known as yet about their physiological roles. Both Arg- and Cys-specific forms of mono(ADP-ribosyl)transferase have been reported in various tissues [3–7], but there is still doubt regarding the existence of Cys-specific eukaryotic mono(ADP-ribosyl)transferases. Arginine-specific mono(ADP-ribosyl)transferases have been purified from various tissues, including turkey erythrocytes [3–6] and rabbit skeletal muscle [8–10]. Turkey erythrocytes contain several different isoforms of the enzyme, which differ in their cellular distribution, regulatory, physical and kinetic properties. In contrast, the enzyme isolated from rabbit skeletal muscle is more restricted in its expression. Several isoforms of eukaryotic mono(ADP-ribosyl)transferase have been cloned, including the mono(ADP-ribosyl)transferases from rabbit skeletal muscle [10],

human skeletal muscle [11], and two isoforms from chicken bone-marrow cells [12]. The deduced amino acid sequence of the rabbit and human skeletal-muscle mono(ADP-ribosyl)transferases revealed proteins with approximate molecular masses of 36 kDa and 40 kDa respectively. They had no significant amino acid similarity to any of the known sequences of the prokaryotic mono(ADP-ribosyl)transferases or poly(ADP-ribosyl)transferases, although there was approx. 50 % sequence similarity to the chicken bone-marrow-derived mono(ADP-ribosyl)transferases and approx. 40 % sequence similarity to the RT6.2 NAD$^+$ glycohydrolase [12].

The deduced amino acid sequence of the rabbit skeletal-muscle mono(ADP-ribosyl)transferase contains a hydrophobic C-terminal region and a site for a glycosylphosphatidylinositol (GPI) anchor, which would secure it in the plasma membrane. This was confirmed by subsequent transfection of the corresponding cDNA into rat mammary adenocarcinoma cells [11]. Furthermore, the GPI anchor could be hydrolysed by phosphatidylinositol-specific phospholipase C (PI-PLC). Other mono(ADP-ribosyl)transferases, including rat cardiac-muscle membrane mono(ADP-ribosyl)transferase [13] and the enzyme from the cell surface of cultured mouse skeletal-muscle cells [14], can be released by hydrolysis of the GPI anchor with PI-PLC. In contrast, an arginine-specific mono(ADP-ribosyl)transferase found in skeletal-muscle sarcolemma has an activity which is predominantly orientated towards the cytoplasm [15].

In many cases, the substrates of mono(ADP-

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ribosyl)transferase were identified in tissue homogenates or isolated membrane fragments in the presence of high concentrations of purified enzyme (for review, see [16]). Many of these candidate substrates have yet to be identified in whole cells or intact tissues, and the presence of a mono(ADP-ribosyl)transferase on the cell surface would suggest that its substrate might have a similar location. To date, only one substrate for this enzyme has been identified on the outer cell surface, namely the 97 kDa laminin-binding protein, integrin $\alpha 7$ of mouse skeletal myocytes [14]. Integrin $\alpha 7$ is developmentally regulated, and forms heterodimers which assemble as a mature laminin receptor ($\beta_1 \alpha_7$). The significance of the ADP-ribosylation of this protein on the cell surface of skeletal-muscle cells remains unclear, but might play a role in integrin signal transduction, the regulation of its expression or cell adhesion.

There is evidence that $G_s \alpha$ is a substrate for mono(ADP-ribosyl)transferase in certain tissues [17–19], which may lead to altered $G_s \alpha$ activity [17–19] or abundance [20]. There are also reports of mono(ADP-ribosyl)ation of the 78 kDa glucose regulator protein [21], non-muscle actin [22,23] and elongation factor-2 [24,25]. It has been proposed in each case that the covalent modification of the acceptor protein alters its biological activity.

Mono(ADP-ribosyl)transferase activity has been described previously in polymorphonuclear-neutrophil-leucocyte (PMN) homogenates and membranes; the substrates have been shown to include 116, 42, 37 and 27 kDa proteins [26–28], and the 42 kDa protein has been identified as actin. The mono(ADP-ribosyl)ation of actin has, however, been shown to be a non-enzymic addition of ADP-ribose on to a cysteine residue [27], which inhibits polymerization of actin filaments. The aims of this study were (i) to identify a mono(ADP-ribosyl)transferase activity on the outer aspect of PMNs, (ii) to determine whether the activity is mediated by an Arg-specific-mono(ADP-ribosyl)transferase, (iii) to determine the kinetic properties of the enzyme and (iv) to determine whether the enzyme is GPI-linked.

METHODS

Materials

[^{32}P]NAD$^+$ (800 Ci/mmol, 29.6 TBq/mmol) was obtained from New England Nuclear (Stevenage, Herts., U.K.). Dulbecco's modified Eagle's minimum essential medium (DMEM) without Phenol Red and Hanks' balanced salt solution (HBSS) were obtained from Gibco BRL (Paisley, Scotland, U.K.). HPLC solvents were obtained from either Fisons (Loughborough, Leics., U.K.) or BDH (Poole, Dorset, U.K.). C-18 Sep-Pak cartridges were obtained from Millipore (Watford, Herts., U.K.). Snake venom phosphodiesterase l (*Crotalus adamanteus*) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Poly(ethyleneimine) (PEI)-impregnated cellulose TLC plates were obtained from Macherey–Nagel (Düren, Germany). All other reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Isolation of PMNs

PMNs were isolated from the blood of normal human volunteers as described by Savill et al. [29]. Whole blood was centrifuged at 500 g for 20 min, and the platelet-poor plasma (PPP) removed and retained. The remaining pellet was mixed with dextran at a final concentration of 0.72 % (w/v), and the red blood cells were allowed to sediment. The supernatant, consisting of leucocyte-rich plasma, was centrifuged at 500 g for 6 min and the resulting pellet was resuspended in PPP, centrifuged through a discontinuous plasma/Percoll gradient, and the PMNs were collected

at the gradient interface. The PMNs were washed once in PPP, followed by two washes with HBSS, and finally resuspended in DMEM without Phenol Red. The cells were allowed to rest at room temperature for 1 h before assay.

Synthesis of diethylamino(benzylidineamino)guanidine (DEA-BAG)

DEA-BAG was synthesized by the method of Soman et al. [30]. The purity of the reaction product was determined by TLC using silica gel 60 F_{254} as stationary phase and methanol as solvent. The identity of the reaction product was confirmed by desorption electron-impact ionization MS on a Finnigan-MAT 4500 quadrupole mass spectrometer. The mass spectrum of the product contained a molecular ion at m/z 233 and prominent fragment ions at m/z 218, 161, 159, 146, 133, 131, 117 and 104.

HPLC of [^{32}P]NAD$^+$

[^{32}P]NAD$^+$ was purified by reverse-phase HPLC in accordance with a modification of the method of Formato et al. [31]. [^{32}P]NAD$^+$ was separated from contaminants on a Nucleosil-10 C-18 reverse-phase column by using a linear gradient from 50 mM ammonium acetate buffer, pH 6, to 90 % (v/v) 50 mM ammonium acetate buffer, pH 6, containing 10 % (v/v) methanol over 30 min at a flow rate of 1 ml/min. Fractions of volume 1 ml were collected and the radioactive peak was monitored by liquid-scintillation counting. The peak fractions were pooled, freeze-dried and resuspended in DMEM as required.

Synthesis of ADP-ribosyl-DEA-BAG

ADP-ribosyl-DEA-BAG was prepared by ADP-ribosylation of the guanidino group of DEA-BAG by using cholera toxin. The reaction mixture contained 50 μg of activated cholera toxin, 2 mM DEA-BAG, 10 mM NAD$^+$ (160 μCi of HPLC-purified [^{32}P]NAD$^+$) in 200 mM phosphate buffer, pH 7.0. This mixture was incubated at 37 °C for 6 h and the reaction was stopped by the addition of an equal volume of ice-cold 10 % (w/v) trichloroacetic acid. The resulting precipitate was removed by centrifugation at 15000 g for 20 min, and the supernatant was retained and stored at −20 °C until required. ADP-ribosyl-DEA-BAG was separated by HPLC by a modification of the method of Peterson et al. [32]. HPLC was performed on a Nucleosil 10 C-18 column by using a linear gradient over 1 min from 0.2 M ammonium acetate/0.1 M NaClO$_4$, pH 6, to 50 % (v/v) 0.2 M ammonium acetate/0.1 M NaClO$_4$, pH 6, containing 50 % (v/v) methanol at a flow rate of 1 ml/min (System I). Thereafter the column was eluted isocratically for 40 min and products were detected at 355 nm.

The fractions containing the ADP-ribosyl-DEA-BAG were retained, freeze-dried, and resuspended in 10 % (v/v) methanol. The salt in the samples was removed by using a C-18 Sep-Pak cartridge. The sample was loaded on to the cartridge and washed with 10 ml of water. The ADP-ribosyl-DEA-BAG was eluted with 50 % (v/v) methanol, freeze-dried, and the identity of the peaks was confirmed by electrospray MS (ES-MS).

ES-MS and microbore HPLC–ES-MS (System II)

Pneumatically assisted ES-MS was performed on a VG Biotech Quattro II tandem quadrupole mass spectrometer (Fisons Instruments). Mass analysis was performed on the first quadrupole (MS1) only. All samples were dissolved in 1 % (v/v) formic acid in methanol and injected via a Rheodyne 8125 injector. For flow-injection analysis, the solvent was 50 % (v/v) acetonitrile at a flow rate of 20 μl/min (Michrom BioResources
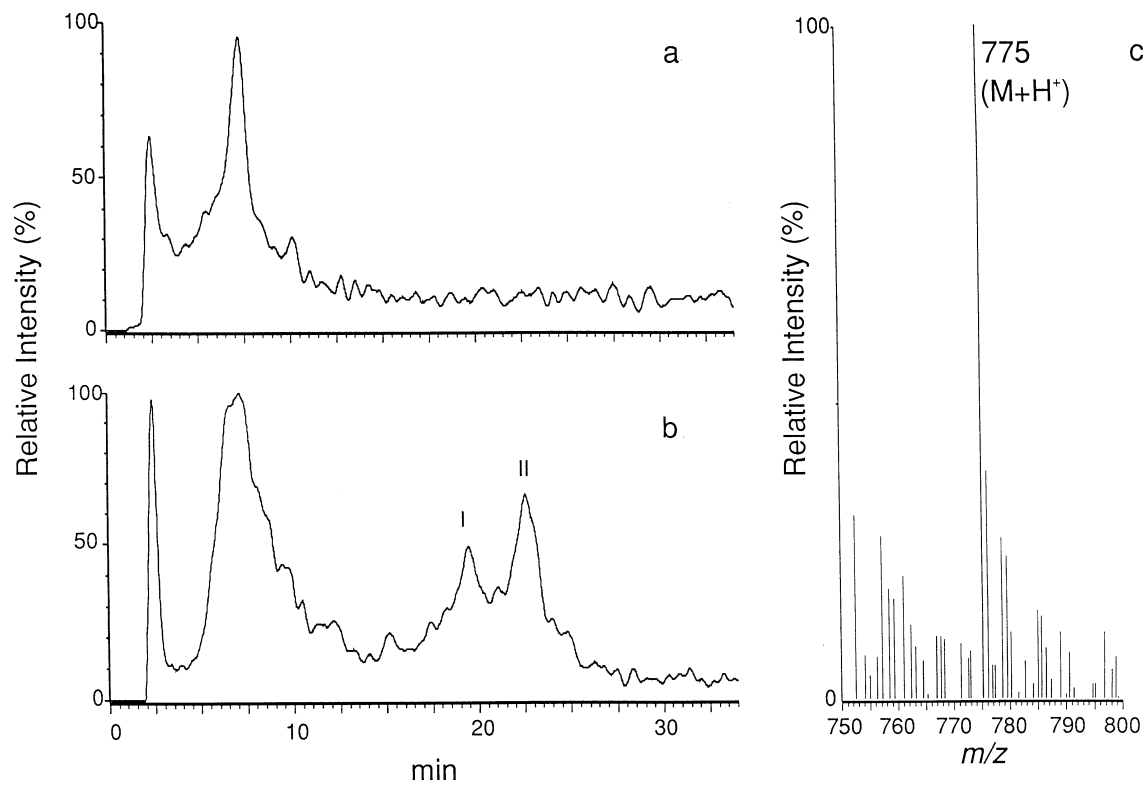
Figure 1   HPLC-ES-MS of ADP-ribosylated DEA-BAG

DEA-BAG and NAD$^+$ were incubated in the absence (**a**) or presence (**b**) of PMN for 17 h at 37 °C. The supernatants were applied to HPLC, and the peaks corresponding to authentic ADP-ribosylDEA-BAG were desalted, dried and applied to HPLC–ES-MS and ion chromatography was monitored at m/z 775. The ion spectrum from m/z 750–800 of peak I is shown in (**c**). The Figure is representative of three separate experiments.

Fluid delivery module, Jones Chromatography) and the instrument (MS1) was scanned at 5 s/scan over the mass range m/z 100–860.

Microbore HPLC–ES-MS was performed on a Hichrom RPB 1 mm (internal diam.) × 150 mm column eluted isocratically with 40 % (v/v) methanol in 50 mM ammonium acetate, pH 6, at 50 μl/min. The HPLC effluent was monitored at 355 nm (Waters model 486 variable wavelength detector fitted with a microbore flow cell) before introduction to the ES source. The MS was scanned at 5 s/scan over the mass range m/z 300–860.

Assay of arginine-specific ADP-ribosyltransferase activity

The assay for ADP-ribosyltransferase activity was modified from that of Moss and Stanley [33] and McMahon et al. [13]. PMNs were resuspended in DMEM (without Phenol Red) buffered with 20 mM Hepes, pH 7.0, containing 750 μM of HPLC-purified [^{32}P]NAD$^+$ (20 μCi/tube) and 50 mM agmatine in a final reaction volume of 300 μl. The tubes were incubated at 37 °C for the times indicated, and the reaction was terminated by addition of an equal volume of 2 M nicotinamide in 20 mM Tris/HCl pH 7.0. The samples were centrifuged at 15000 **g** for 30 min, and 500 μl of the supernatant was loaded on to 3 ml columns of Dowex 1X2-400 ion exchange resin. The columns were then sequentially eluted with 2 × 3.5 ml of 20 mM Tris/HCl, pH 7.0 and then eluted finally with 4 ml of 20 mM Tris/HCl, pH 7.0. The final 4 ml fraction contained ADP-ribosylagmatine, which was collected for liquid-scintillation counting.

Mono(ADP-ribosyl)ation of surface proteins of PMNs

PMNs ($\sim 10^7$ cells) were suspended in 400 μl of DMEM containing 20 mM Hepes buffer, pH 7.4, [α-^{32}P]NAD$^+$ (20 μCi), 1 mM ADP-ribose, 1 mM p-nitrophenylthymidine 5′-monophosphate ester (a cell-surface 5′-nucleotide phosphodiesterase inhibitor) and other reagents as indicated. The reaction was allowed to proceed at 37 °C for the times indicated, and the reaction was terminated by the addition of 1 ml of 20 % (w/v) trichloroacetic acid. The precipitate was washed, and the proteins were resolved on SDS/10 % (w/v)-polyacrylamide gels. The gels were then dried, and the ^{32}P-labelled products located by autoradiography as described previously [19].

The demonstration of a mono(ADP-ribosyl)ated product involved analysis of the protein pellet after trichloroacetic acid precipitation. The pellet was resuspended in 50 μl of 50 mM Tris/HCl buffer, pH 9.0, containing 10 mM MgCl$_2$ and 1 mg/ml snake venom phosphodiesterase 1 (40 units/mg). The mixture was incubated for 17 h at 37 °C, and the reaction terminated by the addition of an equal volume of 20 % (w/v) trichloroacetic acid. The suspension was centrifuged at 14000 **g** for 5 min, and the supernatant retained for TLC analysis. The pellet was solubilized, and the labelled proteins were resolved on SDS/10 %-polyacrylamide gels and located by autoradiography. The ^{32}P-labelled product(s) in the supernatant of the snake venom phosphodiesterase reaction were identified by TLC. The sample was spotted on to PEI-treated cellulose TLC plates, and the spots were resolved in 0.3 M LiCl containing 0.6 M acetic acid. Standards were also spotted on the same TLC plate (ADP-

ribose, ADP, AMP, NAD$^+$ and adenosine). The plate was dried and the location of the product(s) identified by UV quenching (standards) and autoradiography (sample).

Statistical analysis

All results are quoted as means $\pm$ S.E.M. Regression analysis was performed by using the curve-fitting program of Figure P. software (Figure P. Corp.). Analysis of variance was performed by using Statgraphics software (Statgraphics Corp.).

RESULTS

In pilot experiments, the viability of human PMNs maintained in DMEM was examined at times up to 17 h by Trypan Blue dye exclusion. Under the conditions described, cell viability was consistently > 97 % at 17 h, which confirms the results published elsewhere [29,34].

The activity of mono(ADP-ribosyl)transferase on the surface of PMNs was examined in reactions using DEA-BAG as a substrate. DEA-BAG was used as a substrate, as it possesses a chromophore (which facilitates its detection) and, in contrast with arginine and agmatine, it lacks a primary amino group. ADP-ribosylation of DEA-BAG is thus limited to the guanidino group. Authentic ADP-ribosyl-DEA-BAG was synthesized by using cholera toxin and purified by reverse-phase HPLC on a Nucleosil-10 C-18 column (system I). Two UV-absorbing (355 nm) anomeric forms of the product were observed, with retention times of 11 and 13.5 min; these were well separated from NAD$^+$ (6 min) and unchanged DEA-BAG (28 min). Once desalted, each anomer generated an intense electrospray mass spectrum with the base peak at m/z 775 ($M+H^+$) and associated cationized species at m/z 797 ($M+Na^+$), 813 ($M+K^+$) and 819 ($M+2Na^+-H^+$). These fractions were rechromatographed on a microbore RPB HPLC column eluted with methanol/ammonium acetate (system II), and monitored on-line by ES-MS. The anomers were eluted at 19 and 21 min, and each generated a characteristic mass spectrum with $M+H^+$ at m/z 775. Having established the conditions for measurement of ADP-ribosyl-DEA-BAG, the ADP-ribosylation of DEA-BAG in the presence of PMNs was examined.

Approx. 10^6 cells/ml were incubated in a final volume of 10 ml containing 1 mM DEA-BAG and 10 mM NAD$^+$ in HBSS containing 20 mM Hepes buffer, pH 7.4, for 17 h at 37 °C. The cell suspension was centrifuged at 1500 g for 20 min, and the supernatant was retained and desalted. When DEA-BAG was treated in the absence or presence of PMNs, there was no difference in the initial HPLC UV profile (system I) (results not shown). The HPLC fractions which were eluted between 11 and 14 min were combined, desalted and rechromatographed on microbore HPLC/ES-MS (system II). The two ADP-ribosyl-DEA-BAG anomers were observed in the PMN-treated samples (Figure 1b) at 19 min and 21 min respectively. Analysis of the mass spectrum of peak I indicated that the major ion species was m/z 775 (Figure 1c); a similar mass spectrum was obtained for peak II (results not shown). These peaks were absent from the control incubations (Figure 1a).

There was no evidence of ADP-ribosyl-DEA-BAG formation when DEA-BAG and ADP-ribose were co-incubated, over the concentration range 0.1–10 mM ADP-ribose. This result indicates that the PMN-mediated ADP-ribosylation was not simply associated with cell-surface NAD$^+$ glycohydrolase activity and subsequent non-enzymic addition of ADP-ribose to DEA-BAG. These data confirm that human PMNs express an arginine-specific mono(ADP-ribosyl)transferase.
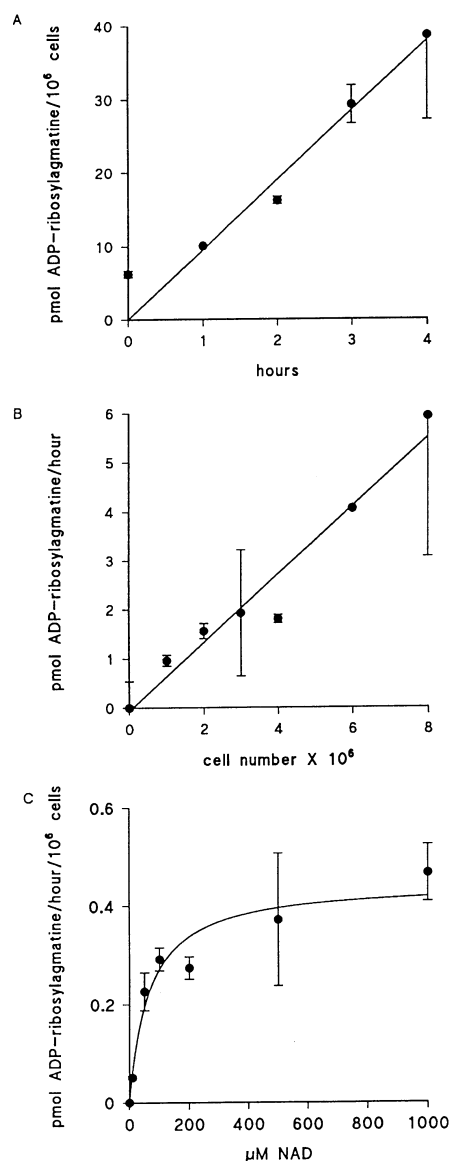

Figure 2   Kinetics of mono(ADP-ribosyl)transferase in human PMNs

PMNs were isolated from human blood and mono(ADP-ribosyl)transferase activity was measured as described in the Methods section. (**A**) The reactions were stopped at selected times and the amount of ADP-ribosylagmatine was measured. (**B**) Selected numbers of human PMNs were incubated for 4 h at 37 °C, the reactions were then stopped and the amount of ADP-ribosylagmatine was measured. (**C**) Mono(ADP-ribosyl)transferase activity was measured in tubes containing 5×10^6 cells and selected concentrations of NAD$^+$ and incubated for 4 h at 37 °C. The data are presented as mean $\pm$ S.E.M. ($n = 3$).

The use of DEA-BAG as a substrate, and two HPLC systems was not a robust assay for human PMN mono(ADP-ribosyl)transferase activity, and the assay for arginine-specific mono(ADP-ribosyl)transferase of Moss and Stanley [33] was modified. This assay uses agmatine as the acceptor for ADP-ribose and measures [^{32}P]ADP-ribosylagmatine as the product, which identifies the enzyme as an arginine-specific mono(ADP-ribosyl)transferase. Mono(ADP-ribosyl)transferase activity was linear with time under these conditions up to 4 h ($r^2 = 0.96$, $P <$ 0.01) (Figure 2A), and remained linear for up to 20 h (results not shown). The effect of cell number on mono(ADP-ribosyl)transferase activity was also examined. With increasing
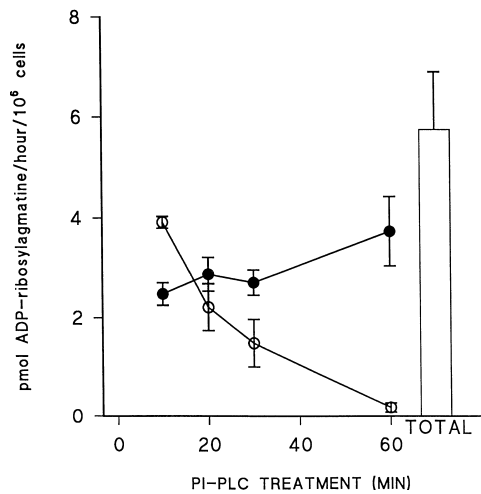
Figure 3   Effect of PI-PLC treatment on mono(ADP-ribosyl)transferase activity in human PMNs

Human PMNs were isolated as described in the Methods section and treated for selected times in the presence of 1.2 units/ml PI-PLC. The cells were centrifuged, and the cell pellet was retained and measured for mono(ADP-ribosyl)transferase activity (○). The supernatant was retained and centrifuged at 40 000 g for 20 min. Mono(ADP-ribosyl)transferase activity was also measured in the resulting supernatant (●). The assay tubes were incubated at 37 °C for 17 h. The bar represents total enzyme activity before PI-PLC treatment. The data are presented as means ± S.E.M. ($n = 3$).

Figure 4   [³²P]ADP-ribosylation of intact human PMNs

PMNs (5×10^6) were incubated at 37 °C in the presence of 10 μCi of [³²P]NAD⁺ for 4 h. The membrane proteins were then precipitated with trichloroacetic acid and the pellet was washed as described in the Methods section. The proteins were solubilized in SDS/PAGE buffer and separated on an SDS/10% (w/v)-polyacrylamide gel. The labelled proteins were identified by autoradiography (left panel). In similar experiments, the ³²P-labelled products in the precipitate were treated with snake venom phosphodiesterase (1 mg/ml) for 17 h. The proteins were precipitated with trichloroacetic acid and the content of the supernatant was analysed by TLC (see the Methods section). The location on the TLC plate of standard solutions of adenosine, NAD⁺, AMP, ADP-ribose and ADP is shown (right panel).

number of cells/tube, the assay remained linear up to and including 8×10^6 cells/tube ($r^2 = 0.95$, $P < 0.001$)(Figure 2B). In subsequent experiments, enzyme activity was measured with 5×10^6 cells/tube and an incubation time of 4 h. The apparent K_m of PMN mono(ADP-ribosyl)transferase was determined by the measurement of enzyme activity as a function of increasing concentrations of NAD⁺. From these experiments an apparent K_m value for NAD⁺ was obtained of 100.1 ± 30.4 μM ($n = 4$) and a V_{max} of 1.4 ± 0.2 pmol of ADP-ribosylagmatine/h per 10^6 cells (Figure 2C).

PI-PLC cleaves the PI side-chain of GPI-anchored proteins, thus releasing them from the membrane. In order to determine whether the mono(ADP-ribosyl)transferase activity on the surface of PMNs was GPI-linked, intact cells were incubated for various times at 30 °C in the presence of 1.2 units/ml PI-PLC, and activity was then measured in both the supernatant and the cell pellet (Figure 3). Increasing the incubation time of the cells with PI-PLC decreased the activity of mono(ADP-ribosyl)transferase in the cell pellet. After 1 h incubation with PI-PLC, approx. 98 % of mono(ADP-ribosyl)transferase activity had been lost from the cell pellet. There was no significant increase in mono(ADP-ribosyl)transferase activity in the cell supernatant after PI-PLC treatment. There was no change in cell-pellet or supernatant mono(ADP-ribosyl)transferase activity over the same time course in cells which were not treated with PI-PLC (results not shown). From these results we conclude that the mono(ADP-ribosyl)transferase of PMN is GPI-linked and is located on the outside of the cell.

³²P-labelled proteins on the surface of PMNs were located autoradiographically (Figure 4, left). Prominent labelled bands were identified at 70, 67, 46, 36 and 26 kDa (Figure 4). The labelled product was identified as a mono(ADP-ribosyl)-protein adduct by snake venom phosphodiesterase cleavage of the bond. The reaction eliminated the ³²P label from the proteins, and the
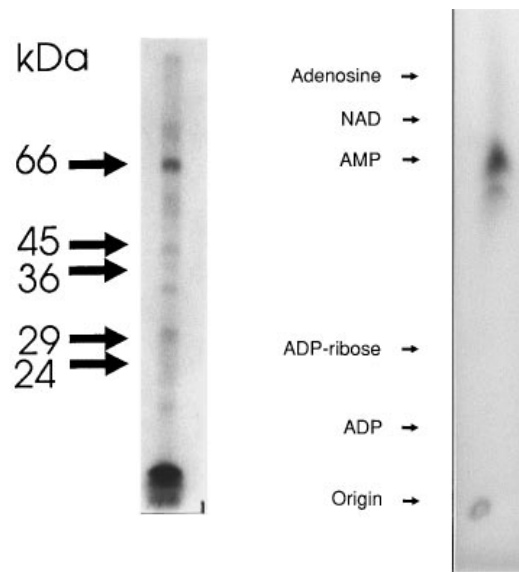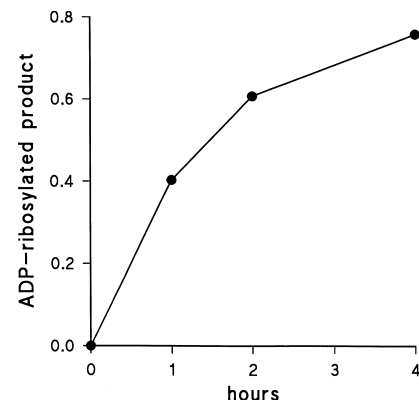

Figure 5   Time course of [³²P]ADP-ribosylation of intact human PMN proteins

PMN (10^7) were incubated at 37 °C in the presence of 20 μCi of [³²P]NAD⁺ for the times indicated. The membrane proteins were precipitated with trichloroacetic acid and the pellet was washed as described in the Methods section. The proteins were solubilized in SDS/PAGE buffer and separated on an SDS/10%-polyacrylamide gel. The [³²P]ADP-ribosylated proteins were identified by autoradiography, and the densities of the bands quantified with an LKB Ultrascan laser densitometer (absorbance units).

cleavage product was identified as 5′-AMP by TLC (Figure 4, right).

The [³²P]mono(ADP-ribosyl)ation of PMN proteins was monitored over 4 h. The results (Figure 5) show a non-linear increase in ADP-ribosylated product. In the same reaction, the DMEM supernatant was analysed for its content of [³²P]NAD⁺

by HPLC (as described for its purification in the Methods section). Under the conditions of the assay, [^{32}P]NAD$^+$ was shown to be unstable, with about 95 % loss within 1 h (results not shown).

DISCUSSION

This is the first demonstration of arginine-specific mono(ADP-ribosyl)transferase activity on the cell surface of human PMNs. Previous measurements of mono(ADP-ribosyl)transferase in PMNs have been made with homogenates [26–28]. In order to determine whether the mono(ADP-ribosyl)transferase activity of PMNs was arginine-specific, the arginine analogue DEA-BAG was used as a substrate. Reaction of PMNs with DEA-BAG yielded ADP-ribosyl-DEA-BAG, whose structure was confirmed in this study. No product could be detected in incubations performed in the absence of PMNs. The two anomeric forms of ADP-ribosylated products have been noted previously [35] and were confirmed by microbore HPLC–ES-MS. The use of a second HPLC system, together with MS identification, makes it unlikely that ADP-ribosyl-DEA-BAG has been misidentified. The possibility was considered that the low level of mono(ADP-ribosyl)transferase activity observed in these experiments was due to non-enzymic ADP-ribosylation. Free ADP-ribose released as a product of NAD$^+$ hydrolysis, possibly as a result of NAD$^+$ glycohydrolase activity, may form a Schiff's base with free amino groups. This reaction is more likely to occur at a primary-amino group than at a guanidino nitrogen atom. Incubation of DEA-BAG with ADP-ribose did not yield ADP-ribosyl-DEA-BAG. This result is similar to that seen when ADP-ribose was incubated with arginine [36], when again no product could be detected. The present results provide irrefutable evidence that the mono(ADP-ribosyl)transferase activity of human PMNs is enzymic.

Routine assay of the mono(ADP-ribosyl)transferase activity of PMNs would be impracticable with the two HPLC systems; therefore the assay for enzyme activity of Moss and Stanley [33], using agmatine as a substrate, was modified. The enzyme activity on the surface of PMNs, as measured by the ADP-ribosylation of agmatine, is much lower than that previously reported for cardiac muscle membrane [13] or mouse skeletal-muscle cells [10]. In our hands, the activity is at least 50-fold lower than that in rabbit cardiac muscle membrane. The apparent K_m of the PMN enzyme was found to be 100.1 ± 30.4 μM, which is comparable with that found previously in rat cardiac muscle, 330 μM [13].

This enzyme assay uses agmatine (decarboxyarginine) as a substrate. This molecule contains a primary-amino group, whence it is possible that non-enzymic ADP-ribosylation could occur at this site. This is unlikely, however, as arginine is not a good acceptor for free ADP-ribose [36]. The assays of mono(ADP-ribosyl)transferase activity were performed with whole cells, which indicates the presence of the enzyme on the outer aspect of PMNs. This is similar to its orientation on cultured skeletal muscle myocytes, where again the enzyme activity was located on the outside of the cell [14]. This orientation of mono(ADP-ribosyl)transferase on the outside of a cell is predicted by the cDNA sequence of muscle mono(ADP-ribosyl)transferase, which suggested the presence of a GPI anchor. The majority of known GPI-linked proteins are found on the outside of the cell, including other NAD$^+$-dependent enzymes such as cyclic ADP-ribose synthase and NAD$^+$ glycohydrolase [25,37,38]. In order to confirm whether the PMN mono(ADP-ribosyl)transferase was indeed a GPI-linked protein, cells were treated with PI-PLC before assay of mono(ADP-ribosyl)transferase activity. Approx. 98 % of the enzyme activity

was lost from the cell surface after pretreatment with PI-PLC. We were unable to measure a significant increase in mono(ADP-ribosyl)transferase activity in the supernatant after PI-PLC treatment, and the reason for this is uncertain. The mono(ADP-ribosyl)transferase in PMNs might be inactivated after loss of its GPI-linked anchor, which under normal circumstances constrains the enzyme at the cell membrane. These results contrast with those from rat cardiac-muscle membrane mono(ADP-ribosyl)transferase, which upon treatment with PI-PLC showed an increase in enzyme activity [13]. Similarly, PI-PLC treatment of cultured mouse skeletal-muscle cells released measurable quantities of the enzyme [10].

Our results have shown the presence of an Arg-specific mono(ADP-ribosyl)transferase on the surface of human PMNs, which led us to examine the possibility of protein substrates for the enzyme on the outer aspect of the plasma membrane. The labelling experiments were performed in the presence of ADP-ribose to minimize the contribution of non-enzymic addition of free [^{32}P]ADP-ribose to protein acceptors. ADP-ribose may be generated rapidly by hydrolysis of NAD$^+$ in the presence of NAD$^+$ glycohydrolase (another GPI-linked cell-surface protein). The stability of the mono(ADP-ribosyl)ated product was maximized by the addition of p-nitrophenylthymidine 5′-monophosphate ester, an inhibitor of 5′-nucleotide phosphodiesterase [39]). This enzyme has been implicated in the hydrolysis of mono(ADP-ribosyl)ated proteins at the cell surface [39]. The results here show multiple protein substrates for a mono(ADP-ribosyl)transferase. This enzyme activity was implicated in the labelling reaction after hydrolysis of the product with phosphodiesterase to yield 5′-AMP.

The time-course experiment for labelling of cell proteins revealed that [^{32}P]NAD$^+$ is unstable under the conditions of the assay. In the earlier experiments, the labelling of DEA-BAG and agmatine were performed in the presence of final concentrations of 10 mM and 0.75 mM NAD$^+$ respectively. However, owing to the limitation of the sensitivity of the method, the labelling of the protein substrates was performed with trace [^{32}P]NAD$^+$ alone (final concn. 670 nM). At present, there are no selective inhibitors of NAD$^+$ glycohydrolase activity, and this problem remains unresolved by all workers in this field [13,14,33,40,41]. Notwithstanding this problem, the labelling reaction increases with time, although slowly after 1 h.

The possibility that mono(ADP-ribosyl)transferases may be involved in white-cell function has been suggested by previous research on cytotoxic T lymphocytes (CTL) [42]. Again, the enzyme is situated on the outer aspect of the cell, and may be released with PI-PLC, indicating the presence of a GPI anchor. In these cells mono(ADP-ribosyl)transferase activity suppresses the ability of CTL cells to proliferate and mediate cell lysis [42].

This is the first report of an arginine-specific mono(ADP-ribosyl)transferase on the external surface of human PMNs. This enzyme most probably contains a GPI anchor, which attaches it to the cell membrane. There are substrates for the enzyme on the cell surface of PMNs, and we are at present exploring the possibility that it could play a role in PMN function.

REFERENCES

1   Ueda, K. and Hayaishi, O. (1985) Annu. Rev. Biochem. **54**, 73–100
2   Wreggett, K. A. (1986) J. Recept. Res. **6**, 95–126
3   Moss, J. and Vaughan, M. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3621–3624
4   Moss, J., Stanley, S. J. and Oppenheimer, N. J. (1979) J. Biol. Chem. **254**, 8891–8894

5   Moss, J., Stanley, S. J. and Watkins, P. A. (1980) J. Biol. Chem. **255**, 5838–5840

6   Yost, D. A. and Moss, J. (1983) J. Biol. Chem. **258**, 4926–4929

7   Tanuma, S. and Endo, H. (1990) FEBS Lett. **261**, 381–384

8   Soman, G., Mickelson, J. R., Louis, C. F. and Graves, D. J. (1984) Biochem. Biophys. Res. Commun. **120**, 973–980

9   Soman, G. and Graves, D. J. (1988) Arch. Biochem. Biophys. **260**, 56–66

10   Zolkiewska, A., Nightingale, M. S. and Moss, J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 11352–11356

11   Okazaki, I. J., Zolkiewska, A., Nightingale, M. S. and Moss, J. (1994) Biochemistry **33**, 12828–12836

12   Tsuchiya, M., Hara, N., Yamada, K., Osago, H. and Shimoyama, M. (1994) J. Biol. Chem. **269**, 27451–27457

13   McMahon, K. K., Piron, K. J., Ha, V. T. and Fullerton, A. T. (1993) Biochem. J. **293**, 789–793

14   Zolkiewska, A. and Moss, J. (1993) J. Biol. Chem. **268**, 25273–25276

15   Klebl, B. M., Matsushita, S. and Pette, D. (1994) FEBS Lett. **342**, 66–70

16   Donnelly, L. E., Boyd, R. S., Clifford, C. P., Olmos, G., Allport, J. R., Lo, G. and MacDermot, J. (1994) Biochem. Pharmacol. **48**, 1669–1675

17   Jacquemin, C., Thibout, H., Lambert, B. and Correze, C. (1986) Nature (London) **323**, 182–184

18   Obara, S., Yamada, K., Yoshimura, Y. and Shimoyama, M. (1991) Eur. J. Biochem. **200**, 75–80

19   Donnelly, L. E., Boyd, R. S. and MacDermot, J. (1992) Biochem. J. **288**, 331–336

20   Donnelly, L. E., Boyd, R. S., Williams, R. J., Kelly, E. and MacDermot, J. (1995) Biochem. Pharmacol. **49**, 767–776

21   Leno, G. H. and Ledford, B. E. (1989) Eur. J. Biochem. **186**, 205–211

22   Matsuyama, S. and Tsuyama, S. (1991) J. Neurochem. **57**, 1380–1387

23   Terashima, M., Mishima, K., Yamada, K., Tsuchiya, M., Wakutani, T. and Shimoyama, M. (1992) Eur. J. Biochem. **204**, 305–311

24   Iglewski, W. J., Lee, H. and Muller, P. (1984) FEBS Lett. **173**, 113–118

25   Lee, H. C., Zocchi, E., Guida, L., Franco, L., Benatti, U. and De Flora, A. (1993) Biochem. Biophys. Res. Commun. **191**, 639–645

26   Clancy, R. M., Leszczynska Piziak, J. and Abramson, S. B. (1993) Biochem. Biophys. Res. Commun. **191**, 847–852

27   Just, I., Wollenberg, P., Moss, J. and Aktories, K. (1994) Eur. J. Biochem. **221**, 1047–1054

28   Obara, S., Mishima, K., Yamada, K., Taniguchi, M. and Shimoyama, M. (1989) Biochem. Biophys. Res. Commun. **163**, 452–457

29   Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. and Haslett, C. (1989) J. Clin. Invest. **83**, 865–875

30   Soman, G., Narayanan, J., Martin, B. L. and Graves, D. J. (1986) Biochemistry **25**, 4113–4119

31   Formato, M., Masala, B. and De Luca, G. (1990) Clin. Chim. Acta **189**, 131–137

32   Peterson, J. E., Larew, J. S. and Graves, D. J. (1990) J. Biol. Chem. **265**, 17062–17069

33   Moss, J. and Stanley, S. J. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4809–4812

34   Lee, A., Whyte, M. K. and Haslett, C. (1993) J. Leukocyte Biol. **54**, 283–288

35   Tsuchiya, M., Tanigawa, Y., Mishima, K. and Shimoyama, M. (1986) Anal. Biochem. **157**, 381–384

36   McDonald, L. J., Wainschel, L. A., Oppenheimer, N. J. and Moss, J. (1992) Biochemistry **31**, 11881–11887

37   Kim, U. H., Rockwood, S. F., Kim, H. R. and Daynes, R. A. (1988) Biochim. Biophys. Acta **965**, 76–81

38   Zocchi, E., Franco, L., Guida, L., Benatti, U., Bargellesi, A., Malavasi, F., Lee, H. C. and De Flora, A. (1993) Biochem. Biophys. Res. Commun. **196**, 1459–1465

39   Zolkiewska, A. and Moss, J. (1995) J. Biol. Chem. **270**, 9227–9233

40   Rankin, P. W., Jacobson, E. J., Benjamin, I. C., Moss, J. and Jacobson, M. K. (1989) J. Biol. Chem. **264**, 4312–4317

41   Honma, T. and Mandel, P. (1986) J. Neurochem. **47**, 972–975

42   Wang, J., Nemoto, E., Kots, A. Y., Kaslow, H. R. and Dennert, G. (1994) J. Immunol. **153**, 4048–4058